Cryoprotection of porcine cornea: a scanning electron microscopy study

A. Neronov[1], P. Giurov[2], M. Cholakova[1], M. Dimitrova[1], E. Nikolova[1]

[1]Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Sofia, Bulgaria

[2]Institute of Experimental Pathology and Parasitology, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT: Porcine corneas were frozen with Me_2SO, glycerol, 1,2-propanediol and PEG-400. The effects of the range of concentrations (5% and 10%) and temperature regimen (1°C/min and 5°C/min) were investigated. The integrity of corneal endothelial cells was evaluated by scanning electron microscopy and trypan blue staining. The presence of 5–10% PEG-400 in the protective medium was the most effective in minimizing changes in the integrity of the corneal endothelium during freezing-thawing procedures.

Keywords: porcine cornea; Me_2SO; glycerol; 1,2-propanediol; PEG-400

A human corneal equivalent has been developed with application in pharmaceutical testing (Baydoun et al., 2004a), biomedical research (Baydoun et al., 2004b; Choy et al., 2004a,b; Spoerl et al., 2004) and instrument design (Grieve et al., 2004; Muller et al., 2004; Tehrani et al., 2004). Foetal porcine corneas have been used to construct the cornea from cultures of three specific corneal cell types and for organotypic corneal constructs (Schneider et al., 1999). The clinical application of irradiated porcine cornea to patients with ocular burns indicated that it might be an ideal therapeutic material for the management of such patients. Reconstructing clinically usable corneas by applying the technology of regenerative medicine can offer a solution to many problems as well as making corneal transplantation a non-emergency surgery and enabling the usage of banked corneal tissue.

Different methods of corneal cryoprotection have been introduced, those employing intracellular cryoprotector such as Me_2SO or glycerol being the most widely favoured. The influence of several freeze-thaw trauma variables on the survival of porcine endothelial monolayer when employing the extracellular cryoprotective agent dextran was also explored and found as a very effective cryoprotector for the freezing of porcine corneas (Halberstadt et al., 2001).

The aim of the present study was to evaluate the feasibility of different programmed freezing methods and cryoprotectors in porcine cornea cryoprotection.

MATERIAL AND METHODS

Corneal material was obtained from the eyes of 43 four-months-old Landrace pigs, about 25–30kg body weight, from Russe region. The animals were sacrificed in a local abattoir and eyes were enucleated from pigs immediately and transferred within 1h to the laboratory in a moist chamber on ice. The corneas were dissected with a 1–2mm rim of sclera. No *post mortem* swelling was detected. The corneal

Supported by the National Fund "Scientific Investigations", Ministry of Education and Science, Bulgaria (Grant L 1102/01).

material was processed according to the method of Capella et al. (1965) and the viability of the endothelial layer was evaluated using a trypan-blue test (0.2% trypan blue in PBS), 10 min incubation at 37°C and inverted microscope. All the corneas were tested prior to cryopreservation and corneas with no stained in blue cells (dead cells) were used.

Freezing protocol. The following compounds were used as cryoprotectors – dimethyl sulphoxide (Me_2SO) (Merck, 102951), glycerol (Gly) (Pharmachim, Bulgaria, 98%), 1,2-propanediol (1,2-PD) (Merck, 822324) and polyethylene glycol – Mw = 380–4 200 g/mole (PEG-400) (ICC, UASci., Kharkov). The cryoprotective media (CPM) were prepared at a temperature of 10°C for Me_2SO, glycerol and 1,2-propanediol, and at room temperature for PEG-400. The cryoprotective media contained 5% or 10% of the cryoprotectors diluted up to 2 ml in medium 199 with 20% human serum albumin (Alb). The final concentration of the cryoprotectors Me_2SO, glycerol and 1,2-propanediol was reached stepwise by 1–3–5% and 1–3–5–7–10% dilution. The isolated corneas were kept in 2 ml cryoprotective medium at 10°C with stepwise increase in the cryoprotectant concentration for 5 min when Me_2SO, glycerol and 1,2-propanediol were added, and directly in 5% or 10% for 15 min when PEG-400 was added. According to our previous studies and investigations of other authors (Jurchenko and Sharlaj, 1979) PEG-400 is not toxic and no stepwise increase in concentration is necessary.

After corneal incubation in final cryoprotective medium they were frozen in 2 ml of it in plastic ware using a programmable freezer (Union Carbide Ml84/II) applying the following temperature regimen: from 10°C to –10°C at 1°C/min or 5°C/min and from –10°C to –80°C at 5°C/min. Samples were then transferred to a liquid nitrogen container for storage (Neronov and Michailova-Bojadjieva, 1989).

Table 1. Effect of the concentration of cryoprotectors in cryoprotective medium (CPM) and cooling rate on the integrity of endothelial cells determined by SEM

Cryoprotector	Concentration of cryoprotectors in CPM % (v/v)	Cooling rate (°C /min)	Damaged endothelial cells (%)
Me_2SO	10	1	3.8 ± 0.4
Me_2SO	5	1	1.2 ± 0.3
Me_2SO	10	5	4.4 ± 0.5
Me_2SO	5	5	0
Gly	10	1	19.5 ± 1.7
Gly	5	1	18.7 ± 2
Gly	10	5	48 ± 6.3
Gly	5	5	25 ± 3.1
1,2PD	10	1	1.2 ± 0.06
1,2PD	5	1	2.4 ± 0.3
1,2PD	10	5	1.2 ± 0.3
1,2PD	5	5	25 ± 3.2
PEG-400	10	1	1.2 ± 0.2
PEG-400	5	1	0
PEG-400	10	5	0.6 ± 0.02
PEG-400	5	5	0
Alb	no	5	17.8 ± 1.5

Note: Number of corneas per group = 5; results are means ± SD. Analysis of variance with three factors (cryoprotectants, concentrations and cooling rates) – ANOVA was used to evaluate the significance of differences. This statistical analysis shows that all combinations of the interactions are significant $P < 0.0001$

Thawing protocol. Thawing was performed in a water-bath at 37°C for 1 min and transferred 3 times in 5 ml medium 199 at 10°C for cryoprotectant media removal. After 24 h incubation at 37°C, 5% CO_2 in medium 199 containing 5% human serum albumin, the corneas were sectioned into two parts. One of them was tested for viability of endothelial cells with trypan blue exclusion test (an average of 300 cells in 2 areas for each cornea were counted for coloured ones in blue – dead cells) and the other processed for scanning electron microscopy as follows: fixing in 5% glutaraldehyde in 0.1M cacodylate buffer for 48 h; washing in buffer three times; cutting into small pieces; treatment with increasing concentrations of ethanol for dehydration; critical point drying and covering with gold for SEM observation under a JEOL-35 scanning electron microscope (Neronov and Michailova-Bojadjieva, 1989). Analysis of variance with three factors (cryoprotectants, concentrations and cooling rates) – ANOVA was used to evaluate the significance of differences.

Control groups. These included fresh corneas without freezing (a) and corneas frozen only in medium 199 containing 20% human serum albumin (b). Both samples were fixed and prepared for scanning electron microscopy as described above.

RESULTS

We applied the following known cryoprotectors – Me_2SO, glycerol, 1,2-propanediol and PEG-400 to elucidate their influence on membrane integrity preservation and on the functional activity of the corneal tissue at freezing and thawing. The supplement of 20% human serum albumin to the cryoprotective medium leads to a slight dehydration of cell layers, which appears to be advantageous during the process of freezing and it became a common practice to be used in such media.

Me_2SO is a well-known cryoprotector with a wide variety of applications in cryopreservation of different cells, tissues and organs. As can be seen in Table 1, it has a significant cryoprotective effect on the recovery of the corneal endothelial layer. In some cases a slight disruption of the endothelial intercellular contacts could be observed (Figure 1).

The use of glycerol as a cryoprotector of the corneal endothelial layer did not seem to be very advantageous as the number of the recovered cells was very low (Table 1) and the SEM investigation showed areas of damaged and exfoliated endothelial layers (Figure 2).

According to the results of our study the cryoprotective effect of 1,2-propanediol was intermedi-

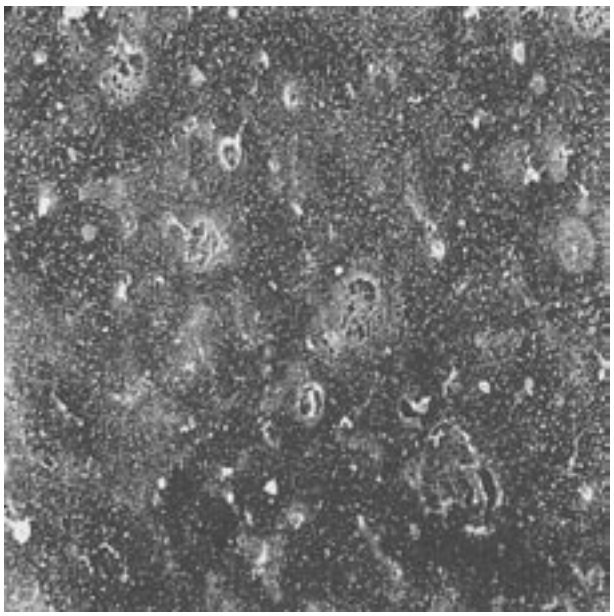

Figure 1. Scanning electron micrograph of the porcine cornea frozen in 10% Me_2SO in cryoprotective medium. The endothelial cell surface is almost intact but the cell boundaries are not clearly distinguished. Some of the cells are destroyed partially. Magnification 1 000×

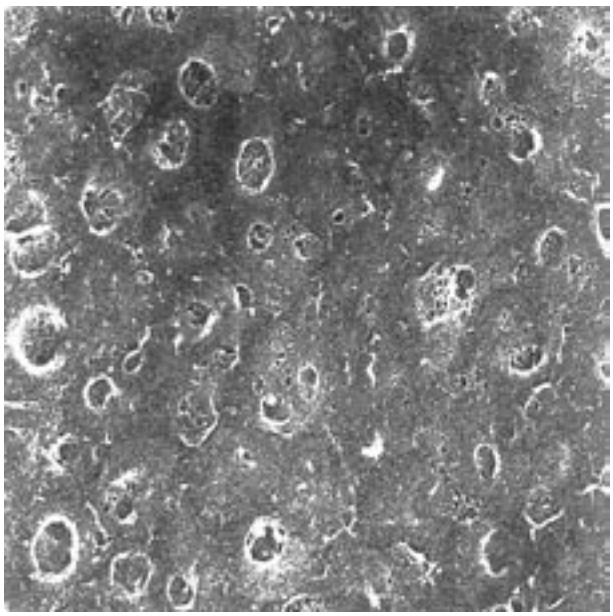

Figure 2. Scanning electron micrograph of the porcine cornea frozen in 10% glycerol in cryoprotective medium. The endothelial cell surface shows an irregular cell pattern and the membranes of some cell are disrupted. Magnification 1 000×

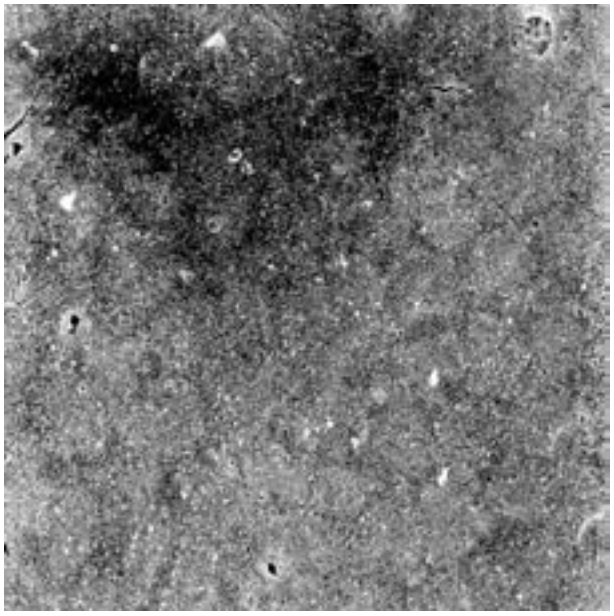

Figure 3. Scanning electron micrograph of the porcine cornea frozen in 10% 1,2-propanediol in cryoprotective medium. The endothelial cell surface appears smooth but the cell boundaries are indistinguishable and the cell junctions are not tight. Magnification 1 000×

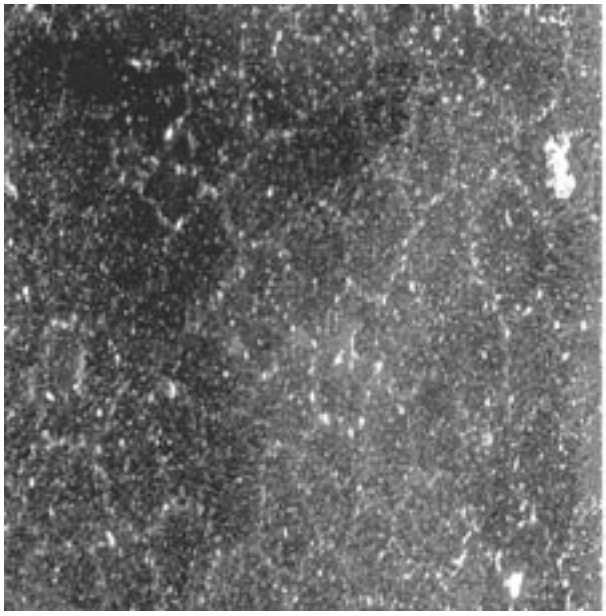

Figure 4. Scanning electron micrograph of the porcine cornea frozen in 10% PEG-400 in cryoprotective medium. The endothelial cell surface is generally smooth and the cell boundaries become more distinguishable. Magnification 1 000×

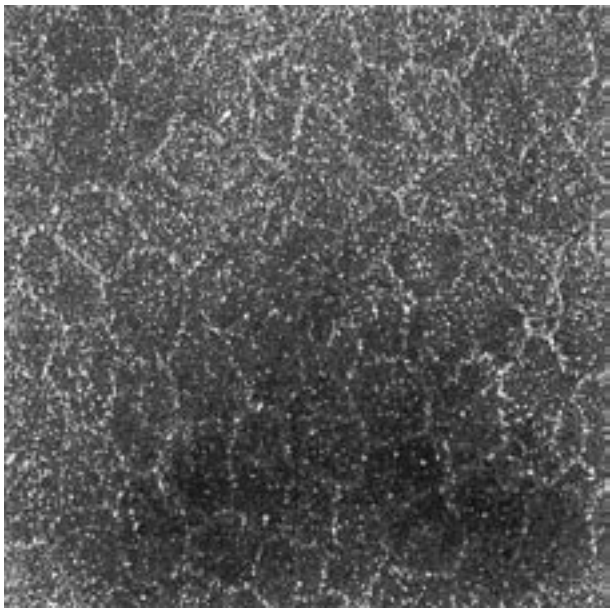

Figure 5. Scanning electron micrograph showing the surface of the normal porcine corneal endothelium. The endothelial cell surface is generally smooth end shows a regular cell pattern. The elevation of the hexagonal cell boundaries is present. Magnification 1 000×

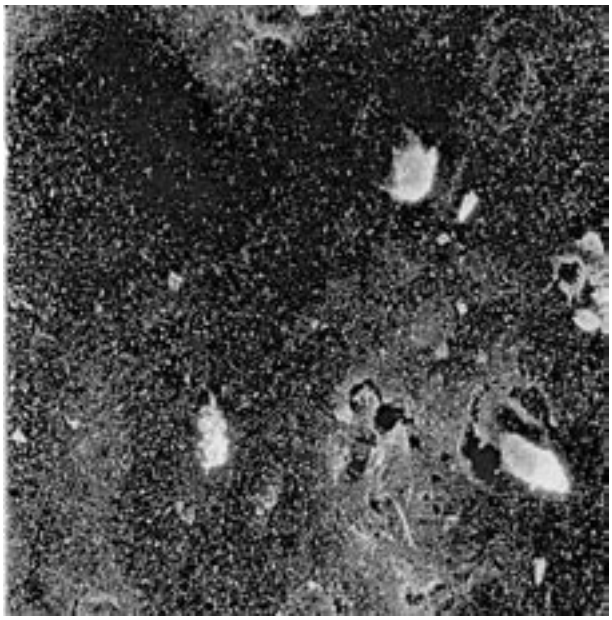

Figure 6. Scanning electron micrograph of the porcine cornea frozen in 20% human albumin as cryoprotective medium (control). The endothelial cell surface is altered, cell boundaries are missing and some cell surface membranes are destroyed. Magnification 1 000×

ary between Me_2SO and glycerol. The cell recovery is higher than that with cryoprotector glycerol (Table 1) and the cell integrity is better preserved. One disadvantage of the use of 1,2-propanediol as cryoprotector is the presence of gaps in a large number of endothelial cells (Figure 3).

The best results were obtained when PEG-400 was used as porcine corneal cryoprotector. The highest percentage of entirely recovered cells (Table 1) with unimpaired cell to cell contacts (Figure 4) in the endothelial layer made PEO-400 the most convenient cryoprotectant for porcine cornea.

In the control group (a) the endothelium surface appears generally smooth, except for the elevation of the hexagonal cell boundaries (Figure 5). In the control group (b) ultrastructural changes are characterized by destruction in the cell membranes and abolishment of the cell boundaries (Figure 6).

All four cryoprotectants in 5% or 10% concentrations were applied for freezing experiments using two different cooling rates – 1°C/min and 5°C/min. The recovery of the endothelial cells favoured the cooling rates of 1°C/min for all of them with the exception of PEG-400 where no difference between the two rates was found (Table 1).

DISCUSSION

The cell membrane is an important element of the cell structural organization. It is extremely important for the biological function and is a prime site for injury or alterations caused by temperature changes and other factors during cooling. During the processes of freezing and thawing the cell membrane could be affected by the ice crystal formation outside as well as inside of the cell walls, depending on their type. The perfect case is a formation of amorphous ice at both sides. This could be determined by the use of different cryoprotectors (Meryman, 1971).

The purpose of the present study was to develop an optimal freezing method for cryopreservation of porcine donor corneas for transplantation comparing four different cryoprotectors in concentrations of 5% and 10% and using cooling rate of 1°C/min or 5°C/min. Studies have been reported on cryoprotection of porcine cornea in dextran and Me_2SO of concentrations higher than those used in our experimental system (Canals et al., 1996; Wusterman et al., 1999). The human cornea has been shown to successfully recover from freezing cryoprotected in Me_2SO (Canals et al., 1999), Me_2SO, formamide and 1,2-propanediol (Bourne and Nelson, 1994), Me_2SO, glycerol and 2,3-butanediol (Bourne et al., 1994). Although the results of these experiments have been satisfactory, the damage cannot be completely avoided using these cryoprotectors.

PEG-400 has a low cytotoxicity, its presence in the freezing medium leads to the formation of amorphous structures and hinders ice crystals, i.e. the formation of ice occurs outside of the cell. PEG-400 forms complexes with the water of the biological structures, stabilizes the hydration mesh surrounding the proteins and decreases the action of the harmful factors during the low temperature preservation. It has been shown to cryopreserve rabbit and human corneas with a high degree of protection of the endothelial monolayer after thawing (Neronov and Michailova-Boiadjieva, 1989).

This study indicates that it is possible to preserve porcine corneas with a high level of structural integrity and viability if PEG-400 is used as a cryoprotector.

REFERENCES

Baydoun L., Duvel A., Daniels R., Drust A., Goldhagen T., Schwan I., Zeidler C., Muller-Goymann C.C. (2004a): Comparison of different ibuprofen-amino acid compounds with respect to emulsifying and cytotoxic properties. International Journal of Pharmaceutics, 274, 157–165.

Baydoun L., Furrer P., Gurny R., Muller-Goymann C.C. (2004b): New surface-active polymers for ophthalmic formulations: evaluation of ocular tolerance. European Journal of Pharmaceutics and Biopharmaceutics, 58, 169–175.

Bourne W.M., Nelson L.R. (1994): Human corneal studies with a vitrification solution containing dimethyl sulphoxide, formamide, and 1,2-propanediol. Cryobiology, 31, 522–530.

Bourne W.M., Shearer D.R., Nelson L.R. (1994): Human corneal endothelial tolerance to glycerol, dimethylsulphoxide, 1,2-propanediol, and 2,3-butanediol. Cryobiology, 31, 1–9.

Canals M., Costa J., Potau J.M., Merindano M.D., Puta D., Ruano D. (1996): Long-term cryopreservation of human donor corneas. European Journal of Ophthalmology, 6, 234–241.

Canals M., Costa-Vila J., Potau J.M., Merindano M.D., Ruano D. (1999): Morphological study of cryopre-

served human corneal endothelium. Cell Tissues Organs, 164, 37–45.

Capella J.A., Kaufman H.E., Robbins J.E. (1965): Preservation of viable corneal tissue. Archives of Ophthalmology, 74, 669–673.

Choy E.P., To T.S., Cho P., Benzie I.F., Choy C.K. (2004a): Viability of porcine corneal epithelium *ex vivo* and effect of exposure to air: a pilot study for a dry eye model. Cornea, 23, 715–719.

Choy E.P., Cho P., Benzie I.F., Choy C.K., To T.S. (2004b): A novel porcine dry eye model system (pDEM) with simulated lacrimation/blinking system: preliminary findings on system variability and effect of corneal drying. Current Eye Research, 28, 319–325.

Grieve K., Paques M., Dubois A., Sahel J., Boccara C., Le Gargasson J.F. (2004): Ocular tissue imaging ultrahigh-resolution, full-field optical coherence tomography. Investigative Ophthalmology & Visual Science, 45, 4126–4131.

Halberstadt M., Athmann S., Hagenah M. (2001): Corneal cryopreservation with dextran. Cryobiology, 43, 71–80.

Jurchenko T.N., Sharlaj TM (1979): On the problem of cornea cryopreservation (in Russian). Kriobiologija i kriomeditsina, 5, 81–88.

Meryman H.T. (1971): Osmotic stress as a mechanism of freezing injury. Cryobiology, 8, 173–183.

Muller B., Boeck T., Hartmann C. (2004): Effect of excimer laser beam delivery and beam shaping on corneal sphericity in photorefractive keratectomy. Journal of Cataract and Refractive Surgery, 30, 464–470.

Neronov A.J., Michailova-Boiadjieva A. (1989): Cryopreservation of rabbit cornea with polyethylene oxide-400. A scanning electron microscopy study. CryoLetters, 10, 279–288.

Schneider A.I., Maier-Reif K., Graeve T. (1999): Constructing an in vitro cornea from cultures of the three specific corneal cell types. In Vitro Cellular & Development Biology – Animal, 35, 515–526.

Spoerl E., Wollensak G., Seiler T. (2004): Increased resistance of crosslinked cornea against enzymatic digestion. Current Eye Research, 29, 35–40.

Tehrani M., Schafer S., Dick H.B. (2004): Evaluation of cut quality using the Amadeus microkeratome with different settings. Journal of Cataract and Refractive Surgery, 30, 2415–2419.

Wusterman M.C., Armitage J.W., Wang L.H., Busza A.L., Pegg D.E. (1999): Cryopreservation studies with porcine corneas. Current Eye Research, 19, 228–233.

Received: 05–02–08
Accepted after corrections: 05–04–26

Corresponding Author

Anatoliy Neronov, Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Acad. G.Bonchev str., bl. 25, 1113 Sofia, Bulgaria
E–mail: neronovan@yahoo.com