The Effects of Topical Nonsteroidal Anti-inflammatory Drugs on Adenoviral Replication

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Objective: To evaluate the antiviral activity of topical diclofenac sodium (Voltaren Ophthalmic) and ketorolac tromethamine (Acular) (2 nonsteroidal anti-inflammatory drugs [NSAIDs]) on adenoviral replication in vitro and in the adenovirus (Ad) 5 McEwen–New Zealand rabbit ocular model.

Methods: The 50% inhibitory concentration of ketorolac and diclofenac and their respective preservative components were determined for common ocular adenoviral serotypes (Ad8, Ad19, Ad1, and Ad5). In a series of experiments, Ad5 McEwen–inoculated New Zealand rabbit eyes were treated topically 4 times daily for 18 days with either ketorolac, diclofenac, prednisolone acetate (Pred Forte), or control vehicle (Comfort Tears).

Main Outcome Measures: Outcome measures included serial ocular tear film titers and the formation of subepithelial immune corneal infiltrates.

Results: In vitro, neither ketorolac nor diclofenac demonstrated significant inhibitory activity against Ad1, Ad5, Ad8, or Ad19. In the rabbit model, there were no statistically significant differences among ketorolac, diclofenac, and the control vehicle with respect to viral replication or the formation of subepithelial immune infiltrates. In contrast, 1% prednisolone prolonged viral shedding and inhibited immune infiltrates (P<.001 for both).

Conclusions: Our experimental study suggests that treatment of epidemic keratoconjunctivitis with topical NSAIDs may be a safer alternative than topical steroids. Only controlled clinical trials can determine whether topical NSAIDs can provide symptomatic relief and not interfere with normal viral clearance.


A denoviral ocular infections are common globally and cause significant individual patient morbidity. Visual disturbances, photophobia, and occasional pain are due to the inflammation induced by replicating adenoviruses (Ad). Ocular surface inflammation is manifested by lid swelling, pseudomembranes, conjunctival hyperemia, chemosis, punctate epithelial keratitis, and secondary anterior iridocyclitis. Epidemic keratoconjunctivitis, pharyngeal conjunctival fever, and follicular conjunctivitis (the 3 most common forms of highly contagious adenoviral ocular infections) cause community and medical facility epidemics that produce significant societal losses from worker and student absenteeism.

The appropriate role of topical anti-inflammatory agents to control patient distress remains an important clinical goal in the management of these patients. The routine use of one category of anti-inflammatory agent—topical corticosteroids—is generally discouraged by most authorities because of the consequences of misdiagnosis (herpes simplex virus 1, Acanthamoeba, or fungal infections) and the complications of cataract, glaucoma, and superinfection associated with long-term use. Nevertheless, during the acute phase, the anti-inflammatory therapeutic effects of topical steroids are effective in providing patient relief. During the chronic phase, the anti-immune therapeutic effects of topical steroids reduce the symptoms associated with subepithelial immune infiltrates (decreased night vision, glare, and halos), but can also facilitate steroid dependence in some patients.

It has been previously reported in the Ad5 McEwen–New Zealand rabbit ocular model that a topical corticosteroid—prednisolone acetate (1% Pred Forte)—was an effective anti-inflammatory and anti-immune agent, but significantly enhanced Ad5 replication and prolonged Ad5
MATERIALS AND METHODS

VIRUS AND CELLS

Several clinical adenoviral isolates were cultured from patients with typical adenoviral keratoconjunctivitis at The Eye and Ear Institute of Pittsburgh, Pittsburgh, Pa. The isolates were serotyped by immunofiltration and serum neutralization and found to be Ad types 1, 5, 8, and 19. The isolates, designated Ad1 Knetz, Ad5 McEwen, Ad8 Gray, Ad8 Edmunds, and Ad19 Kowalski, were grown in A549 monolayers at 37°C in a 5% carbon dioxide and water vapor atmosphere and harvested, divided into aliquots, and frozen as stock virus at −70°C. Before use, the stock viruses were titrated using a standard plaque assay.

A549 cells, an epithelial-like cell derived from human lung carcinoma (CCL-185, American Type Culture Collection, Rockville, Md), were grown and maintained in Eagle minimum essential medium with Earle salts (Sigma Cell Culture Reagents, St Louis, Mo) and supplemented with 6% heat-inactivated fetal bovine serum (Harlan Bioproducts for Science, Indianapolis, Indl), 2.5-μg/mL amphotericin B, 100-U/mL penicillin G sodium, and 0.1-mg/mL streptomycin (Sigma Chemical Co, St Louis).

EXPERIMENTAL DRUGS

Ketorolac tromethamine, 0.5% (Acular, Allergan Pharmaceuticals, Irvine, Calif), is a member of the pyrrolo-pyrrole group of NSAIDs for ophthalmic use. The preservatives contained in ketorolac are 0.01% benzalkonium chloride and 0.01% EDTA. Diclofenac sodium, 0.1% (Voltaren Ophthalmic, CIBA Vision Ophthalmics, Atlanta, Ga), is a member of the phenylacetic acids group of NSAIDs for ophthalmic use and is preserved with 0.1% EDTA. Prednisolone acetate, 1% (Pred Forte, Allergan Pharmaceuticals, Irvine, Calif), is a sterile corticosteroid suspension for ophthalmic use that was used in the present study as a positive control. Artificial tears (Comfort Tears, Barnes-Hind Inc, Sunnyvale, Calif) was used as a negative control drug. The preservative components of the NSAIDs were purchased individually from the Sigma Chemical Co.

DETERMINATION OF 50% INHIBITORY CONCENTRATION

As previously reported,13 100 plaque-forming units (pfu) (0.1 mL) of each adenoviral serotype was added to each well of a 24-well plate containing A549 cell monolayers. After adsorption of the virus suspension for 3 hours, 6 serial 10-fold dilutions of experimental drug were added (1 mL per well) to 3 replicate wells. Controls included the addition of media alone to the 6 remaining virus-infected wells and the addition of the drug concentrations to uninfected A549 cells on a duplicate plate. The wells were examined daily using an inverted microscope for viral cytopathetic effect. When viral plaques were visible in the virus-infected control wells (approximately 7-14 days, depending on serotype), the plates were stained with gentian violet and read under a dissecting microscope (magnification ×25) in a masked fashion. Each well was scored by counting the number of plaques per well, and the 50% inhibitory concentration (IC50) for that adenoviral serotype was calculated graphically. Each determination was performed in duplicate.

ANIMALS

Six-week-old, 1-kg female New Zealand albino rabbits were obtained from Green Meadows Rabbitry, Murrysville, Pa. All animal studies conformed to the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed.

RESULTS

IN VITRO IC50 ASSAYS

Table 2 summarizes the results of duplicate IC50 assays for ketorolac, diclofenac, and their preservative components against various ocular adenoviral serotypes. Compared with the established therapeutic range for proven antiviral agents (0.02-5.00 μg/mL),15 minimal direct antiviral activity was demonstrated only for diclofenac, but it is very unlikely these values have clinical promise. The exact IC50 for ketorolac could not be determined for most serotypes because, at the highest concentrations tested, no antiviral activity could be determined. In one assay,
ASSESSMENT of Ad5 REPLICATION
(TOPICAL INOCULATION ONLY)

This study was performed in duplicate using 12 and 16 rabbits per respective experiment. Following appropriate systemic and topical anesthesia, rabbits were inoculated with 30 µL (1.2 x 10² pfu per eye) of Ad5 in both eyes after 12 cross-hatched strokes of a No. 25 sterile needle. Twenty-four hours later, after the establishment of Ad5 replication, 7 rabbits each were randomly assigned to 1 of 4 treatment groups (artificial tears, ketorolac, diclofenac, or prednisolone). Rabbits were treated in both eyes with the appropriate drug 4 times per day for 14 days, then twice a day for 4 additional days. Ocular swabbing to recover Ad from the tear film and conjunctival surface was performed on days 0, 1, 3, 4, 5, 7, 9, 11, 14, 16, 18, and 21 after inoculation and frozen at −70°C pending plaque assay. Ocular Ad replication in each treatment group was determined using the following outcome measures: the mean ocular titer per day (Figure), the overall mean ocular titer per early phase (days 0–7) or late phase of infection (days 9–21) (no data were generated on day 8), the number of Ad culture–positive eyes per early and late phases of infection, and the mean and median duration of Ad shedding in days (Table 1). The overall mean Ad5 ocular titers were determined for each group for the early and late phases of infection by calculating the mean of all ocular cultures during those respective periods. The number of Ad5 culture–positive eyes per group was determined by calculating the number of Ad5-positive ocular cultures per total number of ocular cultures during the 2 different phases of infection.

ASSESSMENT of Ad5-INDUCED SUBEPITHELIAL INFILTRATES (TOPICAL AND INTRASTROMAL INOCULATION)

Following topical and systemic anesthesia, both eyes of 12 rabbits were inoculated with 30 µL (1.2 x 10² pfu per eye) of Ad5 McEwen intrastromally using a 30-gauge short-beveled needle to form 5 focal blebs (dice pattern, 10 µL per bleb). The corneas were then scarified superficially (8 scratches) with a No. 25 needle to form a square around the central intrastromal injection. Inoculation was completed by applying 30 µL (1.2 x 10⁶ pfu per eye) of Ad5 McEwen. Twenty-four hours later, the 3 rabbits each were randomly assigned to the same treatment groups and regimens as the topical-inoculation-only group. The extent of subepithelial immune infiltrate formation was determined by slitlamp examination of rabbit corneas on day 23 postinoculation. The extent of subepithelial infiltrate formation was scored using a scale of 0 to 4 (0, 0 infiltrates; 1, 1–5 infiltrates; 2, 6–10 infiltrates; 3, 11–15 infiltrates; 4, ≥16 infiltrates).

DETERMINATION of VIRAL TITERS
(PLAQUE ASSAY)

The ocular samples to be titered were thawed, diluted serially (1:10) for 2 dilutions, and inoculated onto A549 monolayers. After 7 days’ incubation, the cells were stained with 0.5% gentian violet, and the number of plaques were counted. The viral titers were then calculated and expressed as plaque-forming units per milliliter.

STATISTICAL ANALYSIS

Following the completion of all experiments, the codes were broken, and the data from each experiment were analyzed statistically. As comparable results were obtained in each experiment, the data were then pooled to obtain a larger subject number and analyzed using analysis of variance (ANOVA), Kruskal-Wallis median test, Duncan multiple range test, and χ² analyses. Significance was established at the P<.05 confidence level.

THE EFFECTS of TOPICAL NSAIDs on ADENOVIRAL REPLICATION

Adenoviral replication for each treatment group is summarized in the Figure. There were no significant differences in the mean ocular viral titer per day among the groups during the early phase of Ad5 infection (days 0–7). During the late phase of infection (days 9–21), there were no significant differences among the ketorolac, diclofenac, and control groups for any day cultures were taken. However, the prednisolone group had a significantly higher mean ocular viral titer than the ketorolac, diclofenac, and control groups (P<.05) for all days cultures were taken (days 9, 11, 14, 16, 18, and 21).

There were no significant differences among any of the treatment groups and the control group for the overall mean Ad5 ocular titer and the ratio of Ad5-culture–positive eyes to the total number of eyes during the early phase of infection (Table 1). In contrast, there were significant differences demonstrated during the late phase (Table 1). While treatment with either ketorolac or diclofenac demonstrated no significant differences from the control group, treatment with prednisolone significantly increased the overall mean Ad5 ocular titer and the ratio of Ad5-culture–positive eyes to the total number of eyes (1.3 x 10² pfu/mL, 71/84 [85%]) compared with the control (1.38 x 10¹ pfu/mL, 24/84 [29%]), ketorolac (8.15 x 10⁰ pfu/mL, 19/84 [23%]), and diclofenac (6.73 x 10⁰ pfu/mL, 18/84 [21%]) groups (mean duration of shedding, P<.001; median duration, P<.001).
Similarly, treatment with either ketorolac or diclofenac demonstrated no significant differences from the control group for the mean and median duration of shedding, whereas prednisolone treatment significantly prolonged shedding by both measures (mean ± SD, 19.6 ± 3.4 days; median, 21 days) compared with the control (10.9 ± 3.3 days; 11 days), ketorolac (9.9 ± 2.6 days; 9 days), and diclofenac (9.7 ± 2.6 days; 9 days) groups (mean duration of shedding, P < .001; median, P < .001).

The appropriate use of established and newer topical anti-inflammatory agents in the treatment of adenoviral ocular infections is currently in evolution. The routine use of potent anti-inflammatory agents (eg, topical corticosteroids) during acute infection is discouraged by most authorities to avoid the well-known complications (misdiagnosis of herpes simplex virus, cataract, glaucoma, or superinfection). Prolonged use of topical corticosteroids for the retreatment of recurrent subepithelial immune infiltrates is also discouraged to avoid “steroid addiction” for a condition that is naturally self-limited. However, the use of corticosteroids to provide for patient comfort and reduce severe inflammation during the acute phase of adenoviral ocular infection is fairly widespread in actual practice. A recent survey reported that 33% of the community ophthalmologists (n = 177) and 42% of the academic ophthalmologists (n = 84) routinely used topical steroids in the treatment of acute epi-

### Table 1. Effects of Topical NSAIDs on Adenoviral Infection in the New Zealand Rabbit Ocular Model

<table>
<thead>
<tr>
<th>Experimental Parameters</th>
<th>Control</th>
<th>Ketorolac Tromethamine</th>
<th>Diclofenac Sodium</th>
<th>Prednisolone Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early phase (days 0-7)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean ± SD Ad5 ocular titer, pfu/mL†</td>
<td>84</td>
<td>1.5 ± 3.7 × 10³</td>
<td>1.1 ± 1.5 × 10³</td>
<td>1.2 ± 1.9 × 10³</td>
</tr>
<tr>
<td>Ad5-culture–positive eyes/total eyes (%)†</td>
<td>84</td>
<td>82/84 (98)</td>
<td>84/84 (100)</td>
<td>83/84 (99)</td>
</tr>
<tr>
<td><strong>Late phase (days 9-21)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD Ad5 ocular titer, pfu/mL‡</td>
<td>84</td>
<td>1.4 ± 4.2 × 10³</td>
<td>8.2 ± 22.3 × 10³</td>
<td>6.7 ± 22.3 × 10³</td>
</tr>
<tr>
<td>Ad5-culture–positive eyes/total eyes (%)‡</td>
<td>84</td>
<td>24/84 (29)</td>
<td>19/84 (23)</td>
<td>18/84 (21)</td>
</tr>
<tr>
<td>Duration of shedding, d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD§</td>
<td>14</td>
<td>10.9 ± 3.3</td>
<td>9.9 ± 2.6</td>
<td>9.7 ± 2.6</td>
</tr>
<tr>
<td>Median¶</td>
<td>14</td>
<td>11.0</td>
<td>9.0</td>
<td>9.0</td>
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<tr>
<td>Subepithelial immune infiltrate score (day 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD#</td>
<td>6</td>
<td>3.0 ± 0.9</td>
<td>2.2 ± 0.8</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Median**</td>
<td>6</td>
<td>3.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*NSAIDs indicates nonsteroidal anti-inflammatory drugs; Ad, adenovirus.
†No significant differences among groups (analysis of variance [ANOVA]; x²).
‡No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has a significantly higher mean Ad5 ocular titer (P < .001) than others (ANOVA).
§No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has significantly more Ad5-positive eyes (P < .001) than others (x²).
¶No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has a significantly longer mean duration (P < .001) than others (ANOVA).
†No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has a significantly longer median duration (P < .001) than others (Kruskal-Wallis).
#No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has a significantly lower mean infiltrate score (P < .005) than others (ANOVA).
**No significant differences among control, ketorolac, and diclofenac groups. Prednisolone has a significantly lower median infiltrate score (P < .01) than others (Kruskal-Wallis).
demetic keratoconjunctivitis. Clearly, there are no universally agreed-on guidelines for the routine use of corticosteroids in the treatment of acute adenoviral ocular infections.

While several studies2,18,19 addressed the effects of topical corticosteroids in the treatment of acute adenoviral ocular disease, there are no reported clinical studies on the role of topical NSAIDs in epidemic keratoconjunctivitis infections. Nevertheless, topical NSAIDs are currently used empirically by individual ophthalmologists for both analgesic and anti-inflammatory effects. In the present study, we report for the first time the effects of 2 popular topical NSAIDs—diclofenac and ketorolac—on adenoviral replication in vitro and in the rabbit ocular model. Our experimental results may be projected to suggest a potential role for their clinical use in the treatment of acute adenoviral ocular infections.

The results of the in vitro IC50 assays for different Ad serotypes (Ad1, Ad5, Ad8, and Ad19) suggested that ketorolac had no direct antiviral inhibitory effect. Diclofenac appeared to have a weak inhibitory effect at a significantly higher IC50 value than those previously reported2 for prednisolone against the same serotypes (IC50 range, 0.01-0.20 µg/mL). Despite these observed in vitro effects for diclofenac, antiviral activity was not confirmed in the animal studies, and it is doubtful that any of these anti-inflammatory drugs have any clinically significant direct antiviral activity against common adenoviral serotypes.

In the Ad5 McEwen–New Zealand rabbit ocular model, topical ketorolac and diclofenac demonstrated (1) no direct adenoviral inhibitory activity, (2) no prolongation of viral shedding, and (3) no effect on the formation of immune infiltrates. In contrast, topical treatment with prednisolone enhanced Ad5 titers, prolonged the duration of viral shedding, and significantly inhibited the formation of subepithelial infiltrates when compared with the ketorolac, diclofenac, and control groups.

The most likely explanation for the observed effects of topical prednisolone are the potent anti-immune and anti-inflammatory properties possessed by this corticosteroid at a 1% concentration. Corticosteroids are known to inhibit the cyclooxygenase and lipooxygenase inflammatory pathways, other cellular enzymes, and direct expression of cytokines and lymphokines that activate the immune system. Recently, the molecular mechanism of action for glucocorticosteroids has been shown to occur through the up-regulation of the complex formed by nuclear factor κB (NF-κB) and its cytoplasmic inhibitor IκBa (IκBa), which in turnNF-κB down-regulates nuclear expression of inflammatory and immune cytokines.20 In contrast, ketorolac and diclofenac, examples of NSAIDs, are simply not as potent as corticosteroids because they block only 1 arm of the inflammatory pathway (ie, the cyclooxygenase pathway)2 and appear to have no direct effect on immune-mediated mechanisms. The limited anti-inflammatory action of ketorolac and diclofenac most likely explains why these agents do not interfere with normal immune-mediated viral clearance. On the other hand, they also have no therapeutic inhibitory effect on the formation of subepithelial immune corneal infiltrates in the rabbit eye.

While it is acknowledged that the pharmacological response in rabbits to topical steroids, NSAIDs, and antidiadenoviral drugs may be different than in humans, the development of successful therapeutic strategies in rabbits for the treatment of patients with herpes simplex virus 1 dendritic keratitis and immunogenic stromal keratitis offers encouraging historical precedent.

Based on the Ad5 McEwen–New Zealand rabbit ocular model, potential clinical guidelines suggest that treatment of patients with acute symptomatic epidemic keratoconjunctivitis using topical NSAIDs for 18 days of continuous therapy may be a safer alternative than using potent topical corticosteroids (eg, 1% prednisolone acetate). Unlike steroids, topical NSAIDs would not be expected to delay viral clearance and, therefore, may not exacerbate the public health problem of community and office epidemics by promoting viral transmission. Furthermore, the established prostaglandin-mediated anti-inflammatory5-8 and topical analgesic effects9-12 may promote patient comfort by reducing ocular inflammation and local pain during the acute stages of infection. Finally, controlled clinical trials remain the only direct way to establish the value of topical NSAIDs in the treatment of symptomatic epidemic keratoconjunctivitis.

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REFERENCES


A look at the past... 

Simplicia sigillum veris is an old saying; but that it is by no means always correct, anybody must admit who has followed the recent development of physiology. There always seems to be a phase in the growth of human sciences, when it appears to be possible to cover a certain subject by a few simple laws; until science advances and finds that these simple laws are only rough deductions and statments from a few limited observations, statements which indeed are often useful and sometimes even necessary for the beginner, but which do not represent the whole truth. The present paper shows the the same development for the physiology of the eye-movements. To draw all the inferences from this new exposition, e.g., with reference to operative procedures, is of course impossible in the present paper, but anybody who has read these lines will admit that the movements of the eyeball are a problem much more complex than it would appear from the present text-books. Only by the introduction of numerical values into our geometrical considerations can there be evolved a real science of the movements of the human eyes.