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Effects of Trypan Blue on Corneal Endothelium and Anterior Lens Capsule in Albino Wistar Rats: An Investigator-Masked, Controlled, Two-Period, Experimental Study

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ABSTRACT

Background: The development of continuous curvilinear capsulorrhexis (CCC) has contributed significantly to the tolerability and effectiveness of cataract extraction and intraocular lens implantation. Staining of the anterior capsule has become a popular method of increasing visibility when performing CCC.

Objective: The aim of this study was to determine, using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the highest concentrations of trypan blue dye that would not cause long-term toxicity after injection into the anterior chamber of rat eyes.

Methods: The eyes of healthy female albino Wistar rats were used in this investigator-masked, controlled, 2-period, experimental study conducted over 12 weeks at the Dicle University Experimental Animal Laboratory, Diyarbakır, Turkey. The rats were randomly divided into 5 groups of 4 using a random-number table. Each rat was administered a 0.05-mL injection of trypan blue into the right eye in 1 of the following concentrations: 0.4%, 0.2%, 0.1%, 0.05%, or 0.025%. A 0.05-mL pH-balanced saline solution was injected into the left eye of each rat to act as a control. At 1 day after injection and 4 weeks after injection (early period), 1 rat from each concentration group was euthanized and their eyes were enucleated. At 12 weeks after injection (late period) the remaining 2 rats from each group were euthanized and their eyes were enucleated. Corneal endothelial cells and the anterior lens capsule of the enucleated eyes were analyzed using SEM and TEM, and the results were compared with those of the control group. In the TEM analysis, the primary end point was the histopathologic changes in the cellular organelles when compared with those in the control group. In the SEM analysis, the primary end point

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was changes in cell shape, intracellular junctions, and density of the microvilli when compared with those in the control group.

Results: Forty eyes from 20 albino Wistar rats (mean [SD] age, 8.2 [1.6] weeks; mean [SD] weight, 175.6 [16.5] g) were used in the study. Each group of rats received a different concentration of trypan blue in the right eye. In the early period, both the 0.4% and 0.2% concentrations were associated with the impairment of the hexagonal structure of corneal endothelial cells and intercellular junctions. Those concentrations were also associated with an increased occurrence of cellular vacuolation, cytoplasmic edema, extensive granulation of the endoplasmic reticulum, pyknotic nuclei, and mitochondrial degeneration. In the late period, these changes were observed as persisting in a decreasing manner. With the 0.1% and 0.05% concentrations, the density of microvilli decreased, nuclei appeared normal, granulation of the endoplasmic reticulum and Golgi apparatus was active, and minimal levels of mitochondrial degeneration were observed.

Conclusions: In this small experimental study in rat eyes, trypan blue at concentrations >0.025% was associated with impaired morphology and structure of corneal endothelial cells after short-term exposure. This effect continued in a decreasing fashion after long-term exposure. No significant changes were noted in the control group or the group administered the 0.025% concentration. (*Curr Ther Res Clin Exp.* 2006;67:366–377)

Key words: trypan blue, corneal endothelium, lens epithelium, capsulorrhexis.

INTRODUCTION

Continuous curvilinear capsulorrhexis (CCC) has contributed significantly to the tolerability and effectiveness of cataract extraction and intraocular lens implantation.[1] This surgical technique produces a strong rim on the anterior capsule that resists tearing, even when stretched during crystalline lens removal or intraocular lens implantation.[1,2] It also ensures reliable intraocular lens centration in the capsular bag. However, accurate capsulorrhexis might be difficult to achieve, particularly in eyes with mature, intumescent cataracts. In those cases, the surgeon may have difficulty seeing the advancing edge of the capsulotomy because of the absence of red reflex when retroilluminated.[3] Poor visualization of the capsule may result in an inadequate CCC; high risk for radial tears toward or beyond the lens equator; and complications such as zonular and posterior capsule tears, vitreous loss, and intraocular lens decentration.[1,4]

Staining of the anterior capsule has become a popular method of increasing visibility when performing CCC. Many staining agents have been used to date, including autologous blood and a variety of dyes[5] (eg, indocyanine green,[6] gentian violet,[7] methylene blue,[8] subcapsular fluorescein, and trypan blue).

Although many pigments have been used to enhance visualization during cataract surgery, complications have been found with the use of some of these dyes. Corneal edema has been reported with 0.1% gentian violet and 1% methylene blue.[9,10] Because fluorescein is administered under the capsule, it stains the cortex as well as the capsule. Thus, the capsule is difficult to distinguish from the cortex, and a blue filter is needed.[11] Nahra and Castilla[12] injected 2% fluorescein into the anterior side of the capsule under air and reported that blue light was not required during surgery. They also reported that fluorescein at that concentration might be toxic to the endothelium. In the study by Melles et al,[9] in 30 human patients with mature cataracts, 0.1 mL of trypan blue 0.1% was found to have no toxic effects after 12 months of follow-up.

Trypan blue is a blue bis-azo dye. The trypan blue molecules are symmetric, consisting of 3 main parts connected by 2 azo bonds. As early as 1967, trypan blue was used to stain the cornea and conjunctiva.[13] It has also been used to examine endothelial cells after cataract surgery and in donor corneal grafts without any documented adverse events.[14]

Bis-azo dyes, such as trypan blue, stain poorly hydrated tissues better than well-hydrated ones.[15] The lens cortex, which consists of lens fibers, has a high water content compared with the acellular basement membrane,[16] which explains why the dye stains the basement membrane without clinically significant staining of the lens cortex. This results in marked color contrast between these 2 structures. The resulting high visibility of the lens capsule allows the surgeon to precisely distinguish it from the underlying cortex during capsulorrhexis for phacoemulsification of the lens.[17] Because the visibility of the anterior capsule is enhanced with trypan blue, this dye is sometimes used when teaching capsulorrhexis to surgeons in training.[18]

Despite changes in staining techniques, trypan blue is the preferred dye in modern cataract surgery.[19] Trypan blue is kept in the anterior chamber for a short time and the chamber is then irrigated before CCC is performed. Thus, in the clinical setting, trypan blue remains in the eye for a short time period. Although trypan blue is reported to be nontoxic to ocular structures over a short period, it is on the list of carcinogenic chemicals in *The Merck Index*.[20] In addition, its long-term effects are unknown, and it should be used carefully in certain groups (eg, pediatric and pregnant patients).[21]

The aim of this study was to determine the highest concentrations of trypan blue dye that would not cause long-term toxicity after injection into the anterior chamber of rat eyes. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to assess histopathologic and morphologic changes in the corneal endothelium and anterior lens capsule.

MATERIALS AND METHODS

Healthy female albino Wistar rats that were free of clinical eye disease were used in this 12-week, investigator-masked, controlled, 2-period, experimental

study. All of the rats were obtained from and housed at the Dicle University Experimental Animal Laboratory, Diyarbakır, Turkey, and assessed before the study by 2 independent ophthalmologists.

All experimental procedures were approved by the Ethics Committee for Experimental Animals at Dicle University and were performed according to the Association for Research in Vision and Ophthalmology Statement[22] for the use of animals in ophthalmic and vision research.

All procedures were performed by the same surgeon (I.Ç.). The rats were randomly assigned using a random-number table to receive trypan blue at 1 of 5 concentrations: 0.4%, 0.2%, 0.1%, 0.05%, or 0.025%. The rats were anesthetized with IM injections of ketamine hydrochloride 30 mg/kg. An operating microscope (OPMI-8, Carl Zeiss, Gottingen, Germany) was positioned over the eye undergoing surgery. The anterior chamber was entered in the superotemporal quadrant through a corneal tunnel using a 28-gauge insulin syringe for dye administration. Each rat was administered a 0.05-mL injection of trypan blue into the right eye at the assigned concentration. A 0.05-mL pH-balanced saline solution (BSS) was inject-ed into the left eye of each rat to act as a control.

At 1 day after injection and 4 weeks after injection (early period), 1 rat from each concentration group was euthanized and their eyes were enucleated. At 12 weeks after injection (late period), the remaining 2 rats from each group were euthanized and their eyes enucleated.

Corneas were completely removed with the aid of an operating microscope. Corneal buttons were cut into 2 equal halves. One half was prepared for SEM analysis and the other half was prepared for TEM analysis. The anterior lens capsule was removed using the CCC method. Histologic changes in the anterior lens capsule were assessed using SEM (LEO 440, Leica Zeiss, Köln, Germany) and TEM (JEOL 1010, Jeol Ltd., Tokyo, Japan).

In the TEM analysis, the histopathologic changes in the cellular organelles were compared with those in the control group. In the SEM analysis, changes in cell shape, intracellular junctions, and density of the microvilli were also compared with those in the control group.

In regard to the group to which the eyes were assigned, investigators performing SEM and TEM analysis were masked.

RESULTS

Twenty albino Wistar rats were included in the study (mean [SD] age, 8.2 [1.6] weeks; mean [SD] weight, 175.6 [16.5] g) (4 rats per study group).

SEM Analysis

Morphologic analysis, in the early period, with SEM revealed that with trypan blue 0.4% and, to a lesser degree, 0.2%, the hexagonal structure of corneal endothelial cells and the intercellular junctions were impaired and microvilli and cellular surfaces were destroyed, whereas in the control group they were

not (**Figures 1** and **2**). In the late period, in the same 2 groups, the hexagonal structure of endothelial cells had been restored and the intercellular junctions and regenerated microvilli were visible in some places. In the early period, in the eyes treated with 0.1% and 0.05% concentrations, normal hexagonal structure of the endothelial cells and intercellular junctions was observed; however, the density of the microvilli was decreased. In the late period, compared with the control group, persistence of the decreased density of the microvilli was observed (**Figure 3**). No significant difference with respect to endothelial cells or density of microvilli was observed in the eyes treated with trypan blue 0.025% compared with those in the control group.

Early-period morphologic analysis of the lens epithelium using SEM revealed loss of cellular borders and microvilli, and degeneration of epithelial cells with protruded nuclei in the 0.4%, 0.2%, and 0.1% groups, with the changes being most noticeable in the 0.4% group (**Figure 4**). In the late period, in the 0.2%- and 0.1%-concentration groups, microvilli were visible in some places. In the early period, in the 0.05%- and 0.025%-concentration groups, cellular borders and microvilli appeared normal, while porous and cytoplasmic constrictions were found in some epithelial cell membranes. These effects were less evident in the late period.

TEM Analysis

Ultrastructural analysis with TEM revealed pyknotic corneal endothelial cell nuclei in the trypan blue 0.4% and 0.2% groups but not in the control group. Degeneration of cellular organelles, cellular vacuolation, intracytoplasmic edema, granulation of endoplasmic reticulum, and mitochondrial degeneration were also noticeable in the 0.4% and 0.2% groups (**Figures 5** and **6**). In the late period, cells were further flattened, nuclei were euchromatic, cellular vacuolation and mitochondrial degeneration had decreased, and intercellular side junctions were normal. In the early and late periods in the groups administered trypan blue 0.1% and 0.05%, the nuclei appeared normal, granulated endoplasmic reticulum and Golgi were active, and there were minimal levels of mitochondrial degeneration (**Figure 7**).

No difference in either the early or late period was found in the 0.025%-concentration group when compared with the control group.

DISCUSSION

The results of this study suggest that intraocular use of high concentrations of trypan blue are associated with irreversible toxic effects in both the short term and the long term. Toxic effects occurring in the short term with the 0.1% and 0.05% concentrations were found to have decreased in the long term. The reason for this is thought to be that intraocular fluid production might decrease the drug concentration and the drug is flushed from the eye via drainage by the Schlemm channel.[23] This study also suggests that the 0.025% concentration of trypan blue is not associated with any toxic effects in the short or long term.

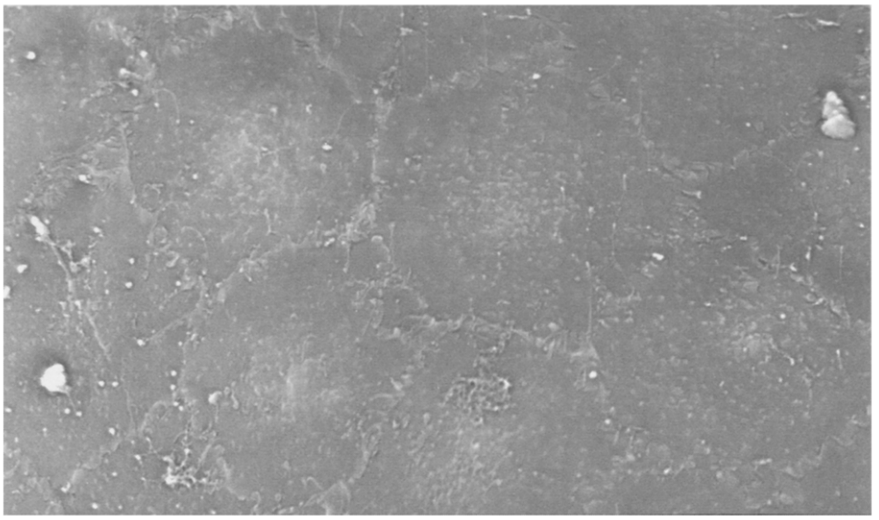

Figure 1. Appearance of corneal endothelial cells in the control group using scanning electron microscopy 1 day after injection of 0.05 mL of pH-balanced saline solution into the anterior chamber of a rat's eye (the hexagonal structure of the cells and intercellular junctions are normal and microvilli on the cell surface are visible). (Magnification rate: Original magnification × 2250. Scale: 1 inch = 10 μm.)

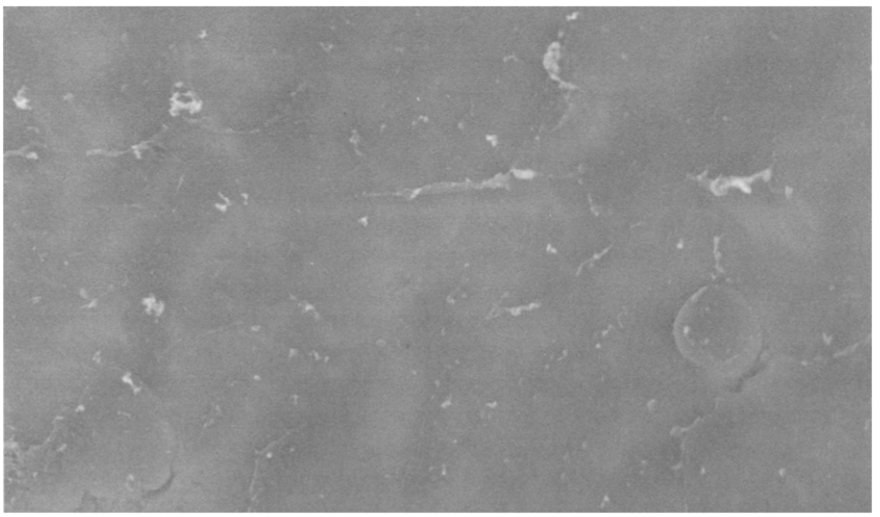

Figure 2. Appearance of corneal endothelial cells using scanning electron microscopy 1 day after injection of 0.05 mL of trypan blue 0.4% into the anterior chamber of a rat's eye (the hexagonal structure of the cells, the intercellular junctions, and the microvilli on the cell surface are not visible). (Magnification rate: Original magnification × 2500. Scale: 1 inch = 10 μm.)

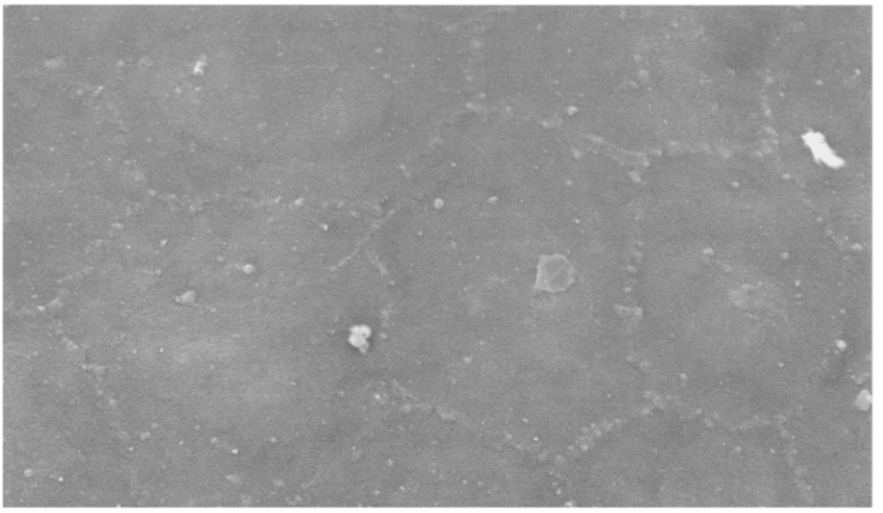

Figure 3. Appearance of corneal endothelial cells using scanning electron microscopy 12 weeks after injection of 0.05 mL of trypan blue 0.1% into the anterior chamber of a rat's eye (the hexagonal structure of the cells and intercellular junctions are visible, but microvillar intensity on the cell surface is decreased). (Magnification rate: Original magnification × 2250. Scale: 1 inch = 10 μm.)

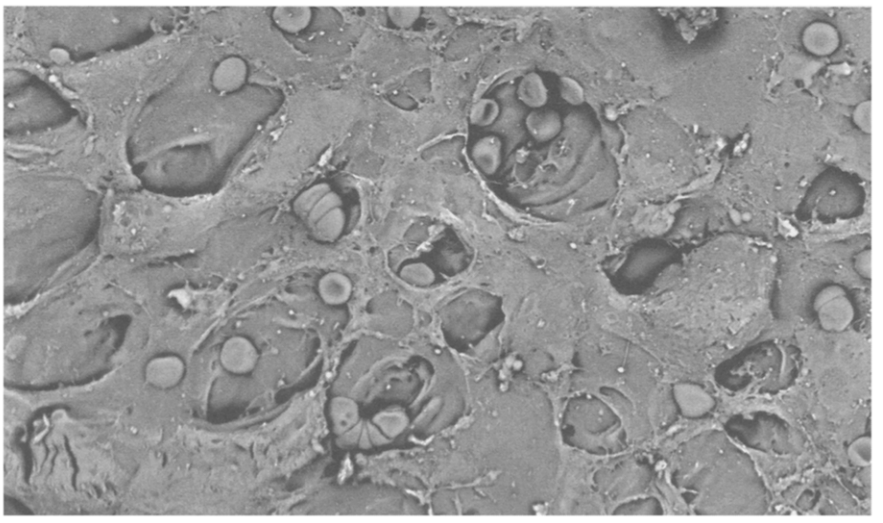

Figure 4. Appearance of lens epithelium using scanning electron microscopy 1 day after injection of 0.05 mL of trypan blue 0.4% into the anterior chamber of a rat's eye (loss of lens epithelial cell borders and microvilli and degenerated epithelial cells with protruded nuclei are seen). (Magnification rate: Original magnification × 1000. Scale: 0.5 inch = 10 μm.)

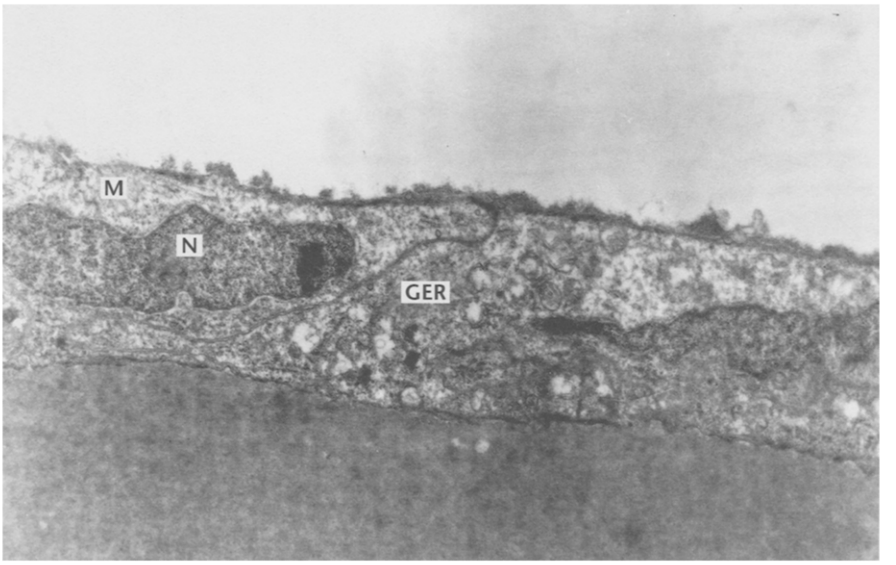

Figure 5. Appearance of mitochondria (M), granulated endoplasmic reticulum (GER), and nuclei (N) of the corneal endothelium in the control group using transmission electron microscopy 1 day after injection of 0.05 mL of pH-balanced saline solution into the anterior chamber of a rat's eye. (Uranyl acetate and lead citrate, original magnification × 4400.)

The most sensitive methods for determining changes that occur at the cellular level are SEM and TEM.[24] We believed that using SEM and TEM to analyze cellular structural changes associated with the use of trypan blue dye might improve the reliability of the study results.

Van Dooren et al[25] used specular microscopy to analyze corneal endothelial cells of patients after phacoemulsification. The patients were divided into 2 groups, 1 of which was administered 0.06% trypan blue and the other was not. They reported that no difference in toxic effects of the corneal endothelium was observed between the 2 groups and, therefore, 0.06% trypan blue was not associated with any toxic effects on corneal endothelium.

In our study, trypan blue was left in the eye for up to 12 weeks to determine whether long-term exposure to the dye was toxic to intraocular tissues. To our knowledge (Web of Science search; all years, all languages; key terms: *trypan blue*, *corneal endothelium*, *SEM*, and *TEM*), this is the first report in which trypan blue was injected into the anterior chamber and the corneal endothelium was then assessed using SEM and TEM.

In a study by Jongebloed et al,[26] lens capsules from patients of advanced age, obtained after extracapsular cataract surgery, were prepared for a combined light microscopy, TEM, and SEM investigation. Using SEM, they were able to observe protruded nuclei in degenerated lens capsule epithelium compared

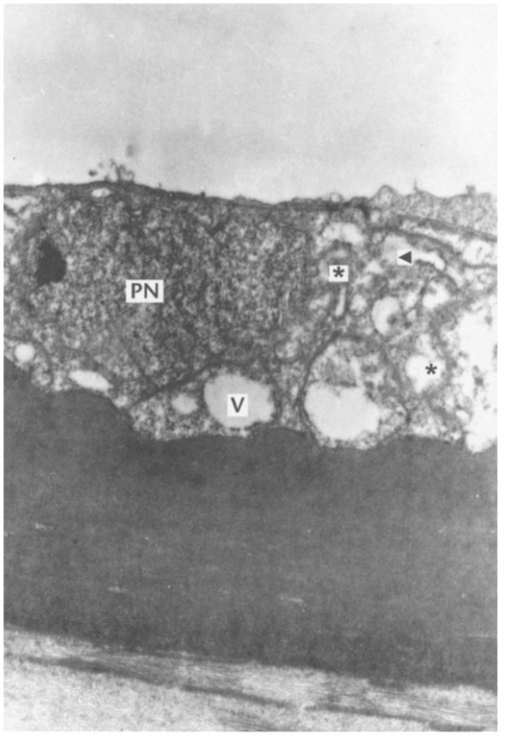

Figure 6. Appearance of corneal endothelial cells using transmission electron microscopy 1 day after injection of trypan blue 0.4% into the anterior chamber of a rat's eye (large and small vacuoles [V], extensive granulation of endoplasmic reticulum [◀], pyknotic nuclei [PN], and common degeneration in mitochondria [*] were observed). (Uranyl acetate and lead citrate, original magnification × 4400.)

with healthy lens capsule epithelium (obtained from clear lens surgery patients at the same facility) and determined that some nuclei were completely detached from the cytoplasm. In our study, loss of epithelial cell borders and microvilli and degenerated epithelial cells with protruded nuclei were most evident in the 0.4% group, and were also observed in the 0.2% and 0.1% groups. Porous epithelial cell membranes and cytoplasmic constriction were observed in the 0.05% and 0.025% groups.

Washing the anterior chamber with BSS soon after injecting trypan blue after protecting the corneal endothelium with sterile air has been associated with a reduction in toxic effects in human eyes.[19] Because the aim of our study was to determine the highest concentration of trypan blue that would not be toxic for ocular use, intraocular air administration and irrigation of the trypan blue dye were not performed. Future studies might use the procedures and results from this study to perform a longer-term comparison of changes.

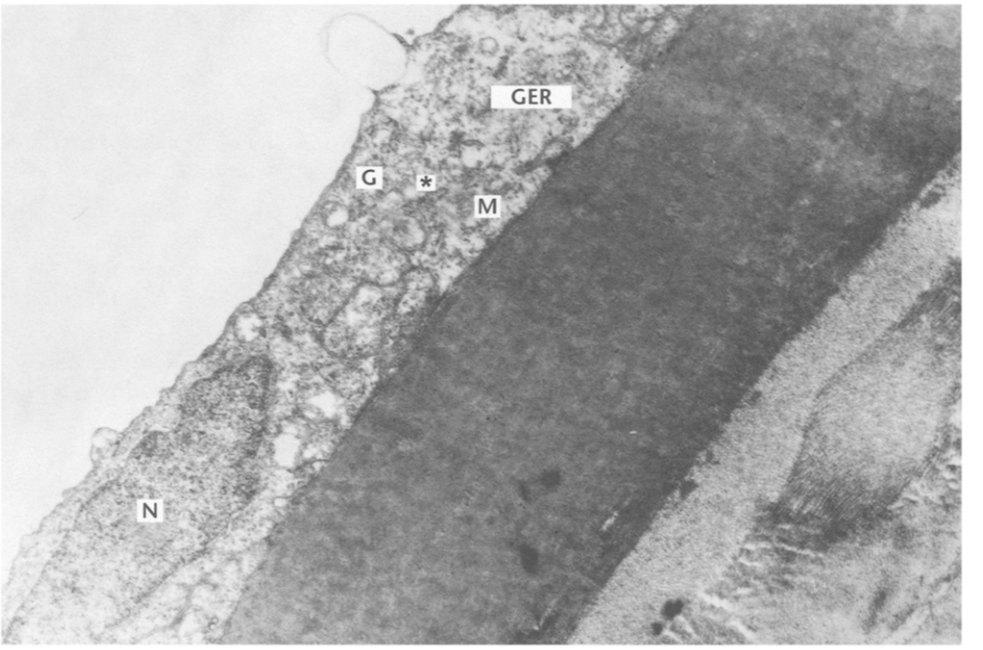

Figure 7. Appearance of corneal endothelial cells using transmission electron microscopy 12 weeks after injection of trypan blue 0.1% into the anterior chamber of a rat's eye (mitochondria [M] and nuclei [N] appeared normal, granulated endoplasmic reticulum [GER] and Golgi [G] were active, and mitochondrial degeneration [*] was observed). (Uranyl acetate and lead citrate, original magnification × 4400.)

CONCLUSIONS

In this small experimental study in rat eyes, trypan blue at concentrations >0.025% was associated with impaired morphology and structure of corneal endothelial cells after short-term exposure. This effect continued in a decreasing fashion after long-term exposure. No significant changes were noted in the control group or the group administered the 0.025% concentration. The use of SEM and TEM in the analysis process was instrumental to observing all changes on the cellular level.

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