

## Effects of intracameral brilliant blue on the corneal endothelium of swine: in vitro study<sup>1</sup>

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**ABSTRACT-** Terzariol M., Hünning P.S., Brambatti G., Albuquerque L., Neumann C. & Pigatto J.A.T. 2016. **Effects of intracameral brilliant blue on the corneal endothelium of swine: in vitro study.** *Pesquisa Veterinária Brasileira* 36(8):775-780. Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: [pigatto@ufrgs.br](mailto:pigatto@ufrgs.br)

The aim was to investigate the ultrastructural changes in the corneal endothelium of pigs induced by intracameral 0.05% brilliant blue. Twenty swine corneas were separated into two groups, the right eye bulbs (control group) and the left eye bulbs (experimental group) of the same animal. All the eye bulbs were evaluated with specular microscopy. The cornea of the right eye bulbs was excised and in the left eye bulbs 0.2ml of 0.05% brilliant blue vital dye (OPH-blue<sup>®</sup>) was injected into the anterior chamber, where it remained for one minute. Then the anterior chamber was cleaned with a balanced salt solution injection and the cornea was excised too. All the corneas were evaluated by scanning electron microscopy to evaluate the changes on the endothelium caused by the brilliant blue dye. There were no significant differences between the right corneal endothelium cells and the left corneal endothelium cells with scanning electron microscopy after intracameral use of 0.05% brilliant blue dye. The 0.05% brilliant blue dye concentration did not cause deleterious effects for the swine corneal endothelium after intracameral use and can be a choice for safe staining of the anterior capsule of the lens in cataract surgery.

INDEX TERMS: Eye, brilliant blue 0.05%, toxicity, corneal endothelium, swine.

**RESUMO.- [Avaliação do endotélio corneano suíno por microscopia eletrônica de varredura após aplicação de azul brilhante a 0,05% na câmara anterior: estudo *in vitro*.]** Com o objetivo de avaliar as alterações ultraestruturais no endotélio corneano suíno induzidas pela aplicação intracameral do corante azul brilhante 0,05%, vinte córneas suínas foram separadas em dois grupos: olhos direitos (grupo controle) e olhos esquerdos (grupo experimental). Inicialmente todos os bulbos oculares foram avaliados por microscopia especular. As córneas dos bulbos oculares do grupo controle foram excisadas, enquanto nos bulbos oculares do grupo experimental foram injetados 0,2ml do corante vital azul brilhante 0,05% na câmara anterior e mantido por um minuto. Após esse período a câmara anterior foi lavada com solução salina balanceada e as córneas foram excisa-

das. Todas as córneas excisadas foram avaliadas por microscopia eletrônica de varredura para verificar a ocorrência possíveis alterações no endotélio corneano causadas pelo corante. Não foram observadas diferenças ultraestruturais durante a avaliação endotelial por microscopia eletrônica de varredura das córneas do grupo controle e experimental após a aplicação do azul brilhante 0,05% na câmara anterior. O corante azul brilhante na concentração 0,05% não causou danos endoteliais após seu uso intracameral em suínos e pode ser uma alternativa segura para a coloração da cápsula anterior da lente na cirurgia de catarata.

TERMOS DE INDEXAÇÃO: Olho, azul brilhante 0,05%, toxicidade, endotélio corneano, suíno.

### INTRODUCTION

Continuous curvilinear capsulorhexis (CCC) is a critical step in cataract surgery, specifically in white cataracts when the red reflex is poor or absent (Chang et al. 2005, Jacobs et al. 2006). In these cases, one of the options to facilitate the visualisation of the anterior capsule is the use

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of vital dyes to stain the anterior capsule of the lens before proceeding with capsulorhexis (Pandey et al. 2000, Dada et al. 2004). The importance of having a better visualisation of the flap during capsulorhexis is that it increases surgery success, decreases the chance of anterior capsule tears during phacoemulsification, and ensures the safe in-the-bag implantation of the intraocular lens (Pandey et al. 2000, Marback et al. 2001).

Another suggested use for vital dyes in cataract surgery is to help trainee cataract surgeons during CCC confection in order to reduce the learning curve for those students (Waziri-Erameh et al. 2006, Udaondo et al. 2007). However, the toxic effects of each vital dye have to be evaluated before the start of its use in the hospital routine (Remy et al. 2008). An ideal vital dye for facilitating CCC during cataract surgery needs to satisfactorily stain the anterior capsule of lens and cause minimal toxic effects to the corneal endothelium (Hisatomi et al. 2006).

The fluorescein sodium solution was the first vital dye used to facilitate capsulorhexis in 1993. From then on, several other vital dyes have been studied and used to stain the anterior capsule of the lens during cataract surgeries, e.g. gentian violet, autologous blood, trypan blue, green indocyanine, and brilliant blue (Melles et al. 1999, Pandey et al. 2000, Ünlü et al. 2000, Yetik et al. 2002, Chang et al. 2005).

The endothelial cell loss due to cataract surgery is inevitable. For this reason, corneal endothelium of the patient should be evaluated before surgery, safer techniques should be chosen and intraocular solutions should not damage the endothelium to avoid large cell loss that can induce corneal decompensation after cataract surgery (Ishikawa 2002, Parikh & Edelhauser 2003).

Some research has studied the use of the brilliant blue vital dye to facilitate CCC and have suggested that this is a safe dye for the corneal endothelium (Hisatomi et al. 2006, Udaondo et al. 2007). However, this is the first research to evaluate the endothelium effects of this dye through scanning electron microscopy in swine eyes.

## MATERIALS AND METHODS

Twenty normal eye bulbs from ten male and female one-year-old Landrace pigs were studied. These eye bulbs were obtained from a licensed Brazilian commercial company (Avisui food company) that breeds these animals for meat production. The eye bulbs were divided into two groups: the control group and the experimental group, consisting respectively of the right eye bulbs and the left eye bulbs. All procedures were performed in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. All animals were sacrificed for reasons unrelated to this study.

The eye bulbs were enucleated and transported to the laboratory in a moist chamber containing physiologic saline immediately after the swine were sacrificed. The eye bulbs were mounted on an eyeball holder and examined using a fluorescein test, biomicroscopy (Kowa, SL 15, Japan), and a contact specular microscope (Celmax, Medical Service, Brazil) with software for corneal endothelium analysis. Specular microscopy was performed on all eye bulbs in order to determine the conditions of the corneal endothelium and those with evidence of alteration were excluded.

Immediately after the specular microscopy, a clear corneal incision with a 15° knife was made in the left eye bulbs using surgical microscopy. One eye bulb received a 0.2ml intracameral injection of 0.05% brilliant blue vital dye (OPHT-BLUE®, Ophthalmos, São Paulo/SP, Brazil) and the fellow eye bulb served as a control. The vital dye remained in the left eye bulb in the anterior chamber for one minute and was then removed with the application of a balanced saline salt solution (Ophthalmos, São Paulo/SP, Brazil).

The corneas and a 2.0mm rim of sclera were excised, transferred to 2.5% glutaraldehyde in 0.1 sodium cacodylate buffer at pH 7.4 and stored for 24 hours at 4°C for fixation. The corneas were washed in a cacodylate buffer and dehydrated through an increasing series of ethanol solutions. Thereafter, the specimens were submitted to critical point drying using liquid carbon dioxide. The corneas were placed on 10 mm aluminium stubs with adhesive tape and sputter coated with gold-palladium. The posterior endothelial surfaces were examined and photographed using a scanning electron microscope (JEOL JSM 6060, Japan) operating at 15 kV.

The photomicrographs were scanned into the computer, and the percentage of cell loss was calculated by observing the surrounding areas which had an absence of endothelial cells. Image analysis was performed with image analyser software (Leica, Quantimed 500 IW, Leica Cambridge Ltd, Cambridge, UK) by a skilled observer who did not know the source of the photographs. Ultrastructural characteristics were described using photomicrographs obtained at 1.000-3.500x. Statistical data analysis was conducted using the F test if intercellular empty spaces were found in the endothelium samples. Values of  $P < 0.05$  were considered significant.

## RESULTS

Specular microscopy showed that a normal swine corneal endothelium was characterised by a continuous monolayer of polygonal cells of uniform size and shape (Fig.1). Reproducible images were obtained from all eye bulbs. The main cell density obtained with the contact specular microscope in control groups and experimental groups was 3,136 cells/mm<sup>2</sup> and 3,150 cells/mm<sup>2</sup>, respectively (Table 1). Regarding the morphology of the endothelium, were found cells with six-sided (68%), five-sided (18%), and seven-sided (14%). The parameters evaluated did not differ significantly between the right eye bulbs and the left eye bulbs from the same pig.

The swine posterior corneal endothelium surface observed on the scanning electron microscope revealed a continuous layer of polygonal cells of uniform size and shape. The scanning electron microscopy images showed that corneas in both the control and experimental sample group

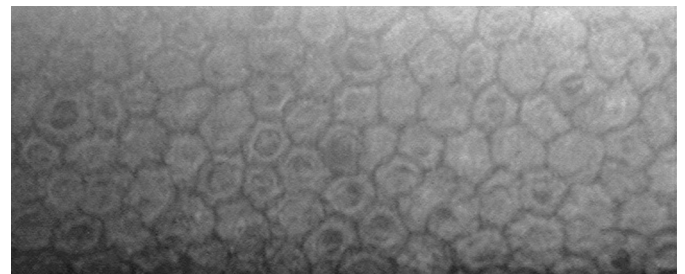


Fig.1. Specular micrograph of a normal corneal endothelium of a pig from the experimental group.

**Table 1. Corneal endothelial cell density in swines obtained from control and experimental group corneas with specular microscopy**

Ocular bulb	1	2	3	4	5	6	7	8	9	10	Average
ROB* (cells/mm <sup>2</sup> )	3097	3185	3008	3078	3199	3761	3267	2352	3062	3355	3136
LOB** (cells/mm <sup>2</sup> )	3381	2991	3778	3338	3156	2810	3902	2940	2342	2868	3150

\*ROB = right ocular bulb, \*\*LOB = left ocular bulb.

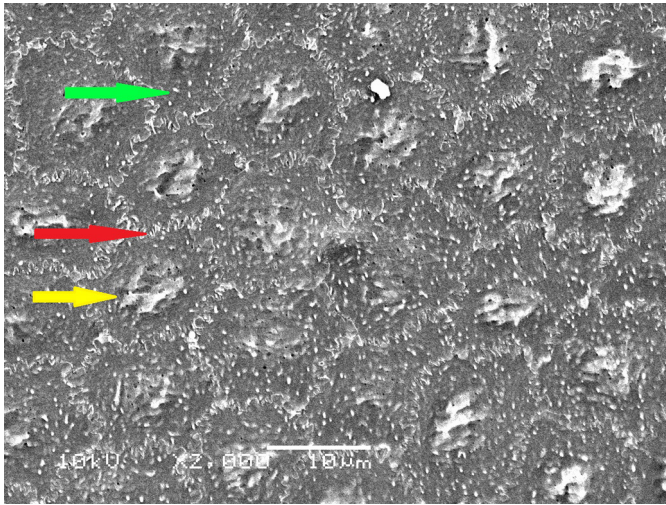


Fig.2. Image of experimental swine ocular bulb corneal endothelium (pig #9) in scanning electron microscope showing the interdigitation (red arrow), microvillus (green arrow), and evident nucleus (yellow arrow). It is possible to note tight intercellular adhesion. 2000x.

characterised similar morphological endothelia. All the samples had high hexagonal cell counts, an evident nucleus, the presence of interdigitation, microvilli, and the absence of intercellular empty spaces. The endothelial cells were interdigitated and adhered in both the experimental group and the control group (Fig.2). In the experimental group and the control group there were no areas with cell loss.

The similarity of the control and experimental scanning electron microscope images obtained during the search are shown in Figure 3, which represents part of the sample images. No intercellular empty areas were evident and the cellular morphology is similar to all the sample images.

## DISCUSSION

The evaluation of the appearance of the corneal endothelium is almost universal nowadays in testing potential toxicity of ophthalmic solutions (Doughty 1989). The brilliant blue vital dye has been considered safe and effective for facilitating CCC in human white cataract surgery and in the training of cataract surgeons (Hisatomi et al. 2006, Udaondo et al. 2007). To describe the corneal endothelial effects of its vital dye, this study evaluated the in vitro effects to the swine corneal endothelial cells with scanning electron microscopy.

Swine eyes are considered the standard experimental model for cataract surgeries (Tamayo-Arango et al. 2009). The ocular bulbs and cornea sizes of pigs, their morphological endothelium characteristics, their wide availability and their low sale prices are some of the biggest advantages of

this choice in medicine and veterinary medicine (Proulx & Brunette 2012). Due to all of these reasons, the ocular models for this research were swine ocular bulbs. Furthermore, pigs were chosen as experimental animals because of the morphological similarities between swine and human corneas (Tamayo-Arango et al. 2009, Proulx & Brunette 2012).

Both ocular bulbs from the same animal were used to get homogeneous samples with similar corneal endothelia. The parameters evaluated by specular microscopy did not differ significantly between the right eye bulb and the left eye bulb from the same pig. It is well established that the density of endothelial cells from both healthy eyes of the same patient is similar (Pigatto et al. 2005, Gum et al. 2007, Tamayo-Arango et al. 2009, Albuquerque et al. 2015, Bercht et al. 2015, Coyo, et al. 2015). The examination of specular microscopy performed before starting of the experiment allowed to only be selected healthy eyes. The results observed in this study show that normal swine corneal endothelium consisted of a continuous monolayer with mainly hexagonal cells, but were also found cells with five sides and with seven sides. Previous studies conducted involving vertebrate animals have shown that there is predominance of cells with hexagonal shape and, in lower proportion, there is presence of five- and seven-sided cells (Collin & Collin, 1998, Pigatto et al. 2005, Gum et al. 2007, Tamayo-Arango et al. 2009, Albuquerque et al. 2015, Bercht et al. 2015, Coyo, et al. 2015).

The endothelial cellular membranes' integrity for scanning electron microscopy evaluation depends on adequate tissue preservation and fixation from the time after the death of the animal until the use of the tissue samples (Collin & Collin 1998). In humans, corneal tissues for transplants must be used within six hours of the donor's death and need to be safely maintained to avoid endothelial damage (Almeida Jr et al. 2007, Franzen et al. 2010). The swine corneas in this research were maintained in moist chamber for less than six hours until glutaraldehyde fixation to avoid endothelial damage. This methodology has also been used with excellent results in previous studies (Pigatto et al. 2005, Franzen et al. 2010, Albuquerque et al. 2015, Coyo et al. 2015). SEM has been widely used to compare the endothelial ultrastructure of vertebrates, and to evaluate the effects of medication, chemicals or surgical procedures on the endothelium (Pigatto et al. 2005, Tamayo-Arango et al. 2009).

The previous studies conducted using SEM demonstrated that the corneal endothelial cells have cilia and microvillus (Tuft & Coster 1990, Pigatto et al. 2005, Pigatto et al. 2008). Humans and rabbits have twenty to thirty microvilli in the cell superficies, and these structures react to toxic substances changing their sizes (Collin & Collin 1998).

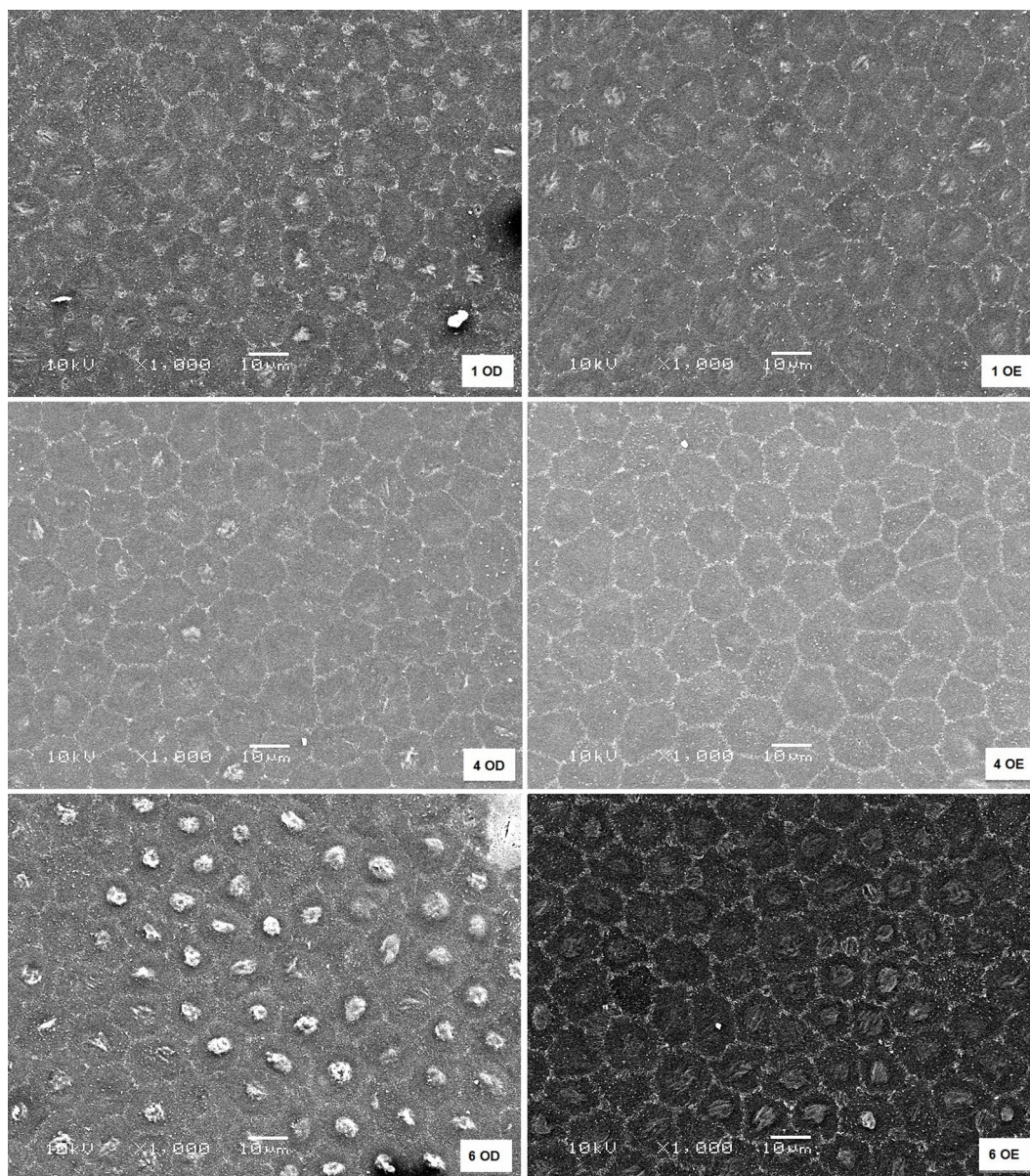


Fig.3. Comparative swine corneal endothelium scanning electron microscope images from each control and experimental group, respectively. 1000x.

Both the endothelium control and experimental groups presented almost the same morphological characters in scanning electron microscopy analyses, and the microvillus and interdigitation were evident in all sample images. The cilia in swine corneal endothelial cells are described by Tamayo-Arango et al. (2009), although in this research neither cell presented these structures. (Tamayo-Arango et al. 2009). The cilia absence or the presence of a few numbers of them in swine endothelium research is due to their fragility during sample production (Collin & Collin 1998, Tamayo-Arango et al. 2009).

To obtain a better visibility of the lens capsule, capsular staining techniques that had to be done using a biocompatible dye (Xiao et al. 2007) were developed. The use of vital dyes in ophthalmology to facilitate CCC is actually indicated in white cataracts without fundus reflex (Marback et al. 2001). The CCC technique can be used in planned extracapsular

cataract extraction and in phacoemulsification to reduce the risks of tearing during nucleus and cortex removal and intraocular lens implantation. Unsuccessful CCC increases the risk of posterior capsule rupture, vitreous loss, nucleus drop and intraocular lens displacement. Rodrigues and collaborators tested the anterior lens stain capacity of the brilliant blue dye in some concentrations and concluded that the concentration of 0.05% was enough to stain the anterior swine lens capsule homogeneously and to promote the observation of the flap portion (tissue being incised) during CCC, although its effects on corneal endothelium are not completely known (Rodrigues 2009).

Several techniques for staining the anterior lens capsules have been used, such as direct vital dye application in the anterior chamber under an air bubble or not, dye application under a viscoelastic substance in the anterior chamber, and subcapsular application (Pandey et al. 2000,

Yetik et al. 2002, Xiao et al. 2004, Allen et al. 2006). The last three techniques are indicated for reducing the possible toxic effects of the dye to the corneal endothelium (Yetik et al. 2002, Allen et al. 2006). In this research, the use of the brilliant blue intracameral dye without another substance or air bubble was done to produce direct contacts between the vital dye and the corneal endothelium.

The vital dyes that have been used to stain the anterior lens capsule are trypan blue, fluorescein, green indocyanine, violet gentian, autologous blood and brilliant blue (Melles et al. 1999, Pandey et al. 2000, Ünlü et al. 2000, Yetik et al. 2002, Chang et al. 2005) although some of them are not considered safe or effective (Pandey et al. 2000, Marback et al. 2001). Trypan blue and green indocyanine selectively stain dead endothelial cells and because the endothelial cells are alive in cataract surgery they do not obstruct the surgeon's view (Horiguchi et al. 1998, Xiao et al. 2007). Nowadays, trypan blue is the vital dye of choice used to stain the anterior lens capsule. Several studies have demonstrated no toxic effects from its use for corneal endothelium and the effective in the staining of anterior lens capsule (Yetik et al. 2002). Both trypan blue and green indocyanine stain the anterior lens capsule uniformly, although (Xiao et al. 2007) considered the staining provided by trypan blue slightly superior. In this study, brilliant blue was effectively for staining the anterior lens capsule and did not provide endothelial cell pigmentation during the experiment, as revealed by Horiguchi et al. (1998) and Xiao et al. (2004) who used trypan blue and green indocyanine to facilitate CCC.

Despite positive points related to its use, the trypan blue is among the carcinogen substances Merck Indices and due to its long-lasting effects, which are not still known, its use in pregnant women and children must be avoided (Yetik et al. 2002). The green indocyanine stains the anterior lens capsule satisfactorily and does not have toxic effects on the corneal endothelium, but its use has been associated with retinal toxicity in lens subluxation after cataract surgeries (Allen et al. 2006). Because of this, when choosing the vital dye to facilitate CCC during cataract surgeries their posterior chamber toxic effect must be considered (Jacobs et al. 2006).

An advantage of the brilliant blue over the trypan blue and green indocyanine for staining the anterior lens capsule is that this vital dye has been shown to have fewer in vitro toxic effects on corneal endothelial cells (Udaondo et al. 2007). Eggeling et al. 2000 examined the potential in vitro damaging effects on swine corneal endothelium of lidocaine in concentrations of 1%, 5%, and 10% with scanning electron microscopy. The 1% concentration did not cause any changes in the corneal endothelium. In the 5% and 10% concentrations, cell borders were hard to distinguish, single groups of two to three necrotic cells were scattered over the entire endothelium and some of them had developed a generally swollen aspect with no intact intercellular borders. Furthermore, extended areas of denuded Descemet's membrane impressed with remainders of detached cells were also seen in the last two groups. In this research, the corneas treated with 0.05% brilliant blue dye showed a normal endothelium in scanning electron microscopy,

similar to that seen in the 1% lidocaine group studied by Eggeling et al. (2000). Endothelial cells in our experimental groups were hexagonal in the majority and presented well-defined cell borders with the typical appearance of the normal cells structures, such as the presence of the nucleus, microvillus, intercellular adhesion and the presence of interdigitation, showing no toxic effects of the 0.05% brilliant blue in the corneal endothelium. Previous studies with a similar methodology using scanning electron microscopy revealed morphological abnormalities in eyes injected with 2% lidocaine, which caused morphological abnormalities, such as decreased corneal endothelial cells with irregularities, damage to the cellular organelles with larger spaces between the endothelial cells, and fewer microvilli (Eggeling et al. 2000).

## CONCLUSION

The results of this study showed that the 0.05% brilliant blue vital dye does not damage swine endothelial cells of the eye after one minute of intracameral injection, and suggested that this vital dye seems to be a safe choice for facilitating CCC in cataract surgery.

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