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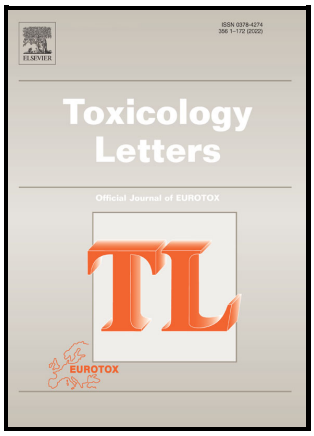

Advancing Ocular Safety Research: A Comprehensive Examination of Benzocaine Acute Exposure without Animal Testing

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Advancing Ocular Safety Research: A Comprehensive Examination of Benzocaine Acute Exposure without Animal Testing

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Abstract

Benzocaine is a widely employed local anaesthetic; however, there is a notable dearth of preclinical and clinical evidence regarding its safety in ophthalmological products. To address this, a comprehensive strategy incorporating *in silico* and *in vitro* methodologies was proposed for assessing benzocaine's ocular toxicity without animal testing. To collect the *in silico* evidence, the QSAR Toolbox (v4.5) was used. A single exposure to two benzocaine concentrations (2% and 20%) was evaluated by *in vitro* methods. Hen's Egg Chorioallantoic Membrane Test (HET-CAM) was performed to evaluate the effects on the conjunctiva. To study corneal integrity, Short Time Exposure test (STE) and Bovine Corneal Opacity and Permeability (BCOP) assay, followed by histopathological analysis, were carried out. Results from both *in silico* and *in vitro* methodologies categorize benzocaine as non-irritating. The histopathological analysis further affirms the safety of using benzocaine in eye drops, as no alterations were observed in evaluated corneal strata. This research proposes a useful combined strategy to provide evidence on the safety of local anaesthetics and particularly show that 2% and 20% benzocaine solutions do not induce eye irritation or corneal damage, supporting the potential use of benzocaine in the development of ophthalmic anesthetic products.

Keywords: Eye drops, Eye irritation, Alternative methods.

1. Introduction

For more than a century, ocular anesthetic has been used in ophthalmology to temporarily decrease pain after surgery, to alleviate annoying procedures, uncomfortable eye conditions or painful corneal damage (Kumar et al., 2015). Proparacaine, tetracaine, benoxinate (oxybuprocaine), and cocaine are some of the most commonly used anaesthetics. For cataract surgery, the drugs of choice are 4% lidocaine and 0.75% bupivacaine (Sun et al., 1999). Despite being well tolerated, all of them have the potential to be harmful to the ocular surface, especially when used improperly. Because there is insufficient knowledge concerning the safety of other anaesthetics, such as benzocaine and procaine, further research is warranted. In fact, some authors advise avoiding the topical corneal application of local anaesthetics since they delay the regeneration of the epithelium and can promote keratitis (Snow et al., 1975).

Benzocaine, 4-aminobenzoic acid ethyl ester, is a topical anesthetic agent well-known for its analgesic potency and pain control in oral mucous membranes, dental and ear discomforts, and local anesthesia in surgical procedures. Benzocaine molecule binds to sodium channels decreasing ion permeability which leads to the inhibition of the neuronal membrane depolarization and the consequence blockade of nerve impulse conduction (Körner et al., 2022). It can be used in combination with other anaesthetics such as lidocaine and tetracaine, however, no standard guidelines exist for optimal use, dosage, formulation, and safety of all topical anaesthetics.

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Unfortunately, there is scarce and conflicting information regarding the possible ocular toxicity caused by the anesthetic benzocaine. A preclinical study in rabbits shows that benzocaine at a concentration of 0.4% does not cause toxicity to the corneal epithelium (Sun et al., 1999). A case reported by Boonsiri et al. (2016) shows severe corneal damage produced by an accidental exposure of a combination of 20% benzocaine, 8% lidocaine and 4% tetracaine (BLT) for dermal use. Evidently, systematic research on this matter is needed.

The cornea is one of the most densely innervated tissues in humans and animals (Medeiros and Santhiago, 2020). Eye trauma, particularly corneal injuries and abrasions, tend to be excruciatingly painful, so the usefulness of eye drops with analgesic properties is beyond dispute. In addition to achieving relief and comfort with local anesthetic and analgesic treatments, it is important to ensure that the drug chosen does not cause damage to the conjunctiva or loss of corneal transparency leading to reduced vision.

In veterinary medicine, obtaining analgesia without adverse effects is of great importance because the relief of pain and/or pruritus can determine the success or failure of a treatment. In animals, particularly in dogs that feel this discomfort in the eye, an injury is usually generated by scratching with the fifth (hanging) finger or spur of the front legs. Mechanical trauma can induce corneal neuroplastic changes and infections. Consequently, a cascade of events involving the corneal wound healing, trophic functions, neural circuits, and the lacrimal product may interfere in corneal homeostasis (Medeiros and Santhiago, 2020).

The toxicological assessment is part of the process of development, evaluation and registration of new drugs or new uses of drugs already on the market. Historically, the gold standard for assessing eye irritation was the Draize rabbit eye test, but it has been criticized due to its subjectivity in scoring, poor reproducibility, anatomical differences between human and rabbit eye and fundamentally for ethical reasons. For many years, specific efforts were dedicated to developing animal-free methods to assess eye irritation (Adriaens et al., 2017; Alépée et al., 2019; Lotz et al., 2016; Scott et al., 2010).

The Quantitative Structure Activity Relationship or (Q)SAR of a molecule constitutes a significant advance in the construction of the weight of evidence as a starting point in a toxicological assessment (Raunio, 2011). (Q)SAR is a method developed to find relationships between the chemical structure and the biological activity of the test compounds (Gallegos et al., 2007). The central axiom of SAR is that the activity of molecules is highly dependent on their structure. Therefore, similar molecules have similar activities. The OECD (Organization for Economic Co-operation and Development) promoted the development of a software tool for this type of analysis, the QSAR Toolbox, commonly used for *in silico* approaches.

The Hen's eggs test on chorioallantoic membrane (HET-CAM), one of the oldest alternative methodologies used to replace the Draize test, was developed by Luepke (1985) and has been considered an adequate model to predict the effects of substances on the conjunctiva of the eye (Debbasch et al., 2005; Derouiche and Abdennour, 2017; Palmeira-de-Oliveira et al., 2018; Presgrave França and Delgado, 2012; Scheel et al., 2011; Spielmann et al., 1996). The CAM is a highly vascularized extra-embryonic structure that includes arteries, capillaries and veins. Adverse effects on CAM induced by a test substance would be correlated with irritation and/or corrosion *in vivo* (Kishore et al., 2008). The HET-CAM method allows the visualization of hemorrhage, lysis, coagulation and hyperemia phenomena in the chorioallantoic membrane. It is considered a replacement method since at this stage of embryo development (seven to nine days)

the peripheral nervous system is immature and there is no perception of pain (Aleksandrowicz and Herr, 2015; Rosenbruch, 1997).

One of the phenomena involved in corneal injury is cytotoxicity (Maurer et al., 2002). Takahashi et al., (2008) developed the STE (short-time exposure) test utilizing the rabbit cornea cell line SIRC (Statens Serum Institut Rabbit Cornea) to reflect a true exposure scenario *in vitro*. The procedure entails cultivating cells and subjecting them to the test substances for 5 minutes. The reduction in cell viability, a criterion that allows classifying compounds as slightly, moderately, or highly irritating, is indicative of the harm produced by the substance. The creation of the formazan salt (blue), which is generated by living cells during the enzymatic conversion of the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium), is quantified to determine cell viability (Mosmann, 1983). The Japan Society for Alternative Animal Experiments (JSAAE) Validation Committee and the Japan Center for Method Validation Alternatives (JaCVAM) both approved the method (Kojima et al., 2013; Sakaguchi et al., 2011).

The BCOP (bovine corneal opacity and permeability) methodology uses corneas isolated from the eyes of recently slaughtered cattle and is based on the evaluation of two parameters: opacity and fluorescein permeability. Corneal opacity is measured as the amount of light transmitted through the cornea using an opacitometer, while permeability as the amount of dye (sodium fluorescein) that passes through the cornea. The BCOP test was originally developed by Muir, (1987) and later improved by Gautheron et al., (1992). Subsequently, the protocol was subjected to rounds of interlaboratory testing and validation (Chamberlain et al., 1997; ECVAM, 2009; Gautheron et al., 1994; OECD, 2020a). To get further information on the effect of the assay item on the corneas employed, a histopathological examination can be performed (IIVS, 2016). For this purpose, fixed and hematoxilin-eosine dyed corneas are examined by an expert pathologist.

The methodologies STE and BCOP are used for identifying extreme categories: i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage. On the other hand, HET-CAM is capable of predicting different levels of irritation, including mild and moderate irritation.

The purpose of this study was to evaluate the potential adverse effects of benzocaine in eye drops for veterinary use applying the methodologies mentioned above other than animal testing. The same vehicle (castor oil) was chosen for our experiments as was intended for the formulation of the therapeutic product. We combined *in silico* evaluation with three alternative methods to evaluate acute ocular irritation and corrosion potential in the short term, after a single application.

2. Materials and methods

Benzocaine (Parafarm, CAS 94-09-7) at 20% and 2% was prepared in castor oil (*Ricinus communis*) (Droguería Saporiti S.A.C.I.F.I.A., CAS 8001-79-4) as vehicle, heating until complete solubility. According to legal GHS classification given by ECHA, Castor Oil is Not Classified and Benzocaine is classified as Danger (causes damage to organs and may cause an allergic skin reaction but no irritation to eyes).

2.1 *In-silico* profiling

Benzocaine and ricinoleic acid, the main constituent of castor oil (Yeboah et al., 2021) were input by their CAS numbers (94-09-7 and 8001-79-4 respectively) into the OECD QSAR Toolbox (v4.5) (https://qsartoolbox.org/). This software contains two rules-based profilers for eye

irritation/corrosion potential: 1) eye irritation/corrosion exclusion rules by the German Federal Institute for Risk Assessment (BfR) and 2) eye irritation/corrosion inclusion rules by BfR.

The exclusion rules relate five physicochemical properties (lipid solubility, octanol water partition coefficient, aqueous solubility, melting point and molecular weight), setting cut-off values for each parameter, to eye damage and/or irritation levels (Gerner et al., 2000). This profiler's logical structure is IF <Physicochemical property> THEN NOT <eye damage level> and it identifies chemicals that do not exhibit eye irritation or corrosion potential (Tsakovska et al., 2005).

The inclusion rules profiler comprise 17 structural alerts based on known mechanisms of action (biochemical reaction within the eye and /or conjunctival tissues) and identifies chemicals that show potential for eye irritation and corrosion, following the logical structure IF <Substructure A> THEN <Effect B> (Tsakovska et al., 2007).

2.2 Hen's Egg Chorioallantoic Membrane Test (HET-CAM)

Fertilized, specific-pathogen free White Leghorn eggs were supplied by Instituto Rosenbusch S.A. (Argentina). Eggs were incubated at 38.0 ± 0.1 °C in a relative humidity of 65 ± 2% and under automatic rotation (Incubator A. Dami). Between 3 and 4 eggs weighing 45–75 g were used for each treatment: 0.9% NaCl (saline solution - negative control), 1% SLS (sodium lauryl sulfate - positive control), castor oil (vehicle control) and 2% and 20% benzocaine. On day 9th the eggshell was opened, the internal white membrane was exposed, moistened with 0.9% NaCl, removed and 0.3 ml of treatment solutions were applied to the CAM. Hemorrhage, lysis and coagulation were registered during 300 seconds as described previously by Rivero et al. (2021). The effects were observed with a Leica S8APO stereoscopic magnifying glass and recorded with a Leica DMC2900 camera. An Irritation Score (IS) was calculated according to ECVAM DB-ALM guideline N◦96 (ECVAM, 2012), and classified as indicated in Table 1.

IS = [((301-H).5/300) + ((301-L).7/300) + ((301-C).9/300))]

H, L y C are the times in seconds of the first appearance of Hemorrhage, vascular Lysis and Coagulation respectively.

Table 1. Classification systems according to ECVAM protocol N◦96.

IS	Irritation category
0–0.9	Non-irritant
1–4.9	Weak or slight irritant
5–8.9	Moderate irritant
9.0–21	Strong or severe irritant

2.3 Short time exposure (STE) test

SIRC cell line

The SIRC rabbit corneal cell line obtained from ATCC® (CCL-60) was cultivated at 37°C with 5% CO2 and a humidified atmosphere. Cells were grown in complete medium (MEMc): Eagel's minimal essential medium (MEM) (Gibco), 10% FBS (Natocor), 2 mM glutamine (Serendipia Lab) and 1% Anti-Anti (Serendipia Lab).

The STE assay

The assay was carried out as described in the OECD TG N°491 (OECD, 2020b). Cells were plated at a density between 3-6x10^3 cells/well in a final volume of 200 µL per well until 80% confluence. The test substances (castor oil, 2% and 20% benzocaine) were dissolved at 5% (v/v) in 0.9% NaCl and two successive logarithmic dilutions were carried out from these solutions until reaching a concentration of 0.05%. A positive control (0.01% SLS), cMEM, 0.9% NaCl controls were included. The cells were exposed to 5% and 0.05% of the test substances or control solutions for 5 minutes at room temperature. After exposure, cells were washed twice with 200 µL of 0.9% NaCl and 200 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added (Invitrogen®) at a concentration of 0.5 mg of MTT/mL of MEMc. After 2 hours of incubation at 37°C, the formazan was extracted with 200 µL of isopropanol-0.04 N hydrochloric acid for 1 hour in darkness and at room temperature. The absorbance of the solution was measured at 570 nm in a 96-well plate spectrophotometer (Thermo Scientific®). Results were expressed as the percentage of cell viability relative to the vehicle control and the mean of three independent assays were used to classified according the UN GHS (United Nations Globally Harmonized System of Classification and Labelling of Chemicals) prediction model (Table 2).

$$\text{Cell viability (\%)} = \frac{(\text{DO test substance}) - (\text{DO blank})}{(\text{DO vehicle control}) - (\text{DO blank})} \times 100$$

Table 2. STE prediction model and UN GHS Classification

Cell viability		UN GHS Classification
At 5%	At 0.05%	
>70%	>70%	Non irritant
≤70%	> 70%	No prediction
≤70%	≤70%	Severe irritant

2.4 Bovine corneal opacity and permeability (BCOP) assay

The BCOP assay was performed according to OECD TG 437 (OECD, 2020a). Briefly, eyes were collected at a slaughterhouse, immersed completely in 0.9% NaCl and refrigerated with ice for transport. Isolated corneas were mounted in holders and filled with Minimum Essential Medium (MEM, Serendipia®). The device was equilibrated at 32 ± 1 °C for 1 h and the baseline opacity of each cornea was read on an opacitometer (DURATEC 3.0). The corneas were treated with 750µl of the negative and positive controls (0.9% NaCl and 100% ethanol, respectively), the vehicle control (castor oil) and benzocaine (2% and 20%) for 10 minutes. After washing three times, holders were incubated for 2 h with MEM and final opacity was measured. Then, 1 mL of sodium fluorescein solution (200 µg/mL) was added to the anterior chamber while the posterior chamber was filled with fresh MEM without phenol red. The holders were incubated for 90 min at 32 ± 1 °C. The amount of sodium fluorescein that crossed into the posterior chamber was quantitatively measured with a spectrophotometer UV/VIS (Multiskan Go, Thermo Scientific®) at 490 nm. The mean opacity and permeability values for each treatment group were combined to obtain an *In Vitro* Irritation Score (IVIS) and classified as the UN GHS prediction model (Table 3):

IVIS = opacity (final opacity – initial opacity) + permeability (15 x DO470)

Table 3. BCOP prediction model and UN GHS Classification

IVIS	UN GHS Classification

≤3	Non irritant
3-55	No prediction
>55	Severe irritant

2.5 Histopathological analysis of corneas employed in BCOP assay

Histopathological analysis was performed as suggested by Guidelines for histopathological evaluation of bovine corneas as an endpoint of the BCOP assay (IIVS, 2016). Once finished the BCOP assay, corneas were fixed in 10% phosphate buffered formaldehyde solution at room temperature. The central area of the corneas was sectioned, dehydrated in graded ethanol (70–100%), cleared in xylene, embedded in paraffin, sectioned at 3 μm using a microtome and stained with hematoxylin and eosin (HE). Histological slices were analyzed using a microscope (NIKON Eclipse E200) at 20x magnification and the photographic representations (Micrometrics 519 camera) of the corneas for each exposed test substance and control group were prepared and analyzed with Micrometrics SE Premium software. Histopathological changes were verified. In particular, the minimal, mild, moderate, or severe presence of parameters in each corneal layer (epithelium-stroma-endothelium) were taken into account. In the epithelium, the presence of: cell loss (erosion), cell coagulation (necrosis), nuclear and cytoplasmic vacuolization, nuclear condensation (pyknosis), partial or complete epithelial detachment of the basal layer from the anterior limiting lamina were considered as pathological parameters. In the stroma, the parameters observed in the extracellular collagenous matrix were taken into account, such as stromal expansion (edema) and stromal coagulation (collagenous hypereosinophilia). In keratocytes, the presence of necrotic cells (pyknosis and karyorrhexis) and cytoplasmic vacuolization were considered. The percentage of stroma compromised by edema was also evaluated as stromal thickness. Total stromal thickness (TST) in control bovine cornea is less than 800 μm, which was considered as the reference value. Finally, in the corneal endothelium, cell loss (denudation) and cytoplasmic vacuolization were considered.

2.6 Data analysis

Values are shown as the mean ± SD (Standard Deviation) for all data. The TST data was subjected to a one-way ANOVA to determine significant difference ($p<0,05$) and Dunnett's post-hoc comparison between each treated group and control group ($p<0,05$). Statistical software GraphPad PRISM® was used for visual representation of the results and statistical analysis.

3. Results

3.1 *In-silico* profiling

Profilers for eye irritation/corrosion were applied to benzocaine and ricinoleic acid, the main constituent of castor oil. For both chemicals, no structural alerts from the inclusion rules by BfR were triggered. However, it's important to note that the absence of an alert from the inclusion rules does not necessarily indicate a negative toxicity result for either benzocaine or ricinoleic acid. For the physicochemical exclusion rules by BfR, benzocaine gave a negative result while ricinoleic acid triggered the rule "Group C Melting point > 55ºC" by having a melting point above 55ºC. While the absence of an alert in the exclusion rules for benzocaine offers no information regarding its toxicity, the presence of the mentioned alert triggered by ricinoleic acid indicates this chemical has no potential of having the risk phrases R34 or R35. These risk phrases are associated with skin irritation, and thus ricinoleic acid wouldn't be classified as a skin irritant, but no assessment regarding its ocular toxicity can be made.

3.2 HET-CAM

HET-CAM according to the ECVAM DB-Alm Prot N°96 (ECVAM, 2012) was carried out. No irritation phenomena were observed in the negative control (0.9% NaCl) while hemorrhage, vascular lysis and coagulation came out in the positive control (1% SLS) as expected. Castor oil and the two concentrations of benzocaine (2 and 20%) did not show irritation phenomena during the 5 minutes of exposure. Results are shown in Table 4 and representative images of the treatments are shown in Figure 1.

Table 4. HET-CAM irritation score and classification. Results are presented as mean ± SD (n=3-4). Severe irritants are identified by score >9. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine. IS: Irritation Score.

Groups	IS		Classification
	mean	SD	
NC (0.9% NaCl)	0	0	Non-irritant
PC (1% SLS)	11.07	0.03	Severe irritant
VH (castor oil)	0	0	Non-irritant
2% BZ	0	0	Non-irritant
20% BZ	0	0	Non-irritant

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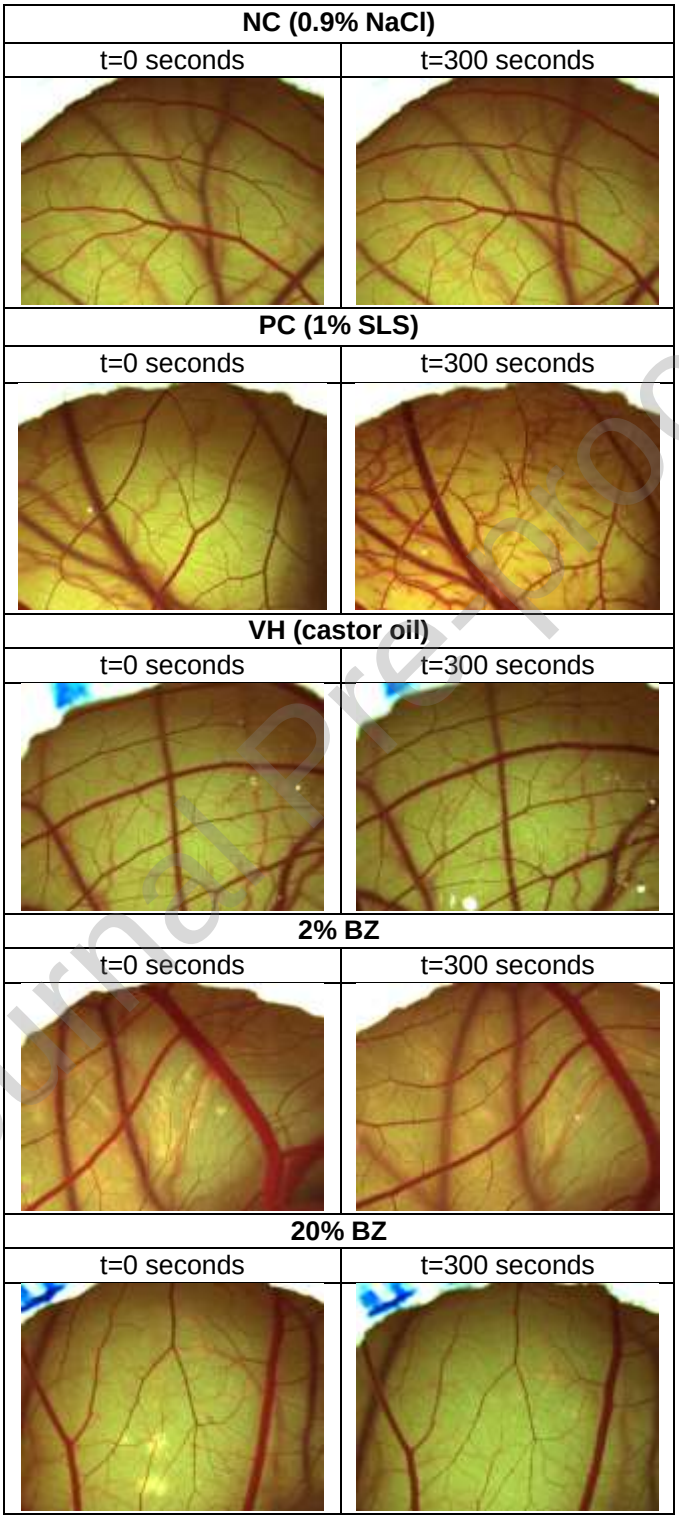
Figure 1. Representative images of the CAM before treatments (t=0 seconds) and final (t=300 seconds) time. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.



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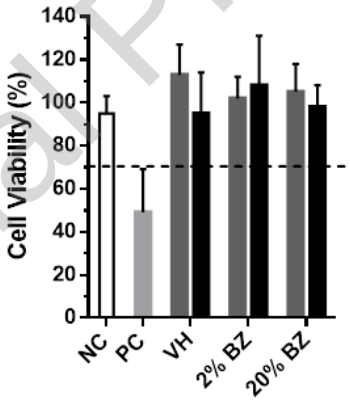
3.3 STE assay

Cell viability was greater than 70% at both test concentrations (0.05% and 5%) for benzocaine and castor oil samples (Figure 2) which were classified as non-irritants (Table 5). The cell viability obtained with the positive and negative controls is within the accepted and expected values.

Table 5. STE cell viability and UN GHS classification. Results are presented as mean ± SD (n=3). Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.

Groups and concentration (%)		Cell viability (%)	UN GHS classification
NC (0.9% NaCl)		95±8	
PC (1% SLS)		49±20	
VH (castor oil)	0.05	113±14	Non-irritant
	5	95±19	
2% BZ	0.05	102±10	Non-irritant
	5	108±23	
20% BZ	0.05	105±13	Non-irritant
	5	98±10	

Figure 2. STE cell viability. Results are presented as mean ± SD (n=3). Solutions at 0.05% and 5% correspond to dark gray and black bars respectively. The dotted line represents the cut-off value used in the prediction model. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.



3.4 BCOP assay

In the BCOP assay, the opacity and permeability values obtained for castor oil and both benzocaine concentrations (2% and 20%) were not significantly different from the negative control (Table 6 and Figure 3). In the positive control, we observed expected opacity and permeability values. The UN GHS categorization obtained from IVIS corresponds to products in which no lesion is observed (no-irritant).

Table 6. BCOP results are expressed as mean ± SD (two independent assays, with 2-4 corneas each). Abbreviations: IVIS; *In Vitro* Irritation Score; NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.

Groups	Opacity	Permeability	IVIS	UN GHS classification
NC (0.9% NaCl)	0.15±1.25	0±0	0.15±1.30	Non-irritant
PC (ethanol)	47.44±6.42	1.83±0.21	74.82±5.64	Severe irritant

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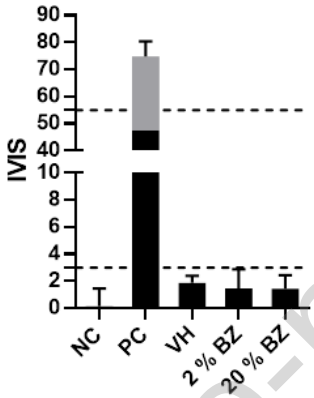
VH (castor oil)	1.89±0.44	0±0	1.84±0.47	Non-irritant
2% BZ	1.47±1.37	0±0	1.54±1.37	Non-irritant
20% BZ	1.44±0.97	0±0	1.31±1.01	Non-irritant

Figure 3: IVIS (*In Vitro* Irritation Score) are expressed as mean ± SD (two independent BCOP assays, with 2-4 corneas each). Opacity (black), permeability as 15xOD490 (gray). The dotted lines represent the cut-off values used in the prediction model. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.



3.5 Histopathological analysis

A histopathological analysis of the corneas was performed. This analysis complements and increases the information details obtained in the BCOP assay. The results show that at the epithelial, stromal and endothelial stratum, the corneas treated with the two concentrations of benzocaine (2% and 20%), castor oil and the negative control, preserved their morphology. The corneas treated with ethanol as a positive control, suffered alterations at the level of the epithelium and the stroma, as detailed in Table 7.

No differences in Total Stromal Thickness (TST) were observed between the benzocaine, castor oil and negative control groups, but the positive control showed a significant increase in the TST (Figure 4).

Table 7. Findings observed in the histopathological analysis of the three corneal layers. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.

Groups	Epithelium	Stroma	Endothelium
NC (0.9% NaCl)	Squamous, middle and basal strata with preserved morphology.	Preserved morphology	Preserved morphology
PC (ethanol)	-Squamous layer: mild intercellular edema -Middle layer: moderate nuclear vacuolization and slight intracellular edema. -Basal layer: mild intercellular edema, slight denudation of the basal layer. Minimal abnormal condensation of the basal and middle stratum chromatin.	Moderate edema	Preserved morphology
VH (castor oil)	Squamous, middle and basal strata with preserved morphology.	Preserved morphology	Preserved morphology
2% BZ	Squamous, middle and basal strata with preserved morphology.	Preserved morphology	Preserved morphology

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20% BZ	Squamous, middle and basal strata with preserved morphology.	Preserved morphology	Preserved morphology

Figure 4. Total Stromal Thickness (TST). Results are expressed as mean ± SD (n=4-5). *p<0,0001 vs. NC. Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.

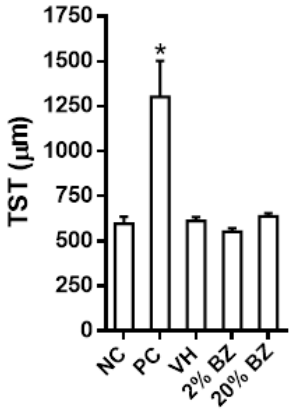
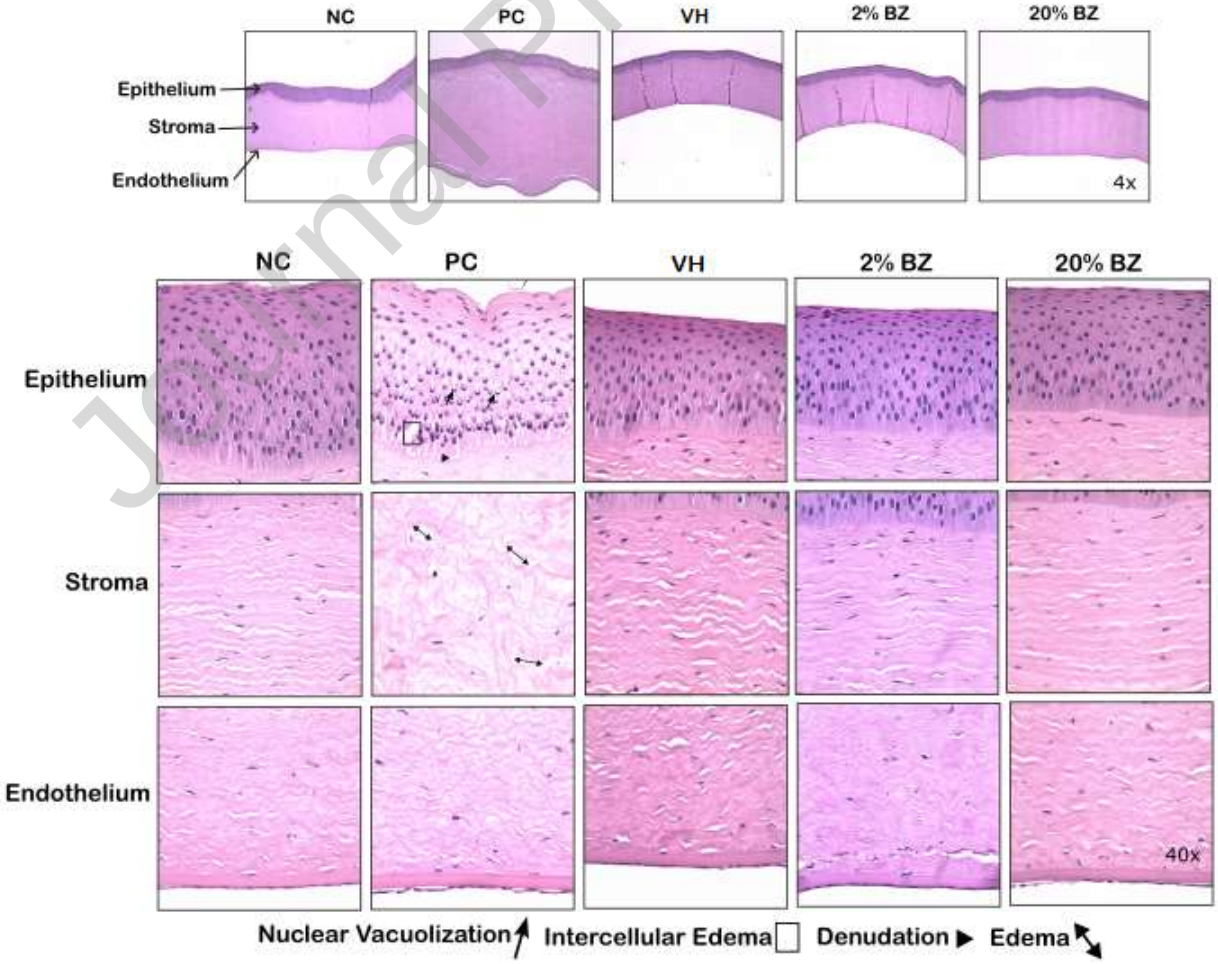


Figure 5. Histological appearance of bovine corneas. Hematoxylin and eosin staining. Epithelium: Nuclear Vacuolization (arrow), Intercellular Edema (rectangle), Denudation (arrowhead). Stroma: Edema (double-headed arrow). Abbreviations: NC, Negative Control; PC, Positive Control; VH, Vehicle; BZ, Benzocaine.



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4. Discussion

There is a need for safer ocular anaesthetics and in this context we proposed the possibility of using benzocaine as part of a formulation intended for ocular application. The potential ocular toxicity of two benzocaine concentrations (2% and 20%) was assessed by a nonanimal integrated approach which involved an *in silico* profiling, two different corneal models (SIRC cell line and bovine corneas follow histopathological analysis) and a conjunctiva model (chorioallantoic membrane).

From the results of the *in silico* profiling done on benzocaine and ricinoleic acid it can be seen that no particular chemical substructures associated with eye irritation/corrosion are present in any of these chemicals. The application of the eye irritation/corrosion exclusion rules indicated that ricinoleic acid would not have the R34 or R35 risk phrases. These results indicate that while ricinoleic acid, the main constituent of castor oil, isn't a skin corrosive, no assessment can be made regarding the potential for eye irritation/corrosion of benzocaine and ricinoleic acid. The BfR Decision Support System regarding eye irritation indicates that if physicochemical rules but not structural alert rules indicate relevant effects, clarification with adequate (if available: *in vitro*) testing for eye irritation is necessary (Gerner et al., 2000).

Then, we tested solutions with a high (20%) and low (2%) benzocaine concentration, as well as the vehicle chosen (castor oil), to determine their toxicity potential on the eye cornea and conjunctiva. The concentration of 2% was chosen considering that could be the concentration of use. The purpose for choosing a concentration of 20% was to maximally challenge and find a safe limit for the drug. However, no adverse reactions were observed at any concentration.

The 2% solution did not show any adverse effect on the different models tested. Prior studies using a 0.4% benzocaine concentration (Sun et al., 1999) had suggested that the substance did not cause other symptoms of corneal toxicity in rabbits. Our test results show that this safety range can be extended to 2% concentration. There are no experimental studies to determine the safety of larger concentration, as the 20% employed in our study. However, there is a case report, which describes an unintentional corneal abrasion induced by the administration of a dermal use preparation containing 20% benzocaine combined with 8% lidocaine and 4% tetracaine (Boonsiri et al., 2016). It is not possible to claim if the reported corneal damage is due to benzocaine or other ingredients, but four cases reported by Franz-Montan et al., (2008) showed ulceration after a 30-minute exposure of the oral mucosa to the local dermal anaesthetic EMLA® (2.5% lidocaine and 2.5% prilocaine). The same authors reported not observing irritation phenomena, measured in terms of coagulation in the HET-CAM test, of the anaesthetic Benzotop® (benzocaine 20%) in comparison with EMLA®, which generated severe irritation (Costa Bezerra et al., 2023). These results could be evidence that the irritation and corneal abrasion reported by the authors in anesthetic combinations is due to other components (e.g. lidocaine, prilocaine, or tratacaine) and not to benzocaine itself. In any case, tests with 20% benzocaine in prolonged and/or repeated exposures and enough time after treatment to observe long-term effects are necessary to guarantee ocular safety.

It is worth noting that our results support the safety of an acute exposition to benzocaine. Since a formulation containing benzocaine could be employed on a chronic basis, further repeated-dose studies should be performed to explore its toxicity under this administration regime.

While there is a lack of empirical support for the effectiveness of 20% benzocaine in ocular anesthesia, a double-blind study in 30 children performed to evaluate and compare the efficacy of two oral topical anaesthetics, demonstrated high significant difference between lignocaine 2% and benzocaine 20%, which provided better pain relief (Nair and Gurunathan, 2019).

The strength of this study lies in its demonstration that acute exposure to two different concentrations of benzocaine are safe at the eye level employing a multiple approach of internationally validated *in vitro* methodologies and satisfy the need to generate new information and power up a conclusion regarding the safety of this product. Furthermore, we highlight that this work proposes a useful combined strategy to provide evidence on the safety of local anaesthetics.

Finally, our *in vitro* results let us conclude that, as previously shown for other topical anesthetic agents, 2% and 20% solutions of benzocaine in castor oil employed as a vehicle do not induce eye irritation or corneal damage. These observations allow us to proceed with clinical trials in order to confirm the safety of this mixture for therapeutic purposes in veterinary medicine.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- Benzocaine is a possible local anesthetic to be used topically in eye drops.
- Benzocaine acute eye damage was evaluated by a non-animal integrated approach.
- 2% and 20% benzocaine solutions do not induce eye irritation or corneal corrosion.

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