# Analysis of the transport of and cytotoxic effects for nalbuphine solution in corneal cells

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**Objective**—To assess the in vitro effects of various nalbuphine concentrations on viability and wound healing ability of corneal cells and potential drug transport through the corneal epithelium.

**Sample**—Cultured canine and human corneal epithelial cells (CECs) and cultured canine corneal stromal fibroblasts.

**Procedures**—CECs and stromal fibroblasts were exposed to nalbuphine (concentration of solutions ranged from 0% to 1.2%) for up to 30 minutes, and viability was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A standard scratch test technique was used. Wound healing of CECs and stromal fibroblasts was evaluated following treatment with nalbuphine solutions < 0.1%. Liquid chromatography–mass spectrometry–mass spectrometry analysis was used to evaluate drug transport across a monolayer and a multilayer of human CECs.

**Results**—A progressive decrease in viability was detected in canine CECs for all nalbuphine treatment groups, whereas treatment with only 0.5% or 1.2% nalbuphine significantly reduced corneal stromal fibroblast viability, compared with results for control cells. Within 24 hours, treatment with 0.1% nalbuphine solution significantly altered the healing rate of both canine CECs and stromal fibroblasts. Continuous increases in transport rates of nalbuphine were detected with time for both the monolayer and multilayer of human CECs.

**Conclusions and Clinical Relevance**—In vitro, nalbuphine potentially could penetrate through corneal tissue, but it may cause damage to the corneal epithelium and stromal fibroblasts. Therefore, nalbuphine potentially may impair corneal wound healing. (Am J Vet Res 2012;73:1987–1995)

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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<tr>
<td>CEC</td>
<td>Corneal epithelial cell</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>TEER</td>
<td>Transepithelial electrical resistance</td>
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The cornea is the most densely innervated structure in the mammalian body, with a population of sensory nerve endings per area up to 600 times as great as that of the skin. Ocular pain in dogs secondary to corneal erosions or ulcers, infectious or inflammatory keratitis, or trauma is often quite severe because of this dense sensory innervation of the corneal tissue. Use of topical solutions for the management of ocular pain, in particular corneal pain, poses many challenges because of the barrier functions of the ocular surface and flushing action of the lacrimal system. These protective mechanisms decrease drug bioavailability by diluting...
applied solutions and rapidly decreasing contact time with the ocular surface.5,7

Furthermore, the options for topically administered ocular analgesics are limited. These include corticosteroids, NSAIDs, sodium channel blocking anesthetics, and cycloplegics.8–11 Each of these medications has benefits and disadvantages for use in the treatment of ocular pain. Corticosteroids have been administered both locally and systemically for their potent anti-inflammatory and immunosuppressive effects, but they have been associated with delayed corneal wound healing, increased susceptibility to infection, and collagenase potentiation in human and veterinary patients.12–14 In canine patients, NSAIDs are routinely used for the prevention and treatment of postoperative inflammation associated with intraocular surgery.15 Reported adverse effects of topically administered NSAIDs, such as ketorolac, are primarily associated with the cornea and include keratomalacia, punctate keratitis, delayed epithelial wound healing, and ocular surface irritation.15–17 Topical anesthetic agents are widely used in ophthalmic practice as an effective method of analgesia for diagnostic purposes and minor surgical procedures. However, long-term use of proparacaine and tetracaine has led to delayed corneal wound healing, keratomalacia, corneal perforation, ocular surface irritation, and persistent epithelial defects in human patients.18

Topical administration of morphine sulfate has been evaluated for its pain-modulating properties and effects on corneal wound healing in rats, rabbits, dogs, and humans.19–22 The effects of topical morphine sulfate on corneal wound healing have been evaluated in rabbit eyes, and investigators found no significant difference in the rate of wound healing in eyes treated with morphine sulfate, compared with the rate for control eyes treated with topically applied saline (0.9% NaCl) solution.19 In that same study,19 investigators evaluated the analgesic effects of topical morphine sulfate in 7 human patients with corneal abrasions following intraocular surgery. Corneal aesthesiometric measurements indicated a significant and relatively rapid onset of corneal analgesia in denuded eyes, compared with results for the clinically normal contralateral eyes.19 Similar properties of morphine sulfate have been reported for canine eyes with experimentally induced corneal ulcers.20 Results of that study21 indicated effective corneal analgesia as evidenced by decreased rates of blepharospasm and increased corneal aesthesiometric values, compared with results for control eyes treated with topically administered saline solution. Additionally, clinical and histologic evaluations did not reveal any difference in the rate of wound healing between treated and control eyes.21

Nalbuphine hydrochloride is a mixed agonist-antagonist semisynthetic opioid labeled for the management of moderate to severe pain in humans. The drug is derived from oxymorphone and is chemically similar to the opioid antagonist naloxone.22,23 Its primary analgesic action is derived from κ-opioid receptor agonism; however, it also has µ-opioid receptor antagonistic effects. Although it has narcotic properties, nalbuphine is not currently listed as a controlled substance by the US FDA. The drug has a wide range of clinical applications in human medicine. Multiple studies24–26 have found that nalbuphine administered IV or IM has analgesic effects similar to those of morphine in postoperative patients. However, in contrast to morphine, IV administration of nalbuphine at doses > 0.15 mg/kg does not cause an increase in respiratory depression, an effect that is likely attributable to nalbuphine’s mixed agonist-antagonist properties, which makes it a more attractive option for use in patients with respiratory compromise.27 Interestingly, administration of low doses of nalbuphine causes a sex-dependent analgesic response in human patients and is more potent in females than males.28 Because of its µ-opioid receptor antagonistic effects, nalbuphine can be used to reverse some of the undesired effects (eg, nausea, pruritus, and respiratory depression) of µ-opioid receptor agonists without totally eliminating analgesia.29,30

Nalbuphine use in veterinary medicine has been limited, with relatively few studies31–33 on its efficacy in animal patients, compared with the number of studies for the more widely used opioid narcotics morphine, hydromorphone, and oxymorphone. Currently, there are a limited number of published reports of the ophthalmic use of nalbuphine in veterinary patients. The corneal sensitivity of clinically normal eyes of horses was evaluated before and shortly after topical application of a 1.0% nalbuphine solution.34 Results of that study34 indicated no difference in corneal sensitivity after application of the drug in clinically normal equine eyes between treated subjects and matched control horses. Additionally, no detrimental effects to the ocular surface were detected in that study.34 Clinical analgesia achieved with topical administration of 1.0% ophthalmic nalbuphine, compared with oral administration of tramadol, was evaluated in male Beagles following experimentally induced corneal ulceration.35 Results of that study35 were based on clinical observation of signs of pain and the need for rescue analgesia; the investigators indicated that topically administered nalbuphine is ineffective, compared with the effects of orally administered tramadol, with regard to analgesia following acute corneal ulceration.

To our knowledge, no studies have been conducted to evaluate the effects of topical ophthalmic administration of nalbuphine on corneal cell viability or the ability of nalbuphine to penetrate corneal tissues. The purpose of the study reported here was to evaluate the impact of topical ophthalmic preparations of nalbuphine on CECs and stromal fibroblasts in vitro and to determine the extent of drug transport through the CEC layer.

Materials and Methods

Cell cultures—Eyes were enucleated from dogs euthanized by administration of an overdose of sodium pentobarbital at a local animal shelter for population control purposes. All eyes were deemed clinically normal prior to enucleation. Globes were collected within 1 hour after dogs were euthanized and immediately placed in 2.0% povodine iodine solution for 5 minutes, then rinsed and immersed in cold PBS solution (pH, 7.2). Within 2 hours after enucleation, primary canine corneal cells were cultured as described elsewhere.17,36
Brieﬂy, corneas were placed in an enzyme solution (2.4 U/mL) and incubated for 1 hour at 37°C and 5% CO2. The CECs were gently scraped off the basement membrane, centrifuged, and then resuspended in Dulbecco modiﬁed Eagle medium–F12 culture media with 10% fetal bovine serum and 1% antimicrobial-antimycotic solution. Cells were plated on collagen-coated culture dishes for cellular propagation and all subsequent experiments.

Primary cultures of canine corneal ﬁbroblasts were prepared from keratectomy samples obtained from the axial region of the cornea. The epithelium was mechanically removed to yield explants composed only of stromal tissue. Explants were allowed to adhere to polymethylmethacrylate culture dishes and then were covered with Dulbecco modiﬁed Eagle medium containing 10% fetal bovine serum and 1% antimicrobial-antimycotic solution. Growth medium was replenished every 3 days.

Because the model for drug transport through multiple CEC layers was of human origin, the comparative effects of nalbuphine on human and canine CECs required evaluation. Human corneal epithelium progenitor cells were purchased and cultivated in accordance with the supplier’s instructions. Human CECs were grown on uncoated culture dishes with supplemented deﬁned corneal epithelium media in a humidiﬁed atmosphere of 5% CO2 at 37°C. For the transport assays, 2.6 × 105 cells/cm2 were seeded in 6-well plates with the insert size of 0.4 μM and the growth area of 4.67 cm2. Medium was changed every 2 days, and experiments were performed 5 days after cells were seeded.

Preparation of nalbuphine solutions—Nalbuphine is a white odorless powder with the name morphinan-3, 6-alpha, 14-triol, 17-(cyclobutylmethyl)-4, 5-aepoxy, hydrochloride. All nalbuphine was obtained from a commercial source. All test solutions were prepared by use of the 1.2% stock solution in cell culture medium incubated at 37°C was to solid-phase extraction. The pH of a 1.2% nalbuphine stock solution (cocaine) at a concentration of 0.2 µg/mL and subjected to solid-phase extraction. The pH of a 1.2% nalbuphine solution in cell culture medium incubated at 37°C was evaluated by use of a pH meter every 4 hours over the course of 24 hours. The pH evaluations were performed on 6 vials of nalbuphine.

Determination of cellular viability—Cultures of CECs and stromal ﬁbroblasts were grown to 90% conﬂuence prior to being serum-starved overnight. A 1-mm scratch was then made on the cellular surface. Cells within the wound area were washed from the culture dish with PBS solution prior to treatment. Cells then were treated by incubation with a nalbuphine solution (3 solutions ranging from 0% to 0.1%) for 24 hours (6 replicates for each treatment). Photomicrographs were obtained at the initial time of wound creation and every 12 hours thereafter. Image analysis software was used to quantify cellular ingrowth into the wound.

In vitro wound healing experiments—Cultures of CECs or stromal ﬁbroblasts were allowed to grow to 90% conﬂuence prior to being serum-starved overnight. The tetrazolium salt, which is taken up only by living cells, is metabolized via the action of succinic dehydrogenase to form a colored formazan product, which was extracted by the addition of dimethyl sulfoxide and quantiﬁed colorimetrically at 570 nm in an automated microplate reader.

Human CEC monolayer experiments—Human CEC monolayers were washed twice with PBS solution before the experiments to remove any remaining culture media. To conﬁrm the integrity of the monolayer, TEER was measured before and after each experiment with an epithelial voltohmometer. Transport studies were performed on cell monolayers 5 days after cells were seeded when the TEER values reached 550 to 650 Ω·cm2. To evaluate transport of 0.25% and 0.5% nalbuphine solutions across human CEC monolayers (n = 3 CEC monolayers/nalbuphine solution), nalbuphine was added to the donor (apical or basolateral) side of each chamber. Experiments were conducted by removing only a single sample from each side of the chamber or by removing multiple samples (repeated sample collection at multiple time points) from the chamber. Volume for each sample was 20 μL.

Human multilayer CEC experiments—A stratified CEC model, isolated from clinically normal corneal tissues of humans, was used to evaluate nalbuphine transport across multiple layers of CECs. Cells were transfected during the ﬁrst passage with human papillomavirus 16 E6/E7 genes to extend their lifespan. Selected cells were evaluated for performance in 2-D cultures and for differentiation capacity in 3-D cultures. Cells were seeded on polyester membranes coated with rat tail collagen type I. Cultures were incubated at the air-liquid interface to achieve stratification of 6 to 9 cell layers. To achieve equilibration, tissue cultures were transferred from the supplied gel-based medium to a sterile plate, and supplemented corneal epithelial differentiation medium was added. Plates were incubated overnight at 37°C with 5% CO2 and 95% humidity. After equilibration, tissue cultures were treated by application of nalbuphine solutions (0.25% and 0.5%; n = 3 tissue cultures/nalbuphine solution) to the apical side. Nalbuphine concentrations were measured on the nalbuphine concentrations for the multilayer analysis were only determined for the basolateral side of the tissues.

Evaluation of nalbuphine permeability—The apparent permeability of nalbuphine was calculated via the following equation:
Apparent permeability = \((\Delta Q/\Delta t) \times (1/60) \times (1/S) \times (1/C_0)\)

where \(\Delta Q/\Delta t\) is the amount of nalbuphine that migrated across the monolayer, \(S\) is the insert membrane surface area, and \(C_0\) is the initial concentration of nalbuphine in the donor chamber. On the basis of the apparent permeability obtained by use of this equation, the efflux ratio was calculated by dividing the apparent permeability for the apical to basolateral direction by the apparent permeability for the basolateral to apical direction.

**Sample preparation via solid-phase extraction**—Nalbuphine was extracted from the matrices (defined corneal epithelium medium) via solid-phase extraction. An internal standard (cocaine, 0.2 µg/mL) was added to 100 µL of matrix along with specified concentrations of nalbuphine. Cartridges (60 mg and 1 mL) were used for solid-phase extraction. The cartridges were conditioned with 1 mL each of methanol and deionized water. To deactivate protein-drug interactions, 100 µL of 0.5% orthophosphoric acid solution was added to the samples. Samples were loaded on the cartridges, and the liquid passed through the cartridges during centrifugation at 350 × g for 2 minutes. The cartridges were washed 3 times with 2 mL of deionized water (centrifugation at 350 × g for 4 minutes after each wash), and analytes retained on the cartridges were extracted with 1 mL of methanol via centrifugation at 12 × g for 4 minutes. Extracted samples were evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 100 µL of 80% methanol containing 10 mM ammonium formate (pH 9.0), and an aliquot (20 µL) of solution was used for analysis.

**Liquid chromatography–mass spectrometry–mass spectrometry analysis**—The analysis was conducted on a triple quadrupole mass spectrometer operated in positive electrospray ionization mode. The mass spectrometer was connected to a pump and autosampler. The analysis was conducted on a C18 column (2.1 X 50 mm in length and 3.5 µm in diameter) coupled with a C18 guard column. The mobile phase (80% methanol containing 10 mM ammonium formate [pH = 9.0]) was used in isocratic mode at a flow rate of 0.2 mL/min. Total run time of the analysis was 5 minutes. The sheath gas and auxiliary gas were adjusted to yield an optimum response. Spray voltage was set at 4.9 kV. Analysts were quantified via single-reaction monitoring with ion transitions of m/z 358→340 and m/z 304→182 (at collision energies of 21 and 18 V, respectively) for the determination of nalbuphine and cocaine, respectively.

**Statistical analysis**—All statistical analyses were performed with commercially available software. Data were analyzed via a 2-way ANOVA with Holm-Sidak post hoc tests, when a significant difference was detected. Values of \(P < 0.05\) were considered significant.

**Results** —To ensure that nalbuphine remained stable and maintained a constant pH for the time periods and temperature used in the experiments, analysis was performed with culture medium as the vehicle. Nalbuphine solutions created in cell culture media did not have significant changes in drug stability at 37°C (data not shown). When pH was assessed over the course of 24 hours, a decrease was detected in the 1.2% nalbuphine solution in cell culture media stored at 37°C. Mean ± SD initial pH was 7.0 ± 0.18, and the mean pH 24 hours later was 6.0 ± 0.37. However, during the brief incubation periods for the cell viability experiments, pH decrease of nalbuphine was negligible.

**Cellular viability**—To determine the effect of nalbuphine on CEC viability, primary canine cultured cells were exposed to various concentrations of nalbuphine (range, 0% to 1.2%). Viability was assessed at each time point and expressed as a percentage. A significant increase in CEC death was detected within 5 minutes after the start of treatment with 0.5% \((P = 0.01)\) or 1.2% \((P < 0.001)\) nalbuphine, compared with results for control cultures (ie, CECs treated with 0% nalbuphine [vehicle only]; Figure 1). Relative to results for control cultures, significant decreases in cellular viability were detected at 10, 15, and 30 minutes when CECs were treated with 0.25% \((P = 0.01)\), 0.5% \((P = 0.01)\) at 10 minutes and \(P < 0.001\) at 15 and 30 minutes), and 1.2% \((P < 0.001)\) nalbuphine solutions. By 30 minutes, few or no viable CECs were detected after treatment with 1.2% nalbuphine. An increase in CEC death was detected at 15 and 30 minutes after treatment with 0.1% nalbuphine, but the increase was not significant. When CECs were treated with 0.01% nalbuphine, no significant changes in cellular viability were detected at any time point. Control cultures had no changes in cell death over time. Nalbuphine exerted similar effects on cellular viability in cultures of human CECs (data not shown).

Cultured canine fibroblasts were exposed to various concentrations of nalbuphine (range, 0% to 1.2%) to determine the effect of nalbuphine on corneal stromal cell viability. Canine stromal fibroblasts treated with 0% nalbuphine solution (vehicle only) had no change in the percentage viability over time (Figure 2). The 0.01% and 0.1% nalbuphine solutions did not significantly reduce cellular viability at any of the time points. Decreases in cellular viability were detected at 10, 15, and 30 minutes when fibroblasts were treated with 0.25% nalbuphine; however, these changes were not significant. A significant increase in corneal stromal fibroblast death was detected at all time points after treatment with 0.5% \((P = 0.01)\) at 5 and 10 minutes and \(P < 0.001\) at 15 and 30 minutes) or 1.2% \((P = 0.01)\) at 5 minutes and \(P < 0.001\) at 10, 15, and 30 minutes nalbuphine solution.

**Corneal wound healing response**—To assess the effect of nalbuphine on the wound healing ability of CECs and fibroblasts, a 1-mm defect was created in cultures prior to treatment with various concentrations of nalbuphine (range, 0% to 1.2%). This assay was conducted over the course of 24 hours; therefore, only nalbuphine concentrations with limited toxic effects were used. At 24 hours, cellular ingrowth into the initial defect (expressed as the percentage healed)
was not impaired for CECs or fibroblasts treated with 0.01% nalbuphine (Figure 3). Cells treated with 0.1% nalbuphine did not migrate into the defect throughout the experimental period; a significant ( \( P < 0.001 \) ) decrease in percentage healed was detected for both CECs and fibroblasts, compared with healing in the control cultures. The negative percentage healed of CECs detected after treatment with 0.1% nalbuphine solution was attributable to loss of cellular viability by 24 hours, which resulted in an increase in the size of the initial defect. Nalbuphine exerted similar effects in cultures of human CECs (data not shown).

Transport of nalbuphine across a human CEC monolayer—Experiments to characterize the ability for a monolayer of human CECs to transport nalbuphine (0.25% solution) from the apical side to the basolateral side (or vice versa) of the cellular monolayer was performed. No significant change was observed in the TEER value (350 to 650 \( \Omega \cdot \text{cm}^2 \)) measured before and after the transport experiment (data not shown). Application of 0.25% nalbuphine on the apical or basolateral side of the chamber with incubation for 5, 15, and 30 minutes resulted in apparent permeability values that were similar for both directions, which yielded values of approximately 1 for the efflux ratios at all 3 time points (Table 1). The nalbuphine concentration was determined on the receiver side which was either the apical or basolateral aspect, depending on where the sample was taken from. In table 1, nalbuphine concentrations are listed for both the apical and basolateral aspects.

To better understand the kinetics of drug transport, the nalbuphine concentration was measured in the basolateral side of each culture tissue at various time points for 2 concentrations (0.25% and 0.5% solutions) of nalbuphine. Because of the previously established toxic effects of 0.5% nalbuphine solution on the CECs, shorter time periods were used, compared with those used for the 0.25% nalbuphine solution. Results for both nalbuphine solutions indicated a similar behavior, with continuous increases in concentrations and transport rates (Figure 4). For both nalbuphine concentrations, apparent permeability was lower at earlier time points and higher at later time points.

Transport of nalbuphine across a human CEC multilayer—A human 3-D tissue culture of the corneal epithelium was used to evaluate the transport of nalbuphine across multiple layers of CECs. Transport of nalbuphine (0.25% and 0.5% solutions) was evaluated at 0, 2, 4, 6, 8, 10, 15, and 30 minutes. Both solutions had continuous increases in transport rates and basolateral concentrations of nalbuphine up to 30 minutes (Figure 5).
4). Histologic examination of the 3-D tissue cultures at the final time point revealed loss of CECs in both treatment groups; however, the 0.25% and 0.5% nalbuphine solutions did not cause complete denuding of the surface (Figure 5).

Table 1—Apparent permeability of nalbuphine and nalbuphine concentrations on the receiver (basolateral) side of human CEC monolayers at various times after application of nalbuphine (0.25% solution) to the apical side.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Direction*</th>
<th>Apparent permeability</th>
<th>Efflux ratio†</th>
<th>Nalbuphine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>A to B</td>
<td>5.1 × 10^{-4} ± 2.9 × 10^{-4}</td>
<td>1.10</td>
<td>0.91 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>B to A</td>
<td>5.5 × 10^{-4} ± 1.6 × 10^{-4}</td>
<td>1.04 ± 0.85</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>A to B</td>
<td>2.4 × 10^{-4} ± 6.2 × 10^{-4}</td>
<td>0.91</td>
<td>1.80 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>B to A</td>
<td>2.2 × 10^{-4} ± 3.2 × 10^{-4}</td>
<td>1.64 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>A to B</td>
<td>2.1 × 10^{-4} ± 3.3 × 10^{-4}</td>
<td>1.40</td>
<td>2.45 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>B to A</td>
<td>2.3 × 10^{-4} ± 3.0 × 10^{-4}</td>
<td>1.64 ± 0.78</td>
<td>3.49 ± 0.91</td>
</tr>
</tbody>
</table>

* A to B represents apical to basolateral, and B to A represents basolateral to apical. † The efflux ratio was calculated by dividing the apparent permeability for the apical to basolateral direction by the apparent permeability for the basolateral to apical direction.

Discussion

The advantage of topically applied medication lies in the ability to deliver drugs directly to the tissue of interest, which ensures access to the target tissue and reduces the likelihood of adverse systemic effects. However, any substance that alters physiologic mechanisms can result in undesired effects, and topically administered ophthalmic medications are no exception. The flushing action of the tear film and barrier function of the corneal epithelium decrease drug bioavailability and necessitate that many topical ophthalmic solutions be applied multiple times each day to achieve therapeutic concentrations by increasing their ocular surface concentrations. It is imperative that any product designed for topical application to the eyes is evaluated for its potential negative impact on external ocular structures so that appropriate risk assessments can be made when choosing among treatment options. Cultured CECs and stromal fibroblasts are commonly used to evaluate the effects of compounds on cellular viability and wound healing. Although caution must be used when translating the results of in vitro studies to in vivo conditions, inferences can be drawn to help guide the use of test compounds in clinical situations.

Exposing cultured CECs to nalbuphine solutions at concentrations ≥ 0.25% caused acute toxicosis; over a 30-minute exposure period, treatment with 0.25%, 0.5%, and 1.2% nalbuphine solutions decreased CEC...
ologic wound healing, whereas dysregulation of these
wound healing was likely the result of cytotoxicosis, particu-
larly with respect to the CECs, where an increase in
cell viability , which indicates dose-dependent cytotoxic
effects. This effect was also observed morphologically,
whereby treatment of cells with 0.5% and 1.2% solu-
tions, in particular, led to cellular shrinkage, rounding, and lifting from the bottom of the culture dish within 5 minutes after onset of exposure, which further indicated a marked decrease in cellular viability.

Several clinical implications could conceivably be related to impaired corneal wound healing and cellular cytotoxic effects. Maintenance of the CEC barrier is of critical importance for ocular health. Epithelial cell death can compromise corneal transparency and leave the underlying stroma vulnerable. Migration and activation of CECs and fibroblasts play an integral role in the closure of corneal wounds. For both CECs and corneal fibroblasts, wound healing experiments in the present study revealed no significant differences in the rates of cellular migration between cultures incubated with a 0.01% nalbuphine solution and control cultures. However, after exposure to nalbuphine for 24 hours, a significant decrease in cellular migration was observed for the 0.1% nalbuphine treatment, compared with results for the control cultures. Such a delay in wound healing was likely the result of cytotoxicosis, particularly with respect to the CECs, where an increase in wound size was detected. Coordination between the corneal epithelium and stroma is required for physiological wound healing, whereas dysregulation of these events can lead to persistent erosions, such as spontaneous chronic corneal epithelial defects in dogs.

Persistent defects within the corneal epithelium may increase the risk for developing infectious keratitis with resultant corneal perforation. Additionally, studies have revealed that activated stromal fibroblasts infiltrate corneal wounds when the corneal epithelium and underlying stroma are damaged. Infiltration of activated stromal fibroblasts into the area of damaged corneal stroma is essential to the production of new collagen fibrils and extracellular matrix needed to regain structural integrity and translucency compatible with appropriate ocular function. Repeated application of solutions that are toxic to corneal fibroblasts could theoretically reduce the population of stromal fibroblasts underlying a corneal epithelial defect and impair tissue repair mechanisms. The result would be corneal wounds that fail to heal properly, which results in a decrease in corneal tissue integrity and leaves affected eyes susceptible to further insult. Canine eyes affected by various disease conditions, including spontaneous chronic corneal epithelial defects and keratoconjunctivitis sicca, in which the overlying corneal epithelium is absent or diseased for a prolonged period, may be at greater risk of cytotoxic effects from topically applied medications such as nalbuphine, compared with effects in healthy eyes.

In contrast, decreased viability of activated stromal fibroblasts may actually have a beneficial role in the healing of corneal wounds, specifically in relation to corneal scarring after injury. Increased numbers of activated corneal fibroblasts have been associated with a condition termed corneal haze, which is an opacity seen in the area of a corneal wound after healing has taken place. Various compounds, including mitomycin C, corticosteroids, and heparin, have been investigated for their ability to decrease the migration and proliferation of activated stromal fibroblasts associated with corneal wounds. These studies, and other similar studies, have found that topical application of antiproliferative and antimigratory agents to corneal wounds can reduce the incidence of corneal haze. Therefore, it is possible that an agent such as nalbuphine, which has similar effects on fibroblast proliferation and migration as indicated by the results of the present study, could have beneficial effects on the development of corneal haze if applied during the wound healing process.

For the conditions of the present study, nalbuphine easily crossed human CEC monolayers. Although not directly tested, we speculate that there would be similar transport of nalbuphine across canine CECs. The apparent permeability of nalbuphine was approximately the same for both directions (apical to basolateral and baso-
lateral to apical) at 3 time points (Table 1). These transepithelial transport results suggest that for the conditions evaluated, nalbuphine was not preferentially transported in either direction across the CEC monolayer.

To further investigate the kinetics of nalbuphine transport across the CEC monolayers, we performed additional experiments to measure transport in only 1 direction (apical to basolateral), and we included additional time points and a higher nalbuphine concentration. We observed that the transport rate of nalbuphine continuously increased throughout the 30-minute experiments. This result may suggest that integrity of the monolayers was compromised, perhaps because of the acute toxic effects of nalbuphine. Although the TEER values did not indicate cell loss at any of the time points, it has been reported that increased permeability is not universally indicated by changes in TEER values. However, this inconsistency does suggest that these data should be interpreted with caution. We used a stratified CEC culture to determine that both 0.25% and 0.5% nalbuphine solutions continuously increased transport rates for at least 30 minutes. These data support the hypothesis that topically applied nalbuphine could penetrate through single and multiple layers of corneal epithelium and possibly enter the stromal tissue. As such, data on toxic effects of nalbuphine on stromal fibroblasts reported here are much more relevant.

Caution should be used when attempting to directly apply the in vitro results of the present study to the in vivo tissue response. Additional studies are needed to assess corneal healing time and potential corneal cytotoxicosis after nalbuphine treatment. In live animals, many factors not accounted for in the present study may influence drug availability to corneal tissues.
References


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