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Proflavine and Light in the Treatment of Experimental Herpetic Ocular Infections

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The effect of purified proflavine and light exposure was assessed in rabbits whose eyes had been infected with one of two strains of herpesvirus. In comparing proflavine-light and placebo-light treatment, 0.1% proflavine administered twice daily for 5 days had a significant effect in suppressing herpetic eye disease, but 0.05% proflavine was less effective. In addition to being effective in infections with either virus strain, the 0.1% proflavine also suppressed intensity of corneal epithelial ulceration and stromal opacity in animals pretreated with subconjunctival corticosteroids to produce more severe disease. Proflavine or idoxuridine (IDU) alone or in combination showed no differences in suppressing herpetic ocular disease, but all were significantly more effective than placebo. Virus recovery rates were approximately the same from eyes treated with proflavine, IDU, or placebo, indicating that viral replication in the cornea and conjunctiva was not completely suppressed by either of the antiviral drugs alone or in combination.

Although the antiviral action of photodynamically active drugs has been recognized for at least 25 years, these agents have only recently been applied to the treatment of herpes simplex infections in humans (3). Photodynamic action is defined as the photosensitization of a biological system by a substance which serves as a light absorber for photochemical reactions in which molecular oxygen takes part (6). Several heterotricyclic vital dyes, namely, neutral red, toluidine blue, proflavine, and acridine orange, have this photodynamic action. In experimental herpetic keratitis in rabbits, one study has reported a favorable result of proflavine and light exposure (9), but another study indicated that the effect was minimal compared with the therapeutic antiviral effect of idoxuridine (IDU) (13).

In the experiments presented here, the effect of purified, topically applied proflavine and light was assessed in experimental primary herpetic ocular infections in rabbits. A further set of experiments compared proflavine, IDU, and a combination of proflavine and IDU.

MATERIALS AND METHODS

Rabbits. Male albino New Zealand rabbits weighing 1.5 to 2.5 kg each were used. All animals were examined before the experiments to exclude those with any ocular abnormalities.

Viruses. Pools of both PH and RE strains of herpes simplex virus (HSV) were prepared in primary cultures of rabbit kidney cells (1, 7, 18). Both pools had titers of 10^6 50% tissue culture infectious doses per ml.

The PH strain of HSV (originally "O" strain) was isolated from a patient with encephalitis in 1948 and has been carried in mouse brain and tissue culture in our laboratory since then (8). The RE strain was originally obtained from Calvin Hanna (4). When inoculated into the cornea with or without prior scarification, the PH strain usually produces only epithelial ulceration with little disease of the corneal stroma and a low (<5%) mortality due to encephalitis; the RE strain regularly produces not only severe epithelial ulceration but also inflammation of deep corneal stroma, in addition to encephalitis and death in about 25% of inoculated animals. Both strains have been identified as type 1 HSV.

Medications. Purified, lyophilized proflavine base (Mead Johnson Research Center, Evansville, Ind.) contained a glycine as a buffer but no preservative. The proflavine base was diluted to 0.1 and 0.05% in 0.7% NaCl in sterile double-distilled water. The saline diluent alone was used as a placebo treatment. Commercial preparations ("Herplex") of 0.1% IDU in 1.4% polyvinyl alcohol with sodium chloride and a preservative of benzalkonium (0.004%) and methylprednisolone acetate suspension (40 mg/ml) were used. One application of proflavine or IDU consisted of one drop onto the cornea twice 5 min apart. Proflavine treatments were administered twice daily approximately 7 h apart. IDU treatments were given four times a day about 2 h apart from 9 a.m. to 3 p.m. Treatments were administered by the same two individuals throughout the study.

Light source. Exposure to light was done with cool blue 15-W (GE F15 58.B) fluorescent bulbs (Mead Johnson Research Center, Evansville, Ind.). The light output of this bulb is high in the region of the spectrum in which proflavine has the maximal absorption. Exposure was done by holding the animals' eyes open 6 to 12 inches (ca. 15.2 to 30.4 cm) from the light source for 5 min. Light exposure was made twice, 30 and 90 min after the application of proflavine or placebo drops. The animals were kept in cages covered at the top in a room illuminated around the clock with fluorescent bulbs.

Ocular inoculation. To infect the rabbits' eyes, 0.05 ml of the undiluted virus pool (approximately 5×10^4 mean tissue culture infective doses) was dropped onto the cornea of each rabbit without previous scarification. Only animals with bilateral infections were used in the treatment experiments.

Clinical examination. All clinical evaluations were made daily, Monday through Friday, for 3 weeks by the same person, using a conventional biomicroscope (slit lamp) modified to hold experimental animals. In each experiment, examinations were performed before viral inoculation and prior to treatment on treatment days.

Clinical grading of ocular disease. The six categories of clinical signs and their grading are presented in Table 1. The grading of epithelial herpetic keratitis is a modified version of that employed by Polikoff et al. (11).

Virus reisolation attempts. Specimens for virus reisolation were made by gently rolling a sterile, dry, cotton-tipped applicator over the corneal surface. The swabs were immediately placed in Eagle minimal essential medium with 5% fetal calf serum and antibiotics. The swabs were placed in storage at −70 C within 1 h and were held for up to 6 months before virus isolation attempts were made in Vero (African green monkey kidney) cells. Specimens for virus reisolation were obtained on days 3, 7, 10, and 14 after virus inoculation.

Bacterial culture. To check for secondary bacterial infection in animals developing purulent corneal ulcers, bacterial cultures were made of the lesions

TABLE 1. *Clinical grading of experimental herpetic ocular infections*

Clinical sign	Grade	Criteria
Corneal epithelial lesions (amount of fluorescein staining)	0	No staining defect
	0.25	One to 10 microdendrites or 25% of cornea covered with punctate or irregular staining
	0.5	Eleven to 20 microdendrites or 50% of cornea covered with punctate or irregular staining
	0.75	Seventy-five percent of cornea covered with greater than 20 microdendrites and/or punctate or irregular staining
	1	All of the cornea covered with greater than 20 microdendrites and/or punctate or irregular staining, or a geographic ulcer involving 25% or less of the corneal surface
	2	Twenty-five to 50% of the cornea with an epithelial ulcer
	3	Fifty to 75% of the cornea with an epithelial ulcer
	4	More than 75% of the cornea with an epithelial ulcer
Corneal opacity (amount)	0	Clear cornea
	1	Some haze but iris details visualized
	2	Iris details obscured
	3	Iris or pupil margin not visualized
Corneal opacity (location)	1	If present in *Epithelium*
	1	If present in *Stroma*
Corneal vascularization (circumference)	0	None
	1	<180°
	2	>180°
Corneal vascularization (penetration)	0	None
	1	0 to 3 mm
	2	3 mm
Iritis	0	None
	1	Mild iris hyperemia
	2	Marked iris hyperemia

with broth-moistened, cotton-tipped applicators which were streaked immediately onto a sheep blood agar plate.

Treatment schedule. All treatments were started the third day after inoculation and continued to the seventh day after inoculation of virus.

Method for evaluation of clinical results. In all experiments the treated and placebo eyes were compared in individual animals. Initially, the observer was never aware of which treatment had been administered to a particular eye. In the first five experiments comparing proflavine-light with placebo, the same (right or left) eye of all animals received the proflavine-light treatment to avoid medication error. The obvious yellow staining with proflavine, however, clearly identified the treated eye in the first five experiments, so the observer was aware of which eye had been treated. In experiment 6 the fur around each eye was painted with the proflavine prior to treatment so that staining would not reveal the proflavine-treated eye. In this experiment, comparing proflavine, IDU, and placebo, the procedure was further refined so that a single treatment was given to the same eye within one of the four treatment groups, but the contralateral eye was used in another treatment group; e.g., in the group comparing proflavine-light and placebo, the right eyes were treated with proflavine, but in the group comparing proflavine and IDU the left eyes were treated with proflavine. Furthermore, animals were presented to the observer in a random order in all experiments.

Since treatment was started on the third day after virus inoculation, only differences on days 4 through 7 (4 days) were assessed for the first week's results, which were based solely on epithelial lesions (Table 1). A one-grade difference between the treated and placebo eye in the grading scale was considered as one point. Five points were required for there to be a difference tabulated in epithelial keratitis between the two eyes in the first week.

During the second and third weeks of ocular herpetic infection, the five other clinical parameters (Table 1) were also evaluated to determine the difference between the drug- and placebo-treated eyes. For each parameter, a one-grade difference in the grading scale between the drug and placebo eye was considered one point. A difference of four points during the five examining days of the week were required for a specific parameter to be considered less severe in an individual rabbit. If three of the six parameters were less severe in one eye, that eye was considered to be better than the other eye.

For each experiment the number of animals better in the treated eye, better in the placebo eye, and with no difference (ties) were tabulated (Tables 2 and 3), and the significance levels were determined by the sign test (5).

RESULTS

Proflavine toxicity. Ten uninfected animals were treated twice daily for 5 days with 0.05% proflavine and light in the right eye and 0.1% proflavine and light in the left eye. A mild punctate fluorescein staining of the corneal epithelium and epithelial edema occurred with both concentrations during the week of treatment, but the signs appeared slightly earlier and were somewhat worse with the 0.1% solution. With both concentrations this mild epithelial keratitis became less severe after 3 days even though the treatments were continued. All eyes were completely clear of punctate epithelial staining and epithelial edema within 72 h after treatment was stopped and remained clear for 1 additional week of observation. Three weeks after inoculation all animals in these experiments were examined for lens abnormalities through a dilated pupil. No discoloration of the lens or cataract formation was found.

Natural course of herpes ocular infection with PH and RE strains of HSV. The course of disease was evaluated in both eyes in 10 animals inoculated with the PH strain and 10 other animals with the RE strain of herpesvirus and in the placebo-treated eyes with both strains. Whereas there was a marked variation in the severity of eye disease from animal to animal with the same strain of herpesvirus, the two eyes of each rabbit generally followed a very similar disease course.

After inoculation with the PH strain, dendritic ulcers (linear, branching lesions) of the corneal epithelium developed by the third day and reached greatest severity on day 7. Iritis, corneal stromal opacity, and corneal vascularization began from day 5 to day 10. The epithelial disease was decreasing by day 14 but stromal opacity and iritis persisted longer with clearing by day 20. Among the total of 142 animals inoculated with this pool of PH strain, 82% developed bilateral disease.

With RE strain epithelial keratitis followed a similar course, but the epithelial disease was more severe, again reaching a peak on day 7. The iritis and stromal opacities appeared earlier (3 to 5 days) with this strain, the residual corneal disease at the end of 21 days was greater than with PH strain, and five of the 10 animals in the initial RE strain experiment died of encephalitis. All 50 animals inoculated with RE strain in the present series of experiments developed bilateral disease.

Comparison of 0.05% and 0.1% proflavine in herpetic keratitis. In 12 rabbits infected bilaterally with PH strain, 0.05% proflavine and light treatments were applied twice daily to the right eye from day 3 to day 7 after inoculation, whereas the left eye received placebo drops and light. There was a significant number of animals treated with proflavine who had less severe

epithelial disease the second week and less severe epithelial and stromal disease combined the second week after infection (Table 2).

In 17 others infected bilaterally with PH strain, 0.1% proflavine produced a significant improvement in epithelial keratitis the first week and in epithelial and stromal disease combined the second week (Table 3). Since the 0.1% proflavine appeared to be more effective than 0.05% and was only minimally more toxic, the 0.1% concentration was used throughout the rest of the study.

The difference between proflavine- and placebo-treated eyes was greatest on days 7 and 10 (Fig. 1). Whereas there was some overlap of scores in the two eyes, the placebo group clearly had more severe disease. Only animals with typical corneal disease in both eyes were inoculated in the treatment groups, so that there was a deliberate bias toward selecting animals who would develop more severe disease.

Proflavine treatment in RE strains of herpesvirus eye infections. In 16 animals infected bilaterally with the more virulent RE strain of herpesvirus, the right eyes were treated with 0.1% proflavine and the left eyes with placebo starting 3 days after inoculation (Table 4). Significantly less severe epithelial disease was observed the first week, and epithelial and stromal disease were observed the second week. As in PH strain infections, the disease tended to clear in both eyes of surviving rabbits the third week, so that differences were no longer apparent.

Pretreatment with corticosteroid in PH- and RE-infected eyes. In animals given 0.25 ml of methyl prednisolone acetate subconjunctively 2 days prior to virus inoculation, the disease was more severe and continued into the third week. In 15 PH strain-infected animals, a significant effect of proflavine was noted in the first and third weeks (Table 4). In 16 rabbits infected with RE strain, this striking effect of proflavine was noted all 3 weeks.

Comparison of 0.1% proflavine and IDU and the two medications combined. In rabbits with bilateral PH strain infections, paired comparisons were made of 0.1% proflavine alone (twice daily), 0.1% IDU alone (four times daily), and proflavine and IDU combined. Each treatment was compared with one of the other treatments or placebo in groups of rabbits as follows: proflavine versus placebo, seven rabbits; IDU versus placebo, five rabbits; proflavine and IDU versus placebo, five rabbits;

TABLE 3. *Differences in combined scores of epithelial and stromal keratitis after treatment with 0.1% proflavine and light*

Outcome	No. of animals		
	1st Week	2nd Week	3rd Week
Placebo—Better	0	0	0
Proflavine—Better	14	7	4
Ties	3	8	9
P value	0.01	0.01	NS[a]

[a] NS, Not significant.

TABLE 2. *Differences in epithelial and stromal keratitis between placebo- and 0.05% proflavine-treated eyes in herpes-infected rabbits*

Corneal disease	Outcome	No. of animals		
		1st Week	2nd Week	3rd Week
Epithelial keratitis	Placebo—better	1[a]	0	0
	Proflavine—better	4	5	2
	Ties	7	5	6
	P value	NS[b]	0.05	NS
Stromal opacity	Placebo—better	0	0	0
	Proflavine—better	1	3	2
	Ties	11	7	6
	P value	NS	NS	NS
Epithelial and stromal disease combined	Placebo—better	1	0	0
	Proflavine—better	4	5	1
	Ties	7	5	7
	P value	NS	0.05	NS

[a] Number of animals in which the placebo-treated eye was better than the treated eye on 4 of 5 observation days.

[b] NS, No significant difference; $P = 0.05$.

proflavine versus IDU, 13 rabbits; proflavine versus proflavine and IDU, 14 rabbits; IDU versus proflavine and IDU, 13 rabbits.

In this experiment, the proflavine and IDU used separately or as a combination were equally effective in suppressing clinical disease (Table 5). The combination of IDU and proflavine was no better than either drug used alone.

Effect of proflavine or IDU treatment on virus isolation. In all experiments virus was recovered as frequently from treated eyes as from control eyes (Table 6). More than 90% of rabbits yielded virus 5 days post-inoculation during treatment with placebo or proflavine. Even on day 10, after 1 week of proflavine treatment, the recovery rates in treated and placebo groups were not significantly different. In animals pretreated with corticosteroids, the virus recovery rate was no higher (247 positive in 301 attempts) than in animals who were not treated with steroids (248 isolations in 304 attempts). In the comparison of proflavine and IDU, no agent was recovered on day 14 in the placebo-treated eyes, although about 20% of drug-treated eyes yielded virus (Table 6). Virus recovery was usually from eyes with dendritic keratitis, except for four isolates from proflavine-treated eyes which did not have this typical corneal lesion.

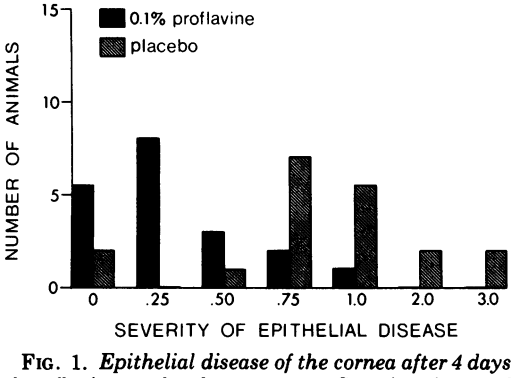

FIG. 1. *Epithelial disease of the cornea after 4 days of proflavine or placebo treatment. Severity of corneal ulceration in rabbits infected 7 days previously with PH strain HSV. Whereas there is some overlap of the scores, the proflavine-light treatment appears to have suppressed the clinical intensity of disease.*

Secondary bacterial corneal ulcers in proflavine and control eyes. Among animals receiving proflavine, only one of 97 eyes developed a purulent bacterial corneal ulcer confirmed by bacterial culture. Among the placebo-treated eyes, however, 10 of 97 developed such secondary infections. This difference may have been due to the antibacterial effect of the

TABLE 5. *Clinical evaluation of IDU and proflavine in herpetic eye infections in rabbits*[a]

Treatment	No. of animals	1st Week	2nd Week	3rd Week
All three treatments versus placebo	17	0.05	0.01	NS[b]
IDU versus proflavine	13	NS	NS	NS
IDU versus IDU and proflavine	13	NS	NS	NS
Proflavine versus IDU and proflavine	14	NS	NS	NS

[a] Data from experiment 6.
[b] NS, Not significant.

TABLE 6. *Virus recovery in treated herpetic ocular infections*

Treatment	% Specimens yielding isolates			
	Day 5	Day 7	Day 10	Day 14
Exp 1-5				
Placebo	92	87	35	ND[a]
Proflavine	93	84	50	ND
Expt 6				
Placebo	ND	94	55	0
IDU	ND	84	71	23
Proflavine	ND	94	50	22
Proflavine and IDU	ND	74	66	19

[a] N.D., Not done.

TABLE 4. *Probability that differences between proflavine-treated eyes and control eyes were due to chance*

Exp. no.	HSV Strain	Proflavine conc (%)	No. of animals	*P* Value		
				Week 1	Week 2	Week 3
1	PH	0.05	12	NS[a]	<0.05	NS
2	PH	0.1	17	<0.01	<0.01	NS
3	RE	0.1	16	<0.01	<0.05	NS
4	PH (steroids)	0.1	15	<0.01	NS	<0.05
5	RE (steroids)	0.1	16	<0.01	<0.01	<0.01

[a] NS, Not significant.

proflavine or to the increased severity of herpetic disease in placebo eyes, which created more favorable conditions for secondary bacterial invasion.

DISCUSSION

The use of photodynamically active drugs in the treatment of herpetic keratitis has been considered for a number of reasons: the mechanism of drug action is different from that of IDU; the drugs may penetrate the corneal stroma, inactivate virus there, and thus prevent the later occurrence of stromal keratitis; and there is a definite need for another clinically effective form of chemotherapy in patients who have developed IDU toxicity.

In this experimental model of primary ocular infection with herpesvirus, we found that proflavine and light had a significant effect in suppressing herpetic disease of the cornea and that the effect was similar to that produced with IDU given four times daily. The apparent failure of proflavine to interfere with recovery of virus may be due to a partial suppression of virus in the cornea and to the widespread herpes infection of the conjunctiva, which would not have received adequate light exposure. These observations would support the original observations of Moore et al. that proflavine does have a beneficial effect on this disease (9). On the other hand, Varnell and Kaufman (13) showed only a minimal effect of proflavine on herpetic keratitis in rabbits, but differences in virus strains and treatment schedules may account for the discrepancy in the effect of proflavine.

Pretreatment with a depot corticosteroid appeared to produce more corneal disease in the deep corneal stroma the second and third week after inoculation. This late stromal keratitis was suppressed by either proflavine or IDU given early in the course of infection. Deep herpetic keratitis may be caused by either growth of virus in stromal cells or by the diffusion of viral antigens from the epithelium. In either case early treatment during the most active phase of viral proliferation is necessary to prevent the later occurrence of stromal keratitis in this animal model.

In the experiments comparing proflavine and IDU alone or in combination, virus was recovered later in the course of the disease from drug-treated animals than from controls (Table 6). Although quantitative estimates of virus were not made and virus recovery was not eliminated entirely in proflavine- and IDU-treated eyes, the decrease in corneal inflammation was very likely a consequence of suppression of viral replication. If anomalous persistence of virus in treated eyes is due to a decrease in the amount of virus during the first week of infection, it may be that local factors play a role in limiting viral proliferation by immune mechanisms in this model of primary HSV infections.

Although there was a mild degree of epithelial edema and fluorescein staining with the proflavine-light treatment, this sign of toxicity cleared during continued applications. Since the only other antiviral in clinical use, IDU, also has a certain amount of toxicity for the corneal epithelium (12), this degree of proflavine-light toxicity could not be detected in herpes-infected rabbit eyes and was not thought to influence the clinical evaluations. The acceptibility of this particular proflavine preparation for the treatment of eye infections in humans is outside the scope of this study, but would depend in part on a comparison of the preparation with other antivirals used to treat HSV infections of the eye.

Because of the wide variability between animals in both epithelial and stromal keratitis, the efficacy of treatment in these studies was based on the differences between treated and control eyes in individual animals. Moreover, it was required that the observed differences be present on several examination days during each week, so that the estimate of differences between eyes is relatively conservative. Evaluation by nonparametric statistical procedure is an alternative method of dealing with multiple observations that are tabulated as ordinal values and avoids the arbitrary weighting of certain signs, which is carried out in the Draize method for estimating ocular inflammation (2).

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