THE ASSESSMENT OF ENDOTHELIAL VIABILITY*

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The testing of viability is an important aspect of transplantation and there are many available methods of doing this. It is especially essential in the case of the corneal endothelium, where integrity is an important factor in the success of a penetrating corneal graft. With the development of new methods of long-term preservation, the viability of the endothelium must be accurately assessed, because small differences in the percentage of preservatives, and in the rates of freezing and thawing, may have marked effects on viability. The more accurate the methods of assessment, the better will be the techniques of preservation.

The definition of viability is difficult, as witnessed by recent attempts to define death from a cardiac donor point of view. The problem is difficult because there are grades of viability, i.e. a cell which is damaged may yet be capable of recovery, and so be viable. Thus, irreversible damage is the key word in considering what is not viable, and the problem remains to define the irreversible, which in itself is not difficult, but which, in the experimental application, presents great problems. It is not known which particular function or process, when lost, causes irreversible damage to the integrity of the cell.[1]

A cell is dead if it loses all or most of its vital processes and does not react to any experimental technique which would normally demonstrate a vital process. One may attempt to define a viable cell as one which is capable of performing its normal functions.

The corneal endothelium, because it is a monolayer structure, lends itself to many techniques of demonstrating viability which are not normally used for solid tissues. It is important to remember that when one uses a particular technique for viability testing, one is only testing one aspect of a cell's metabolic activity.

VIABILITY TESTING

Over 40 different methods have been described for viability testing. They can be broadly divided into 6 categories:[2]

Structural Alteration, Chemical Composition and Electro-Conductivity

Most of these techniques are not applicable to the corneal endothelium, but some have been used.

(a) *Ordinary microscopy*. This is not practical, but does help to distinguish gross appearances such as pyknosis, etc. However, one cannot really assess which changes are reversible and one is furthermore examining cells under highly artificial conditions, the cells having already been killed before examination.

(b) *Electron-microscopy*. This is feasible, provided preparation of the specimen does not destroy organelles, ribosomes, etc. Some work has already been done along these lines.[3] The disadvantage is that it is time-consuming and does not express the viability of a whole sheet of cells.

(c) *Phase-contrast microscopy*. This technique is neat and shows decomposition of the cell under various conditions.[4] The apparatus described, however, is elaborate and cannot be applied to the assessment of factors such as the type and nature of the containers used for storage.

(d) *Motility*. This indicates retention of a vital property, but is not seen in the endothelium.

(e) *Phagocytosis and pinocytosis* might be applied to endothelial cells, but this has not yet been done.

Tissue Culture Techniques

This is the time-honoured technique of assessing viability. Cells may be studied as a monolayer culture or the whole organ may be observed as an explant. The disadvantages of this technique are: (*a*) A group of cells may be the only ones viable and thus may start multiplying, giving a false idea of viability even when other cells are dead, i.e. it is only a qualitative test (e.g. 75% of the cells of the kidney may be dead, yet one can still obtain a positive culture); (*b*) it is time-consuming; (*c*) it is subject to technical difficulties; and (*d*) organized tissues, such as an explant, are difficult to assess.

Utilization of Membrane Permeability

The semi-permeability of the cell membrane allows assessment of viability in a manner which has become increasingly exploited over the last few years.

(a) *The membrane as a barrier* (dye exclusion studies).[1] Certain substances cannot penetrate the cell membrane while it functions normally, but, should this membrane become disturbed, then normally-excluded substances can penetrate the cell and so stain it. Examples of these substances are eosin, lissamine green, acridine orange, trypan blue and nigrosin. The technique in all instances consists of layering the substance onto the endothelium, washing this off, and then fixing and assessing the endothelium. In the presence of an intact endothelium no cells will be stained. If the cells are damaged they will be stained. The technique has the advantage that it is easy to perform and requires the minimal amount of tissue handling. The obvious disadvantage is that cells which are damaged are not necessarily dead. When assessing a preservation technique, however, this is not important because the best technique is that which gives the minimal amount of staining.

A further disadvantage is that many of the substances used may be toxic and can themselves cause damage to the cell membrane. It is difficult to gain an accurate idea of the number of cells viable—only an assessment can be made and it is often difficult to assess the peripheral areas of the cornea. Even counting by means of a reticule is not accurate, and thus only an impression is gained. This technique has been effectively used by Stocker and his associates who used trypan blue,[5] although they mistakenly called their method a supravital technique. The use of trypan blue has been seriously questioned in the literature. Even in the presence of total nuclear deterioration, trypan blue may still be excluded.[6] The specificity of results using trypan blue has thus been questioned. We would, however, agree with Stocker that

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it is by far the simplest of the available techniques. I have used nigrosin with considerable success.[7]

(b) *The membrane as a transport structure (supravital staining).* Various components of the cell may be selectively stained without impairment of the cell's vitality. The viability of the cell can thus be assessed by observing the ability of the membrane to transport labelled substances or stains into its interior.

The vital dyes used to stain the organelles of a living cell are laid down in the cytoplasm.[1] The cell takes up dye or concentrates it from a dilute solution. Janus green, neutral red, methylene blue and trypan blue have been used. The disadvantage of many of these is their toxicity in higher concentrations. Acridine orange is said to be superior because of its ultraviolet fluorescence. It stains nuclei green and cytoplasm red. Thus, cells which have lost their cytoplasm lack red fluorescence. Staining of the total endothelial sheet with these techniques has not yet been attempted.

Enzymatic Techniques

Hydrogen-ion acceptors, when added to an intracellular dehydrogenase system, will change their colour if the appropriate enzyme system is active. This is the basis of the popularly used tetrazolium techniques.[8] First used in 1941, it was found that a colourless tetrazolium salt was changed to a red formazan salt by a reduction reaction.

This was not due to the usual reducing agents, such as cysteine or ascorbic acid, and worked best at a pH of 7·2. These salts act as an electron acceptor in enzyme-catalysed reactions. Since 1941 far more sophisticated tetrazoliums have been developed.

The intact membrane of the mitochondria is a barrier to the penetration of tetrazolium salts, and therefore this barrier must be broken down to allow a reasonable concentration of tetrazolium salts at the site of electron transfer. Most of the enzymes which can be estimated are situated within the mitochondria, and the types of injury which can damage these enzymes are important because of their application to endothelial storage and survival. Pearse lists the following:[8]

1. Anoxia.
2. Thermal—room temperature rapidly destroys the enzymes and therefore cooling is essential.
3. Osmotic—hypertonic media should be used.
4. Chemical—the presence of ATP and magnesium is important.

The purpose of these assays is to show Krebs-cycle activity, and, where DPN is used as a substrate, the activity of DPN-linked systems. The enzyme usually tested by most workers in corneal endothelial work is DPN diaphorase. This is thought to be DPNH cytochrome c reductase.

In using these tests the cornea is incubated with a substrate and a tetrazolium salt. Para-nitro-blue tetrazolium is the most commonly used salt. If the appropriate enzyme is present, then a blue formazan will result. The technique was first described by Kaufman and his colleagues,[9] and it is to this group of workers that the credit must go, not only for stimulating interest in viability techniques, but also for their pioneering studies on the preservation of frozen tissue.

There are some disadvantages of these techniques. It is frequently difficult to correlate enzyme activity of the cell with retention of vital properties. The tests used by most workers are rough, qualitative experiments. The degree to which an enzyme being tested is essential is not known with certainty. For purposes of endothelial viability testing, however, the enzyme assayed in the Krebs cycle and its terminal pathways may be considered essential. In practice, they obviously yield applicable results because with some preservation techniques they are not present, while with others they are, and these results have been statistically correlated by grafting.

More practical objections are that the use of the technique involves much manipulation which will certainly cause damage. Also, as Stocker has pointed out,[5] there is a considerable difference in observer interpretation of the degree of staining. In our experience most cells do stain, because even if the cell is nearly dead the fragmented mitochondria may still give a positive reaction. If a cell is homogenized to the extent that its structure is no longer recognizable as cellular, the mitochondrial respiratory enzyme system still shows great activity. The enzymes are exposed to far more favourable contact with the hydrogen ion acceptor. In fact, it has been suggested[10] that mitochondria should be tested within cells as isolated intact mitochondria and as fragmented mitochondria.

The endothelium may be mechanically lost, and then no comment can be made on the viability of the endothelium. Compared with tissue culture, wide individual differences can occur[11] and the tests are therefore by no means critically decisive.

The tetrazolium dyes have also been used by O'Neil *et al.*[12] to assess viability. However, they have used the tetrazolium as a dye-exclusion test. They incubated corneas with tetrazolium, without preliminary preparation, and then assessed the amount of staining. If the membrane was permeable and enzyme was present, then the cell stained and they considered this a non-viable cell. This technique is open to objection, as has been pointed out by Capella and his associates.[13] The fact that the membrane is permeable but that enzymes are still present, shows that the cell still retains some of its functions. A non-staining cell may be a cell with a permeable membrane but with complete loss of respiratory enzymes, and so can be mistaken for a viable cell. The dye itself is toxic and can damage the cell.

Nucleotide Incorporation

Radioactive formate and tritiated thymidene tests are not as yet applicable to the assessment of endothelial viability.

Transplantation

This is the only single approach which, by its success, confirms viability. However, it is time-consuming and the experiments are subject to technical errors. It is obvious that ultimately any preservation technique must have the endothelial viability results confirmed by animal transplantation before they can be used in the human situation.

DISCUSSION

Of the many methods available, the 2 most commonly used for corneal viability testing are enzyme studies and

dye-exclusion studies. The advantages of these individual techniques have been pointed out.

There are also certain disadvantages common to all techniques. Often, because of the nature of the preparation, only impressions of the number of viable cells are formed, and thus the total number is expressed as a percentage based on an impression. In fact, every cell should theoretically be counted; obviously this is almost impossible, but by means of a reticule at least 5 fields should be assessed (4 peripheral and one central).

If the endothelium is lost in preparation, one cannot be certain if this is mechanical damage, or non-viable cells which have sloughed off Descemet's membrane. Generally this can be decided by the nature of the break.

The 2 techniques are to a certain extent complementary. Thus, if a cell does not take up a dye such as nigrosin, it is presumably viable, e.g. if x% stain, then 100—x% are viable. Similarly, cells which do not stain with tetrazolium must be dead, because they have lost their oxidative enzymes, e.g. if y% stained, then 100—y% are non-viable. On this basis we may say that 100—x% are viable, 100—y% are non-viable and the remainder of the cells, i.e. x + y—100, are those cells which are damaged but still viable. Ideally one should perform this type of histochemical analysis on the same cell, but this is technically difficult and so we have done this on paired eyes and have found that there is always a definite number of cells which show this intermediate state.

Of the two types of testing, the simplest to use are the dye-exclusion studies.

As the need for increased accuracy increases, a test will have to be perfected which allows exact and total counting of all cells. To this end we have been experimenting with techniques which involve counting the endothelial cells in a counting chamber after they have been removed from Descemet's membrane, and staining them with a mild non-toxic dye.

SUMMARY

The techniques used for the assessment of endothelial viability have been reviewed and their advantages and disadvantages discussed.

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