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Original Article

Effect of intraoperative trypan blue on lens epithelial cells – Histomorphological analysis

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Abstract

Introduction: Trypan Blue is an acid azo dye commonly used as a stain to distinguish viable from non-viable cells. It is a vital stain used intra operatively during cataract surgery to stain the external surface of the anterior lens capsule for better visualization.
Aim: To analyze the histomorphological effects of trypan blue on Lens Epithelial cells and the Basement Membrane on direct exposure by staining the internal surface of the anterior lens capsule during Small Incision Cataract Surgery.
Methods: Analytical cross sectional case control study. Anterior capsule specimens of 14 Patients undergoing small incision cataract surgery at Department of Ophthalmology, Govt Medical College Hospital, Thrissur were studied. Two specimens of anterior capsule taken from the same eye form the case and control. Control specimen **(sample A)** was removed first, after the routine *external* staining with trypan blue 0.06% (w/v) for 10 seconds. The stain was washed off by balanced salt solution in every case. Then trypan blue was injected under the remaining anterior capsule and case (Test) specimen (**sample B)** was obtained after direct contact of trypan blue to the internal surface (lens epithelial cells) for 1 minute. Histomorphological (qualitative and quantitative) examination of both specimens done.
Results: **Qualitative data** analysis was done by EPI INFO software.*v*.7. Intactness of LECs throughout the length was statistically significant in Sample A ($p = 0.000027$). Partial and complete detachment of Lens Epithelial Cells, degeneration, and nuclear smudging were significantly higher in Sample B. Qualitative analysis of the basement membrane showed significant edema of the basement membrane in sample B. Basement membrane splitting observed in sample B was not statistically significant. **Quantitative data** analyzed using independent *t* test. There was a statistically significant decrease in cell density in sample B with *p* value less than 0.05.
Discussion: Our study demonstrated that direct staining of the internal surface of anterior capsule with trypan blue affected LECs and the basement membrane. There were reduction in cell density, irreversible degeneration of Lens Epithelial Cells and basement membrane edema. Hence treating the internal surface of capsular bag with trypan blue may reduce incidence of Posterior capsular opacification.

Keywords: Lens capsule, Lens epithelial cells, Trypan blue

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Introduction

Trypan Blue is an acid azo dye commonly used as a stain to distinguish viable from non-viable cells.[1] Use of Trypan blue in Ophthalmology dates back to the 1970s, when it was used to stain the corneal endothelium preoperatively.[2] It emerged in the late 1990s as one of the known staining material used intra operatively for visualization of the anterior lens capsule during cataract surgery.[3] FDA approved staining of anterior lens capsule with Trypan blue in 2004(NDA 21-670). The routine method is staining the external surface of anterior capsule for 10 seconds for better visualization during capsulorrhexis and for capsule-cortex differentiation during cortical aspiration.

The lens epithelium is a single layer of cuboidal cells located on inner surface of the anterior capsule. These cells are not present in the posterior capsule. Lens epithelial cells are responsible for production of lens fibres. The cells show morphological variations according to the location. The centrally located are polygonal, in the pre equatorial zone cuboidal, at the equator, they are columnar. These cells serve as progenitor for new lens fibres by a mechanism of epithelial mesenchymal transition. Posterior capsular opacification (PCO) usually develops from the lens epithelial cells that remain on the lens capsule after cataract surgery. Direct exposure in vivo by staining the internal surface of the anterior lens capsule by trypan blue during Small Incision Cataract Surgery (SICS) is found to destroy the lens epithelial cells in various studies.

In this study we are analyzing the histomorphological (qualitative) and quantitative effects of trypan blue on Lens Epithelial Cells (LECs) on direct exposure in vivo by staining the internal surface of the anterior lens capsule during Small Incision Cataract Surgery (SICS).

Methodology

This was an analytical cross sectional case control study done in the Department of Ophthalmology and Department of Pathology, Govt. Medical College, Thrissur, for a period of 3 months after getting ethical committee clearance.

Subjects

Patients undergoing small incision cataract surgery for congenital cataract, pre senile cataract and immature senile cataract were included in this study after informed consent. Cases of lens induced glaucoma, hyper mature cataracts, complicated cataracts, traumatic cataracts and subluxated cataracts were excluded. Inadequate samples for histomorphological analysis were also excluded from the study. Sample size was calculated using the formula for analytical comparative study.

Sample size calculated by using the formula

$$n = \frac{(Z\alpha + Z\beta)^2 \times 2 \times SD^2}{d^2}$$

$Z\alpha$ = Alpha error at 5% level
Value is 1.96
$Z\beta$ = Beta error at 10%
Value is 1.284
SD = (SD of mean cell density in 2 groups)
d = Mean difference (mean difference of same- mean cell density in 2 groups)

By this formula the sample size calculated was 8.

This was based on similar studies by Nanavati et al (Ref. 7) & Mathan et al (Ref. 9).

Anterior capsule specimens (case and control from the same patient) of 14 patients were studied. 4 cases were congenital cataract, 4 cases were presenile cataract and 6 cases were immature senile cataract.

Specimen collection

During SICS, after routine external staining of the anterior lens capsule with Trypan blue 0.06%(w/v) for 10 seconds, continuous curvilinear capsulorrhexis (CCC) was initiated using double bend 26 G needle and continued halfway and that half of anterior capsule was removed by cutting with Vannas scissors. This was kept in a formalin bottle and labeled as sample A (control). Then, Trypan blue 0.06% (w/v) was injected under the remaining half of anterior lens capsule and the LECs were directly exposed to the dye for 1 min. Capsulorrhexis was completed to remove that half of anterior capsule and was kept in another formalin bottle and labeled as sample B (case/Test). This comparative cross sectional study was conducted between two samples from the same patient's lens capsule, thereby eliminating all the other variables like age and sex of the patient, type of Cataract etc. All surgeries were done by same surgeon using same technique, again minimising other bias. Both specimens were sent for histomorphological (qualitative and quantitative) analysis.

Pathology specimen preparation and parameter observation

The specimens were received in the pathology department as 2 samples from each patient in separate bottles as sample A-Control and sample B-Case/test. Routine processing for histopathological examination were done, embedded in paraffin wax and 5 micron thick sections were stained with Hematoxylin and Eosin stain. Histomorphological observation was done under Leica-DM 750 image analysis microscope. The following observations were made in both control and case samples.

Qualitative data

Histomorphology of lens epithelial cells (LECs) (Figs. 1–5)

1. Intactness of LECs throughout the basement membrane
2. Partial or complete detachment of LECs from basement membrane
3. Intermittent fall off LECs
4. Evidence of degeneration of cells (cytoplasmic vacuolation, cell membrane rupture)
5. Nuclear smudging (loss of character and blurring).

Histomorphology of basement membrane (BM) (Figs. 6–8)

1. Intactness of basement membrane throughout
2. Splitting of basement membrane
3. Edema

Fig. 1A. Sample A-Control (anterior lens capsule after routine external staining with trypan blue for 10sec) H&Ex400.

Fig. 1B. Sample B -Case (anterior lens capsule after internal staining with trypan blue for 1 min) (H&Ex400).

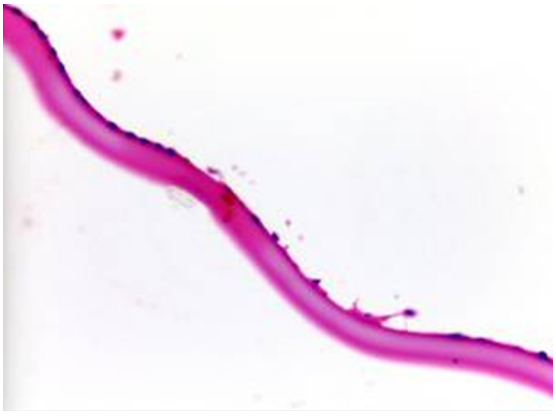

Fig. 2. (Case) show intermittent detachment of LEC (H&Ex400).

Numerical Data was measured by quantitative morphometry with Leica's LASEZ software. Under high power objective (40x) of the microscope, number of cells in the most and least cellular areas were counted in a fixed distance of 100 micrometer length for each sample (A&B) to get an average number of cells, since the number of cells were not uniform throughout the length of specimen B. Thickness of the basement membrane was also measured.

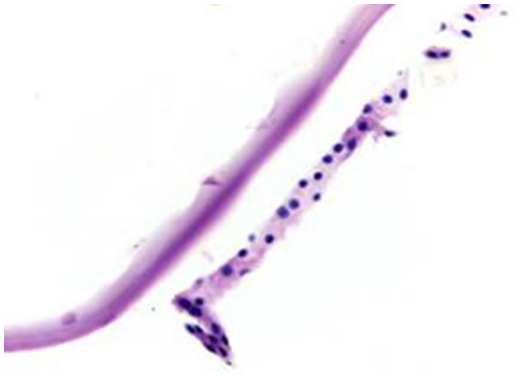

Fig. 3. (Case) Degeneration of LEC with complete detachment from the capsule (H&Ex400).

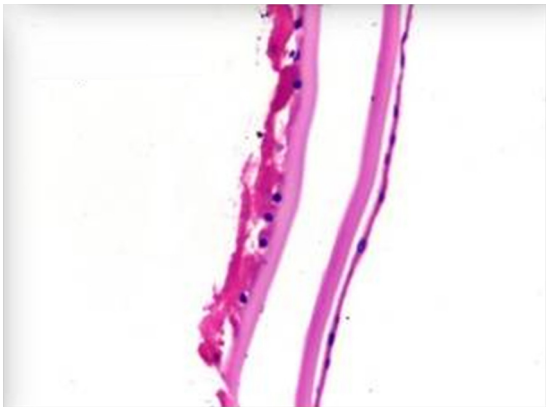

Fig. 4. (Case) show varying stages of LEC degeneration (H&Ex400).

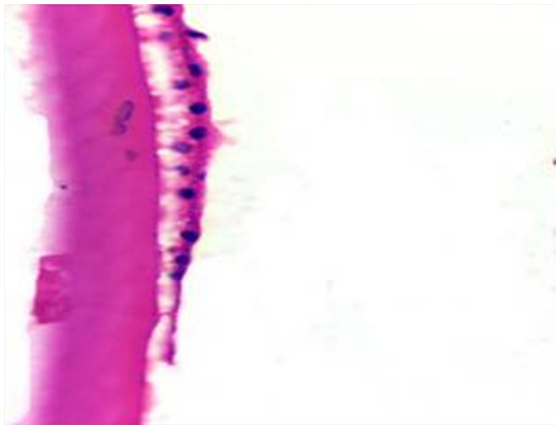

Fig. 5. (Case) show varying stages of LEC degeneration (H&Ex400).

Results

Anterior capsule specimens of 14 patients were studied. Histomorphology of lens epithelial cells, histomorphology of basement membrane and quantitative morphometry were analyzed and compared between Sample A and Sample B.

Qualitative data (histomorphology of lens epithelial cells and basement membrane**)** analysis was done by EPI INFO SOFTWARE version7. Data analyzed using proportions.

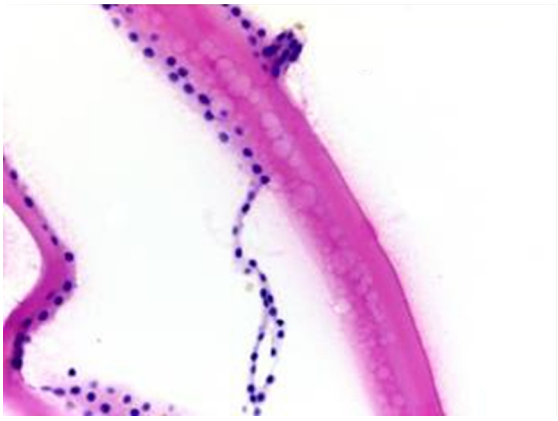

Fig. 6. (Case) show basement membrane oedema (H&Ex400).

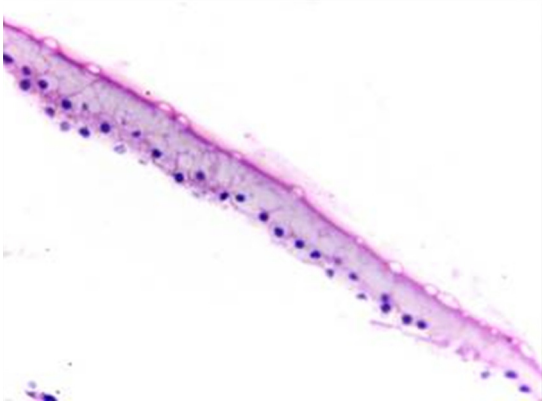

Fig. 7. (Case) show basement membrane oedema (H&Ex400).

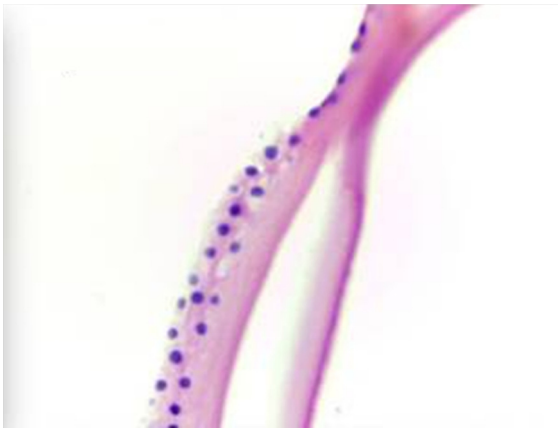

Fig. 8. (Case) Splitting of capsule (basement membrane) (H&Ex400).

Association of different variables was analyzed using X^2 test (Tables 1 and 2).

Qualitative analysis of LECs showed statistically significant intactness of LECs throughout the length of the capsule in the control group **Sample A**. Partial and complete detachment of LECs, degeneration of LECs and nuclear smudging were significantly higher in the case group **Sample B.** There was no statistically significant difference between the groups regarding intermittent fall off of cells.

Qualitative analysis of the basement membrane showed statistically significant edema in Sample B. Basement membrane was intact in most of the samples in both groups. Basement membrane splitting observed in 28.5% of Sample B was not statistically significant.

Quantitative Data: Number of LECs per 100 micrometer length of the capsule and thickness of basement membrane were analyzed using mean and standard deviation. Comparison was analyzed using independent t test. Significant level was kept at 5% Chart 1.

There was statistically significant difference in the number of lens epithelial cells in the most cellular area, number of cells in the least cellular area and average cells per 100 micrometer length of capsule. LECs were significantly less in **Sample B.** The average width of basement membrane was more in sample B. But the difference was not statistically significant (Table 3).

Discussion

Posterior capsular opacification (PCO) popularly known as ''after cataract'', is a major problem following cataract surgery, especially in young patients even with modern microsurgical techniques. It has been demonstrated that the remaining lens epithelial cells (LECs) of the anterior capsular rim and equatorial region of the capsular bag undergo hyperplasia and change to spindle shaped myofibroblast-like cells.[4] Histopathologically, these changes in the epithelial cells are accompanied by formation of multi-layered basement membrane material composed of proteoglycans and collagen fibrils.[5] This PCO causes visual axis obscuration, capsular contraction, IOL decenteration etc.

Studies showed that trypan blue has got some effect in the count and viability of LECs, structure of lens capsule, and incidence of PCO. But most of these studies were done after external staining of the anterior capsule. Since LECs are located in the internal surface of anterior lens capsule, there was no direct exposure of the cells to the dye. In our study, internal staining of the anterior capsule with trypan blue 0.06% (w/v) resulted in direct exposure of LECs to the dye for a period of 1 minute in the case Sample B. The effects were compared to that of the control Sample A taken from the same patient which was initially removed after routine external staining for 10 seconds. 'Intactness of LECs throughout the length of anterior capsule' was significantly high in the control group Sample A where only routine external staining was done. There were statistically significant histomorphological changes like detachment of cells from basement membrane, irreversible degenerative changes in LECs and basement membrane edema in Sample B. Cell density decrease in Sample B was also statistically significant. All these changes may be due to the effect of the dye since all other variables affecting the study has been excluded.

A similar study was done by Pankaj Sharma, et al. In their study-''Trypan blue injection into the capsular bag during phacoemulsification: Initial postoperative posterior capsule opacification results.'' They found that Intraoperative injection of trypan blue 0.1% into the capsular bag reduced PCO after phacoemulsification with hydrophilic acrylic IOL implantation.[6] Here they have done internal staining of the anterior lens capsule, though histomorphology was not assessed. Our results very well explain their finding.

In another study, Nanavaty MA et al. assessed the effect of anterior capsule staining with trypan blue 0.0125% on the

Table 1. Comparison of **histomorphology of lens epithelial cells** among controls (sample A) and cases (sample B).

Histological parameters-LEC	Control **sample- A** present no (%)	Case **sample- B** present no (%)	X^2 value	*p*-value
LECs-Intact Throughout	12 (85.7%)	0 (0%)	17	0.000027*
LECs Partial Detachment/Complete Detachment (PD/CD)	0 (0 PD&0CD) (0%)	11 (9 PD&2CD) (78.5%)	14.9	0.00011*
LECs Intermittent Fall Off	2 (14.28%)	4 (28.5%)	0.2	0.64
LECs Evidence of Degeneration	0 (0%)	14 (100%)	24	0.000005*
LECs Nuclear Smudging	0 (0%)	14 (100%)	24	0.000005*

* Denotes significant *p*-value.

Table 2. Comparison of **histomorphology of basement membrane** among controls (sample- A) and cases (sample- B).

Histological parameters-basemet membrane	Control **sample A** present no (%)	Case **sample B** present no (%)	X^2 value	*p*-value
B M Intact throughout	14 (100%)	10 (71.4%)	2.6	0.105
B M Splitting	0 (0%)	4 (28.5%)	2.6	0.105
B M Edema	0 (0%)	14 (100%)	24	0.000005*

* Denotes significant p Value.

Table 3. Comparative analysis of **quantitative data of lens epithelial cells and basement membrane** among controls (sample-A) and cases (sample- B).

Variable	Mean ± SD		Mean difference	p value
	Controls **sample A**	Cases **sample B**		
Cells in most cellular area	8 ± 0.96	5.07 ± 2.6	2.929	0.001*
Cells in least cellular area	6.93 ± 1.42	1 ± 1.03	5.929	0.000*
Average cells	7.46 ± 1.08	3.04 ± 1.47	4.429	0.00*
Width of basement membrane	0.015 ± 0.0049	0.021 ± 0.0099	0.00590	0.062

* Denotes significant p Value.

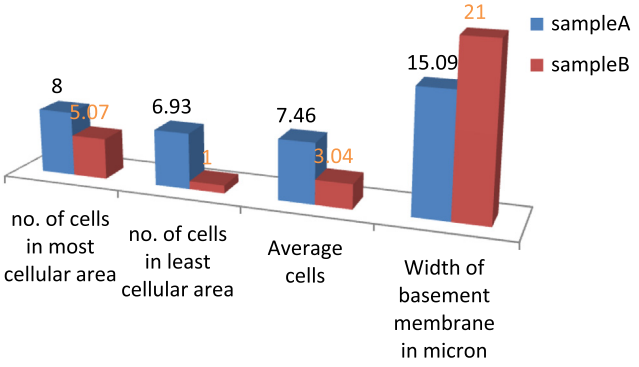

Chart 1. Comparative analysis of quantitative data.

density and viability of the lens epithelial cells and concluded that staining the anterior capsule with trypan blue affected the density and viability of LECs.[7] This study was done after routine external staining of anterior capsule. Our study also showed definite decrease in cell density though viability was not directly assessed.

The effects of trypan blue staining in the mechanical characteristics on anterior lens capsule have been investigated by Dick et al.[8] Staining led to the decrease in elasticity but increase in the stiffness of the membrane when measured with a modified rheometer. Minu M Mathan et al studied the effect of concentration of trypan blue and the length of exposure of trypan blue on the human anterior lens capsule using Raman spectroscopy. They found that trypan blue staining of the lens capsule leads to increased cross linking and reduction in the hydrogen bonding leading to increased capsule stiffness and reduced elasticity. We also had changes in the collagen of the basement membrane in the form of edema and splitting.

André Luís F. Portes, MD et al. in their study found that the TEM images of sub capsular epithelium cells showed mitochondrial rupture, dilation of the cisterns of the endoplasmic reticulum, increased cytoplasmic and nuclear electron density, and abnormalities in the nuclear profile of trypan blue–stained cells.[10] This study supports the hypothesis that staining with trypan blue 0.1% can help reduce the incidence of posterior capsule opacification after cataract surgery. In our study under Leica-DM 750 image analysis microscope we found that there were partial and complete detachment of LECs from basement membrane, degeneration of cells (cytoplasmic vacuolation, cell membrane rupture) and nuclear smudging (loss of character and blurring). Our comparative study results showed that LECs are much more affected by adequate direct exposure to trypan blue than routine external staining as there were significantly increased irreversible degeneration of LECs, reduced density of LECs and basement membrane edema in Sample B. This supports our hypothesis that direct staining of LECs with trypan blue can reduce the incidence of posterior capsule opacification after cataract surgery.

Conclusion

In our study we have found that partial and complete detachment of LECs, degeneration of LECs, nuclear

smudging and basement membrane edema were significantly higher in the case group where internal staining and direct exposure of LEC to trypan blue was done for 1 minute. This shows that adequate direct exposure of the internal surface of anterior capsule with trypan blue promoted irreversible degeneration of LECs and reduced the density of LECs. Hence intra operative exposure of the internal surface of capsular bag to trypan blue may be helpful to enhance the loss of LECs and prevention of Posterior capsular opacification as It has been demonstrated that the remaining lens epithelial cells of the capsular bag is the main cause for Posterior capsular opacification. More studies are needed for further evaluation of this hypothesis.

Conflict of Interest

The authors declared that there is no conflict of interest.

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