

## A Rabbit Model of Corneal Endothelial Injury Induced by Intracameral Injection of 0.05% Benzalkonium Chloride

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### ABSTRACT

**INTRODUCTION:** Rabbits are currently not suitable to be used as an animal model of corneal endothelial disorders because their corneal endothelial cells (CEC) maintain a highly proliferative capacity *in vivo*. A recent *ex vivo* study suggests that intracameral injection (ICI)

of 0.1 mL 0.05% benzalkonium chloride (BAC) in rabbits produces a selective endothelial damage. This study was aimed at evaluating the effects of BAC ICI in rabbits *in vivo* to define the most appropriate BAC dose for inducing a rabbit model of corneal endothelial disease.

**METHODS:** Twenty-four healthy adult New Zealand White rabbits were divided into 4 groups ( $n = 6$ ) based on the compound injected unilaterally into the anterior chamber: 0.025% BAC, 0.05% BAC, 0.1% BAC, and BSS (Control group). Complete physical and ophthalmic examinations, pachymetry and specular microscopy were performed at 0, 2, 7 and 14 days. After 14 days, rabbits were euthanized and subconjunctival enucleation of the injected eye was performed. Excised corneas were vital-stained and observed under the light microscope to assess the corneal endothelial cells (CEC) morphology and mortality rate. The entire eye globe was sent for histopathology.

**RESULTS:** Compared to BSS, concentrations of  $\geq 0.025\%$  BAC led to significant increases in corneal edema, corneal thickness, mean CEC area and polymegathism. CEC mortality was significantly higher with 0.05% and 0.1% BAC. CEC density and hexagonality were significantly decreased with all BAC concentrations. Higher incidence of conjunctival congestion and corneal ulceration was detected with 0.1% BAC. Histopathology revealed CEC loss and ruled out alterations affecting the rest of the ocular structures.

**CONCLUSION:** BAC ICI *in vivo* leads to dose-dependent corneal endothelial toxicity in rabbits, being 0.05% the minimum required concentration to achieve a sufficient, but not excessive, selective corneal endothelial damage. This method could consequently be used for inducing a rabbit model of corneal endothelial disease.

**Key words:** Animal model; Endothelial dysfunction; Corneal endothelial cell; Specular microscopy; Pachymetry

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## INTRODUCTION

The corneal endothelium (CE) is a monolayer of polygonal cells located on the inner surface of the cornea, whose main role is to maintain corneal transparency by regulating stromal hydration. The *in vivo* proliferative capacity of corneal endothelial cells (CEC) in humans, primates and small animals is very limited. In humans, CEC are arrested in the G1 phase of the cell cycle and remain in a non-proliferative state<sup>[1-6]</sup>.

Endothelial cell density (ECD) decreases with age, trauma, surgery, or stress caused by disorders such as diabetes, glaucoma, or endothelial dystrophies. The remaining CEC respond with stretching and migration, increasing their cell size (polymegathism) and altering their shape (polymorphism). When loss of CEC is excessive, transparency function can be compromised, and endothelial decompensation results in corneal edema and vision loss<sup>[1,3,7-11]</sup>.

Currently, corneal transplant is the main treatment available for restoring vision after irreversible endothelial decompensation<sup>[5,6]</sup>. However, since this procedure often involves difficulties and complications, there is a need for novel therapeutic options for managing endothelial dysfunction which should first be evaluated in preclinical trials. The ideal animal model for the development of techniques to repair endothelial lesions should be anatomically and physiologically similar to the human model<sup>[12]</sup>. Because of its high availability, low cost, small size and easy handling, rabbits are very frequently used in research, including that of human ophthalmology<sup>[13-15]</sup>. However, rabbits are currently not a good model for studying diseases of the CE because, unlike humans, primates and dogs, rabbit CEC maintain a high proliferative capacity throughout almost all their life<sup>[1,13,15,16]</sup>.

Despite being the most commonly used preservative in ophthalmic preparations, several *in vitro* and *in vivo* studies in different species have shown that both topical and intraocular administration of benzalkonium chloride (BAC) can cause significant corneal damage, affecting especially the CE especially. The safety of this quaternary ammonium compound when used as a preservative in eye drops for humans has been questioned in several publications. Its use has even been reported to decrease the efficacy of hypotensive agents for the management of glaucoma, hence the current tendency to recommend the use of BAC-free formulations<sup>[2,17-24]</sup>.

The reported CE toxicity of BAC led our research group to recently perform an *ex vivo* study to evaluate the corneal endothelial damage caused by BAC intracameral injection (ICI) into the enucleated rabbit eyes<sup>[25]</sup>. Using specular microscopy and vital staining in addition to ophthalmic examination, a dose-dependent toxic effect of intraocular BAC on the CE was confirmed. Results showed a direct relationship between BAC concentrations and central corneal thickness (CCT), mean cell area (MCA), degree of corneal edema, formation of endothelial holes, and CEC mortality; and an inverse relationship between BAC concentrations and ECD and CEC hexagonality (increased pleomorphism). It was concluded that 0.05% BAC was the most suitable concentration for further *in vivo* studies aimed at developing an animal model of endothelial disease, given that a significant effect on the CE was achieved with this dose but higher concentrations would probably cause too much damage and affect the rest of ocular structures if administered to live animals<sup>[25]</sup>.

Several techniques aimed at selectively and deliberately destroying the CE have been described, including transcorneal freezing, CE abrasion and application of toxic compounds, but they all failed to achieve either a selective action when targeting the CE or a durable effect<sup>[4,11,15,16]</sup>. In the present study, we hypothesized that a single 0.1 mL ICI of 0.05% BAC can selectively destroy the CE without affect-

ing the rest of the ocular structures. Based upon this hypothesis, the purpose of this study was to evaluate the toxic effects of intracameral BAC on CEC in rabbits, in order to validate *in vivo* a repeatable and reproducible animal model of corneal endothelial disease.

## MATERIALS AND METHODS

All procedures performed in this study followed the ARVO (Association for the Research in Vision and Ophthalmology) guidelines. Ethics approval for the study was obtained from the Ethics Committee of the Autonomous University of Barcelona (Comissió d'Ètica en l'Experimentació Animal i Humana, CEEAH, UAB) with reference number "CEAAH 1969", and from the Ministry of Agriculture, Livestock, Fisheries and Food (DARP, Generalitat de Catalunya) with reference number "DARP7240".

### Animals and study compounds

Twenty-four healthy adult New Zealand White rabbits weighing from 2.0 to 2.5 kg, between 8 and 12 weeks of age (Animals Experimentals Riera, Barcelona, Spain) were included. During the study, each rabbit was accommodated in a 0.9 m<sup>2</sup> individual hutch and environmental factors were controlled (20 °C environment temperature and 60% relative humidity). Standard maintenance rabbit diet (Oxbow Adult rabbit food, Oxbow Animal Health, Murdock, NE, USA) once daily and *ad libitum* water and hay were provided. Rabbits were also kept in these conditions for three days before starting the study itself, with daily physical examinations, in order to ensure a healthy status prior to entering the trial and also to allow the study subjects to get used to the environment and the handling by the researchers involved in the study.

A commercially available balanced salt solution (BSS, BSS sterile irrigation solution, Laboratoris Alcon, El Masnou, Barcelona, Spain) was used for the Control group and as diluent for the BAC preparations. The three different BAC compounds used in the study were prepared under sterile conditions (Farmàcia Xalabarder, Barcelona, Spain).

### Presurgical evaluation

Before entering the study, a complete general physical examination was performed in all rabbits to confirm that only healthy animals were included. Bilateral ophthalmic examination, pachymetry and specular microscopy were performed in all rabbits. For all these procedures, rabbits were properly handled and positioned by an experienced handler. Only animals with healthy eyes were included in the study. Ophthalmic examination included neurophthalmic evaluation, slit lamp biomicroscopy (SL-15 Portable Slit Lamp Biomicroscope®, Kowa Co., Ltd, Tokyo, Japan), tonometry (Tonovet®-P, Icare Finland Oy, Helsinki, Finland) and indirect ophthalmoscopy (Indirect ophthalmoscope, Omega, Heine, Germany). By means of biomicroscopy, corneal edema was graded from 0 (absence) to +4 (severe) and conjunctival congestion from 0 (absence) to 3 (severe conjunctival congestion). Pachymetry and specular microscopy were performed simultaneously using a non-contact specular microscope (SP-2000P®; Topcon, Tokyo, Japan) in automatic mode, maintaining a working distance of 25 mm. Rabbits were excluded from the study if their eyes presented corneal decompensation, damaged CEC, corneal endothelial cell loss, or presence of inflammatory cells on the CE at time 0 days. Three microphotographs were obtained from the central area of the cornea. Thirty well-defined CEC from each microphotograph were analyzed to obtain the MCA (expressed in μm<sup>2</sup>), ECD (expressed in number of cells/mm<sup>2</sup>) and the coefficient

of variation (CV; expressed in %, and used to measure the degree of polymegathism). The CCT (expressed in  $\mu\text{m}$ ) was measured using the digital pachymeter of the same specular microscope.

#### Intracameral injection

After the presurgical evaluation, rabbits were randomly divided (random.org) into 4 different study groups ( $n = 6$ ), based on the compound injected into the anterior chamber (AC): 0.025% BAC, 0.05% BAC, 0.1% BAC, and BSS (Control group). Anesthesia was induced by administering an intramuscular combination of 0.5 mg/kg butorphanol (Turbogestic®, Fort Dodge Veterinaria, Girona, Spain) and 2 mg/kg midazolam (Midazolam Hospira, Hospira productos farmacéuticos y hospitalarios SL, Alcobendas, Spain), and maintained using oxygen and 2-3% isoflurane (Isoflurane®, Abbott Laboratories, Abbot Park, IL, USA) delivered through a facemask. Prior to the ICI, the area was disinfected using a diluted povidone-iodine solution, and a drop of topical anesthetic (Colircusí anestésico doble, Laboratoris Alcon, El Masnou, Barcelona, Spain) was instilled. Under general anaesthesia, an ICI of 0.1 mL of the selected compound was performed into one eye, with the fellow eye serving as a control. Assignment of the injected eye as well as of the concentration group was also performed randomly (random.org), so that a total of 12 rabbits received an ICI into the OS and 12 into the OD, being equally distributed within each concentration group (3 OS and 3 OD in each study group).

In all rabbits, a single injection was performed at the corneoscleral limbus of the assigned eye. A magnification loupe (Loupes 4x, Zeiss, Germany) and a 27 G needle were used for this purpose. One or two drops of aqueous humor were allowed to passively egress through the hub of the needle, then 0.1 mL of the appropriate compound were carefully injected into the AC. The needle was removed immediately after the injection and pressure was then applied at the injection site with conjunctival forceps for 20 seconds to avoid fluid leakage. After injection, one drop of tobramycin (Tobrex®, Alcon cusí laboratories, El Masnou, Barcelona, Spain) was instilled. After anesthesia, subcutaneous 0.5 mg/kg metoclopramide (Primperan®, Sanofi Aventis, Barcelona, Spain) and 2 mg/kg ranitidine (Ranitidina Normon, Laboratorios Normon SA, Madrid, Spain) were administered every eight hours for a total of 24 hours, to enhance intestinal motility. A special recovery food (Oxbow Critical Care, Oxbow Animal Health, Murdock, NE, USA) was given postoperatively.

#### Post-injection assessment

A complete physical examination was performed daily to check the health status of the study subjects and to identify any possible alterations caused by the study compounds. Pachymetry, specular microscopy, and basic ophthalmologic examination including biomicroscopy and tonometry were performed 2, 7 and 14 days post-injection, following the presurgical evaluation protocol.

#### Vital staining and histologic evaluations

After the 14-days post-injection assessment, rabbits were anesthetized using the previously described protocol and euthanized with an overdose of intravenous pentobarbital (Dolethal®, Vetoquinol, Alcobendas, Madrid, Spain). A subconjunctival enucleation of the injected eye was performed. The cornea was dissected 360° at the corneoscleral limbus with a 2-3 mm scleral ring, and placed endothelial side up in a Teflon holder to minimize wrinkling and distortion. Damage to CEC was determined by using trypan blue (Trypan blue powder, Sigma-Aldrich, St Louis, Missouri) and alizarin red S (Alizarin red S powder, Sigma-Aldrich, St Louis, Missouri) vital stains, following

a protocol described elsewhere<sup>[26]</sup>. After the staining procedure, several radial incisions were made in each sample in order to flatten the cornea before examination under the light microscope. Corneas were then flat-mounted endothelial side up and evaluated under the light microscope with a 40x objective (Motic® BA210, Motic Group, Spain). For each sample, three photographs of the central cornea were taken (Nikon Eclipse TE 2000, Nikon Corporation, Japan) and processed with image analysis software (Image J, National Institutes of Health, Bethesda, Maryland, USA) to measure the mortality rate -classified into low (0-33%), medium (34-66%) and high (67-100%) - and the degree of pleomorphism (based on the percentage of hexagonal CEC, or hexagonality). When appropriate, 10x images were also taken.

After microscopic observation of vital-stained samples, corneas were placed in 4% formaldehyde with the rest of the eye globe, and eyes were then sent to a histology laboratory (IDEXX Laboratories, Barcelona, Spain). Then, eyes were dehydrated, embedded in paraffin and cut in 4  $\mu\text{m}$ -thick sections. Thereafter, sections were stained with hematoxylin-eosin and evaluated under the microscope by a pathologist specialized in ocular clinical pathology (CN) in order to assess whether ICI of the different compounds affected any of the ocular structures and hence prove their selective action on the CE. Histologic evaluations allowed the semi-quantitative assessment of CE damage for each cornea, based on the loss of CEC, from 1+ (none or minimal loss of CEC) to 4+ (marked reduction in the number of CEC).

#### Statistical Analysis

No formal calculation of sample size was done due to this study being the first *in vivo* approach to a BAC dose finding using this technique. It was expected that 6 animals per group would allow obtaining appropriate results. Results were presented by groups according to BAC concentrations. Qualitative and ordinal variables were summarized with absolute and relative frequencies. For quantitative variables median and interquartile range [25<sup>th</sup>, 75<sup>th</sup> percentiles] and range (minimum, maximum) were used for continuous variables. Analysis of degree of corneal edema was performed by comparisons of each BAC concentration to BSS by Mann-Whitney U test stratified by time. The analysis for longitudinal evaluation for CCT, ECD, MCA and CV was performed using a rank-transformation of dependent variables in order to obtain a non-parametrical approach prior to applying a Generalized Estimating Equations (GEE) model with an estimation of within-subject correlation from AR(1) approach. These models included BAC group, time and their interaction as factors with baseline results of independent variables as covariate. All statistical analyses were performed with IBM SPSS version 20, Armonk, NY and the significance level alpha was set at 0.05 two-tailed.

## RESULTS

#### Ophthalmic examination

The different degrees of corneal edema detected during the study are depicted in Table 1.

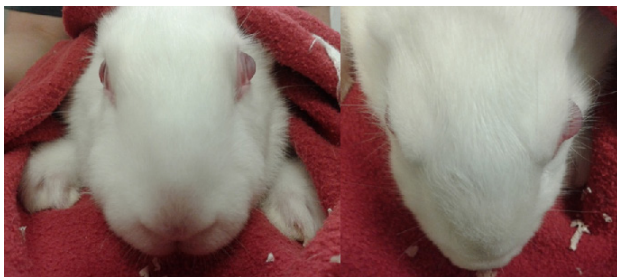
After 2 days of ICI, a significant increase of corneal edema was observed in groups 0.025%, 0.05% and 0.1% BAC, when compared to the BSS group. However, this significant effect was maintained throughout the rest of the study only with 0.05% and 0.1% BAC. Compared to BSS, a significantly higher number of cases presenting conjunctival congestion was seen within all BAC groups 2 days after ICI, but it remained significant until 7 and 14 days only in the 0.1% BAC group (Table 2).

During the study period, cases of corneal ulceration (8/24), cataracts (6/24) and AC fibrin formation (10/24) were observed (Table 3). Corneal ulcers, which were epithelial in all cases, occurred in a higher significantly number with 0.1% BAC ( $p=0.015$ ), compared to BSS. No significant differences were seen between groups regarding the number of cases developing cataracts (which were of immature anterior cortical nature, in all cases) and AC fibrin. All cases featuring corneal ulcers and/or AC fibrin formation resolved without any medication within less than three days, without exhibiting signs of pain at any time.

No remarkable changes were found in intraocular pressure values between groups (data not shown), even though the day after ICI, the injected eye of some of the rabbits (even with lower BAC concentrations) showed a pseudo-buphthalmic appearance (shown in Figure 1). This lesion resolved gradually two to three days after ICI in all cases.

**Corneal pachymetry and specular microscopy**

In the present study, the Topcon SP-2000P specular microscope provided clear images of the CE in healthy and mildly abnormal corneas (shown in Figure 2), but obtaining adequate images from moderate to severe edematous corneas was not possible. A dose-dependent BAC effect was seen on specular microscopy (shown in Figure 3), and pachymetric measurements. After 2 days of ICI, a significant increase ( $p < 0.01$ ) in CCT was observed in all BAC groups, compared to the BSS group. However, the difference remained statistically significant at  $p < 0.01$  throughout the rest of the study only with 0.05% and 0.1% BAC. Compared to BSS, a significantly decreased ECD and an increased MCA were seen in all BAC groups from 2 days after ICI ( $p < 0.05$ ). CV was significantly higher in all BAC groups 2 days after ICI, but this difference remained significant at  $p < 0.01$  after 7 and 14 days only with 0.05% and 0.1% BAC as shown in Figure 4.



**Figure 1** Rabbit #1205 belonging to the 0.025% BAC group, 2 days after intracameral BAC injection. The study subject showed an abnormal pseudo-buphthalmic left eye (injected eye) with obvious keratoconus. This lesion resolved by itself two days after injection.



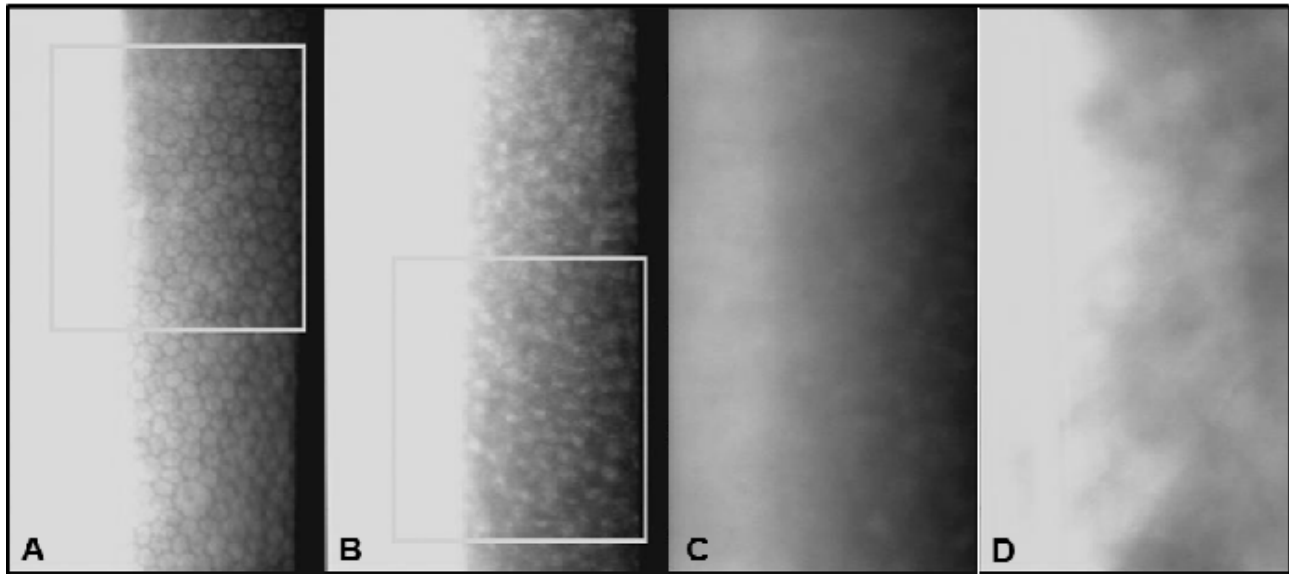
**Figure 2** Specular microscopy images with (right) and without (left) zoom including CCT (T), ECD (CD), CV and MCA (AVG) of eye OS from rabbit #1110, 14 days after ICI of BSS (SP-2000P specular microscope). Normal rabbit CE was visualized as a monolayer of polygonal cells of uniform size and shape. The zoom image shows 30 well-defined selected CEC marked with white dots, which were used to obtain the ECD, MCA and CV.

**Table 1** Incidence of various degrees of corneal edema [0: no edema; +1: mild corneal edema; +2: moderate corneal edema; +3 severe corneal edema; +4 very severe corneal edema] per group, at 0, 2, 7 and 14 days after intracameral injection. The level of statistical significance (p-value) of each group, when compared to the BSS group, is also shown. Bold text indicates a statistically significant difference with a p-value less than 0.01.

Day	Corneal edema	BSS (n=6)	0.025% BAC (n=6)	0.05% BAC (n=6)	0.1% BAC (n=6)
0	0	6;100%	6;100%	6;100%	6;100%
	1	0;0%	0;0%	0;0%	0;0%
	2	0;0%	0;0%	0;0%	0;0%
	3	0;0%	0;0%	0;0%	0;0%
	4	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		1	1	1
2	0	6;100%	0;0%	0;0%	0;0%
	1	0;0%	0;0%	0;0%	0;0%
	2	0;0%	4;66.7%	1;16.7%	0;0%
	3	0;0%	2;33.3%	5;83.3%	6;100%
	4	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		<b>0.002</b>	<b>0.002</b>	<b>0.002</b>
7	0	6;100%	1;16.7%	0;0%	0;0%
	1	0;0%	5;83.3%	1;16.7%	0;0%
	2	0;0%	0;0%	5;83.3%	4;66.7%
	3	0;0%	0;0%	0;0%	2;33.3%
	4	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		<b>0.015</b>	<b>0.002</b>	<b>0.002</b>
14	0	6;100%	5;83.3%	0;0%	0;0%
	1	0;0%	1;16.7%	5;83.3%	1;16.7%
	2	0;0%	0;0%	1;16.7%	4;66.7%
	3	0;0%	0;0%	0;0%	1;16.7%
	4	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		1	<b>0.002</b>	<b>0.002</b>

**Table 2** Incidence of various degrees of conjunctival congestion (ranging from 0 to 3) per group, at 0, 2, 7 and 14 days after intracameral injection. The level of statistical significance (p-value) of each group, when compared to the BSS group, is also shown. Bold text indicates a statistically significant difference with a p-value less than 0.05.

Day	Conjunctival congestion	BSS (n=6)	0.025% BAC (n=6)	0.05% BAC (n=6)	0.1% BAC (n=6)
0	0	6;100%	6;100%	6;100%	6;100%
	1	0;0%	0;0%	0;0%	0;0%
	2	0;0%	0;0%	0;0%	0;0%
	3	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		1	1	1
2	0	4;66.7%	0;0%	0;0%	0;0%
	1	2;33.3%	4;66.7%	3;50%	0;0%
	2	0;0%	2;33.3%	3;50%	2;33.3%
	3	0;0%	0;0%	0;0%	4;66.7%
p-value vs BSS		<b>0.032</b>	<b>0.022</b>	<b>0.002</b>	
7	0	6;100%	5;83.3%	3;50%	0;0%
	1	0;0%	1;16.7%	3;50%	0;0%
	2	0;0%	0;0%	0;0%	4;66.7%
	3	0;0%	0;0%	0;0%	2;33.3%
	p-value vs BSS		1	0.182	<b>0.002</b>
14	0	6;100%	6;100%	4;66.7%	0;0%
	1	0;0%	0;0%	2;33.3%	2;33.3%
	2	0;0%	0;0%	0;0%	4;66.7%
	3	0;0%	0;0%	0;0%	0;0%
	p-value vs BSS		1	0.455	<b>0.002</b>



**Figure 3** Specular microscopy images of injected eyes belonging to groups BSS (A), 0.025% BAC (B), 0.05% BAC (C) and 0.1% BAC (D) 7 days after ICI (SP-2000P specular microscope). A dose-dependent effect of BAC can be seen on the CE structure.

**Table 3** Incidence of corneal ulcer, cataracts or anterior chamber fibrin formation during the study period. The level of statistical significance (p-value) of each group, when compared to the BSS group, is also shown. (NE: not estimable). Bold text indicates a statistically significant difference with a p-value less than 0.05.

		BSS (n=6)	0.025% BAC (n=6)	0.05% BAC (n=6)	0.1% BAC (n=6)
Ulcer	No	6;100%	6;100%	3;50%	1;16.7%
	Yes	0;0%	0;0%	3;50%	5;83.3%
	p-value vs BSS		NE	0.182	<b>0.015</b>
Cataract	No	6;100%	5;83.3%	4;66.7%	3;50%
	Yes	0;0%	1;16.7%	2;33.3%	3;50%
	p-value vs BSS		1	0.455	0.182
Fibrin	No	4;66.7%	4;66.7%	4;66.7%	2;33.3%
	Yes	2;33.3%	2;33.3%	2;33.3%	4;66.7%
	p-value vs BSS		1	1	0.567

**Vital-stained microscopic images**

The degree of pleomorphism (median hexagonality) and mortality (percentage of dead CEC) for each study group 14 days after ICI are summarized in Table 4.

A dose-dependent toxic effect was confirmed by both CEC hexagonality and mortality as shown in Figure 5. Significantly lower mean CEC hexagonality percentages were found in groups 0.025% BAC (50%;  $p = 0.001$ ), 0.05% BAC (14.2%;  $p < 0.001$ ) and 0.1% BAC (2.8%;  $p < 0.001$ ) 14 days post-ICI, compared to BSS (82.5%). CEC mortality was significantly increased with 0.05% and 0.1% BAC ( $p = 0.002$ ), but not with 0.025% BAC ( $p = 0.180$ ).

**Histologic evaluation**

A higher CEC loss occurred in all groups treated with BAC, but there were no significant differences in the degree of CEC loss between groups (data not shown). These abnormalities were not equally distributed throughout the whole cornea as shown in Figure 6. The lens appeared unaffected in most cases, except for the sporadic findings of Morgagnian globules in two eyes (one belonging to the group 0.05% BAC and one to the 0.1% BAC group). The epithelial corneal ulcers observed during the ophthalmic examinations were not observed in the sections used for histologic evaluations. There were no significant intraocular abnormalities in any injected eye and study group.

**Table 4** Median hexagonality for each study group and percentages of cases per group classified as having a low (0-33%), medium (34-66%) or high (67-100%) percentage of dead CEC, 14 days post-injection. The level of statistical significance (p-value) for the values of each group compared to the BSS group is also shown. Bold text indicates a statistically significant difference with a p-value less than 0.01.

		BSS (n=6)	0.025% BAC (n=6)	0.05% BAC (n=6)	0.1% BAC (n=6)
Hexagonality		82.50%	50%	14.20%	2.80%
	p-value vs BSS		0.001	<0.001	<0.001
Mortality	Low	6;100%	3;50%	0;0%	0;0%
	Medium	0;0%	3;50%	1;16.7%	0;0%
	High	0;0%	0;0%	5;83.3%	6;100%
	p-value vs BSS		0.18	0.002	0.002

**DISCUSSION/CONCLUSION**

The present study confirms a dose-dependent CE toxicity of BAC in live rabbits, seen on ophthalmic examination, pachymetry, specular microscopy and vital staining. Furthermore, the study reported herein demonstrates that a single 0.1-mL ICI of 0.05% BAC can selectively destroy the CE without affecting the rest of the ocular structures in rabbits, and validates this animal model for its potential use in corneal endothelial research.

Rabbits are very frequently used in eye research<sup>[13-15]</sup>, but unfortunately, they are currently not a good model for studying diseases of the CE, because their CEC maintain a high proliferative capacity throughout nearly all their life<sup>[13,15,16]</sup>. In an attempt to avoid this problem, and based on results from a previous *ex vivo* study<sup>[25]</sup>, we aimed to provoke a selective and controlled CE damage in rabbits by BAC ICI *in vivo*.

Corneal edema can occur as a result of corneal decompensation due to loss of CEC. In the current study, a complete ophthalmic examination of the study subjects was used to identify changes such as increased corneal edema attributable to BAC toxicity. A concentration of 0.05% BAC was the minimum required to cause a significant increase in corneal edema that will last for at least 14 days. This effect was also achieved with 0.1% BAC, but ICI of this higher concentration also involved undesired complications in other ocular structures including a significant higher number of cases with

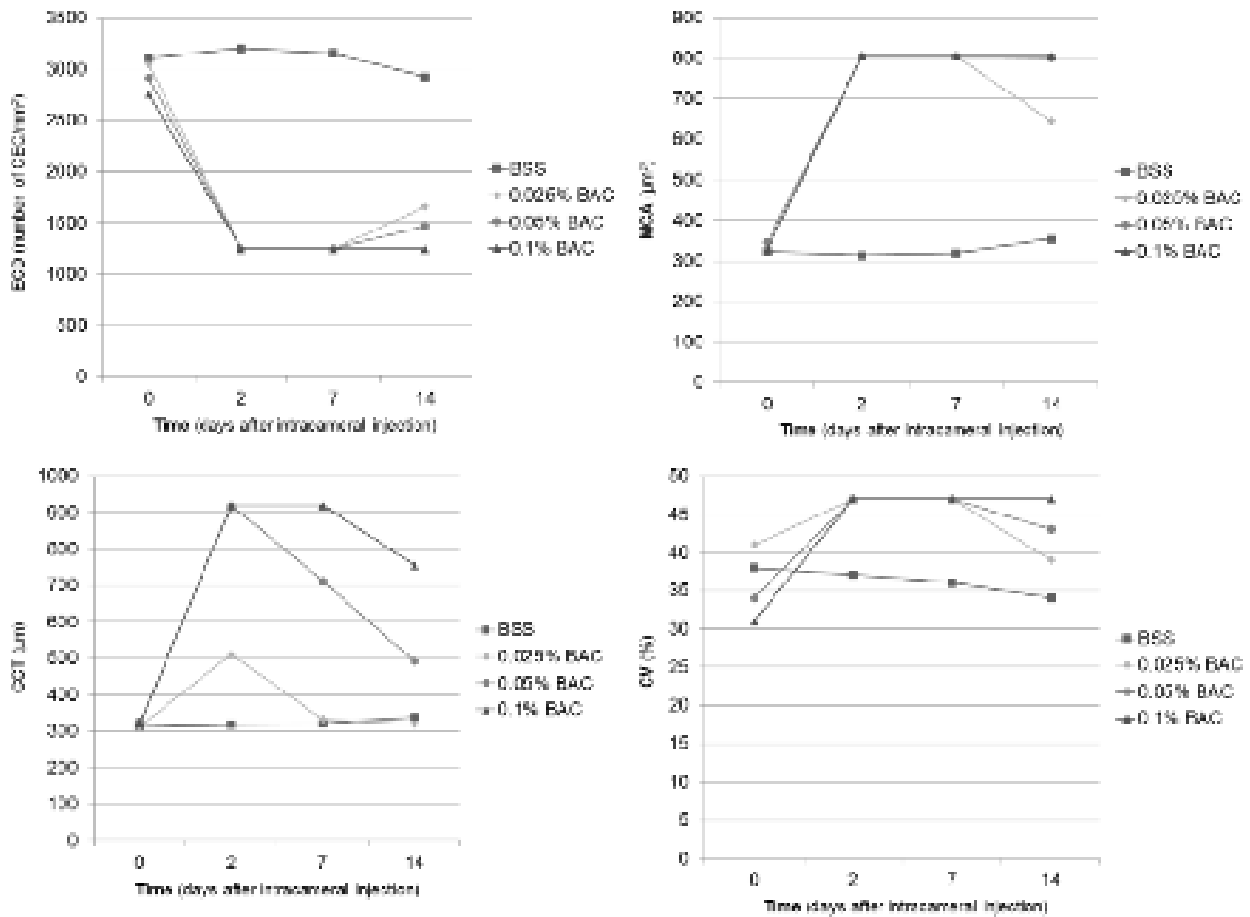


Figure 4 Median ECD (number of CEC/mm<sup>2</sup>), MCA (μm<sup>2</sup>), CCT (μm) and CV (%) found in rabbit eyes from the different experimental groups over time.

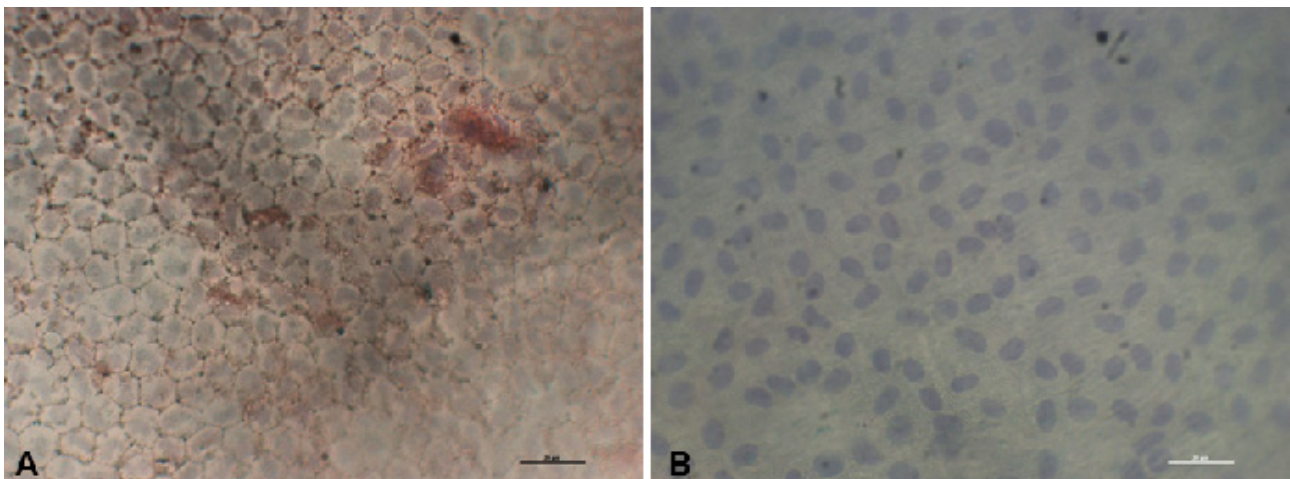
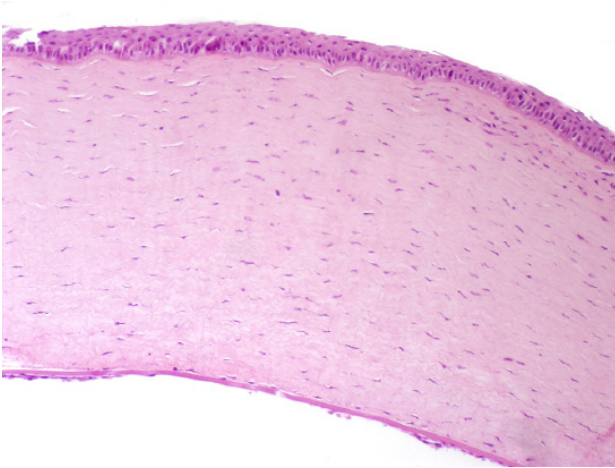


Figure 5 Trypan blue and alizarin red vital-stained images of eyes #I101 (A; BSS group) and #I405 (B; 0.1% BAC group) at 14 days post-injection, obtained under the light microscope at 40x. Note that the higher BAC dose used in the study resulted in reduced CEC hexagonality and increased mortality, when compared to the control group (A).

corneal ulcers and conjunctival congestion. Moreover, although it did not reach the level of statistical significance, a higher number of cases developing cataracts during the study was seen with all BAC compounds in a dose-dependent manner, and more cases with AC fibrin formation were seen with 0.1% BAC. Outpouring of fibrin into the AC is an effect that had already been reported previously after BAC ICI<sup>[27]</sup>. Ophthalmic examination also allowed the observation of eyes with a pseudo-buphthalmic appearance due to an abnormally thickened and deformed cornea in some rabbits the day after ICI.

This lesion resolved gradually by itself two to three days after ICI in all cases, which probably indicates that this temporary alteration can be counteracted by the regenerative capacity of rabbit CE.

The TOPCON SP-2000P® specular microscope was used in this study to obtain pachymetric and specular measurements simultaneously although, as previously reported<sup>[25,28]</sup>, obtaining adequate images from moderate to severe edematous corneas was not possible. This device confirmed a dose-dependent BAC toxic effect on the CE characterized by a significant increase in CCT, MCA and CV, and a



**Figure 6** Histological section at 10x of the left eye (injected eye) of rabbit #1310, belonging to the 0.05% BAC group, 14 days post-ICI. A marked CEC loss can be observed.

significant decrease in ECD. However, 0.05% and 0.1% BAC were the only concentrations with which these effects were maintained until the end of the study.

Vital staining combining trypan blue and alizarin red has been associated with a good visualization of CEC and assessment of damage to the CE<sup>[26,29]</sup>. In the present study, this technique provided very useful information, especially in those eyes in which specular microscopy could not be performed due to excessive corneal edema. The toxic effect of BAC was shown as an increased CEC pleomorphism with all BAC compounds, and an increased CEC mortality that was only seen with 0.05% and 0.1% BAC.

Since the aim of the study was to selectively destroy the CE, histologic evaluations were performed to complement the findings from ophthalmic examinations and to guarantee that BAC ICI would not negatively affect the rest of the ocular structures. Histologic results confirmed that after BAC ICI considerable damage to the CE is produced, but no significant alterations occurred in the rest of the eye globe at none of the tested concentrations.

In the present study, ICI of 0.05% BAC was found to be the most appropriate concentration to induce a selective toxic effect on the CE, causing a significant increase in corneal edema, CCT, MCA, CV, CEC pleomorphism and CEC mortality, and a significant decrease in ECD without significantly affecting other ocular structures. The next higher dose tested in our study, 0.1% BAC, appeared to be excessively toxic, since it was associated with a significantly higher number of cases developing corneal ulcers, as well as a tendency to an increased frequency of conjunctival congestion. On the other hand, based on our findings, using 0.025% BAC appears to be insufficient to provide a sustained and significant corneal edema, and it was not high enough to provoke a significant increase in CEC mortality.

These findings confirm our initial research hypothesis, being in line with prior *ex vivo* observations from our research group, which suggested 0.05% BAC as the most appropriate dose for an *in vivo* experiment<sup>[25]</sup>. Both studies point towards 0.05% being the minimum threshold for achieving significant CE damage based on changes in CEC pleomorphism and mortality, although a significantly increased pleomorphism was already seen *in vivo* with 0.025% BAC. Regarding specular microscopy and pachymetry, even though a dose of 0.1% BAC was needed to provoke a significantly increased CCT *ex vivo*, a concentration of 0.05% BAC led to significant changes in ECD, MCA and CV. This was confirmed both *ex vivo* and *in vivo*, except for CV which was only evaluated *in vivo*.

The present study consequently validates the results from the

above-mentioned *ex vivo* study and further supports the use of the described technique for inducing an animal model of corneal endothelial disease. This new rabbit model provides researchers with a novel and relatively easy-to-perform procedure that could be used for testing new therapeutic options for restoring the CE. The choice of the administration route of BAC should also be highlighted, as the irritation caused by topically applied BAC has been described as higher. Prior studies have already reported the toxic effects of BAC injected into the AC at similar concentrations<sup>[27,30]</sup>. The present study further supports the convenience of using a low BAC dose if injected into the AC to develop an animal model.

In conclusion, BAC ICI induces dose-dependent corneal endothelial toxicity in rabbits *in vivo*. A concentration of 0.05% BAC is the minimum required to achieve a sufficient, but not excessive, selective corneal endothelial damage, which is in line with prior *ex vivo* observations. An animal model of corneal endothelial disease can be induced in rabbits by ICI of 0.1 mL 0.05% BAC.

#### Author Contributions

SS, ML and TP participated in the design of the study. SS supervised the study and prepared the draft of the manuscript. JR performed the statistical analysis. CN performed the histologic evaluations. DC, NC and MS-A participated in data collection and examination of study subjects. SS, ML and TP participated in data analysis and interpretation of results. All authors read and approved the final manuscript.

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