Dual staining of corneal endothelium with trypan blue and alizarin red S: importance of pH for the dye-lake reaction

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SUMMARY Evaluation of corneal endothelial integrity by combined staining with the vital stain trypan blue and the intercellular stain alizarin red S provides a simple, quick technique for visualisation of both damaged and normal cells, thereby permitting the quantification of endothelial cell damage. Adjustment of the pH of the alizarin red S reagent to 4.2 is important for optimum dye-laking at the intercellular borders, and brief fixation with glutaraldehyde maintains the staining effect of both dyes.

The transparency of the cornea unquestionably depends upon the integrity of its endothelium, and this is especially important for the success of penetrating keratoplasty. The viability of the endothelium has been assessed by a number of techniques.¹⁻⁴ Most recently a great deal of attention has been given to use of the specular microscope both in vitro⁵⁻⁸ and in vivo.⁹⁻¹² This technique not only enables the function of the endothelium to be evaluated by measuring the control of corneal thickness^{6,13} but also allows some assessment of morphological integrity^{14,15} and endothelial cell density.^{11,16} Nevertheless simpler, indirect methods of assessing endothelial status such as the observation of changes in cell membrane permeability using vital stains, for example, trypan blue¹⁷⁻²¹ or indocyanine green²² in dye-exclusion tests provide a simple, quick, and practical method of detecting damage in the corneal endothelium. In our studies of factors important for the cryopreservation of corneas we recognised the value of a quick general assessment of the percentage of intact cells that vital staining offers. There have been a number of reports describing the combined staining of devitalised cells with trypan blue^{23,24} or rose bengal²⁵ together with delineation of healthy cells with a so-called 'intercellular stain'—alizarin red S. This technique allows a proportional count of damaged and undamaged cells in the endothelial monolayer. However, we were

unable to obtain a clear outline of the margins of normal cells by these methods until we introduced some important refinements to the technique which are described in this paper.

Materials and methods

Corneas used in this study were mainly from New Zealand albino rabbits weighing 2-3 kg which had been killed by an intravenous overdose of sodium pentobarbitone. Other corneas were taken from pig eyes which had been enucleated immediately after exsanguination of the animals. The cornea from one human eye which had been stored in a moist chamber at +4°C for 3 days was also used. Prior to staining, the rabbit corneas and some of the pig corneas were excised from the enucleated eyes by the method of Dikstein and Maurice⁶ and held on a plastic support ring to minimise wrinkling and distortion. Alternatively, corneas from pig and human eyes were removed with a 2 mm rim of sclera in the way described by Capella.²⁶

The method of preparation of reagents and the staining procedure finally adopted in this work is both simple and rapid.

SOLUTIONS

A 0.25% solution of trypan blue was prepared by dissolving 0.25 g of the dye in 100 ml of 0.9% saline. A stock solution of alizarin red S (0.2%) was prepared

by stirring 100 ml of 0.9% saline containing 0.2 g of stain for several hours on a magnetic stirrer. The deep-iodine coloured solution was filtered (8 μ m filter) to remove any undissolved sediment. This stock solution was stored at room temperature and each day a 5 ml sample was taken and adjusted to pH 4.2^{27 28} with dilute ammonium hydroxide (0.1% solution in normal saline)²³ using a glass/calomel pH-cell (Radiometer).

A solution of 2.48% glutaraldehyde in sodium cacodylate buffer²¹ (osmolality 301 mosm.kg⁻¹; pH 7.2) was prepared as a fixative.

STAINING PROCEDURE

Isolated corneas were placed endothelial side up in a Teflon corneal cup, and trypan blue was added dropwise to cover the endothelium. After 1½ minutes the stain was poured off and the cornea briefly rinsed twice in normal saline, drained to remove excess saline, and then replaced in the corneal cup. The endothelial layer was then covered with alizarin red S (0.2%; pH 4.2) for 1½ minutes and again rinsed twice in saline after pouring away the staining reagent. After the staining procedure some corneas were immersed in the glutaraldehyde fixative solution for 10 minutes.

Finally a 7.5 mm button was cut from the centre of each fixed and unfixed cornea using a surgical trephine, and this disc of tissue was mounted, endothelium uppermost, in saline, under a coverslip, on a microscope slide having a central cavity to accommodate the thickness of the corneal button. 400 ASA (27 DIN) film was used for photomicrography.

Results and discussion

The delineation of both damaged and undamaged cells on the basis of dye exclusion is shown in Figs. 1 and 2. Changed cells that have become permeable to trypan blue show deep blue staining of their nuclei in contrast to the unstained condition of normal cells. Staining with trypan blue alone, as demonstrated by Spence and Peyman,²³ shows only cells that have become permeable to the dye; undamaged cells are rarely discernible under the light microscope, which precludes determination of the proportional cell damage. Alizarin red S has been advocated in a number of studies^{17 23-25} as a specific indicator of the intercellular borders of endothelial cells. We have to report repeatedly unsuccessful attempts to achieve delineation of healthy cells by alizarin red S using these methods. At best we occasionally saw a very faint outline of the endothelial cell pattern, which can in any case sometimes be seen in the absence of specific staining of the intercellular borders.

In 1930 Cameron²⁹ concluded that the anthra-

quinone dye sodium alizarin sulphonate (alizarin red S) was a specific reagent for calcium and subsequently McGee-Russell²⁷ in a detailed study of histochemical methods for calcium described the dye-lake reaction of such anthraquinone dyes. McGee-Russell records difficulties in producing the laking effect, at known sites of calcium deposits, using simple aqueous or alcoholic solutions prepared from a variety of sources of the alizarin compound. However, his study revealed that the key to success was the observation that the reaction has a critical pH range for the formation of the insoluble lake precipitate. McGee-Russell's tests confirmed the findings of Dahl³⁰ that the laking reaction occurs only between pH 3.9 and 8.05; he concluded that the most satisfactory pH for the staining reagent is between 4.1 and 4.3.

As shown in the photomicrographs the alizarin red S solution prepared in this work and adjusted to pH 4.2 with dilute ammonia solution produced a prominent outline of both the normal cells in the endothelial layer and those which had very clearly become permeable to trypan blue. Table 1 shows a comparison of the measured pH values of alizarin red S solutions prepared by different methods. The methods of Sperling²⁴ and Spence and Peyman²³ gave pH values at the extremes of the range in which the dye-lake reaction occurs, and this might explain the variable and vague staining we achieved using these techniques. McGee-Russell pointed out that some differences in manufacture or in the impurities present in samples of the stain could have given rise to the significant differences in pH he observed in aqueous solutions of several samples, and he attributed the variable success of earlier workers to significant differences in pH.

We are able to confirm the completely reproducible and consistent results obtained using an alizarin red S solution the pH of which is carefully adjusted to 4.2. Under these conditions we found that the time of staining was not nearly so critical as claimed by Spence and Peyman²³ but that about 1½ minutes was optimal for a reproducibly dense outline of the polygonal cells. This staining technique not only

Table 1 A comparison of the measured pH values of alizarin red S staining reagent prepared by different methods

Solution	pH	Reference
1. 70 ml of 0.2% alizarin red S in 0.9% saline + 30 ml 0.1% NH ₄ OH	7.34	23
2. 1% alizarin red S in deionised water + sucrose (2 g per 10 ml)	3.15	24
3. 0.2% stock solution of alizarin red S (Sigma) in 0.9% saline (pH unadjusted)	3.8	Present work
4. 0.2% solution of alizarin red S, pH adjusted with 0.1% NH ₄ OH (~250 μ l per 10 ml of stock staining reagent)	4.2	Present work

Fig. 1 Combined staining of rabbit endothelia with trypan blue and alizarin red S (pH 4.2). (a) An area of damage occurring after 7½ hours in-vitro perfusion included individual cells with dark blue nuclei and areas of exposed Descemet's membrane stained red (D). Staining of the intercellular borders with alizarin red S shows the mosaic pattern of normal cells. The shadowing effect across the endothelium is probably due to minute folds in the cornea ($\times 100$). (b) Endothelium of a frozen and thawed rabbit cornea showing a high proportion of trypan blue stained cells and the exposure of Descemet's membrane at the site of missing cells (arrowed D). Points of junctional separation (arrowed Js) may also be indicated ($\times 100$).

provides a contrast between damaged and undamaged cells but also shows areas where Descemet's membrane has been exposed owing to the detachment of endothelial cells; these areas show a diffuse pink-red staining as shown in Fig. 1 and Figs. 2a and 2d.

Examples of the combined staining effect in a number of differently treated corneas are shown in Figs. 1 and 2: an area of damaged endothelial cells identified under the specular microscope after 7½ hours in vitro perfusion of a rabbit cornea with glutathione bicarbonate Ringer's solution (GBR) is

shown in Fig. 1a. Fig. 1b shows part of the endothelium of a rabbit cornea stained immediately after thawing and rehydration in rabbit serum after having been frozen and thawed by the method of Capella *et al.*³¹ A high proportion of cells have stained with trypan blue and there are many detached or necrosed cells revealing the diffuse red staining of the underlying Descemet's membrane. Poor apposition of endothelial cells, or junctional separation, may be indicated by the darkly stained areas and the numerous discrete red spots at the cell borders.

An air bubble in contact with the endothelium of a

Fig. 2 Combined staining of a variety of corneas with trypan blue and alizarin red S. (a) Damage induced in a rabbit cornea by an air bubble in contact with the endothelium for 5 minutes during in-vitro perfusion ($\times 85$). (b) Giant cells in a rabbit endothelium at low power ($\times 104$). (c) Giant cells in a rabbit endothelium at high power ($\times 354$). (d) Damage induced in a pig cornea by drawing a silk suture across the endothelium ($\times 64$). (e) Endothelial cells of a pig cornea showing stained nuclei and cell margins at high power ($\times 336$). (f) Human cornea after 3 days' storage in a moist chamber at $+4^{\circ}\text{C}$ ($\times 95$). Arrows indicate areas where Descemet's membrane is exposed.

rabbit cornea for only 5 minutes during in vitro perfusion at 34°C induced a ring of cells to become permeable to trypan blue, and in one area detached endothelial cells have exposed the underlying basement membrane (Fig. 2a). Some giant endothelial cells in rabbit corneas are shown under low power ($\times 104$) in Fig. 2b and under high power ($\times 354$) in Fig. 2c. Damage caused by drawing a silk suture lightly over the endothelial layer of an isolated pig cornea is shown in Fig. 2d, and some of the stained nuclei are shown under high power in Fig. 2e.

One of the problems of using trypan blue for staining the endothelium, as pointed out by Stocker,¹⁸ is that the stained preparations fade rapidly. However, Van Horn²¹ showed that this problem can be overcome by fixing the tissue in glutaraldehyde buffered with sodium cacodylate. We found that the very sharp

and dense staining achieved in unfixed tissue immediately after staining does fade noticeably within 1 hour, but that brief fixation for 10 minutes in 2.5% glutaraldehyde solution preserves the intensity of the trypan blue without affecting the staining due to alizarin red S. The endothelium of the frozen and thawed rabbit cornea in Fig. 1b was photographed 2½ hours after staining and fixation.

Fixation in the way described also reduces the folding of the cornea which can interfere with exact focusing of the entire field of view. This is particularly useful for the flaccid rabbit cornea which can be kept on a plastic support ring⁶ from the time of isolation throughout the staining and fixation procedure. After 10 minutes in fixative the cornea will maintain its shape on removal from the support ring. The effect of folds in the endothelium of a human cornea stored for

3 days in a moist chamber are shown in Fig. 2f, where cells on the ridge of a fold have stained with trypan blue and cells in the remainder of the field appear out of focus.

This dual staining technique may be used in addition to other viability assays on the same cornea, for example, after measuring corneal thickness during a period of in-vitro perfusion under the specular microscope.

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