Biocompatibility of Trypan Blue With Human Corneal Cells

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Objective: To quantify the toxicity of trypan blue on human corneal cells according to exposure time and concentration.

Methods: Three in vitro experiments were performed. (1) We exposed cultured human corneal fibroblasts to trypan blue (0.0001% to 0.1%) in Eagle modified minimum essential medium (EMEM) or phosphate-buffered saline (PBS) for 15 minutes to 24 hours. Cytotoxicity was evaluated by Mosmann's colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. (2) We exposed human corneas in EMEM for 24 hours to trypan blue (0.001% to 0.1%). Fellow donor corneas served as controls. Endothelial survival was evaluated morphologically and by cell density assessment. (3) We morphologically compared the endothelial viability of human donor corneas after exposure to 0.1% trypan blue for 5 to 30 minutes with control corneas.

Results: In experiment 1, trypan blue in EMEM was not significantly toxic at concentrations of 0.005% or lower. Higher concentrations were toxic only after exposure to trypan blue for at least 6 hours. In PBS, significant toxicity was found after exposure to 0.1% trypan blue for 30 minutes or longer. Lower concentrations were toxic after longer exposures. In experiment 2, exposure to 0.01% and 0.1% trypan blue for 24 hours resulted in significant loss in cell density. At lower concentrations, the endothelium was affected only morphologically. In experiment 3, endothelial morphology changed in control corneas and after exposure to 0.1% trypan blue for as little as 5 minutes. After 30-minute exposure, morphologic deterioration was more pronounced.

Conclusions: Trypan blue was toxic in vitro to corneal endothelium and corneal fibroblasts at higher concentrations and notably longer exposure times. Toxicity was less in EMEM than in PBS.

Clinical Relevance: At commonly used concentrations, both during cataract surgery and in the cornea bank, trypan blue is safe for corneal cells. At higher concentrations or longer exposures, however, caution is warranted.


TRYPAN BLUE VITAL STAINING for the evaluation of the endothelium of donor corneas was first described by Stocker et al.1 Trypan blue stains the nuclei of severely damaged and dead endothelial cells of donor corneas, as well as areas of Descemet membrane denuded of endothelial cells. It does not stain viable endothelial cells with an intact cell membrane.1-4 Sperling et al.5-7 introduced trypan blue vital staining in combination with induced dilation of intercellular spaces (with 0.45% and 0.9% sodium chloride, or with 1.8% sucrose) for visualization of endothelial cell borders, thus allowing light-microscopic assessment of both endothelial damage and endothelial cell density (ECD) on potentially transplantable donor corneas. Since 1982, this technique has been used by the Cornea Bank of the Netherlands Ophthalmic Research Institute (NORI) for the evaluation of the endothelium of human donor corneas after storage by organ culture preservation.8,9 Variations of this technique, all using trypan blue, are applied in many cornea banks in Europe.

Norn10-12 was the first to use trypan blue intraoperatively, during intracapsular cataract surgery, to evaluate endothelial status. Recently, intraoperative application of the dye has gained new interest: trypan blue anterior lens capsule staining aims at improving visualization of the capsulorrhexis during phacoemulsification in patients with absent red fundus reflex, eg, in mature cataracts.13

To expand the existing body of knowledge on the toxicity of trypan blue on human corneal cells, concentration- and exposure time-related effects of trypan blue were studied in 3 in vitro experiments on human donor corneas and human corneal fibroblasts.
CYTOTOXICITY OF TRYPAN BLUE TO HUMAN CORNEAL FIBROBLASTS

METHODS

Trypan Blue Solutions

Trypan blue (Gurr; BDH Chemicals Ltd, Poole, England) was dissolved in sterile phosphate-buffered saline (PBS) (Azua Pharmacy, Amsterdam, the Netherlands) unless otherwise stated. The Cornea Bank NORI has used a 1.2% solution from this stock for routine purposes since 1998. The photometric extinction value of this solution is 0.227 at 560 nm. This value is the same for the 0.3% trypan blue solution, obtained from another stock, and described and applied in previous years.8,11,15

Human Corneal Fibroblast Cultures and Their Experimental Incubation

Corneal fibroblasts were obtained from normal human donor eyes as described previously.16,17 In short, corneal buttons were excised and fragmented. Three to 4 corneal pieces were implanted per well of a 6-well plate (Nunc, Roskilde, Denmark) and incubated with 1 mL of Eagle modified minimum essential medium (EMEM) (ICN Biomedicals Inc, Costa Mesa, Calif) supplemented with 10% fetal bovine serum (FBS) (Gibico Life Technologies Ltd, Paisley, Scotland), 100-U/mL penicillin (Gist Brocades, Leiderdorp, the Netherlands), and 50-µg/mL streptomycin (Biochemie GmbH, Vienna, Austria) at 37°C. When the pieces adhered firmly to the bottom of the well by the outgrowing cells, 5 mL of medium per well was added. Medium was renewed twice a week. Outgrowing fibroblasts were subcultured after 25 to 28 days and fibroblasts of the second passage were stored in liquid nitrogen. Cells were defrosted, subcultured for 1 passage, and used for experiments. At confluency, the cells were removed from the culture flasks by incubation with a trypsin-EDTA solution, collected by centrifugation (200g), resuspended in medium, and plated out at 5 × 10^4 cells per well in 96-well flat-bottomed tissue culture plates (Nunc). The cells were cultured at 37°C in a humidified atmosphere with 5% carbon dioxide before exposure to freshly prepared sterile trypan blue solutions, varying in concentration from 0.0001% to 0.1%, in a vehicle of EMEM (half of the wells per tray) or sterile PBS. Wells were filled with 100 µL of study preparations, either with the trypan blue solution as described or vehicle (EMEM or PBS) without added trypan blue as control.

Quantitative Cytotoxicity Assessment With Human Corneal Fibroblasts

Cytotoxicity was determined with Mosmann’s colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay directly after exposure to trypan blue.18 The MTT assay was performed as described previously.19,20; it basically measures the capacity of mitochondrial enzymes to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Corp, St Louis, Mo) into a formazan product and thus reflects the mitochondrial activity of corneal fibroblasts. After exposure for 24 hours, all corneas were rinsed and preserved in fresh EMEM at 31°C for another 21 days. At 9 to 10 and 20 to 21 days, corneal endothelium was evaluated by the technicians of the cornea bank. Storage of donor corneas in EMEM, as well as endothelial evaluation, was performed under sterile conditions using standard eye bank techniques, as described previously.8,14,15 Endothelia of experimental and control corneas were compared on ECD and morphologic status. Endothelial morphology was graded normal (score of 3) when a normal, “string of pearls”–like swelling pattern of the intercellular space indicative of a well-functioning cell membrane was present; dubious (score of 2) was assigned to endothelia with irregular or locally poor swelling of the intercellular space, and with the presence of many vacuoles within the endothelial cells; and abnormal morphology (score of 1) was used for endothelia with rounding up of the cells, indicative of loss of intercellular contacts, and with generally poor swelling of the intercellular space (endothelia were called dead, with a score of 0, when no recognizable endothelial cells could be discerned).14,15

Statistical evaluation of ECD was performed by means of a mixed-model analysis of variance with ECD as the dependent variable, adjusted for its baseline measurements and time of investigation as covariate in the model. There was a within-subject factor (trypan blue vs control) and a between-subjects factor (concentration) in the model. Interest was in the interaction between these latter 2 factors. No structure was assumed for the within-subject correlations. Endothelial morphology scores were evaluated by means of a nonparametric Wilcoxon signed rank test. Experimental corneas, all taken together, were tested against all control corneas for both time points. Testing by concentration group was considered not to be meaningful because of small group size.

ENDOTHELIAL VIABILITY BY MORPHOLOGIC ASSESSMENT AFTER APPLICATION OF TRYPAN BLUE

Human donor corneas were used that were judged unsuitable for transplantation because of corneal scars, low ECD (between 1800 and 2300 cells/mm²), or signs of abnormal mor-
phologic characteristics such as significant polymegathism.14,15 These corneas were briefly (1-2 days) stored at the Cornea Bank NORI under sterile conditions at 31°C in EMEM with 2% FBS and supplemented with 5% dextran before shipment to the Rotterdam Eye Hospital, Rotterdam, the Netherlands, for the experiment.

Both at the beginning and at the end of the experiment, the endothelium of all corneas was evaluated by 0.1% trypan blue vital staining and provoked intercellular swelling with 1.8% sucrose solution (hospital pharmacy of the Rotterdam Eye Hospital), and photographed with an inverted light microscope. Corneas with endothelia that showed severe degenerative changes (ie, trypan blue–stained nuclei, vacuoles in the cells, poor swelling of the intercellular space) before the experiment were excluded from the experiment. The remaining corneas were randomly allotted to one of the treatment groups or the control group.

The control corneas (n=8) were merely incubated for 2 hours in EMEM with 2% FBS and 5% dextran at room temperature. In 3 of the experimental groups, 0.1% trypan blue solution was applied to the endothelium of the corneas directly after the preexperimental evaluation for 30 (n=7), 10 (n=9), and 5 (n=7) minutes. A fourth experimental group (n=6) was exposed to 0.01% trypan blue for 10 minutes. Then, after trypan blue was thoroughly rinsed off with balanced saline solution, all experimental corneas were incubated in EMEM for 2 hours with 2% FBS and 5% dextran at room temperature.

The preexperimental and postexperimental photomicrographs of the endothelium of control and experimental corneas were mixed and subjected to a masked assessment by 5 laboratory technicians of the Cornea Bank NORI. They used a grading scale for each morphologic assessment score was obtained for each photomicrograph and for each of the observers, a product score ranging between 1 and 12 was calculated from the scores on the 2 scales. Graph and for each of the observers, a product score ranging from 1 to 12 was calculated from the scores on the 2 scales. By averaging the product scores of the 5 observers, an endothelial morphology assessment score was obtained for each cornea exposed to 0.1% trypan blue had already been de- served damage to the fibroblasts was irreversible.

**RESULTS**

**CYTOTOXICITY OF TRYPAN BLUE TO HUMAN CORNEAL FIBROBLASTS**

The relationship between the mean CI, trypan blue concentration, and exposure time is shown in the 3-dimensional graphs in Figure 2A and B for EMEM and PBS, respectively. Significant CIs (>20%) are represented by the gray bars in the graphs. Figure 2A shows that in EMEM, trypan blue concentrations of 0.01% and higher resulted in significant toxicity after exposure for 6 hours or longer. At 24 hours' exposure, a trypan blue concentration of 0.005% showed a threshold significant CI. Lower concentrations did not cause significant toxicity with exposures up to 24 hours. The graph in Figure 2B shows that in PBS significant toxicity was observed at exposures to 0.1% trypan blue of 30 minutes or longer. The exposure times needed to reach significant CIs decreased with decreasing trypan blue concentrations. With 0.0001% trypan blue in PBS, no significant CI was observed, regardless of exposure times.

Dose-response relationships for trypan blue in the 2 vehicles, EMEM and PBS, were plotted for each of the different exposure times. Response was expressed as the mean CI obtained in the 3 (repeated) experiments. Figure 3 shows a representative dose-response curve, in this example after 18 hours' exposure to trypan blue in EMEM. Using a sigmoidal fit program (Origin, version 4.0, Microcal Software Inc, Northampton, Mass), the maximal toxicity (CI) levels and L50s (50% lethal concentration, ie, concentration resulting in half-maximal response) were calculated for all exposure times and for both of the vehicles. These data are summarized in Table 1. The L50s appeared to be independent of exposure time and vehicle and was found at a concentration of 0.02% to 0.04%. In contrast, the calculated maximal toxicity levels were strongly dependent on exposure time, and higher calculated maximal toxicities were found in PBS than in EMEM.

At days 2 to 3 and days 7 to 8 after exposure, the results (not shown) were essentially the same as the results directly after exposure, indicating that the observed damage to the fibroblasts was irreversible.

**ENDOTHELIAL CELL SURVIVAL AFTER 24-HOUR EXPOSURE TO TRYPAN BLUE**

The results after 9 to 10 days and 20 to 21 days of preservation of the donor cornea pairs were expressed as mean ECDs, endothelial morphology scores, and percentages of ECD loss (Table 2). All corneas exposed to 0.1% or 0.01% trypan blue suffered total endothelial cell loss at 20 to 21 days of preservation; the endothelium of one cornea exposed to 0.1% trypan blue had already been destroyed at day 9.

Analysis of variance outcomes showed a significant interaction of the within-subject factor (trypan blue vs control) and the between-subject factor (concentration) (P<.001), as well as separate significant main effects of the factor “concentration” (P<.001) and the factor “trypan blue vs control” (P<.001). These results indicate a significant concentration-dependent effect of trypan blue on ECD when experimental corneas were compared with their fellow controls. Estimated effects by this model of the interaction (concentration–trypan blue vs control) on ECD loss, with 95% confidence limits, were 60 cells/mm² for trypan blue concentration of 0.001% (P=.57), 93 cells/mm² for 0.005% trypan blue (P=.38), 2078 cells/mm² for 0.01% trypan blue (P<.001), and 1894 cells/mm² for 0.1% trypan blue (P<.001) (Table 2).
On both observation points (days 9-10 and days 20-21), the Wilcoxon signed rank test showed a significant effect of trypan blue (all concentrations together) on endothelial cell morphologic characteristics ($P = .03$ and $P = .02$, respectively) as compared with the endothelial cell morphology scores of all control corneas.

### ENDOTHELIAL VIABILITY BY MORPHOLOGIC SCORES AFTER APPLICATION OF TRYPAN BLUE

Endothelial morphology assessment and change scores are summarized in **Table 3**. Cronbach $\alpha$, a coefficient for interobserver reliability, was 0.942; this confirms strongly the validity of the grading scales.

Morphologic change scores were normally distributed. Unpaired, 2-tailed $t$ test showed no significant difference ($P = .33$) between morphologic change scores in the corneas in the control group ($n=8$) and the corneas in all the other groups together ($n=29$), indicating an overall detrimental effect on the endothelium of manipulation of the donor corneas by merely performing this experiment. A linear regression model was constructed of the morphologic change values for the 0.1% trypan blue concentration. The overall contribution of exposure time was not significant ($P = .18$); however, comparison of the different exposure times showed the following: controls vs 5 minutes, $P = .88$; 5 minutes vs 10 minutes, $P = .92$; controls vs 30 minutes, $P = .08$; 5 minutes vs 30 minutes, $P = .07$; and 10 minutes vs 30 minutes, $P = .06$. This is an indication that exposure to 0.1% trypan blue for 30 minutes may cause additional deterioration of endothelial morphologic features.

### Figure 1.
Examples of endothelial morphometric assessment by grading scales. A, Mean product score of 11.2: score 4 (<5% dead cells) $\times$ score 2.8 (completely normal morphologic characteristics according to 4 of 5 observers). B, Mean product score of 6.4: score 4 (<5% dead cells) $\times$ score 1.6 (abnormal to dubious morphologic characteristics, poor swelling [star], and Descemet fold [arrowheads]). C, Mean product score of 6.2: score 2.6 (5%-25% dead cells [white arrowheads], over Descemet folds and diffusely) $\times$ score 2.4 (Descemet fold [black arrowheads], relatively normal morphologic characteristics in areas without dead cells). D, Mean product score of 2.6: score 1.6 (±25% dead cells [white arrowheads], diffusely and over Descemet fold) $\times$ score 1.6 (abnormal morphologic features: poor swelling [stars] and Descemet fold [black arrowheads]).
The average morphologic change score of the group corneas exposed to 0.01% trypan blue appears to be lower than those of the groups exposed to 0.1% trypan blue (Table 3).

**COMMENT**

The results of our 3 experiments, in which different methods were applied to assess toxic effects of trypan blue for corneal cells, endothelial cells in particular, demonstrated the following effects: (1) a concentration-related effect of trypan blue on corneal cell viability; (2) an effect of exposure time to trypan blue on cell survival; (3) an effect of the vehicle on the degree of toxicity exhibited by trypan blue; and (4) damaging effects of (experimental) manipulation of cells or endothelia.

Each of the methods used in the 3 different experiments has its advantages and disadvantages. The first experiment assessing the cytotoxicity of trypan blue for corneal fibroblasts used an objective, quantitative evaluation method and homogeneous material of good quality, and had closely controlled experimental circumstances. However, no endothelial cells but human fibroblast cell cultures were used, and therefore this was not a true assessment of the toxicity of trypan blue on human corneal endothelium in situ. The second, paired experiment met this latter criterion. Furthermore, its design, using organ culture preservation as a controlled condition "stress" test for endothelial cell survival on paired experimental vs control endothelia, provided for a powerful quantitative assessment of long-term endothelial cell viability after exposure to trypan blue, obviating the need for large amounts of tissue. On the downside, relatively subjective assessment methods were needed to obtain quantitative measures, a dose-response relationship was much more difficult to evaluate, and effects of exposure time were not examined. In the third experiment, several short exposure times were studied, and the design, application of trypan blue to donor cornea endothelium, reflected the actual situation in eye banks rather well. However, statistical evaluation suffered from lack of power due to relatively small numbers of corneas per treatment group, because of restricted supply of suitable donor material. Second, the assessment of endothelial morphometrics was subjective; however, the use of 5 independent masked experienced observers and standardized grading scales that provided for a very high interobserver reproducibility both improved on the reproducibility of this semiquantitative assessment. Third, the surprising outcome with exposure for 10 minutes to 0.01% trypan blue (Table 3) indicates a limitation of the use of morphometric measures on viable endothelium. When the osmolarity of the solutions tested differs, eg, by the use of a different concentration of trypan blue in PBS, as was the case in this experiment, the endothelium may become temporarily edematous and more vulnerable to a subsequent swelling of the intercellular space necessary to visualize living endothelial cell borders.

**Figure 2.** Cytotoxicity indices of human corneal fibroblast cultures after exposure to trypan blue. A, Eagle modified minimum essential medium vehicle. B, Phosphate-buffered saline vehicle. The gray bars indicate significantly toxic cytotoxicity indices (cutoff point, 20%). Bars with black tops indicate indices of less than 0%.

**Figure 3.** Example of a sigmoid concentration-response curve of human corneal fibroblasts exposed to trypan blue (in this case, 18-hour exposure in Eagle modified minimum essential medium [EMEM] vehicle [dashed curve] and phosphate-buffered saline [PBS] vehicle [solid curve]). The x-axis uses a logarithmic scale.
fore, concentration-dependent effects were not further tested in this experiment. Finally, preexperimental status of the endothelium was rather heterogeneous because of the selection criteria and preexperimental conditions. This was compensated for by carefully evaluating preexperiment morphometric status and discarding morphologically clearly abnormal endothelia before the experiment started. This preexperimental evaluation appeared to have succeeded, for Table 3 shows that the preexperiment endothelial assessment scores were quite good, and similar in all experimental and control groups.

Although each of the experiments may have had its own flaws, each showed consistent results that furthermore were complementary to and in concordance with those from the other experiments. This study demonstrated a consistent relationship between concentration, exposure time, vehicle of trypan blue, and/or application circumstances. Under favorable conditions (the second experiment, and EMEM in the first experiment), 24 hours of exposure to a trypan blue concentration of 0.005% was found to be a threshold at and below which there was no significant toxicity. Under unfavorable conditions (PBS in the first experiment, and the repeated manipulation in the third experiment), the toxicity threshold was found at an exposure to 0.1% trypan blue for 30 minutes; this threshold shifted toward longer exposure times as concentrations decreased and vice versa.

A recent experimental study confirms concentration-related toxicity and the existence of a threshold of toxicity related to trypan blue application. Interest had arisen in the application of trypan blue as a surgical tool to facilitate visualization in epiretinal membrane surgery. Trypan blue application in a 0.06% concentration in the vitreous cavity of rabbit eyes for up to 4 weeks did not cause any recognizable damage to the rabbit retina. In contrast, the application of 0.2% trypan blue caused considerable damage to retinal cells and architecture in areas that were more exposed to the dye. It is, however, not feasible to compare in more detail the results of this study with our own findings on endothelial cells because of the different nature of the cells of interest, the different circumstances under which the exposure took place (in vivo vs in vitro), the far longer exposure times used, and the different techniques applied for the determination of toxicity.

The results in our study seem to be in line with the literature and with experience with the dye in eye banks. Sperling and Stocker et al stained the endothelium of human donor corneas briefly (1 and 1.5 minutes, respectively) with 0.3% and with 0.25% to 0.5% trypan blue solutions, respectively. Both authors found no indications for endothelial cell loss caused by the staining. For many years, the Cornea Bank NORI applied 0.3% trypan blue to the endothelium for 30 to 60 seconds for routine donor evaluation. In 1998, colorimetric investigations of a new stock of trypan blue disclosed higher extinction values of the 0.3% solutions than before; the concentration of trypan blue solution for donor cornea evaluation was then changed to 0.12% to use a solution with the same extinction value as before. For almost 20 years and more than 14000 transplanted corneas, adverse effects to the endothelium have not been observed with application of 0.3% or 0.12% trypan blue solutions (E.P., unpublished results, 2000). Our present experimental findings may be considered to be congruous with our experience with trypan blue in the Cornea Bank NORI and an extrapolation of the findings in the older studies. Although the reported concentrations of trypan blue were often higher than those used in our experiments, endo-

### Table 1. LC50 and Maximum Toxicity Levels, as Calculated With the Sigmoidal Fit Program, for Fibroblasts Exposed to Trypan Blue

<table>
<thead>
<tr>
<th>Exposure Time</th>
<th>LC50, Mass %</th>
<th>Maximum Toxicity Level (CI), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMEM</td>
<td>PBS</td>
</tr>
<tr>
<td>15 min</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>30 min</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>60 min</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>3 h</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>6 h</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>12 h</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>18 h</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>24 h</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Abbreviations: CI, cytotoxicity index; EMEM, Eagle modified minimum essential medium; LC50, 50% lethal concentration; PBS, phosphate-buffered saline.

### Table 2. ECD Loss and Morphologic Scores of Donor Corneal Pairs, Exposure to Trypan Blue for 24 Hours vs Controls

<table>
<thead>
<tr>
<th>Trypan Blue Concentration, %</th>
<th>Before Experiment</th>
<th>Days 9-10</th>
<th>Days 20-21</th>
<th>Estimated Effects on ECD Loss*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ECD, Mean MS</td>
<td>Mean ECD, Mean MS</td>
<td>Mean ECD, Mean MS</td>
<td>Estimated Effects on ECD Loss*</td>
</tr>
<tr>
<td>0.1</td>
<td>2200 2.5</td>
<td>1100 0.5</td>
<td>0 0</td>
<td>1894 cells/mm², P &lt; .001</td>
</tr>
<tr>
<td>Control (n = 2)</td>
<td>2200 2.5</td>
<td>2200 2.3</td>
<td>2150 1.8</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>2600 2.3</td>
<td>2150 1.3</td>
<td>0 0</td>
<td>2078 cells/mm², P &lt; .001</td>
</tr>
<tr>
<td>Control (n = 2)</td>
<td>2600 2.3</td>
<td>2550 2.8</td>
<td>2600 2.3</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>2300 2.5</td>
<td>2280 2.3</td>
<td>2080 1.5</td>
<td>93 cells/mm², P = .38</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>2280 2.5</td>
<td>2280 2.3</td>
<td>2060 2.0</td>
<td></td>
</tr>
<tr>
<td>0.001</td>
<td>2560 2.5</td>
<td>2460 2.7</td>
<td>2300 2.3</td>
<td>60 cells/mm², P = .57</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>2500 2.5</td>
<td>2440 3.0</td>
<td>2400 2.9</td>
<td></td>
</tr>
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</table>

Abbreviations: ECD, endothelial cell density; MS, morphologic score.
*Calculated by analysis of variance, compared with controls.
thelia were exposed for shorter times, which probably prevented possible toxic effects from taking place.

Observed toxicity limits in this study allow the intraoperative application of trypan blue. In lens capsule staining with trypan blue during cataract surgery, a concentration of 0.06% is used to obtain adequate visualization of the lens capsule in the absence of a red fundus reflex. The dye is applied to the anterior lens capsule under an air bubble in the anterior chamber, which prevents dilution of the trypan blue by aqueous before staining the capsule and also impairs to some extent direct contact of the dye with the endothelium. Immediately after staining, the excess dye is washed out of the anterior chamber by continuous washout effect through aqueous before staining the capsule and also impairs to some extent direct contact of the dye with the endothelium. Immediately after staining, the excess dye is washed out of the anterior chamber by co-

miously irrigation, after which a viscoelastic substance is injected. The dye on the anterior lens capsule is partly removed with the anterior lens capsule after the capsulorrhexis is completed. The remaining dye is partly washed out with irrigation during the operative procedure, and remaining traces are thought to leave the anterior chamber by the aqueous route. The dye cannot be observed anymore on the first postoperative day. Thus, an initial concentration of 0.06% trypan blue may partly contact the endothelium for a few seconds. Thereafter, only diluted and steadily decreasing concentrations of trypan blue are thought to be present within the anterior chamber because of a continuous washout effect through aqueous flow and resorption. The results of our in vitro study support the view that concentrations of trypan blue in the anterior chamber after anterior lens capsule staining with trypan blue in cataract surgery do not reach a toxic level. Confirmation of safety for the corneal endothelium of trypan blue capsule staining was published recently in a prospective, randomized clinical trial on endothelial cell loss. At 1 year postoperatively, no deleterious effects could be observed, either on corneal ECD or on endothelial morphometric measures. Submitted for publication February 3, 2003; final revision received October 22, 2003; accepted November 21, 2003.

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Table 3. Mean ± SD Endothelial Morphology Assessment Scores After Direct Exposure of Donor Corneal Endothelia to Trypan Blue

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 8)</th>
<th>30 min (n = 7)</th>
<th>10 min (n = 9)</th>
<th>5 min (n = 7)</th>
<th>10 min (n = 6)</th>
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<tr>
<td><strong>Before experiment</strong></td>
<td>9.5 ± 2.6</td>
<td>9.6 ± 2.2</td>
<td>9.5 ± 2.3</td>
<td>9.5 ± 1.4</td>
<td>9.4 ± 1.5</td>
</tr>
<tr>
<td><strong>After 2-h incubation in EMEM</strong></td>
<td>6.7 ± 3.3</td>
<td>4.7 ± 2.5</td>
<td>6.7 ± 2.9</td>
<td>6.9 ± 3.2</td>
<td>5.3 ± 2.8</td>
</tr>
<tr>
<td><strong>Morphologic change score</strong></td>
<td>-2.7 ± 2.3</td>
<td>-4.8 ± 2.4</td>
<td>-2.7 ± 2.1</td>
<td>-2.6 ± 2.1</td>
<td>-5.1 ± 1.9</td>
</tr>
</tbody>
</table>

Abbreviations: EMEM, Eagle modified minimum essential medium; TB, trypan blue.

*From the linear regression model: 30 minutes vs controls, P = .08; 30 minutes vs 10 minutes (0.1% TB), P = .06; and 30 minutes vs 5 minutes, P = .07.

REFERENCES


