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13. ABSTRACT (Maximum 200 words) Two blinded studies were conducted to evaluate the effects of selected systemic analgesics on the Sereny Test in outbred Hartley guinea pigs. Study 1 evaluated the recommended dosages for two systemic analgesics; study groups consisted of those receiving butorphanol tartrate (n=16), those receiving buprenorphine hydrochloride (n=16), and untreated controls (n=5). Study 2 evaluated a low-dose buprenorphine hydrochloride group (n=16) and an untreated control group (n=5). All animals were inoculated with <i>Shigella flexneri</i> , strain 2a 2457T, onto the cornea and conjunctiva of each eye. At the onset of clinical signs, analgesics were administered to test groups. The degree of keratoconjunctivitis was evaluated per standard procedure; animals were weighed daily. After 7 days, animals were euthanatized and the eyes were removed for histologic morphometric evaluation. Clinical observations of keratoconjunctivitis in both studies were not significantly different. Histologic morphometry confirmed clinical observations when each analgesic treatment group was compared with the corresponding untreated control group. Although heavy buildup of periorbital mucopurulent discharge in the buprenorphine study-1 group complicated clinical observations, the lower dose of buprenorphine (study-2) appears compatible for use.					
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## Evaluation of Butorphanol Tartrate and Buprenorphine Hydrochloride on the Inflammatory Reaction of the Sereny Test

James R. Swearingen, Rebecca A. Cockman-Thomas, Judith A. Davis, and Peter J. Weina

**Abstract** | Invasion of the ocular epithelia of guinea pigs by virulent *Shigella* organisms, eliciting keratoconjunctivitis, is the basis of the Sereny Test (ST). This test has been used to ascertain the virulence of *Shigella* strains and more recently to screen candidate *Shigella* vaccines for efficacy. This test undoubtedly causes pain in test animals; however, recommendation for use of local analgesics/anesthetics has not been accepted because of concern that these topical agents may affect the ability of the *Shigella* organisms to invade the ocular epithelia or have a physiologic effect on the inflammatory process. Similarly, investigators are hesitant to use systemic analgesics in conjunction with the ST.

Two blinded studies were conducted to evaluate the effects of selected systemic analgesics on the ST in outbred Hartley guinea pigs. Study 1 evaluated the recommended dosages for two systemic analgesics; study groups consisted of those receiving butorphanol tartrate (n = 18), those receiving buprenorphine hydrochloride (n = 16), and untreated controls (n = 5). Study 2 evaluated a low-dose buprenorphine hydrochloride group (n = 16) and an untreated control group (n = 5). All animals were inoculated with *Shigella flexneri*, strain 2a 2457T, onto the cornea and conjunctiva of each eye. At the onset of clinical signs, analgesics were administered to test groups. The degree of keratoconjunctivitis was evaluated per standard procedure; animals were weighed daily. After 7 days, animals were euthanized and the eyes were removed for histologic morphometric evaluation.

Clinical observations of keratoconjunctivitis in both studies were not significantly different. Histologic morphometry confirmed clinical observations when each analgesic treatment group was compared with the corresponding untreated control group. Mean individual weight gains were less in all analgesic groups when compared with their untreated control group and were attributed to opioid-induced sedation. Our findings suggest that agonist-antagonist opioid analgesics do not interfere with the inflammatory response of the ST. Although heavy buildup of periorbital mucopurulent discharge in the buprenorphine study-1 group complicated clinical observations, the lower dose of buprenorphine (study 2) appears compatible for use with the ST, on the basis of non-interference with the inflammatory reaction, logistical advantages over butorphanol, and minimal interference with making clinical observations.

The Sereny Test (ST) has been used in the study of virulence of the *Shigella* species of bacteria for over 35 years (1). Ability of *Shigella* organisms to invade the conjunctival and corneal epithelium, proliferate, and induce keratoconjunctivitis in mice, rabbits, and guinea pigs provides a model system that imitates the natural process of *Shigella* invasion of the intestinal mucosa (2). Development of keratoconjunctivitis is most reproducible in the guinea pig (2), making this model highly popular for testing the virulence of *Shigella* strains as well as for measuring the protective efficacy and immunogenicity of candidate vaccine strains (3).

Researchers in our institute have used the ST as a model to test efficacy of candidate vaccine strains against shigellosis. Previously, the only models available for vaccine efficacy testing were nonhuman primates and humans (4, 5). These vaccine trials are both expensive and difficult to per-

form; therefore, researchers have used the ST in the guinea pig as a small animal model to evaluate efficacy of vaccine candidates by intraocular challenge. Guinea pigs are inoculated with the candidate vaccine at specified time intervals. Following significant antibody titer development, the ST is conducted and the candidate vaccine evaluated on the basis of the degree of inflammation that develops. Our Institutional Animal Care and Use Committee (IACUC) raised concerns about the lack of analgesics in conjunction with the ST because of the extensive ocular lesions that result from the procedure in naive and hypimmune animals. Topical analgesics were ruled out because the ability of the organisms to invade the ocular epithelium could be disrupted with the addition of topical ointments or solutions. However, the IACUC was interested in whether systemic analgesics would alter the inflammatory response. The IACUC pointed out that there is an increasing awareness of the need to provide adequate analgesia for experimental animals and unless

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documentation proves otherwise, pain relief must be considered essential. At the request of the IACUC, this study was designed to objectively evaluate the use of two different systemic analgesics with the ST.

## Materials and Methods

**Animals:** Thirty-four male and 33 female outbred Hartley guinea pigs (CrI:(HA)BR, VAF/Plus<sup>®</sup>, Charles River Laboratories, Wilmington, MA), weighing 250 to 300 g, were used in this study. The guinea pigs were purchased antibody-free to Sendai virus, pneumonia virus of mice, reovirus type 3, lymphocytic choriomeningitis virus, and *Mycoplasma pulmonis* and underwent a 10-day quarantine. They were singly housed in polycarbonate cages (Lab Products, Inc., Maywood, NJ) measuring 18 x 9 x 8 cm on hardwood bedding (Beta-Chip<sup>®</sup>, Northeastern Product Corporation, Warrensburg, NY). The guinea pigs were provided Guinea Pig Chow/5025<sup>®</sup> (Purina Mills, Inc., St. Louis, MO) and tap water ad libitum. Environmental conditions provided 10 to 15 changes per hour of 100% conditioned fresh air, a temperature range of 24 to 25°C, relative humidity between 40 and 70%, and a 12-hour light:dark photoperiod with no twilight. All animals were individually identified with the Electronic Laboratory Animal Monitoring System (ELAMS<sup>®</sup>, BioMedic Data Systems, Inc., Maywood, NJ).

**Biohazard precautions:** Since the strain of *Shigella* used was virulent and capable of causing clinical disease in humans, appropriate biohazard precautions were taken throughout the course of the study.

**Analgesics:** Systemic opioid analgesics were chosen for this study because they are generally considered the most potent analgesics. They also act specifically on opiate receptors, with highest binding affinity in the central nervous system. Two systemic analgesics chosen for evaluation were butorphanol tartrate (Torbutrol<sup>®</sup>, Fort Dodge Laboratories, Fort Dodge, IA) and buprenorphine hydrochloride (Buprenex<sup>®</sup>, Norwich Eaton Pharmaceuticals Inc., Norwich, NJ). Comparative anesthetic efficacy was not a consideration in these studies. The primary concern was to investigate the potential use of systemic analgesics without modifying the inflammatory ocular response in the ST.

**Experimental design:** Two studies were performed. In study 1, both analgesics were evaluated at recommended dosages. Each analgesic test group contained 16 animals, 8 female and 8 male animals. An untreated control group of two males and three females was used. A small untreated control group was justified because of a near 100% positive reaction rate seen with the ST when using a virulent *Shigella* strain. Seven uninoculated animals were used for histologic morphometric comparisons at the end of the study. Study 2 was performed to evaluate a lower dosage of buprenorphine; test and control groups were the same size with two uninoculated animals used for morphometry comparisons. Each of the analgesic test groups and untreated control groups were inoculated with 0.05 ml of *Shigella flexneri*, strain 2a 2457T (approximately  $5 \times 10^8$  organisms), onto the cornea and conjunctiva of each eye. The eyelids were massaged open and shut for approximately 30 seconds to ensure even distribu-

tion of the organisms over the conjunctiva and cornea. Ocular inoculations and treatments were performed independently from observations of inflammation by separate individuals. The identification of the test and control group animals were confidential until the end of each study.

Each animal was observed twice daily for 7 days following conjunctival inoculation. All observations were performed by one trained observer. At the first signs of ocular inflammation (hyperemia of conjunctiva, epiphora, exudation), each affected guinea pig received either butorphanol tartrate or buprenorphine hydrochloride according to their assigned group. Butorphanol was administered at a dosage of 0.043 mg/kg, q 8 h (6), subcutaneously into the dorsal aspect of the neck. Buprenorphine was administered in the same manner at a dosage of 0.05 mg/kg, q 12 h (7) in study 1 and at a dosage of 0.025 mg/kg, q 12 h in study 2. A clinical description of each affected eye was made during each observation and the animals were weighed daily.

Clinical descriptions of eyes were categorized into one of three types: negative, weak positive, and positive. Weak positive eyes had only signs of conjunctival inflammation that included conjunctival hyperemia and epiphora or purulent exudate, but no corneal opacities. Positive eyes had corneal opacities or ulcerations in addition to conjunctival inflammation.

At the end of the 7-day observation period, each animal was euthanatized in a carbon dioxide chamber. The eyes were removed and fixed in buffered 10% formalin. Following fixation, each eye was embedded in paraffin and sectioned through the center of the cornea on a longitudinal axis at 5-micrometer increments and prepared with Movat stain. Histologic morphometry was used to calculate the thickness of the cornea centered on the anterior chamber. Measurements for each cornea were made in 0.01-micrometer increments. Two sections of each eye were measured for a total of four sections per animal. After measurements were recorded, animal identifications were revealed and measurements collated according to assigned groups.

**Statistical analysis:** Clinical observations were evaluated by arranging the data into ordered categories and comparing groups with the Wilcoxon rank sum test. Each eye was given a numerical score based on clinical signs as follows: 0 - negative; 1 - weak positive; and 2 - positive. The score for each animal was then determined by averaging the scores for both eyes.

Comparisons of individual weight gain between groups were also evaluated with the Wilcoxon rank sum test. Morphometry measurements were compared by calculating the mean and standard deviation for each group and using Student's *t* test to determine differences between groups. An unexpected finding of increased incidence of periorbital mucopurulent buildup was evaluated with Fischer's exact test.

## Results

Clinical observations of all inoculated groups followed normal ST progression patterns (Table 1). One animal in each study had a weak positive reaction at postinoculation (pi) hour 8. The majority of animals (96.5%) had either weak

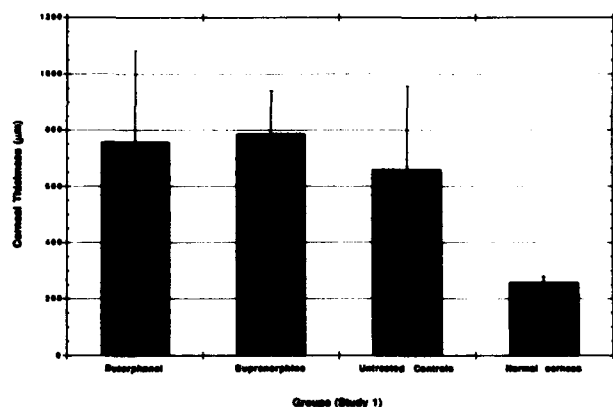
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**Table 1.** Progression of ocular lesions in guinea pigs after inoculation of *Shigella flexneri*, strain 2a 2457T, onto the cornea and conjunctiva of each eye

Hours after inoculation	Graded response <sup>a</sup>					
	Study 1			Study 2		
	0	1	2	0	1	2
8	73 <sup>b</sup>	1	0	41 <sup>b</sup>	1	0
24	5	60	9	0	2	40
36	3	12	59	0	2	40
48	1	3	70	0	0	42
72	1	2	71	0	0	42
168	0	2	72	0	0	42

<sup>a</sup>Numerical score based on ophthalmic clinical signs: 0 = negative, 1 = weak positive, 2 = positive.

<sup>b</sup>Number of eyes, no differences were found between study and control groups, therefore data were pooled.



**Figure 1.** Corneal thickness measurements for groups of guinea pigs in study 1.

positive or positive reactions between pi hour 8 and 24. On day 7 of study 1, 97.3% of the eyes were graded as positive and 2.7% weak positive. There was 100% positive reaction on day 7 of study 2. Only the data from the endpoint of each study were used for statistical comparison. The mean score for each animal was calculated for the three groups of study 1 and the two groups of study 2 (Table 2). Clinical observations were not significantly different ( $P > 0.25$ ) between any analgesic treatment group and the corresponding untreated control group of either study or between the two analgesic treatment groups of study 1.

The means and standard deviations were calculated for morphometry measurements of corneal thickening for each study group. A group of normal guinea pig corneas was also evaluated with each study to demonstrate the extent of corneal involvement in the inoculated groups. Statistical analysis of results of study 1 revealed no significant difference between the butorphanol group and the untreated control group ( $P > 0.25$ ), between the buprenorphine group and the untreated control group ( $P > 0.12$ ), or between either analgesic group ( $P > 0.25$ ; Figure 1). Results of study 2 were also not significantly different ( $P > 0.25$ ) between the low-dose buprenorphine group and the untreated control group (Figure 2). All inoculated groups had obvious differences ( $P < 0.0001$ ) in corneal thickening, compared with normal corneas.

**Table 2.** Sereny Tests scoring of keratoconjunctivitis

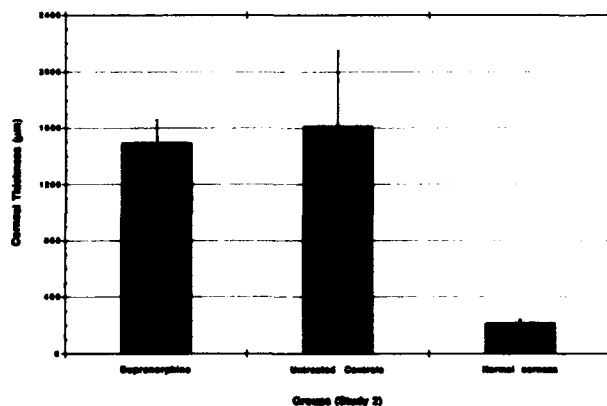
Study group	Mean eye score <sup>a</sup>	
	1.5	2.0
Butorphanol	1	15
Buprenorphine (study 1) <sup>b</sup>	0	16
Buprenorphine (study 2) <sup>c</sup>	0	16
Untreated	1 <sup>d</sup>	9

<sup>a</sup>Eye scores for each animal were averaged to obtain final score.

<sup>b</sup>Buprenorphine dosage of 0.05 mg/kg.

<sup>c</sup>Buprenorphine dosage of 0.025 mg/kg.

<sup>d</sup>Control animal, study 1.



**Figure 2.** Corneal thickness measurements for groups of guinea pigs in study 2.



**Figure 3.** Representative ocular lesions, day 7 after inoculation. Notice hyperemia and corneal thickening.

Study-2 inoculated animals had an obvious increase in corneal thickening when compared with study-1 inoculated animals (Figure 2). Also, a faster conversion to a positive status was apparent in study 2. These observations suggested that a more virulent *Shigella* inoculum was used in study 2. The presence of an apparently more virulent *Shigella* organism resulted in greater corneal thickening in both treatment and control groups of study 2, but the differences between the two groups were not significant.

The mean individual weight gain (MIWG) varied significantly between study groups in both study 1 and study 2 (Table 3). The MIWG of the buprenorphine and butorphanol groups of study 1 was less than the MIWG of the untreated control group. The buprenorphine group had the lowest MIWG in study 1. The low-dose buprenorphine group of study 2 also had a lower MIWG than the untreated control group. Differences between the MIWG of the buprenorphine group of study 1 and the low-dose buprenorphine group of study 2 were not significant; however, there was a more consistent weight gain in the low-dose group of study 2 as seen by the considerably smaller standard deviation (8.34 versus 14.23).

An ancillary observation was made during the course of these studies. Some guinea pigs tended to develop a noticeably heavier buildup of mucopurulent discharge around the eye than others. This exudative deposition would often dry and lead to the inability of the guinea pig to voluntarily open its eye, requiring manual separation of the eyelids to allow clinical observations of the cornea and conjunctiva. To further analyze these findings, any animal that had an inability to open either eye during the 7-day observation period of either study was categorized according to type of study group. The buprenorphine group receiving the recommended dose and the butorphanol group of study 1 had an 88% and 12% incidence of occurrence, respectively. The low-dose buprenorphine group of study 2 had a 25% incidence of occurrence and untreated control animals exhibited a 20% incidence. The buprenorphine group receiving the recommended dose in study 1 had a significantly higher incidence of occurrence than any of the other groups ( $P < 0.001$ ). No difference was found between any of the other groups.

## Discussion

The normal progression of a positive ST result in a naive guinea pig includes acute edema and a mucopurulent discharge within 12 hours of inoculation. By pi hour 48, the entire cornea is hazy and quickly becomes totally opacified and a heavy fibrin layer is formed over the cornea (Figure 3). Extensive corneal ulceration with purulent debris is seen within 72 hours after inoculation; extensive vascularization followed by severe bleeding can develop as complications (1).

In the literature of experimental models that use the ST, analgesics are not mentioned; yet pain is clearly associated with keratoconjunctivitis in humans. One study (8) concluded that in eye irritation studies, the use of topical anesthetics may increase eye irritation but reduction of volume of test material instilled into the eye can reduce ocular injury while maintaining test sensitivity. No information could be found concerning the specific nociceptive neuroanatomic features of the guinea pig eye, but human pathways are well documented. In humans, the sensory innervation of the cornea is from A-delta and C fibers, which contribute fine axon terminals to the basal layer of the corneal epithelium. These endings are presumably responsible for triggering reflex actions such as blinking as well as transmitting nociceptive impulses to the brain. Pain is characteristic of corneal inflammation or injury in humans (9), and we have no objective evidence to suggest that that is not the case in guinea pigs.

Table 3. Mean individual weight gain of guinea pigs

Study group	Weight (g) <sup>a</sup>	
	Study 1	Study 2
Butorphanol	31.75 ± 15.87	NE
Buprenorphine	18.31 ± 14.23	24.81 ± 8.34
Untreated	49.20 ± 12.89	44.80 ± 4.49

<sup>a</sup>Mean weight gain ± SD, over 7-day study period.

NE = not evaluated in study 2.

Buprenorphine hydrochloride is a C-bridged oripavine derived from thebaine (10) and is a partial agonist with high affinity for  $\mu$ -subclass opiate receptors in the central nervous system (11). It is increasingly used for pain relief and is one of the most useful agents that can provide effective analgesia in a wide number of species with a longer treatment interval than most other opioid analgesics (12). Butorphanol tartrate, a narcotic analgesic, is a member of the phenanthrene series. Butorphanol appears to be a partial agonist for  $\kappa$ -subclass opiate receptors in the central nervous system. Because both of these analgesics are also active as an antagonist at the alternate analgesic receptor, they are classified as agonist-antagonist analgesics (13).

The clinical observations and subjective morphometry data obtained in this study support the null hypothesis: that butorphanol (0.043 mg/kg) and buprenorphine (0.05 or 0.025 mg/kg) do not significantly affect the inflammatory process of a positive ST result. The progression of ocular lesions in all groups followed documented descriptions of the typical response to conjunctival inoculation of a naive guinea pig with a virulent *Shigella* strain. Even with the speculative increase in virulence of the organism and greater increase in corneal thickening seen in study 2, buprenorphine (0.025 mg/kg) did not appear to affect the inflammatory response. The morphometric measurements of corneal thickening provided an objective comparison of the inflammatory process between groups. Morphometry is not usually performed when interpreting ST reactions but was used in this study to confirm the clinical observations routinely used to classify each animal's response.

Logistical considerations must be considered when implementing additional steps to any research protocol. The use of butorphanol on a TID schedule has increased logistical considerations, compared with the BID schedule of buprenorphine. The BID dosing of buprenorphine appears more reasonably incorporable to protocol design and function.

The increase in exudative deposition around the eye observed in the buprenorphine group of study 1 could not be explained by any observed or measured increase in inflammation. The exudative deposition did make the ST evaluation more time-consuming in that the affected eyelids had to be manually separated on occasion to allow clinical observations of the conjunctiva and cornea. Fully effective doses of opioid analgesics usually cause sedation in humans. Drowsiness and clouding of the sensorium and mental processes are the most prominent central effects of opioids (14). Most individuals can be easily aroused to an alert state. We speculate that the high incidence of eye closures in the group given the recommended dose of buprenorphine was due to a decrease in normal grooming behavior as a result of a heav-

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ily induced opioid sedation. Similarly, we propose that sedative effects were not noticed when the animals were handled during observation and weighing because of easy arousal to the alert state. The butorphanol and low-dose buprenorphine groups had incidences of eye closure similar to the untreated control animals. This would indicate that the dosages used for the butorphanol and low-dose buprenorphine groups had less severe sedative effects.

The significantly smaller weight gains seen in the analgesic groups of both studies also indicate that some sedative effects resulted at the dosages used. Mixed agonist-antagonist analgesics have been shown to significantly reduce food intake in rats, with no apparent effect on water intake (15). Although not statistically significant, the MIWG of the low-dose buprenorphine group of study 2 was higher than the group given the recommended dose of buprenorphine in study 1. The MIWG also appeared more consistent with the low-dose buprenorphine group as evidenced by a considerably smaller standard deviation. These findings suggest that using weight gain as an indicator of the presence or absence of pain is not valid when opioid analgesics are used in guinea pigs.

Analgesia should always be a consideration with any procedure that could induce pain, and only through appropriate justification should analgesics not be used. Lack of documentation in the literature of the effects of analgesics on various biological variables is a common and sometimes applicable justification for not using them. This study provides scientific data that may be beneficial to help make determinations on the use of analgesics with the ST. We believe that buprenorphine hydrochloride and butorphanol tartrate are both acceptable systemic analgesics for use in conjunction with the ST, on the basis of their noninterference with the inflammatory response. Buprenorphine has obvious logistical advantages over butorphanol, with the lower dosage of buprenorphine (0.025 mg/kg) resulting in minimal interference with clinical observations.

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The authors thank Dr. Antoinette Hartman for assistance in working with the Sereny Test. The views and opinions expressed are solely those of the authors and do not necessarily reflect those of the Department of Defense or the United States Army. This study was approved by the Institutional Animal Care and Use Committee. All animals used in this study were maintained and handled in accordance with the National Institutes of Health Publication 86-23 (1985), *Guide for the Care and Use of Laboratory Animals*.

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