Arch. Soc. Amer. Oftal. Optom. (1981)-15-61

REFRACTIVE KERATOPLASTY CRYOBIOLOGIC CONSIDERATIONS

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Lamellar refractive keratoplasty, conceived and developed by Dr Jose I. Barraquer, is a most innovative contribution to ophthalmic surgery. Its possible clinical ramifications are vast, and investigation in this area is growing rapidly around the world.

Lamellar refractive keratoplasty includes several surgical procedures that alter the refractive state of the eye by changing the anterior corneal radius of curvature. This is accomplished by the specific lathing of corneal tissue that has been hardened by freezing.

Reported clinical results of patients undergoing lamellar refractive keratoplasty have been most encouraging, and significant complications have been minimal [1, 3]. Nevertheless, research into the effects of these procedures upon the eye at the histologic and ultrastructural level is only beginning, and many questions are unanswered.

The author has been involved in refractive keratoplasty for three years and has performed keratophakia and both myopic and hypermetropic keratomileusis. A major aspect of these procedures that immediately interested him was visual rehabilitation. Final visual rehabilitation is often delayed for many months, especially in keratophakia, and the delay may be related to adverse effects upon the corneal tissue produced by the freezing process. The author is actively investigating the effects of cryopreservation upon the ocular tissue. This paper will present an overview of cryopreservation as related to lamellar refractive keratoplasty.

Cryopreservation began with Luyet and Hodapp, who in 1938 showed that sucrose protected the viability of frog spermatozoa frozen to low temperatures [4]. Polge et. al., in 1949, protected spermatozoa of various species from the damaging effects of freezing by using glycerol [5], and Lovelock proposed a mechanism for such protection in 1953 [6]. In 1954, Eastcott attempted corneal cryopreservation and performed the first successful penetrating keratoplasty using donor material that had been protected with glycerol and frozen to -79ºC [7].

Based upon theoretical considerations, Lovelock introduced dimethyl sulfoxide (DMSO) to cryobiology and showed its protective effects upon frozen spermatozoa [8]. Later, Smith found DMSO to be superior to glycerol for protecting frozen corneal endothelium, and he successfully performed the first penetrating keratoplasty from a donor eye frozen in DMSO at -79ºC [9]. Mueller then utilized donor eyes frozen in both glycerol and DMSO for penetrating keratoplasty with good results [10]. Capella et. al., in 1965, derived what has been the standard protocol for corneal cryopreservation using sucrose, DMSO, and human serum [11]. In this technique, the cornea is cooled very slowly to the eutectic point at which time the cooling rate is increased. Ashwood-Smith, a basic scientist cryobiologist, has severaly criticized this technique by stating that the osmotic stress imposed upon the cornea is unnecessarily high [12]. More recently, Sperling appears to have improved upon the Capella standard method [13].

There have been numerous articles on the biochemical and biophysical consequences of subjecting animal cells to freezing and thawing. The external and internal cell fluids contain a variety of molecules whose solubilities and eutectic points vary. During cooling, the pH is altered significantly and various salts are concentrated as extracellular ice forms. Thus, the cell is exposed to an increasing salt concentration on the outside, and water leaves the cell to compensate for this.

At least four discrete events occur during freezing: namely, 1) removal of water as ice; 2) concentration of high and low molecular weight solutes; 3) decrease in celly volume; 4) precipitation of solutes. Many theories for freezing damage indicate that osmotic shock, probably during thawing, may be a major cause of cellular death. The osmotic shock results from membranes whose lipoproteins have been damaged by high concentrations of salts.

For most cells, there are optimal rates of cooling and thawing. These rates vary greatly, and the optimal rates also vary for specific cell types,

and sometimes for the same cell type in different species. Optimal rates also vary with the type of cryoprotective additive, the suspending medium, the freezing vial, and the freezing volume.

The means by which cryoprotective agents exert their beneficial effects upon frozen cells are unknown. Substances such as glycerol and DMSO enter cells rather rapidly, while such agents as polyvinylpyrrolidone, dextran, and hydroxyethyl starch do not. Cryoprotectants may stabilize membranes, increase protein buffering capacity, or dehydrate the cell and decrease intracellular ice formation.

Because of the critical importance of the endothelium in corneal grafting, corneal cryopreservation studies in the past have centered on this structure. The results of penetrating keratoplasty with corneal tissue preserved by the Capella method indicate that its success rate is comparable to that of fresh tissue [14]. The in vivo survival of cryopreserved endothelial cells has also been documented by histology [15], specular microscopy [16] and scanning electron microscopy [17]. Corneal cells may thus be cryopreserved with maintenance of in vivo viability.

It has been known for many years that non-viable stroma is adequate for lamellar keratoplasty, and one study of 1,457 cases of non-viable stromal lamellar grafts stated that the final results were comparable to those with fres tissue [18].

Specific study of corneal stromal cryopreservation was firts carried out by Dr. Jose Barraquer, during his development of refractive keratoplasty [19]. Barraquer stated that stromal cells disappeared within eight days when treated with preserving solutions alone, and that attempts to save the keratocytes were unfruitful. Dr. Barraquer chose his present cryopreserving solution on the basis of preservation of the collagen matrix. He stated that the volumetric properties of his solution were similar to those of corneal tissue and decreased tissue alteration. Though non-viable tissue results, the stroma is gradually repopulated by viable lost keratocytes. Considering that the term "cryopreservation" implies maintenance of cellular viability, it is perhaps best not to apply this term in refractive keratoplasty unless definitive evidence of cellular viability is demostrated.

In addition to the work of Barraquer, there have been other studies on stromal cell survival following freezing, but much of the data appears to be in conflict. For many years it has been believed that rapid in situ freezing of the cornea, without cryoprotection, results in complete loss of

keratocytes[20]. More recently, however, it was found that such treatment produced non-viable keratocytes only when the epithelium was removed by the freezing process[21].

Most studies on corneal stromal cell cryopreservation have used the Capella technique. In vitro studies with this technique have demonstrated keratocyte survival both ultrastructurally and metabolically in human eye bank corneas[22] and fresh rabbit corneas[23]. In vivo studies, however, have been less successful. In 1969, Polack and McEntyre studied cryopreserved penetrating grafts in rabbits and concluded on the basis of histologic and autoradiographic studies that keratocyte survival was minimal[24]. In 1970, Gallu et. al. studied cryopreserved intramellar stromal grafts in rabbits[25]. They found that keratocytes did not maintain viability unless the cryopreservation was carried out after first removing the epithelium. A more recent study by Clifton and Hanna in 1974 demonstrated that keratocytes failed to survive in cryopreserved penetrating grafts in monkeys[26]. At the present time, therefore, there is no controlled study documenting that a method has been found to cryopreserve corneal stroma with maintenance of keratocyte viability in vivo.

It is the opinion of this investigator that the freezin protocol as presently practiced should undergo intensive investigation. Despite the fact that long term clinical results appear satisfactory, little has been published concerning the effects of these procedures upon the cornea at the histologic or ultrastructural level. As stated previously, the keratocytes are presumed to die and the frozen tissue is later repopulated from the surrounding viable tissue. The whole process, however, may have deleterious effects upon the tissue. Necrosis of large numbers of cells may release toxic products detrimental to the endothelium.

Only recently have the effects of various dyes and cryoprotectants upon the surrounding normal keratocytes or the endothelium been studied in detail. The pH of these solutions can be very acidic and recent studies have demonstrated the harmful effects upon the endothelium of solutions with highly acidic or basic pH[27]. For this reason we have been experimenting with modified solutions and feel that a buffered solution is preferable.

In addition to direct toxic effects, massive cellular necrosis is likely to promote leukocytic infiltration during the initial stages, and this process is felt by some to play a role in inducing corneal vascularization[28].

There may be definite advantages in developing a new cryopreservation protocol in addition to minimizing adverse effects. If keratocyte viability could be maintained in vivo, one would have a much more physiologic result. Wound healing should be improved significantly as it is well known that mucopolysaccharide production and collagen formation is delayed during the stage of cellular migration and repopulation into previously acellular stroma [24]. This may improve visual rehabilitation considerably. It is my clinical impression that visual acuity returns more quickly when viable rather than non-viable tissue (e.g. glycerin stored) is used in lamellar keratoplasty. Increased viability of the epithelium may also decrease the incidence of epithelial abrasion or erosion following keratomileusis.

Maintaining cellular viability may thus offer distinct advantages over non-viable tissue. However, one must consider the question of immune phenomena. Increasing cellular viability may also increase antigenicity in keratophakia. Documented rejection of a keratophakia lenticle has not been reported, although some cases of slow visual rehabilitation could conceivably represent a sub-clinical manifestation of rejection. There have been a number of studies documenting lack of difference in antigenicity between fresh and cryopreserved grafts [29, 30]. Most of these utilized xenografts. It is known that both cellular and non-cellular antigens exist in corneal tissue. The former seem to be of primary importance in homograft situations where as the latter also play a role in xenografts. These studies are not, therefore, directly applicable in our situation and keratophakia with a viable lenticle may indeed promote an immune reaction. Nevertheless, the lenticle is sequestered within the cornea and is considerably smaller in size than tissue routinely used for routine lamellar keratoplasty, a procedure known to have a very low rate of rejection. Of course, viable tissue in keratomileusis would presumably not have this drawback unless tissue antigenicity was altered by the procedure.

There may be other disadvantages to modifying the protocol. It has been shown by Edelhauser et. al., that the kinetics of DMSO uptake and release from corneal tissue proceeds at a relatively slow rate [31]. True cryopreservation may well demand a much longer incubation period than the one minute now used. This could make keratomileusis lengthy or even impractical to perform. Prolonged incubation would not be such a problem with keratophakia where patient surgery need not begin until the lenticle is carved or with lenticles being prepared at a centralized bank. Other potential disadvantages could be cost and inefficiency.

These are some of the areas we have been working in and we hope to provide you with some definitive answers in the very near future. We are just beginning an adventure into one of the most exciting areas in ophthalmology, and it is a great tribute to the genius of Dr. Jose I. Barraquer that one man alone could develop the abstract concept of refractive keratoplasty into the reality as we know it today.

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