

The impact of culture media on the endothelial viability of corneas

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Abstract

Background: The complexity of cryopreservation and its potential to damage the endothelium make it so that it is rarely used in routine eye banking, except for occasional, clinically urgent transplants, where the main objective is to save the eye. The culture medium was the method of choice in the Eye Bank.

Material and methods: The study group was divided into 3 subgroups. For penetrating keratoplasty and anterior lamellar keratoplasty, corneas taken up to 24 hours, with a number greater than 2000 endothelial cells per mm², are preferably used.

Results: In the first group we found: weakly edematous epithelium; thin, transparent stroma; thin Descemet's membrane; transparent endothelial layer, endothelial cell density greater than 2800 cells/mm². The corneas of the second group presented: edematous, but with uncompromised integrity epithelium; slightly edematous, transparent stroma; slightly folded Descemet's membrane; intact endothelial layer and an average of 2600 cells/mm². Corneas of the third group: evidently edematous epithelium, with exfoliations in some areas and Bowman's membrane detachment; considerable edema of stroma in all layers; pronounced folds of Descemet's membrane; interrupted endothelial layer along the outline of the folds.

Conclusions: The age of the donor and the preservation time are important factors that influence corneas in culture media and determine the state of endothelial cells. Although the number of endothelial cells usually decreases with age, there are still many corneas from donors over 80 years of age who meet the minimum criteria for transplantation.

Key words: minimum essential medium Eagle, fetal bovine serum.

Introduction

Out of 62 eye banks included in the Association of European eye banks of 2010, 47 – have used culture media, 9 – have used the hypothermic method and 6 – have used both methods: generally, 70% of corneas were stored in culture media [9,13]. The most common organ culture medium is the Eagle Essential Medium (MEM) with 2% fetal bovine serum (FBS), although up to 8% FBS is used by some banks [14]. Most eye banks also include in their medium: penicillin, streptomycin and amphotericin B, but there are alternative antibiotics including biklin, amukin, tazocin and nystatin [12,15]. Another difference in methodology is that the medium is changed during storage; however, 40% of eye banks do not alter the medium during storage, while others refresh the culture medium every 1-2 weeks [11]. The concentration of dextran, used to reverse the stromal edema, which occurs during cultivation in the medium, ranges from 4 to 8%. Regardless of these changes, it would seem that the results of the grafts are similar. Corneas are normally stored for up to 4 weeks [1], but there have been reported successful cornea transplants stored in organ cultures for 7 weeks [2].

The culture medium was the method of choice in the eye bank in the city of Bristol, since it was established in the mid-1980-s, and illustrates the general technical descrip-

tion [3]. Before cutting corneoscleral discs, the eyes are cleaned by rinsing in sterile saline and immersion in povidone-iodine to reduce bacterial and fungal contamination of the surface of the eye. Corneas are suspended in 80 ml of HEPES buffer MEM Eagle, 26 mmol/l NaHCO₃, 2% FBS, 2 mmol/l L-glutamine, penicillin, gentamicin and amphotericin B and stored at 34°C [23]. The cornea contains viable cells and cannot be sterilized; however, in comparison with hypothermic storage, culture medium increases the chance of detecting the bacteria and fungi that might cause a post-operative infection, and the addition of antibiotics in the culture medium is more effective, allowing a higher storage temperature [16,17,18]. The first sample probe is taken for bacteriological examination after 7 days of cultivation to examine bacteria and fungi, the second probe – at the end of storage, before the release of the cornea for transplantation [24].

There are three main approaches to preservation and storage of corneas containing living cells: via organ culture, hypothermia, and cryopreservation. Only the latter currently offers the prospect of an unlimited storage time [19]. Techniques for corneal cryopreservation were developed in the 1960s and applied clinically [20, 21]. More recently, retention of endothelial function was reported after ice-free cryopreservation by vitrification of rabbit cornea in a high

concentration of propane – 12-diol [23]. But the complexity of cryopreservation and its potential to damage the endothelium make it so that it is rarely used in routine eye banking, except for occasional, clinically urgent transplants, where the main objective is to save the eye [23].

Material and methods

The study is based on dystrophic changes of endothelial cells in culture medium at different periods of time till sampling and evaluation of cellular conditions during 60 days storage in culture medium “Tissue C”, containing Eagle MEM with fetal bovine serum, 2% (FBS), a mix of antibiotic/antimycotic which guarantees effective protection against bacteria and fungi, phenol red indicator which allows a quick view of the changes in pH. During the corneal cultivation, the medium remains unaltered.

250 corneas were collected from 126 donors who died of cardiac arrest. The sampling was performed by the team of the Human Tissue Bank from the Republic of Moldova, Chisinau. All sampling stages have been completed in accordance with the regulations of standard operation protocol (SOP) [4]. The criteria of donor selection [5], the screening of the corneal morphological defects, congenital abnormalities, postoperative scars, post-mortem structural changes were observed. The study group was subdivided into 3 groups, group I – corneas collected up to 20 hours, 160 donors – the stroma is completely transparent, thin, the contours of the boundaries between the iris and pupil are visible; group II – corneas collected from 20 to 24 hours, 60 donors – the surface epithelium is swollen, turbid, its integrity is dubious, the borders of the iris look good; group III – corneas collected after 24 hours, 30 donors – the contour of the iris is poorly distinguished with the presence of an opalescent ring. The thickness of the cornea is proportional to the time the organ is retrieved. Sampling of eye tissue is preferably to be carried out up to 24 hours after cardiac arrest. Corneas were morphologically assessed on transparency. The vast majority of the corneas were excised with surgical instruments specific to ophthalmic interventions (fig 1) [6].

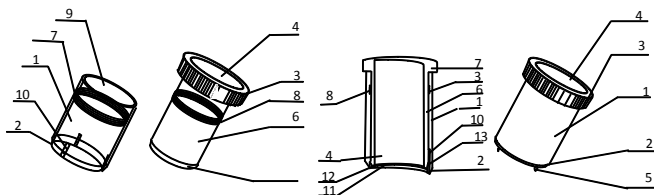


Fig. 1. Device for cutting cornea consists of two tubes, external and internal, made of inoxidable steel, external (1), with the sharp edge (2), the distal end being straight (13), which assembles the blade (5), internal diameter of 18 mm (9), internal tube (6) with distal external thread (8), at the far end of the threaded handle (3), internal diameter of 17 mm (4), proximal external edge (12) and internal (11).

Sampling methods: after aseptic processing of the eye, the eyelids were fixed with blepharostat. The sectional area

was set in a ring-shaped manner, afterwards, using a scalpel, a small incision was made in the eye and one of the scissors blade was inserted into the eye section, and the tissue was incised along the line.

Using the ophthalmic tweezers, the edge of the cornea-sclera complex disk was fixed, and the irido-crystalline diaphragm was limited. Using the device for cutting the cornea, the cornea is incised in the center, allowing to make an incision of 2 mm from the edge of the limb, and rotating it clockwise until the conjunctiva and the cornea are completely separated. Subsequently, the device is removed and the cornea is fixed with tweezers, and then the irido-crystalline complex is separated. The corneas obtained were washed with a sterile isotonic solution at a temperature of 20-25 °C in a sterile plastic container with a screw cap. Subsequently, the transport medium, Eussol C", was changed by immersing the tissue in a sterile 50 ml tube with a screw cap containing MEM medium, antibiotics and antifungal agents. Then it is placed in a specialized container for the transportation to the tissue bank.

After receiving the results of the serological test, was assessed the state of the endothelial cells on an inverted contrast microscope with a blunt trepan, in which cells with a uniform redistribution are determined, preferably at the edge of the cornea and in the middle region. In the extracellular space, dead cells are visualized (necrosis/apoptosis) and the state of the Descemet's membrane is observed.

Endothelial cell density should be higher than 2000 cells/mm². Moderate or severe symptoms, such as cell pleo-

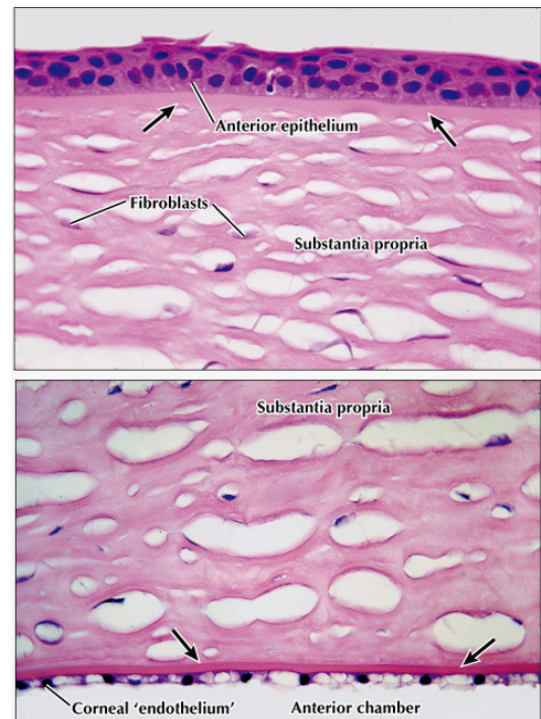


Fig. 2. Patient B, 64 years old. Histological examination of cornea preserved by Tissue C, after 4 days. Anterior epithelium. Layered non-keratinized with planocellular 5-7 cell lines (a), continued with the conjunctival epithelium covering the corneal limbus – injury to stem cells induces fibrosis of the cornea. H-E, x 90.

morphism, significant cell loss during long-term storage, or the presence of dead cells, are considered contraindications to transplantation. The number of cells is calculated in 10 (ten) of 100 squares, diagonally, the average number of endothelial cells is indicated in the calculation.

In the first group, microscopic examination most commonly reveals edematous epithelium, absolutely transparent stroma, rare short membrane folds, thin Descemet's layer; endothelium is completely transparent, intact throughout the area (fig. 2). Areas with a uniform redistribution of cells, mainly on the edge of the cornea and the middle region, the intercellular space and dead cells (necrosis/apoptosis), which occupy small spaces, are identified. The density of endothelial cells is greater than 2800 cells/mm² with moderate cell pleomorphism, which is considered as indications for penetrating keratoplasty.

Corneas from group II show: the surface of the epithelium is slightly edematous (Fig. 4), its integrity is not compromised (the exception may be a slight mechanical separa-

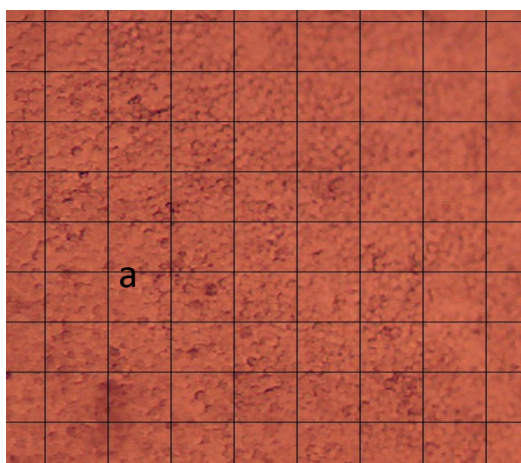


Fig. 3. Patient, 71 years old. Morphological examination of cornea with electron microscopy with inverted phase contrast. Endothelial cells arranged in mosaic form (a), including 26 in a square, constituting 2800 cells/mm².

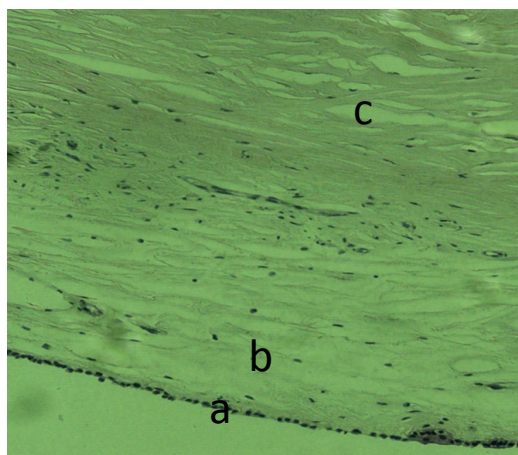


Fig. 4. Patient C, 67-years old. Histological examination of the cornea in the second group, preserved in the culture medium Tissue C, after the 4th day, stroma with initial signs of edema in the lower layers, a thickened, transparent (c) posterior membrane (a) has one central-radial flat shell; endothelial layer is intact (b), usually, along the membrane folds. H-E, x 90.

tion). The stroma is thin, transparent with initial signs of edema in the lower layers. The Descemet's membrane has a single flat shell located centrally and radially; the endothelial layer is intact, and usually, over the folds of the Descemet's membrane, a subtle swelling in the form of an opaque opalescence is allowed. Endothelial layer is arranged evenly, with persistent tesserae (fig. 5), which includes 26 cells per square, giving on average 2600 cells/mm².

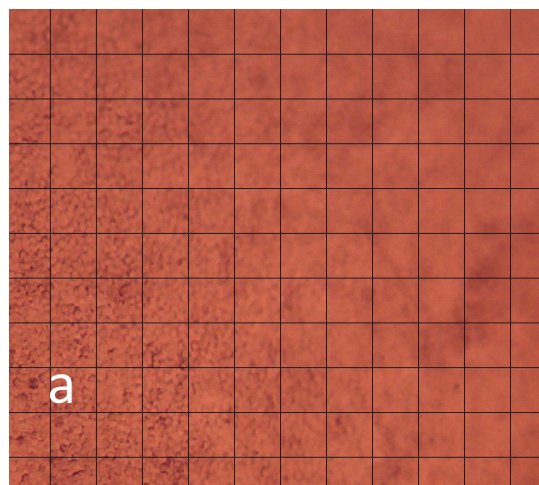


Fig. 5. Patient D, 70 years old. Morphological examination of cornea by electron microscopy with inverted phase contrast. Endothelial cells arranged in mosaic form (a), including 26 cells in a square, totalling 2600 cells/mm².

Multi-layered squamous epithelium is isolated from the anterior layer (Fig. 6), highlighting the blast cells at different phases of division along with stem cells, the sources of regeneration of the anterior epithelium.

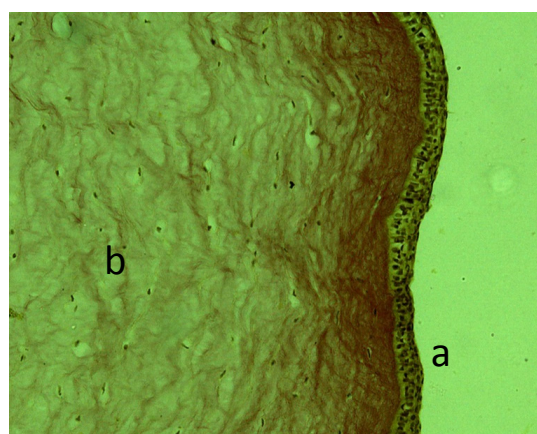


Fig. 6. C. 53-year-old patient. Across the cornea – Tissue “C” preserved on the 5th day. Surface epithelium is edematous (a), its integrity is not compromised (exception can be a minor mechanical exfoliation). Stroma (b) with the initial signs of edema in the lower layers, thickened, transparent. Picrofuxin, x 140.

Corneas of the group III – anterior epithelium edema-tion in some areas with Bowman's membrane exfoliation (fig. 7). Stroma is edematous in the whole layer and matte colored. Descemet's membrane has pronounced folds,

creases pointing in different directions, giving the aspect of "parquet floor" or "chessboard". Endothelial layer is matte, discontinued along the outline of the folds (fig. 8).

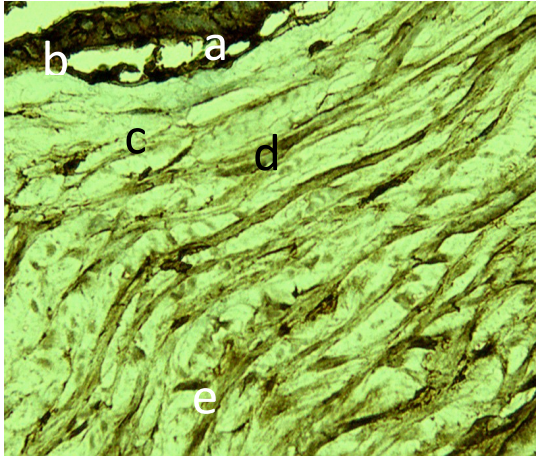


Fig. 7. Patient E, 54 years old. Corneas in the group III. Anterior layer consisting of weakly contoured epithelium (a), enhanced color (+++) with AE1 / AE2 (b), detachment in some areas of the anterior layer (c), the stroma has a frameless thickened fibrillated structure (d), weak fibroblasts (e) AE1/AE2, x 140.

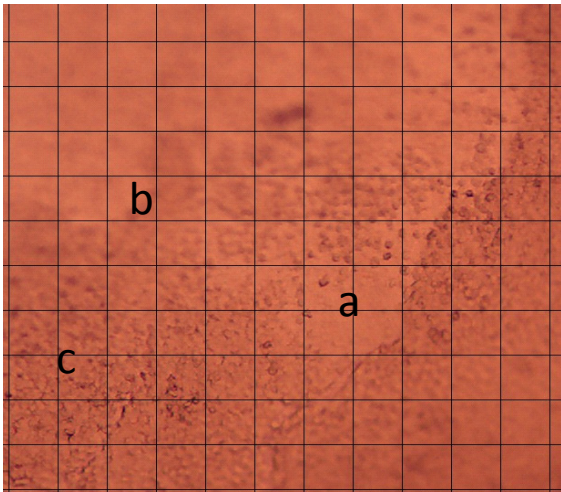


Fig. 8. Patient D, 69 years old. Morphological examination of cornea by electron microscopy with inverted phase contrast. Endothelial cells are sectorally stable with the disappearance of the mosaic (a), which contains 20 cells per square (c), constituting 2000 cells per square millimeter, and detachment of the epidermis (b).

Results and discussion

Corneas are stored for up to 4 weeks, and their suitability for transplantation shall be based on the examination of the corneal endothelium through luminescent microscopy. This allows assessment of endothelial cells (ETC). In Bristol, a minimum of 2200 ETC cells/mm² is considered acceptable for transplants, which require a viable endothelium. Other abnormalities and endothelial damage may be also identified and taken into account [25]. Corneas stored in the culture medium for up to 4 weeks have been shown to preserve the integrity of both endothelial and epithelial layers. Although

corneal cells may be lost through apoptosis, this seems to affect the epithelial layer more than endothelial cells [7]. Important factors influencing corneas from culture media and determining the condition of the endothelial cells are the age of the donor and, to a lesser extent, the storage duration [3]. Although ETC typically declines with age, there are still many corneas from donors of over 80 years of age who meet the minimum criteria for transplantation.

When the culture medium was introduced in the United Kingdom in the mid-1980s, it has had a major impact on supply of corneas to the hospitals across the country [26]. There were two opportunities in the first place, a national distribution service of the cornea similar to an existing one for organs, established within a few years; and, secondly, the culture medium of extended storage time from 2 (whole eyes or corneas in M-K medium) to 4 weeks [8]. This gave time for medical testing and evaluation of donors, as well as for surgical demand. Application of medium culture in the United Kingdom has prompted the storage of corneas in the Bank of eye and they have been made available for elective surgery and for urgent transplants [27]. These benefits of the culture medium have improved the quality, safety and availability of the corneas in the UK and other European countries.

A different technique from corneal organ culture is used for ex-vivo expansion of corneal epithelial stem cells. These cells are extracted from the limbal region between the cornea and the sclera, and they are used in the treatment of ocular surface diseases where the corneal epithelium is defective. This is a painful and sight-threatening condition that is difficult to treat. The most successful application of this technique has been for unilateral ocular diseases where sheets of stem cells can be established from a small limbal biopsy taken from the healthy fellow eye [32]. As with organ culture of corneas, the development of culture media free of bovine serum for limbal stem cell expansion is considered desirable [32, 33]. These tissue constructs echo the trend in corneal transplant surgery for replacing only the defective parts of a cornea rather than relying on full-thickness grafts as the treatment of choice for all corneal deficiencies [34, 35].

The potential for modification of corneas during storage is being investigated. Since allograft rejection is a major cause of corneal graft failure, immunomodulation to reduce the immunogenicity of corneas is being attempted by transfection of endothelial cells to over-express down-regulatory cytokines, such as IL-10 and IL-12 [28]. Another approach is to encourage endothelial cells to divide during corneal storage by transfection with transcription factors such as E2F2, which has been reported to stimulate cell-cycle progression and endothelial replication [30]. There is also the prospect of a tissue-engineered corneal construct [31].

Conclusions

1. The most preferred cornea for penetrating keratoplasty and anterior lamellar keratoplasty is the cornea taken up to 24 hours, with more than 2000 endothelial cells per mm².

2. The most effective preservative medium remains the culture medium with the addition of growth factors and antibiotics.

3. Corneas with a low content of endothelial cells of up to 2.000 per mm² are used for tectonic anterior keratoplasty.

4. Endothelial cells are thin-walled and require a proper diet with permanent nutrients and oxygen, with more frequent change of media preservation, which gives the possibility to prolong their viability.

References

- Pels E, Schuchard Y. Organ-culture preservation of human corneas. *Doc Ophthalmol.* 1983;56:147-153. [PubMed].
- Ehlers H, Ehlers N, Hjortdal JO. Corneal transplantation with donor tissue kept in organ culture for 7 weeks. *Acta Ophthalmol Scand.* 1999;77:277-278. [PubMed].
- Armitage WJ, Easty DL. Factors influencing the suitability of organ-cultured corneas for transplantation. *Invest Ophthalmol Vis Sci.* 1997;38:16-24. [PubMed].
- Cociug A, Macagonova O, Nacu V, Cusnir V Jr, Procopciuc V, Cusnir V Sr. The influence of tissue retrieval period on the viability of corneal endothelial cells. In: Proceedings of the the Annual Congress of the Contact Lenses and Ocular Surface Society (RCLSO); 2018 Nov 2-4. Sibiu; 2018. p. 89-90.
- European Directorate for the Quality of Medicines & HealthCare (EDQM). Guide to the quality and safety of tissues and cells for human application. 3rd ed. Strasbourg: Council of Europe; 2017. Part B Specific requirements, Chapter 16 Ocular tissue; p. 185-193.
- Cociug A, Nacu V, Macagonova O, inventors; Nicolae Testemitsanu State University of Medicine and Pharmacy (Republic of Moldova). Cornea incision device. Republic of Moldova patent (MD) 1048 (13) Y. A61F 9/01 (2006.01), A61F 9/013 (2006.01). 2016 Feb 11. BOPI. 2016;(6):34-36.
- Crewe JM, Armitage WJ. Integrity of epithelium and endothelium in organ-cultured human corneas. *Invest Ophthalmol Vis Sci.* 2001;42:1757-1761. [PubMed].
- Williams KA, Brereton HM, Coster DJ. Prospects for genetic modulation of corneal graft survival. *Eye (London).* 2009;23:1904-1909. [PubMed].
- Claerhout I, Maas H, Pels E. European Eye Bank Association Directory Report. 18th ed. 2010. Available at: www.europeaneyebanks.org
- Dikstein S, Maurice DM. The metabolic basis to the fluid pump in the cornea. *J Physiol.* 1972;221:29-41. [PMC free article] [PubMed]
- Meek KM, Quantock AJ. The use of x-ray scattering techniques to determine corneal ultrastructure. *Prog Retin Eye Res.* 2001;20:95-137. [PubMed].
- Joyce NC, Mekler B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci.* 1996;37:645-655. [PubMed].
- Armitage WJ, Tullo AB, Larkin DFP. The first successful full-thickness corneal transplant: a commentary on Eduard Zirm's landmark paper of 1906. *Br J Ophthalmol.* 2006;90:1222-1223. [PMC free article] [PubMed].
- Zirm E. Eine erfolgreiche totale Keratoplastik. [A successful total keratoplasty]. *Albrecht von Graefes Archiv für Ophthalmologie.* 1906;64(3):580-593. German.
- Filatov VP. Transplantation of the cornea. *Arch Ophthalmol.* 1935;13:321-347.
- Schroeter J, Rieck P, Maier P, Reinhard T. Augenhornhaut – Banken und klinische Anwendung [Cornea – banking and clinical application]. *Transfus Med Hemother.* 2008;35:431-437. German.
- Eastlund T. Infectious disease transmission through tissue transplantation: reducing the risk through donor selection. *Cell Transplant.* 1995;4:455-477. [PubMed].
- Armitage WJ, Tullo AB, Ironside JW. Risk of Creutzfeldt-Jakob disease transmission by ocular surgery and tissue transplantation. *Eye (London).* 2009;23:1926-1930. [PubMed].
- Prub A, Caspari G, Krüger DH, Blümel J, Nübling CM, Quenzel E-M, Kalus U, Gerlich W, Gürtler L. Nukleinsäure-Amplifikationstests für HIV, HBV und HCV bei Gewebespendern: Sinnvoll oder überflüssig? [Nucleic acid amplification tests for HIV, HBV, and HCV in tissue donors: useful or dispensable?]. *Transfus Med Hemother.* 2008;35:421-430. German.
- Gal RL, Dontchev M, Beck RW, Mannis MJ, Holland EJ, Kollman C, Dunn SP, Heck EL, Lass JH, Montoya MM, Schultze RL, Stulting RD, Sugar A, Sugar J, Tennant B, Verdier DD. The effect of donor age on corneal transplantation outcome. *Ophthalmology.* 2008;115:620-626. [PMC free article] [PubMed].
- Thuret G, Manissolle C, Acquart S, Garraud O, Campos-Guyotat L, Maugery J, Gain P. Urgent need for normalization of corneal graft quality controls in French eye banks. *Transplantation.* 2004;78:1299-1302. [PubMed].
- Ruggeri A, Grisan E, Schroeter J. Evaluation of repeatability for the automatic estimation of endothelial cell density in donor corneas. *Br J Ophthalmol.* 2007;91:1213-1215. [PMC free article] [PubMed].
- Williams KA, Lowe M, Bartlett C, Kelly TL, Coster DJ. Risk factors for human corneal graft failure within the Australian corneal graft registry. *Transplantation.* 2008;86:1720-1724. [PubMed].
- Capella JA, Kaufman HE, Robbins JE. Preservation of viable corneal tissue. *Cryobiology.* 1965;2:116-121. [PubMed].
- O'Neill P, Mueller FO, Trevor-Roper PD. On the preservation of cornea at 196 degrees C for fullthickness homografts in man and dog. *Br J Ophthalmol.* 1967;51:13-30. [PMC free article] [PubMed].
- Schultz RO, Matsuda M, Yee RW, Glasser DB, Sabin SM, Edelhauser HF. Long-term survival of cryopreserved corneal endothelium. *Ophthalmology.* 1985;92:1663-1667. [PubMed].
- Armitage WJ, Hall SC, Routledge C. Recovery of endothelial function after vitrification of cornea at 110 °C. *Invest Ophthalmol Vis Sci.* 2002;43:2160-2164. [PubMed].
- Brunette I, Le Francois M, Tremblay MC, Guertin MC. Corneal transplant tolerance of cryopreservation. *Cornea.* 2001;20:590-596. [PubMed].
- Fuller BJ. The effects of cooling on mammalian cells. In: Fuller BJ, Grout BWW, editors. *Clinical Applications of Cryobiology.* Boca Raton: CRC Press; 1991. p. 1-22.
- Kuwahara Y, Sakanoue M, Hayashi M, Akiya S, Komoto M, Kumano-mido A, Obazawa H, Nakano T, Hara T, Komukai M, Saga U, Tamura H, Ishihara K, Shimizu K, Takeda K, Ooshima T, Ozawa H. Studies on the long-term preservation of the cornea for penetrating keratoplasty. *Nippon Ganka Gakkai Zasshi.* 1965;69:1751-1840.
- Stocker FW. Preservation of donor cornea in autologous serum prior to penetrating grafts. *Am J Ophthalmol.* 1965;60:21-24. [PubMed].
- Kuwahara Y, Sakanoue M. Preservation of donor eyes for penetrating keratoplasty (regarding medium-term preservation). *Nippon Ganka Kyo.* 1968;19:1319-1327. [PubMed].
- McCarey BE, Meyer RF, Kaufman HE. Improved corneal storage for penetrating keratoplasties in humans. *Am Ophthalmol.* 1976;8:1488-1492. [PubMed].
- Lindstrom RL, Kaufman HE, Skelnik DL, Laing RA, Lass JH, Musch DC, Trousdale MD, Reinhart WJ, Burris TE, Sugar A, Davis RM, Hirokawa K, Smith T, Gordon JF. Optisol corneal storage medium. *Am J Ophthalmol.* 1992;114:345-356. [PubMed].
- Chen CH, Rama P, Chen AH, Franch A, Sulewski M, Orlin S, Chen EH, Tseng SH, Lee H, Wang CC, Hung GY, Chan MY, Huang MS, Chen SC. Efficacy of media enriched with nonlactate-generating substrate for organ preservation: in vitro and clinical studies using the cornea model. *Transplantation.* 1999;67:800-808. [PubMed].