1968

Critique of two staining techniques for the study of corneal endothelial viability and their importance in keratoplasty

Donald Max Gentry
University of Nebraska Medical Center

A CRITIQUE OF TWO STAINING TECHNIQUES FOR THE STUDY OF CORNEAL ENDOTHELIAL VIABILITY AND THEIR IMPORTANCE IN KERATOPLASTY

By

Donald M. Gentry

A THESIS

Presented to the Faculty of

The College of Medicine in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Medicine

Under the Supervision of John C. Filkins, M.D.

Omaha, Nebraska

February 1, 1968

TABLE OF CONTENTS

Introduction

The endothelium of the cornea has been found to be the most important layer for successful full thickness keratoplasties. These cells are known to be one of the most delicate types in the human body. Immediately after cessation of the circulation, changes in the structure of the cytoplasm start to take place. The endothelial cells are also easily altered by routine fixation and staining methods. Therefore, it is very difficult to get an accurate picture of their normal and pathologic structure using the routine fixation and staining methods. The slit lamp is the only method of examining the unaltered endothelium that is at our disposal today. This method is limited in its possibilities, inasmuch as the magnification for accurate observation may not exceed 60 to 70 times. Therefore, a better means of studying the endothelial cells needs to be found.

Two new promising staining techniques have recently been introduced for the study of the corneal endothelium by microscopic means. A critique of these two staining techniques will be discussed in this thesis.

History of Corneal Transplants

Tissue transplantation has interested man for a long time. The use of grafting has been attempted in plants and lower animals with high success. When it comes to higher animals, including man, tissues and organs are so specialized that transplantation has not been very effective. The problems with transplantation or grafting have stemmed mainly from the antigen-antibody reaction leading to rejection of the donor tissue. It is thought that the antibodies produced in the human body are transported by lymphocytes found in the blood. The antibodies are therefore transported to the area of the antigens by way of the blood vessels.

Probably the most successful transplantation so far developed in man has been blood transfusions. A method of type and cross match has been developed which has helped to screen out antigens in the donors blood which might cause an antigen-antibody response in the recipient.

Another successful transplantation in man has been keratoplasty. This is probably due to the fact of the structure and physiology of the cornea which contains no blood vessels. Therefore, the antigen-antibody reaction cannot easily take place since the antibodies cannot reach the corneal tissue directly.

There are three main types of corneal transplants used today. The first and least used is a total keratoplasty involving the transplantation of the entire cornea. This type of transplant, suggested by Wagenmann (1888) is regarded clinically impractical. The grafts will opacify in about three weeks. The iris usually adheres to the cornea, and the eye, in each case performed, degenerates either through phthisis bulbis or secondary glaucoma. The second type of transplant is the lamellar which was not widely employed until after World War II, largely because its field was considered to be limited. This is performed by removal of a circumscribed superficial lamella of an opaque cornea and its replacement by a similar lamella of transparent donor cornea. It was not until 1888 when Arthur von Hippel (Figure 1) introduced his method of using a guarded trephine controlled by clockwork that the technique became feasible.

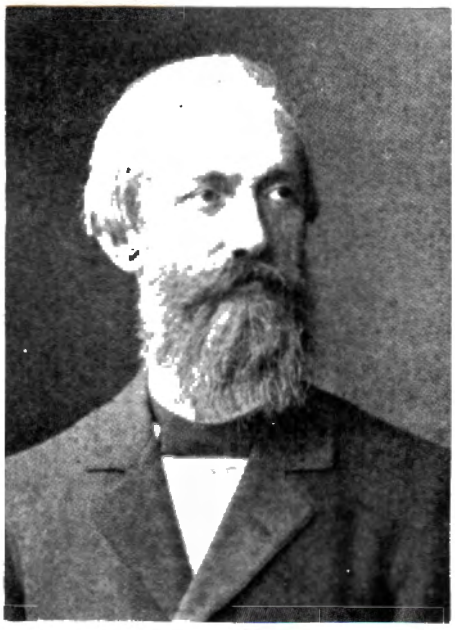


Figure 1. Arthur von Hippel (1841-1916) Taken from Duke-Elder: System of Ophthalmology, Vol. VIII Part 2, 1965, p 649

He used discs of animal corneas as donor material so that the visual results were not good. It was not until comparatively recent years that, as a result of the brilliant work of the French School represented by Paufigue, Sourdille and Uffret (1940-60), the value of lamellar keratoplasty was established beyond dispute, not only as a preparatory, tectonic or therapeutic procedure but also as a method of choice for some optical grafts. Although the ultimate visual acuity is not usually as good as that achieved after a perforating graft, the relative simplicity of the technique and the immunity from many surgical hazards which attend perforating keratoplasty render lamellar keratoplasty an increasingly popular and valuable operation. A third type of keratoplasty is the penetrating circumscribed wherein a window of the entire thickness of the opaque cornea is removed and replaced by a corresponding piece of transparent donor cornea. This type was pioneered by Zirm (1906) and popularized by Anton Elsching of Prague (1920-30). It undoubtedly gives the best optical results and is the only practical method when the cornea is entirely opaque.

Donor material for corneal transplants may theoretically be derived from the same individual (autogenous), from another individual of the same species (homogenous), from the eye of another species (heterogenous), or be constituted from inert substances as glass or plastic (alloplastic). Alloplastic material has not proved to be successful for keratoplasty. Autogenous material is undoubtedly the ideal,

but the material is limited. There have been a few successful lamellar grafts reported with heterogenous material, but none have been reported successful in perforating keratoplasty. Homogenous grafts remain as the only large and reliable source of supply.

The technique of corneal grafting was first mentioned in ophthalmic literature by Franz Reisinger (1824), who got the idea from his teacher, Karl Himly. Reisinger worked with rabbits alone. Richard S. Kissam of New York in 1844 seems to have been the first to operate on man. He used a pigs eye as the donor material and was not successful. Kissam was inspired in doing such an undertaking by a report published in 1837 of the experiences of S. L. Bigger, an Irishman held prisoner in the Sahara Desert. Bigger successfully grafted a traumatized eye of his pet gazelle with the cornea of another animal. After negative results occurred using other animals eyes for human corneal transplants, inert materials were attempted, particularly glass. The problems of infection and expulsion of the glass were too great to be practical.

In the following years, a considerable amount of work was undertaken using living tissues, notably by Henry Power (1872-78) and subsequently by Wolfe (1879) both of whom showed that only the homogenous graft would remain clear. Arthur von Hippel (1877-78), after many years of experimental failure, was the first to improve vision permanently by the technique of lamellar grafting. The possibility of practicing

whole thickness grafts was due in large measure to the inspiring success of the extensive work of Elsching, who was responsible for converting a technique which before this time had almost invariably met with disappointment and disaster to one full of promise for the future. With the improvement of operative techniques, the development of exquisite instruments, the introduction of antibiotics, an increasing experience of the type of case in which the operation may be of value, and the establishment of eye banks for storage and distribution of cadaver material, keratoplasty has now become a routine procedure in many ophthalmic centers throughout the world.

Importance of the Corneal Endothelium in Transplants

The importance of the corneal endothelium in keratoplasty cannot fully be appreciated without knowing some facts about the histology, embryology and physiology of the cornea itself.

The cornea constitutes the anterior one-sixth of the eye and is more curved than the remainder of the bulb. It joins the sclera at a transition zone known as the limbus. The human cornea is slightly thicker than the sclera, measuring 0.8 to 0.9 mm in the center and 1.1 mm at the periphery. The cornea is made up of five layers. (Figure 2)

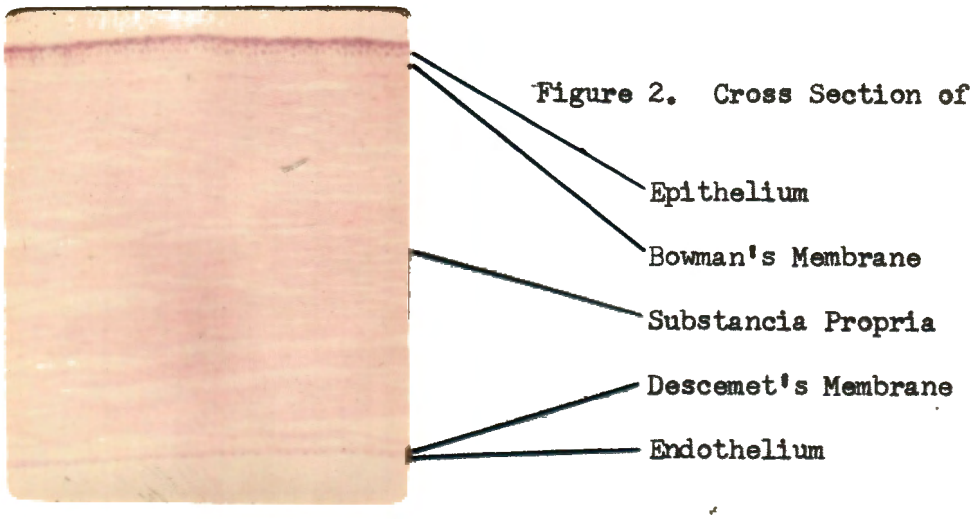


Figure 2. Cross Section of Cornea

The epithelium, about 50 microns thick, is made up of stratified squamous epithelial cells, which consists as a rule of five or six layers. It is endowed with a remarkable capacity

of regeneration. The epithelium rests upon Bowman's membrane which is 6 to 9 microns thick. Bowman's membrane consists of a feltwork of randomly arranged fibrils, apparently of collagen. The substancia propria forms about 90% of the cornea. It is made up of regular connective tissue whose bundles form lamellae arranged in many layers. In each layer the direction of the bundles intercross at various angles. Descemet's membrane, 5 to 10 microns thick, consists chiefly of fibrous proteins of collagen type. It is probably a basement membrane secreted by the corneal endothelial cells. The endothelium consists of a singular layer of large squamous cells arranged in a hexagonal shape. The cornea is devoid of blood vessels, deriving its nutrition from the superficial marginal plexus of vessels and aqueous humor.

Embryologically, the epithelium is considered as ectoderm in origin and the substancia propria as mesoderm in origin. The origin of endothelium is still of some question. One school feels it is ectodermal in origin. They feel that as the ectoderm invaginates to form the lens that some ectodermal cells migrate between the lens and cornea and form the endothelial layer of the cornea. The other and presently more popular school feels that the endothelium is mesodermal in origin. As the cornea develops, the inner portion of the mesoderm differentiates into the endothelium. It has been found that when corneal endothelium is damaged and partially destroyed, the cells will regenerate in

various shapes and forms looking similar to that of fibroblasts which are of mesodermal origin.

The cornea has two main functions in the physiology of the eye. The curvature of the cornea plays an important role in the optics of the eye, helping the lens in focusing images on the retina. The other and more obvious thing the cornea does is allow light to enter the eye so that it reaches the retina in the first place. This last function is the one that can be retained by keratoplasty in those eyes that have corneal pathology with the remainder of the eye being in tact.

The fundamental facts concerning the function of the corneal endothelium were demonstrated by Theodore Leber (Figure 3) as far back as 1873. Before Leber's work it was

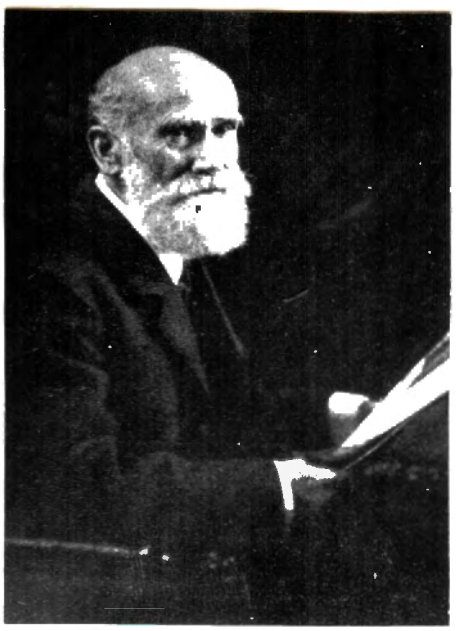


Figure 3. Theodore Leber (1840-1917)
Taken from Duke-Elder: System of Ophthalmology, Vol. II, 1961, p 340

thought that Descemet's membrane was the barrier preventing the cornea from becoming cloudy with aqueous fluid. It was found that when the endothelium was stripped away from

the cornea, the cornea became cloudy sooner than when the endothelium remained in tact. There is still speculation as to how the endothelium prevents fluid from infiltrating the stroma of the cornea. Leber stated that the mechanism must be connected with the living properties of the cells, since he found that the endothelium loses its protective function gradually and progressively after death.

The importance of the endothelium of the cornea is shown clinically in the entity called Fuchs' dystrophy. When Ernest Fuchs (1851-1930) (Figure 4) described the

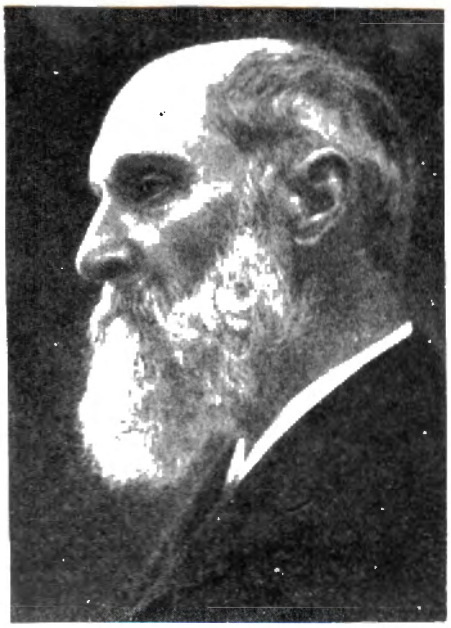


Figure 4. Ernest Fuchs (1851-1930) Taken from Duka-Elder: System of Ophthalmology, Vol. II, p 168

clinical picture of what he called "dystrophia corneae epithelialis," he did not realize the fact that the origin of the disease was in the endothelium. H. H. Chi and associates[6] have done an extensive study on the pathology of the cornea in Fuchs' dystrophy. The most characteristic histologic findings that they found were Descemet's membrane

and endothelial changes. The endothelial cells are distorted by globular bodies of different sizes called guttata. These bodies are nodular and found to be excrescences of Descemet's membrane. As a whole the endothelial cells become reduced in number, although they do increase in number around these globular bodies as if these bodies crowd in between the endothelial cells. As the dystrophy progresses, the stroma and the epithelium becomes involved secondarily.

The integrity of the endothelium in donor corneas have been shown to be very important in successful keratoplasty. Chi and associates[8] have shown the importance of this fact. Using rabbits as their subjects, they did corneal transplants using the sex chromatin of the endothelial cells as a cell marker. The corneas were again removed from 4 to 23 months after operation and studied. It was found that the donor endothelial cells were retained for a period of at least 21 months in homografts that cleared within a few days. It was also found that the donor endothelial cells were almost completely replaced by host cells in grafts which had been hazy for the first 10 to 14 days postoperatively but regained transparency before removal of the cornea at various intervals from 4 to 21 months. Three permanent opaque grafts occurred in their study, in all of which the donor cells had been completely replaced by host cells. Similar studies by Hanna and Irwin[14], Polack and Smelsher[27] using donor material tagged with

radioactive labeled thymidine show that endothelial donor cells of successful grafts persist indefinitely.

Most ophthalmologists today feel that donor cells remain viable permanently in most successful corneal grafts. Some successful keratoplasties have been noted in Fuchs' dystrophy if good donor material is used and the dystrophy has not advanced too far supporting this theory.

Procedure of Staining Corneas

The two types of staining methods to be reviewed in this thesis are nitroblue tetrazolium, a formazan dye, and trypan blue, a benzidene dye. The nitroblue tetrazolium procedure was first applied on corneal endothelial cells by H. E. Kaufman and associates,[5, 17, 18, 19, 30] to find a long term preservation of the corneal endothelium by an appropriate freezing technique. The cadaver eyes are placed in a fitted hole cut into a piece of foam rubber, which keeps the eye in place making it easier to work on the eye. The epithelium is rubbed off with cotton. The cornea is then removed with a small portion of sclera as atraumatically as possible. (Figure 5) The cornea is placed

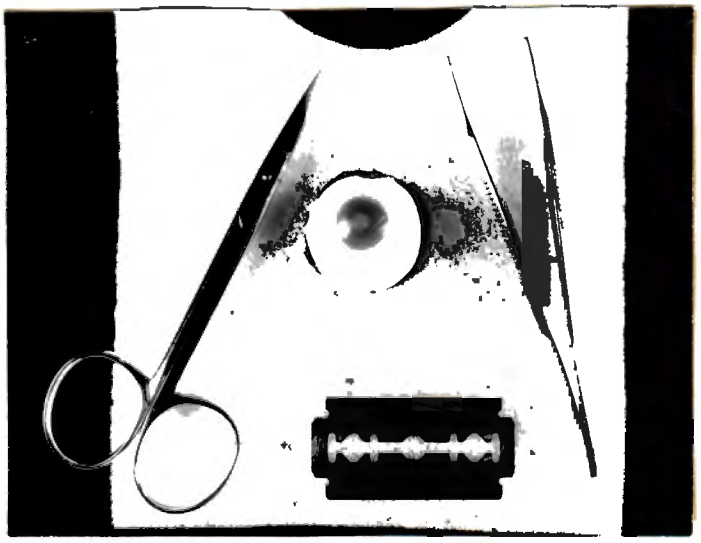


Figure 5. This is the equipment necessary to remove the cornea: a piece of foam rubber with a fitted hole cut in it, scissors, a thin double-edge razor blade, and forceps.

on a glass slide, endothelium up. The slide is in turn placed in a glass tube and stoppered with cotton. Then the glass tube is placed in a wide-mouth thermos bottle which is filled with acetone and dry ice. The cornea remains there for 30 minutes for freezing after which time it is removed and placed into a fitted parafin block. The basic staining media, made up of 0.3 mg/ml of p-nitroblue tetrazolium dissolved in a 0.25 M phosphate buffer solution of pH 7.3, is stored in the refrigerator in 10 ml aliquates until used. This media must be refrigerated since it is unstable at room temperature. An aliquate of the media, after incubated to 37°C, is placed over the cornea, and 15 mgms of DPNH in powder form, which is also unstable at room temperature and must be kept in the freezer to prevent oxidation, is added. The staining media is then washed off after 20 to 30 minutes. (Figure 6) The corneas are observed micro-

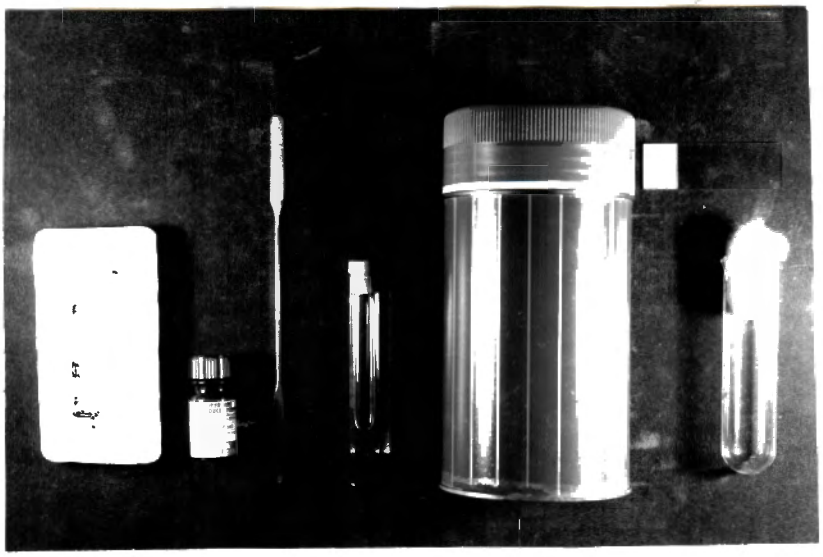


Figure 6. This is the equipment needed for the nitroblue tetrazolium staining method: parafin block for slide, DPNH powder, spatula, staining media, wide-mouthed thermos bottle, glass slide, and glass tube with cotton stopper.

scopically and then stored in 10% neutral formalin for further examination. (Figure 7) The purpose of freezing the corneas

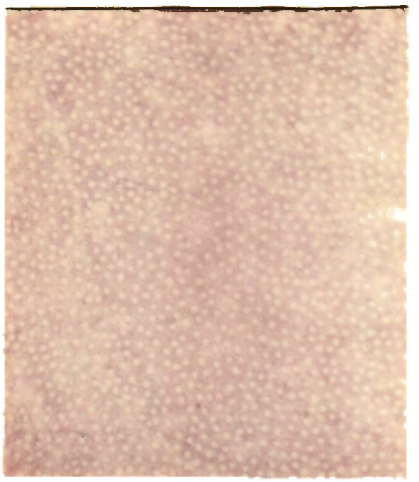
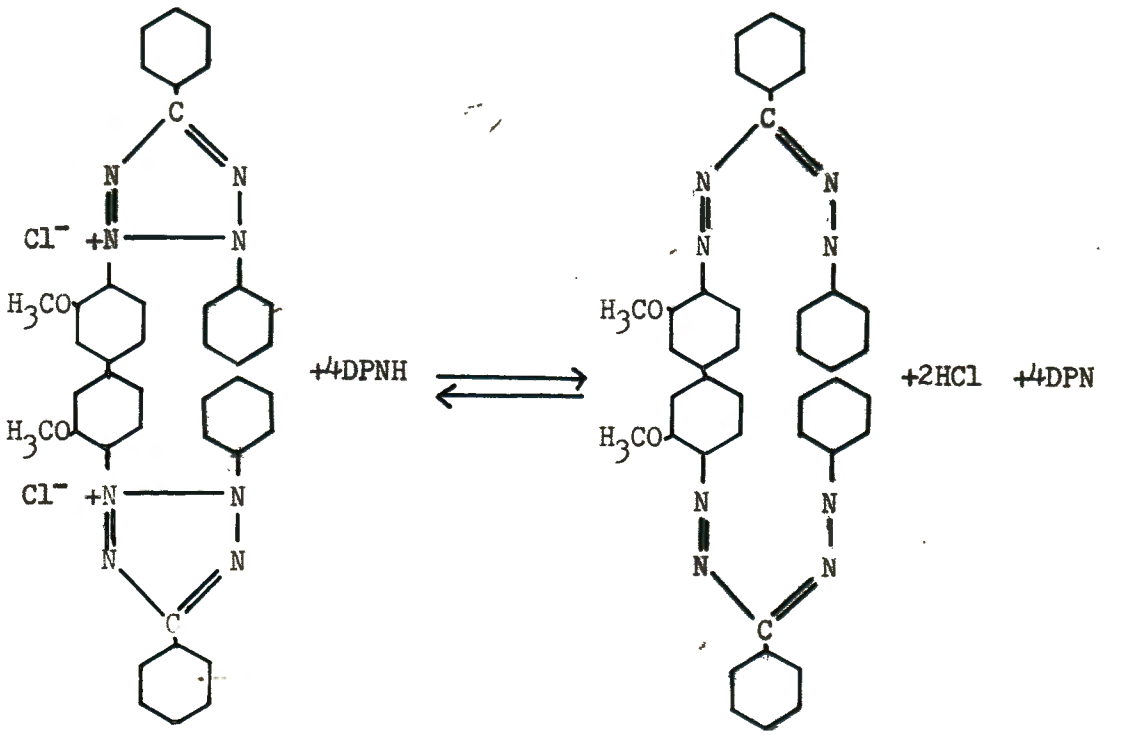


Figure 7. This is an example of the microscopic view of the nitroblue tetrazolium staining method. Note: nuclei of the endothelial cells do not take up the stain.

is to rupture the cell membranes of the endothelial cells so that the tetrazolium stain can enter the cells. Chemically the following is how it stains the cells:



(Yellow Solution) Formazan (Blue Precipitate)

The cells must be metabolically active so that the DPNH added to the cornea and staining media may be converted to DPN, releasing hydrogen ions which can react with the staining medium to form the blue precipitate in the cells. The dead, or severely damaged cells are not stained by this method since these cells are theoretically not metabolically active.

The second staining procedure used is that of the trypan blue, which was introduced for corneal endothelial study by Stocker and associates.(33, 34) Stocker developed this method to try to show that human serum helps to keep the endothelium of stored corneas viable for a longer length of time than if the corneas are stored in Neosporin solution. The cornea is again removed with a portion of sclera using the method described before. It is placed endothelium up on a glass slide. The cornea is then filled but not overflowing with a 0.25% aqueous solution of trypan blue. (Figure 8)

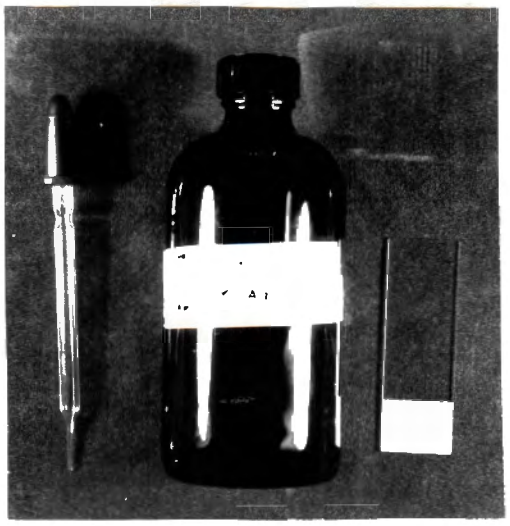
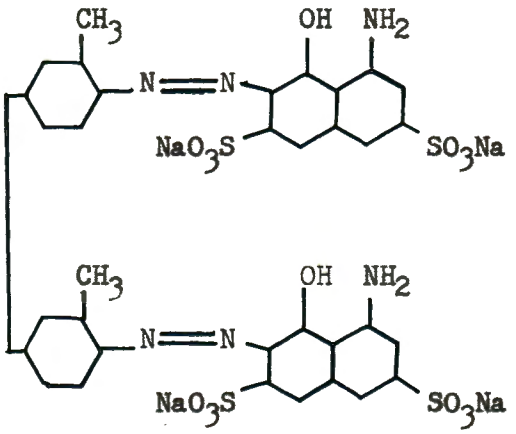


Figure 8. This is the equipment needed for the trypan blue staining method: glass pipette, staining media, and glass slide.

It is important not to over fill the cornea with stain since the excess solution will stain the stroma making it more difficult to study the endothelial layer. After one to two minutes the dye is washed off with saline. The endothelium is studied under the microscope. The structure of the trypan blue stain is as follows:



This dye enters only the dead or damaged cells, staining the nucleus of these cells blue. (Figure 9)

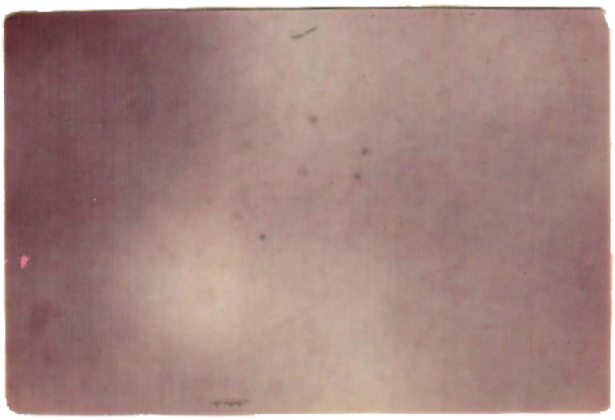


Figure 9. This is an example of the microscopic view of the trypan blue staining method. Note: nuclei of only non-viable cells take up the stain.

In summary, the tetrazolium technique stains the cytoplasm of viable endothelial cells whereas the trypan blue technique stains the nuclei of dead or damaged endothelial cells.

Discussion of the Staining Results

Thirty-eight pairs of human autopsy eyes were used for this study. Twenty-five pairs of eyes were studied using the nitroblue tetrazolium staining technique. One cornea of eight pairs were used for keratoplasty. The fellow cornea of these eight were stained for study. The corneas of one pair were stained at different times. The remaining corneas of pairs were stained at approximately the same time so that these corneas could be comparable. Ten pairs of eyes were studied using the trypan blue staining technique. One cornea of one pair was used for keratoplasty. The fellow cornea was stained and studied. The corneas of two pairs of eyes were stained at different times. Again the remaining corneas of pairs were stained at approximately the same time. Three other pairs of corneas were studied, staining one of each pair with the tetrazolium method and the fellow cornea with the trypan blue method. After observing the results, the table on the following page shows the advantages and disadvantages of both staining methods. Each of these areas will be presented separately.

Stain	Advantages	Disadvantages
Tetrazolium	1) Study cell morphology 2) Study intactness of endothelium	1) Long technique 2) Difficult to count individual dead cells
Trypan blue	1) Short technique 2) Easier to count individual dead cells	1) Cannot study cell morphology 2) Cannot study intactness of endothelium

Studying Cell Morphology

Since the nitroblue tetrazolium method stains the cytoplasm of the living endothelial cells, the morphology of these cells can be studied. Although the nuclei do not stain by this method, the size and shape of the nuclei can be observed.

The trypan blue staining method stains only the nuclei of the dead cells. The nuclear size and shape of the dead cells can be observed but the morphology in the cytoplasm of these cells cannot be studied. A distinct disadvantage of this method is that the morphology of the the living cells cannot be seen. It stands to reason that the living cells are actually more important to study morphologically than the dead cells when determining endothelial viability.

Some specific morphological changes in endothelial cells were noted by the tetrazolium staining method; such as, the presence of endothelial guttata, the size and shape of the nuclei, the maintenance of the typical helical structure, the cytoplasmic to nuclear ratio and the presence of cytoplasmic vacuolization. (See Chart #1) As one can

CHART #1

Age (Years)	Cause of Death	Time (Hours)	Guttata	Nuclei	Helical	Cyt. to Nuclei	Vac. Per H.P.F.	Gross	Slit Lamp
15 F	Encephalitis	16	None	Good	Good	Good	12% 14%	Clear	Wrinkled
*23 F	Brain Tumor	26	None	Good	Good	Good	0-25%	Clear	Wrinkled
*31 M	CHF	18½	None	Severe	Marked	Good	20-75%	Clear	Clear
*38 M	CVA	12	None	Good	Good	Good	0-30%	Clear	Clear
#39 F	Uremia	14½	None	Good	Good	Good	60-80%	Clear	Clear
#39 F	Uremia	39½	None	Slight	Slight	Good	60-80%	Clear	Clear
*40 F	Cardiac Arrest	21	None	Good	Good	Good	Insign.	Clear	Clear
40 F	Rt. Heart Failure Sec. to Pneumonia	3½	None	Good	Slight	Good	50-100% 5-20%	Clear	Clear
*42 M	Fat Embolism	40	None	Slight	Good	Good	20-60%	Clear	Clear
52 F	Metastatic Ca. of Uterus	5	None	Good	Good	Good	50-80%	Clear	Clear
59 F	Septicemia Sec. to Rupture of Colon (Ca.)	16½	None	Slight	Slight	Slightly Greater	0-50%	Clear	Wrinkled
60 M	Pseudomonas Pneumonia	13¼	None	Good	Good	Good	80-90%	Cloudy	Clear
60 M	Liver Cirrhosis	10	Few	Moderate	Moderate	Slightly Greater	50% 20-80%	Clear	Clear

*62 M	MI	10	None	Slight	Slight	Good	70-80%	Clear	Clear
63 M	MI	8	None	Slight	Slight	Slightly Greater	Insign.	Clear	Clear
64 M	MI	103	Few	Moderate	Moderate	Greater	40-100% 50-100%	Cloudy	Cloudy
70 M	MI	37½	Few	Slight	Slight	Slightly Greater	50-80%	Clear	Wrinkled
72 M	Septicemia	40	Few	Slight	Moderate	Slightly Greater	40-50%	Cloudy	Cloudy
*72 M	GI Hemorrhage	43½	None	Moderate	Moderate	Markedly Greater	10-90%	Clear	Clear
*74 M	CHF	21½	Few	Moderate	Marked	Markedly Greater	Insign.	Clear	Clear
75 F	Guillain-Barré Syndrome	6½	None	Good	Slight	Slightly Greater	50-90% 20-60%	Cloudy	Wrinkled
75 F	Cerebral Emboli	3½	Many	Marked	Marked	Greater	10-30%	Clear	Clear
85 M	MI	4	Few	Moderate	Moderate	Greater	80-90% 60-80%	Clear	Clear
90 F	GI Hemorrhage	7½	None	Slight	Slight	Slightly Greater	5-10%	Clear	Clear
90 F	Unknown	38½	Great	Quite Marked	Quite Marked	Markedly Greater	50-100%	Clear	L) Wrinkled R) Filamentous Scars

* Fellow Eye for Keratoplasty
\# Same Pair of Eyes Stained at Different Times

see from studying the chart, all the morphological changes noted except one are associated with the age of the patient. The only exception is the vacuolization in the cytoplasm. As was described before, the endothelial cells characteristically form a helical structure and the nuclei are typically round in shape and equal in size from cell to cell. As the cornea ages, the endothelial layer starts to lose its typical helical structure (Figure 10) and the nuclei become more oval with a greater variation in size from cell to cell. (Figure 11) Areas of endothelial guttata

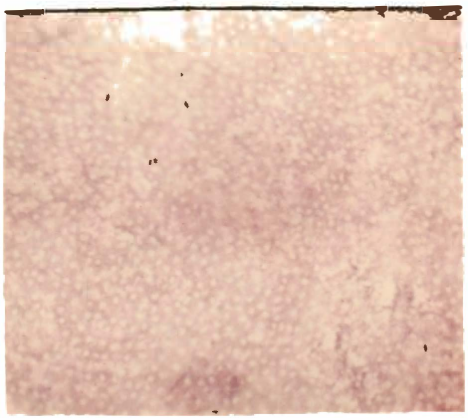


Figure 10. This is an example of disruption of the typical helical structure of the endothelium. Compare this with Figure 7 which has a good helical structure.

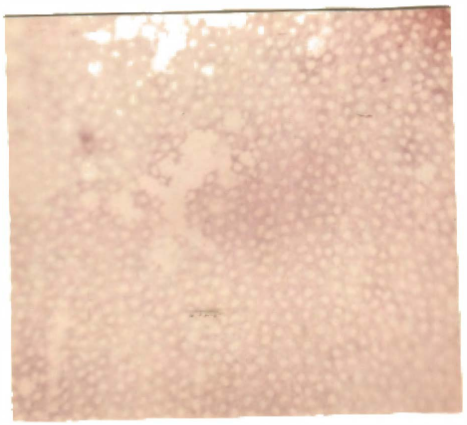


Figure 11. This is an example of variations in nuclear size and shape.

are fairly common in some of the older aged corneas even when there was no evidence of Fuchs' dystrophy. (Figure 12)

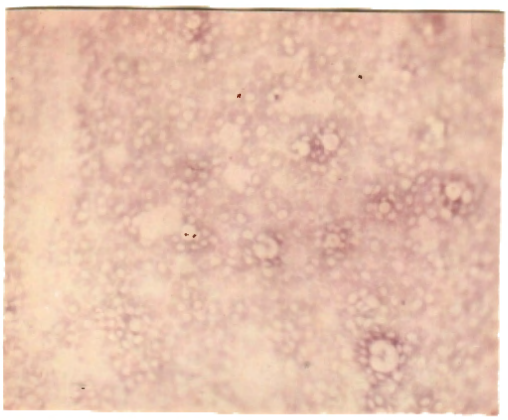


Figure 12. This is an example of endothelial guttata typically seen in Fuchs' dystrophy. It is of interest that the gross and slit lamp appearance of this cornea was essentially negative.

Fuchs' dystrophy may therefore be an early aging of the corneal endothelium. As the age of the cornea progresses there is more cytoplasm present in comparison to the nuclear area. This is what will be called cytoplasmic to nuclear ratio. As the age increases this ratio seems to increase. (Figure 13)

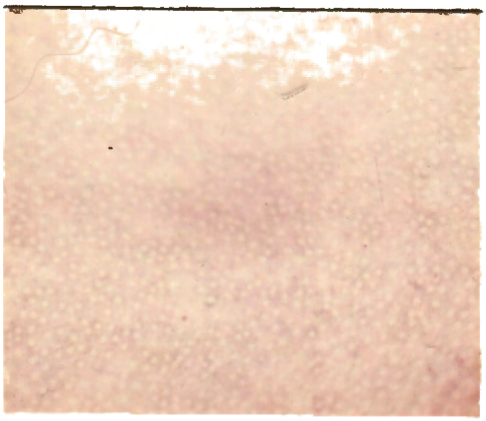


Figure 13. This is an example of a greater cytoplasmic to nuclear ratio. Compare with Figure 7. Note that not all the cells have a greater cytoplasmic area, but rather the involvement is scattered.

It is interesting to note that cytoplasmic vacuolization of the cells is not particularly associated with the age of the patient, the cause of death, or the time between

death and staining. It is also interesting to note that vacuolization is quite a common occurrence. The importance of the vacuolization of the cells cannot be ascertained without further study. (Figure 14)

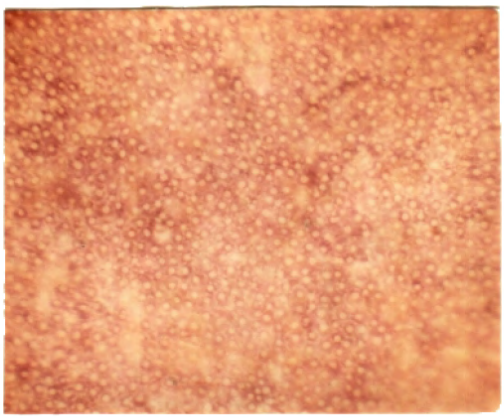


Figure 14. This is an example showing numerous cytoplasmic vacuoles. Note: the color of this picture is altered due to faulty lighting.

It should be noted that the gross and slit lamp appearance of the corneas cannot be related to the age of the patients nor the morphology of the endothelial cells alone.

Intactness of the Endothelial Layer

The intactness of the corneal endothelial layer is a very important factor in studying its viability. This cannot be adequately determined with the trypan blue staining method since only the nuclei of the dead cells are stained consisting of usually less than 10% of the entire endothelial layer. The tetrazolium method gives an excellent picture of the intactness of the endothelium both grossly and microscopically since it stains viable cells only. Torn

areas with complete loss of the corneal endothelium can be observed. (Figure 15)

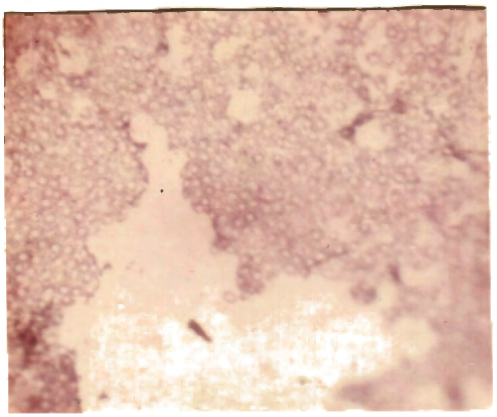


Figure 15. This is an example showing a large area and some smaller areas where the corneal endothelium has been lost.

It was noted (See Chart #2) that the intactness of the endothelium seems to be somewhat related to the time between death of the patient and the staining of the endothelium. As the time increases, the intactness of the endothelium decreases. If this fact is true, this alone should show the importance of fresh donor material for corneal transplantation. It should be noted that most of the corneas with loss of endothelial intactness have gross and/or slit lamp anomolies also. However, not all the corneas with gross and slit lamp anomolies have loss of endothelial intactness. The gross and slit lamp appearance seems to get worse as time progresses, although, this is not absolute.

Technique of Staining

The trypan blue staining technique has many advantages over the tetrazolium staining technique. The trypan blue method is much shorter and simpler. The tetrazolium

CHART #2

Time (Hours)	Cause of Death	Age (Years)	Gross	Slit Lamp	% Missing
3½	Cerebral Emboli	75 F	Clear	Clear	None
3½	Rt. Heart Failure Sec. to Pneumonia	40 F	Clear	Clear	L) 30% R) None
4	MI	85 M	Clear	Clear	None
5	Metastatic Ca. of Uterus	52 F	Clear	Clear	None
6½	Guillain-Barré Syndrome	75 F	Cloudy	Wrinkled	None
7½	GI Hemorrhage	90 F	Clear	Clear	None
8	MI	63 M	Clear	Clear	None
* 10	MI	62 M	Clear	Clear	None
10	Liver Cirrhosis	60 M	Clear	Clear	L) 20% R) None
* 12	CVA	38 M	Clear	Clear	None
13¼	Pseudomonas Pneumonia	60 M	Cloudy	Wrinkled	None
# 14½	Uremia	39 F	Clear	Clear	None
16	Encephalitis	15 F	Clear	Wrinkled	None
16½	Septicemia Sec. to Rupture of	59 F	Clear	Wrinkled	L) None R) 25%

	Colon (Ca.)				
* 18½	CHF	31 M	Clear	Clear	10%
* 21	Cardiac Arrest	40 F	Clear	Clear	None
* 21½	CHF	74 M	Clear	Clear	10%
* 26	Brain Tumor	23 F	Clear	Wrinkled	25%
37½	MI	70 M	Clear	Wrinkled	None
38½	Unknown	90 F	Clear	L) Wrinkled R) Filamentous Sears	None
# 39½	Uremia	39 F	Clear	Clear	None
* 40	Fat Embolism	42 M	Clear	Clear	None
40	Septicemia	72 M	Cloudy	Cloudy	L) None R) 40%
* 43½	GI Hemorrhage	72 M	Clear	Clear	10%
103	MI	64 M	Cloudy	Cloudy	L) 60% R) 75%

* Fellow Eye Used for Keratoplasty
\# Same Pair of Eyes Stained at Different Times

technique requires the cornea to be frozen. This enters another step, which in turn adds an area for error and artifact, that the trypan blue technique does not have. The trypan blue stain is stable at room temperature and therefore can be made in larger quantities. On the other hand, the tetrazolium stain must be made in smaller quantities and kept in refrigeration to prevent the formation of formazan, the blue precipitated form. The DPNH used in the tetrazolium staining method must be kept in a freezer and away from moisture to prevent the reduced DPNH to the oxidized DPN. As noted before, the hydrogen ion in DPNH is a very important substance for the tetrazolium staining. The DPNH also adds an additional substance in the tetrazolium method which the trypan blue method does not have; an area for error and artifact to occur.

In summary, the trypan blue method is quicker and simpler, requiring less equipment and material than the tetrazolium method. (Compare Figures 6 and 8) It stands to reason that less error and artifact during staining should occur with the trypan blue technique in comparison to the tetrazolium staining technique.

Counting Dead Cells

The number of dead cells in the corneal endothelium is one of the most important, if not the most important, factor in determining endothelial viability. It is one of

the more obvious factors and one of the easier ones to study by either staining method.

The trypan blue staining has a considerable advantage over the tetrazolium stain in counting dead cells, though. Since the tetrazolium stain does not stain the dead cells, unstained patches are recognized as dead cells. Quite often it is hard to determine how many dead cells are actually present at one of these unstained areas. (Figure 16)

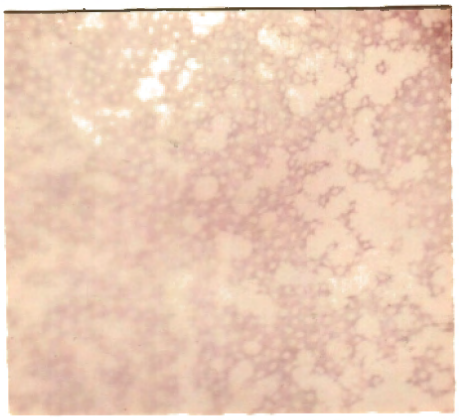


Figure 16. This is an example of large areas of unstained endothelial cells using the tetrazolium staining method. Note that it is difficult to determine the exact number of unstained cells.

With the trypan blue stain, there is no great guess work involved in the counting of these dead cells. Each individual dead cell's nucleus is stained separately and therefore can be counted separately. (Figure 17)

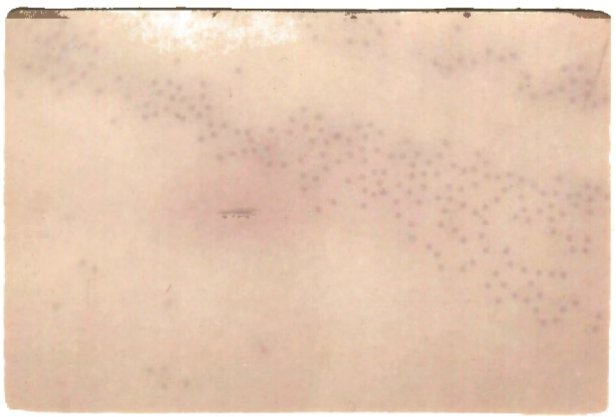


Figure 17. This is an example of a large area of nonviable endothelial cells using the trypan blue staining method. Note that each cell can be counted individually. Compare it to Figure 16.

It is interesting to note that the number of dead cells is not particularly associated with the gross or slit lamp appearance of the cornea. (See Charts #3, #4, #5.) It is also not particularly associated with the age of the patient, the cause of death or the time between death and stain. The importance of these relations in determining good corneal donor material cannot be ascertained without further study. If further studies show that better results in keratoplasty occur with corneas of lesser dead cells, the slit lamp and gross appearance of the cornea does not give an accurate picture of selecting good corneal donor material.

CHART #3

% of Dead Cells	Gross	Slit Lamp	Time (Hours)	Age (Years)	Cause of Death
* 0.2%	Clear	Clear	12	38 M	CVA
* 0.3%	Clear	Wrinkled	26	23 F	Brain Tumor
* 0.3%	Clear	Clear	21	40 F	Cardiac Arrest
* 0.4%	Clear	Clear	10	62 M	MI
0.4% 0.6%	Clear	Wrinkled	37½	70 M	MI
* 0.5%	Clear	Clear	18½	31 M	CHF
0.5% 0.5%	Clear	Clear	5	52 F	Metastatic Ca. of Uterus
# 0.6%	Clear	Clear	14½	39 F	Uremia
* 0.7%	Clear	Clear	10	62 M	MI
0.7% 0.8%	Cloudy	Wrinkled	13¼	60 M	Pseudomonas Pneumonia
0.7% 0.8%	Clear	Wrinkled	16½	59 F	Septicemia Sec. to Rupture of Colon (Ca.)
# 0.8%	Clear	Clear	39½	39 F	Uremia
0.8% 0.8%	Clear	Clear	3½	75 F	Cerebral Emboli
0.9%	Cloudy	Cloudy	103	64 M	MI

0.0%					
1.2% 0.3%	Clear	Clear	4	85 M	MI
0.9% 1.1%	Cloudy	Wrinkled	6½	75 F	Guillain Barré Syndrome
1.3% 1.1%	Cloudy	Cloudy	40	72 M	Septicemia
* 1.25%	Clear	Clear	43½	72 M	GI Hemorrhage
1.1% 3 %	Clear	L) Wrinkled R) Filamentous Scars	38½	90 F	Unknown
3.9%	Clear	Clear	7½	90 F	GI Hemorrhage
1.9% 4.5%	Epith. Rough	Clear	8	63 M	MI
9.7% 6.4%	Clear	Clear	10	60 M	Liver Cirrhosis
12 % 14 %	Clear	Wrinkled	16	15 F	Encephalitis
40 % 4 %	Clear	Clear	3½	40 F	Rt. Heart Failure Sec. to Pneumonia
*50 %	Clear	Clear	21½	74 M	CHF

* Fellow Cornea used for Keratoplasty
\# Same Pair of Eyes Stained at Different Times

CHART #4

% of Dead Cells	Gross	Slit Lamp	Time (Hours)	Age (Years)	Cause of Death
* 1.375%	Cloudy	Cloudy	14 1/2	43 M	Ca. of Colon with Septicemia
# 1.9%	Clear	Clear	3	72 M	Acute Renal Failure
5.3% 5.3%	Clear (Arcus Senilis)	Clear	3 1/2	76 F	Cor Pulmonale
5.85% 9.25%	Cloudy	Folds	18 1/2	25 F	Multiple Sclerosis
9 % 5.5%	Clear	Clear	48	76 M	Ruptured Aortic Aneurism
** 7.5%	Clear	Few Folds	16	67 F	MI & CVA
# 7.75%	Cloudy	Cloudy	27	72 M	Acute Renal Failure
11 % 13 %	Clear	Folds	25	75 F	Pulmonary Emboli
12.5% 15 %	Clear	Many Folds	1 3/4	77 F	Pulmonary Emboli
*15 %	Cloudy	Cloudy	29 1/2	43 M	Ca. of Colon with Septicemia

* Same Pair of Eyes Stained at Different Times
\# Same Pair of Eyes Stained at Different Times
** Fellow Cornea used for Keratoplasty

Conclusion

The disadvantage of one staining procedure seems to be advantageous to the other. The nitroblue tetrazolium method has an advantage over the trypan blue method in studying the morphology of the endothelial cells and the intactness of the corneal endothelium. On the other hand, the trypan blue method has an advantage over the tetrazolium method in being a simpler and quicker technique, and being easier to count the dead endothelial cells.

To study the viability of the endothelium completely and accurately with the methods that are available to us today, both the trypan blue and tetrazolium techniques can be used on the same cornea. The trypan blue staining technique should be used first allowing the dead cells to be stained. These cells can be counted immediately. (Figure 18) The cornea can then be stained by the tetra-

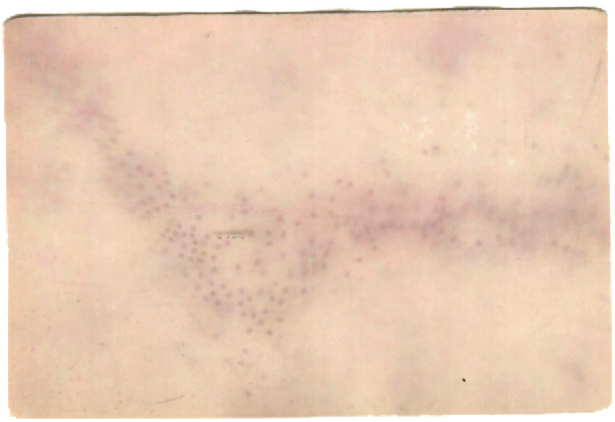


Figure 18. This is a cornea stained with trypan blue. Of side interest note that the nonviable cells seem to occur in a line. This was of common occurrence, the reason of which is not known.

zolium technique so that the morphology of the living cells and intactness of the endothelium may be better studied. (Figure 19) By using both methods one would have the ad-

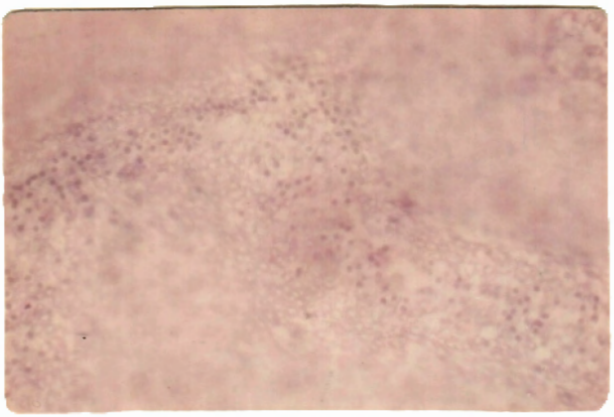


Figure 19. This is the same cornea shown in Figure 18 after being stained with nitroblue tetrazolium.

vantage of counting the dead endothelial cells with the trypan blue stain and the advantage of studying the morphology and the endothelial structure with the tetrazolium stain all on the same cornea. The only disadvantage in using the two procedures together is that the technique would be longer and, therefore, would add to more possible error and artifact. It should be remembered that when using both methods, the trypan blue method should be used first. After the cornea is frozen during the tetrazolium method, all the cell membranes will be disrupted allowing the trypan blue stain to enter all cells. The dead cells should probably be counted first before the cornea is stained with the tetrazolium method since the nuclei can be seen better without the blue cytoplasmic background. There is also some evidence that the trypan blue stain may be washed out during the tetrazolium procedure.

(Figures 20, 21) A series of corneas needs to be done to

Figure 20. This shows a cornea stained with trypan blue before being stained with nitroblue tetrazolium.

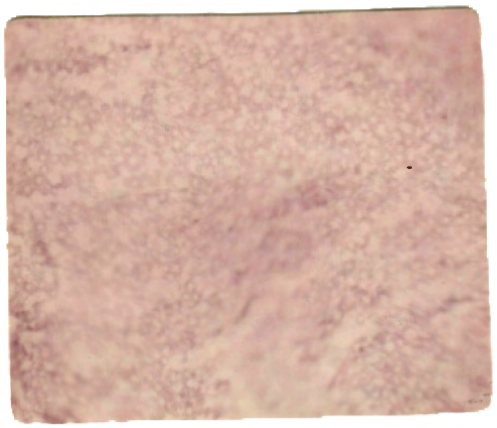


Figure 21. This shows the same cornea as in Figure 20 after using the nitroblue tetrazolium. Note that many unstained cells here have lost their trypan stain.

determine this factor.

These staining procedures used together or alone still are not the ideal method in studying the viability of the corneal endothelium. Although they do give an advantage over the slit lamp study of the corneal endothelium since individual cells can be observed, the corneas when once stained cannot be used for keratoplasty. The fellow eye must therefore be used. In this way much donor material is wasted which could possibly be used for corneal transplantation. Therefore, an ideal stain would be one by which

the cornea can be used for keratoplasty after the endothelium is stained and studied. The tetrazolium method destroys all cells and therefore is not very ideal at all. The trypan blue method approaches the ideal stain more closely. It may be possible to do corneal transplantation with a trypan blue stained cornea but experimentation needs to be done before attempts are made on man.

After observing a few corneas that were stained by both methods, it was noticed that the tetrazolium stain is much lighter than when a cornea was stained by the tetrazolium method alone. Whether the trypan blue interferes with tetrazolium staining or whether the trypan blue stain is somewhat toxic to the endothelial cells cannot be determined at this time.

Summary

This thesis is a critique of two new staining methods which have been recently introduced for studying the viability of the corneal endothelium. A history of corneal transplantation shows that keratoplasty has moved out of its experimental stage having a high rate of success. The importance of the donor's corneal endothelium is noted, showing it to be the most important layer for successful keratoplasties.

The drawbacks of selecting donor material for transplantation by noting its gross and slit lamp appearance shows that a staining technique to study the endothelial cells directly needs to be found. Two such staining techniques, the trypan blue method and the nitroblue tetrazolium method, are introduced noting their advantages and disadvantages in studying the viability of the corneal endothelium. In conclusion, it is formulated that the two techniques can be used on the same cornea so that the advantages of both can be utilized. Neither stain used separately nor together can be considered as ideal. More work needs to be done to find such a corneal endothelial stain.

REFERENCES

1. Ahuja, O. P., and Nema, H. V.: Experimental Corneal Vascularization and Its Management, Am J Ophthal 62:707-710, 1966

2. Baum, J. L.: A Histochemical Study of Corneal Respiratory Enzymes, Arch Ophthal 70:59-68, 1963

3. Bergman, G. D.: Posterior Polymorphous Degeneration of the Cornea, Am J Ophthal 58:125-128, 1964

4. Bloom, William, and Fawcett, Don: A Textbook of Histology, Philadelphia: W. B. Saunders Co., 1962, pp 637-640

5. Capella, J. A., Robbins, J., and Kaufman, H. E.: Preservation of Viable Corneal Tissue, Arch Ophthal 74:669-673, 1965

6. Chi, H. H., Teng, C. C., and Katzin, H. M.: Histopathology of Primary Endo-epith. Dystrophy of Cornea, Am J Ophthal 45:518-535, 1958

7. Chi, H. H., Teng, C. C., and Katzin, H. M.: Histology of Corneal Endothelium, Am J Ophthal 53:215-235, 1962

8. Chi, H. H., Teng, C. C., and Katzin, H. M.: The Fate of Endothelial Cells in Corneal Homografts, Am J Ophthal 59:186-191, 1965

9. DeOcampo, G.: Embryonation: A Biological Test for Corneal Stroma Cell Nonviability, Orient Arch Ophthal 2:18-20, 1964

10. Duke-Elder, Sir Stewart, and Leigh, Arthur George: System of Ophthalmology, Vol VIII Part 2, St. Louis: C. V. Mosby Company, 1965, pp 648-659

11. Evans, H. M., and Schulemann, W.: The Action of Vital Stains Belonging to the Benzidene Group, Science 39:443-454, 1914

12. Gurr, Edward: Encyclopedia of Microscopic Stains, Baltimore: William and Wilkins Co., 1960, pp 397-398

13. Gurr, Edward: Encyclopedia of Microscopic Stains, Baltimore: William and Wilkins Co., 1960, pp 445-448

14. Hanna, Calvin and Irwin, Edward: Fate of Cells in the Corneal Graft, Arch Ophthal 68:810-817, 1962

15. Irvin, A. R.: The Role of the Endothelium in Bulbus Keratoplasty, Arch Ophthal 56:338-351, 1956

16. Iwamoto, T., and Smelser, G. K.: Electron Microscopy of Human Corneal Endothelium with Reference to Transplant Mechanisms, Invest Ophthal 4:270-284, 1965

17. Kaufman, H. E., Robbins, J., and Capella, J. A.: A Study of Enzyme Activity in Corneal Repair, Invest Ophthal 3:34-46, 1967

18. Kaufman, H. E., Robbins, J., and Capella, J. A.: Endothelium in Normal and Abnormal Corneas, Am Ac of Ophthal and Otolaryn 69:931-942, 1965

19. Kaufman, H. E., Robbins, J., and Capella, J. A.: Human Corneal Endothelium, Am J Ophthal 61:835-841, 1966

20. Klein, B. A.: Fuchs' Epithelial Dystrophy of the Cornea, Am J Ophthal 46:297-304, 1958

21. Klouman, O. F.: A Peculiar Type of Corneal Endothelial Dystrophy, Acta Ophthal 43:534-538, 1965

22. Langham, M. E.: The Interrelationship of the Metabolism and the Deturgenscence of the Living Cornea, Invest Ophthal 1:187-194, 1962

23. Lowry, George M.: Corneal Endothelium in Vitro Characterization by Ultrastructure and Histochemistry, Invest Ophthal 5:355-365, 1966

24. Muehler, F. O., Casey, T. A., and Trevor-Roper, P. D.: Use of Deep Frozen Human Cornea in Full Thickness Grafts, Brit M J 2:473-475, 1964

25. Nicholas, J. P.: Whole Mounts of the Corneal Endothelium, Arch Ophthal 73:555-558, 1965

26. Pena-Carillo, Jr., and Polack, F. M.: Histochemical Changes in Endothelium of Corneas Stored in Moist Chamber, Arch Ophthal 78:811-816,1964

27. Polack, F. M. and Smelsher, George K.: Long-term Survival of Isotopically Labeled Stromal and Endothelial Cells in Corneal Homografts, Am J Ophthal 57:67-78, 1964

28. Potts, A. M.: Some Aspects of the Interrelation Between Corneal Hydration and Corneal Transparency, Invest Ophthal 1:163-169, 1962

29. Pregiosi, Victor A.: An Apparatus for Phase-Contrast Microscopic Observation of Viable Corneal Endothelium During Freezing and Thawing, Am J Ophthal 62:19-26, 1966

30. Robbins, J. E., Kaufman, H. E., and Capella, J. A.: Study of Endothelium in Keratoplasty and Corneal Preservation, Arch Ophthal 73:242-247, 1965

31. Stocker, F. W.: Successful Corneal Graft in a Case of Endothelial Dystrophy, Am J Ophthal 35:349-352, 1952

32. Stocker, F. W.: The Endothelium of the Cornea and Its Clinical Implications, Tr Am Ophth Soc 51:669-786, 1953

33. Stocker, F. W., King, E. H., Lucas, D. O., and Georgiade, N.: A Comparison of Two Different Staining Methods for Evaluating Corneal Endothelial Viability, Arch Ophthal 76:833-835, 1966

34. Stocker, F. W., King, E. H., Lucas, D. O., and Georgiade, N.: A Comparison of Two Principles for Evaluating Corneal Endothelial Viability, Arch Ophthal 76:836-838, 1966

35. Sugar, H. Saul: Prognosis in Stripping of Descemet's Membrane, Am J Ophthal 63:140-143, 1967

36. Wolter, J. R., Henderson, J. W., and Gates, K.: Endothelial and Epithelial Dystrophy of the Cornea, Am J Ophthal 44:191-199, 1957

37. Wolter, J. R.: Secondary Cornea Guttata, Am J Ophthal 50:17-25, 1960

38. Wolter, J. R.: Secondary Cornea Guttata in Interstitial Keratoplasty, Ophthalmologica 148:289-295, 1964

39. Yanoff, M., and Tsou, K. C.: Demonstration of Ocular Reduced DPNH and TPNH Diaphorase Using Tetrazolium Salts, Am J Ophthal 60: 310-317, 1965