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Evaluation of the herpes simplex virus antiviral activity of pyrethrins

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Summary

Pyrethrins, complex esters extracted from *Chrysanthemum cinerariaefolium*, exhibit only minimal in vitro activity against herpes simplex virus (HSV). Employing a guinea pig model of HSV genital infection, no in vivo activity could be demonstrated. Although purported to be an effective remedy for the treatment of genital herpes, we were unable to demonstrate efficacy for either the oral administration of an alcoholic solution of pyrethrins or the topical application of pyrethrins in mineral oil.

herpes simplex virus; pyrethrins; guinea pigs

Introduction

Alcohol extraction of the dried chrysanthemum (*Chrysanthemum cinerariaefolium*) produces at least six complex esters collectively referred to as pyrethrins, biologically active compounds which have been widely employed as insecticides [1,2,4]. While pyrethrins have no proven medicinal value, other chrysanthemum extracts have been employed as folk remedies in Europe and the Americas for a variety of ills [3,7,9,14]. Despite the lack of scientific studies exploring the pharmacological properties of pyrethrins we recently learned that over 120 000 doses of oral pyrethrins have been administered to patients in a Mexican clinic with illnesses clinically characterized as genital herpes simplex virus (HSV) infection. Many of these patients have purportedly experienced clinical improvement when treated with orally administered pyrethrins. This apparent success has prompted plans for possible open trial testing in Spain (H. Wilson, personal communications). When administered orally and in small amounts, the animal toxicity of pyrethrins is low, however, in high concentrations, or when administered parenterally, pyrethrins may be neurotoxic [1,2,4,8,13]. We were intrigued by the prospect that infections produced by a neurotropic virus such as HSV

might be influenced by neurotoxic agents such as the pyrethrins. Because of the extensive use of oral pyrethrins in the Mexican clinic and the absence of data on the antiviral activity of pyrethrins we undertook to explore the effects of pyrethrins upon HSV infection in vitro and in vivo.

Methods and Materials

Drugs

Two pyrethrin formulations were supplied by Harmax Laboratories, El Paso, Texas. An alcoholic pyrethrin formulation containing 10 mg/ml pyrethrins in a 35% ethanol solution was prepared for oral administration. A second formulation containing 200 mg/ml pyrethrins in mineral oil was prepared for topical administration. Dilutions of the alcoholic pyrethrin formulation were prepared with Eagle's basal media (BME) or distilled water whereas the topical pyrethrin formulation was diluted with mineral oil.

Cell culture

Human foreskin fibroblasts (HFF), fetal guinea pig (FGP), continuous Rhesus monkey kidney (MA-104) and rabbit kidney (RK) cells were prepared as described previously [10,11,16]. Cells were grown in BME with 10% fetal bovine serum (FBS). HFF cells were utilized in the 12th–15th passage, FGP and RK cells, in the second passage and MA-104 cells were used between passages 25 and 30.

Viruses

Work pools of HSV-1, MacIntyre strain (ATCC No. VR-539, passaged twice in HFF) and HSV-2, MS strain (ATCC No. VR-540, passaged three times in HFF and three times in RK cells) were prepared in RK cells, clarified, and stored at –70°C until used.

Plaque reduction assays

Varying dilutions of HSV-1 or HSV-2 (containing from 1–100 pfu) were added to 6-well plates containing confluent tissue culture monolayers. After adsorption for 1 h at 37°C, the cell monolayers were overlaid with medium containing 0.75% methylcellulose. The number of plaques were enumerated at 48 h. In some experiments the virus was treated with varying concentrations of the alcoholic pyrethrin formulation for 2 h prior to the virus being added to the cell monolayers. In other experiments, varying concentrations of the alcoholic pyrethrin formulation were included in the methylcellulose overlay. In one series of experiments HFF cells were pretreated for 2 h at 37°C with varying concentrations of the alcoholic pyrethrin preparation prior to the addition of virus and pyrethrins were included in the methylcellulose overlay.

Guinea pig studies

We utilized the guinea pig model of initial and recurrent genital HSV infection [15]. Briefly, weanling female Hartley guinea pigs (Charles River Breeding Laboratories,

Wilmington, MA) were inoculated intravaginally with 5×10^5 pfu HSV-2, MS strain. The clinical course of initial genital infection was assessed by lesion score and the course of vaginal HSV-2 replication was determined by plaque assay of secretions collected by vaginal swabs on days 1, 3, 5, and 7 following intravaginal inoculation. Undiluted alcoholic pyrethrins (0.2 ml three times daily) were administered by gavage beginning 8 h following intravaginal inoculation. The undiluted topical pyrethrin preparation (0.2 ml three times daily) was administered as a vaginal douche with smearing on the external genital skin beginning 8 h after intravaginal virus inoculation. Both the oral and topical drug treatment was continued for a total of 10 days. To investigate the possible prophylactic usefulness of topical pyrethrins, an additional group of animals were administered topical pyrethrins from 24 h prior to and for 72 h following intravaginal HSV-2 inoculation. Control animals received either no therapy or the appropriate vehicle without drug. In addition to evaluating the clinical and virologic course of the initial infection, guinea pigs used in the prophylaxis experiment were evaluated daily after recovery from the initial infection for evidence of recurrent herpetic lesions on the external genital skin.

Results

In vitro studies

On ice, the alcoholic pyrethrin formulation was diluted 1:500 in 2 × Eagle's basal medium (BME) followed by serial 10-fold dilutions in 2 × BME to working concentrations. Equal volumes of drug in 2 × BME were diluted with 2 × (1.5%) methylcellulose. To observe for toxicity, final concentrations varying from 0.01 to 100 μg/ml pyrethrins in BME and methylcellulose were overlaid onto primary RK cells. The overlay was tested both with and without the addition of 10% FBS. In the absence of FBS, the pyrethrin preparation was toxic (as evidenced by degeneration of the cell monolayer) at concentrations as low as 1 μg/ml without FBS and at 100 μg/ml when FBS was present.

To examine for antiviral activity, HSV-1 or HSV-2 was adsorbed for 1 h at 37°C to one of four cell lines then overlaid with methylcellulose containing varying concentrations of the alcoholic pyrethrin formulation. Little effect on plaque number was observed (Table 1) at the concentrations tested, although a 50% plaque reduction for HSV-2 was observed in FGP at 20 μg/ml. In addition, the plaque size was smaller in all assays where drug was incorporated in the overlay medium.

We conducted an experiment to determine whether pretreatment of HFF cells with pyrethrins would affect the plaque assay. The cells were pretreated for 2 h at 37°C with varying concentrations of the alcoholic pyrethrin formulation in BME. Prior to applying the pyrethrins, the cells were washed with medium containing the drug and then washed following incubation with medium without drug. Virus was then adsorbed for 1 h and the cells overlaid with methylcellulose containing various concentrations of pyrethrins. Employing pretreatment in this fashion, we observed no appreciable effect on plaque number (Table 2).

The final in vitro experiments addressed possible direct virucidal activity of the

TABLE 1

HSV plaque reduction by alcoholic pyrethrin formulation

Virus	Cell type[a]	Drug concentration (μg/ml)[b]					
		1	10	20	40	80	100
HSV-2	RK	-0.2[c]	-0.2	ND[d]	ND	ND	Toxic
(MS strain)	HFF	-0.1	-0.2	ND	ND	ND	ND
	FGP	ND	-0.1	-0.3	Toxic	Toxic	ND
	MA-104	ND	0.0	0.0	Toxic	Toxic	ND
HSV-1	RK	+0.1	Toxic	Toxic	ND	ND	ND
(MacIntyre strain)	HFF	0.0	0.0	-0.1	ND	ND	ND

[a] RK = rabbit kidney; HFF = human foreskin fibroblasts; FGP = fetal guinea pig; MA-104 = continuous monkey kidney.

[b] Final concentration of pyrethrins in methylcellulose overlay.

[c] Log_{10} pfu change in plaque numbers. - denotes reduction, and + an increase in plaques compared to cultures without drug. Cultures were inoculated in triplicate with varying concentrations of HSV ranging from 1-100 pfu/well.

[d] ND = not done.

TABLE 2

HSV-2 (MS strain) plaque reduction[a] by alcoholic pyrethrin formulation

Pretreatment of cells			Pretreatment of virus	
Pretreatment concentration[b] (μg/ml)	Concentration in overlay medium (μg/ml)	Changes in No. of plaques[c]	Pretreatment concentration[d] (μg/ml)	Changes in No. of plaques[c]
5	0	0.0	10	+0.2
5	5	+0.2	20	+0.2
10	0	-0.1	40	+0.1
10	10	0.0	80	-0.1
20	0	0.0		
20	20	0.0		

[a] Cells used were human foreskin fibroblasts.

[b] 2 h at 37°C.

[c] Log_{10} pfu change in plaque number. - denotes reduction, and + an increase in plaques compared to cultures without drug. Cultures were inoculated in triplicate with varying concentrations of HSV ranging from 1-100 pfu/well.

[d] 2 h at 4°C.

alcoholic pyrethrin preparation. The preparation was diluted in tissue culture media to provide concentrations ranging from 10 to 80 μg/ml. Virus was added to the drug solution and incubated for 2 h, then assayed in HFF cells. This study failed to demonstrate any direct virucidal action of the alcoholic pyrethrin preparation against HSV (Table 2).

Guinea pig studies

The first set of in vivo experiments explored the effect of the oral pyrethrin preparation on genital herpes in female guinea pigs. Weanling animals were inoculated intravaginally with HSV-2 and 8 h later six animals per group were randomized to receive either oral pyrethrins (0.2 ml three times daily), alcohol placebo (0.2 ml three times daily) or no treatment. Therapy was continued for a total of 10 days. The effect of oral pyrethrins on genital skin disease in female guinea pigs is shown in Fig. 1A. The results demonstrate no difference between the clinical disease seen in animals receiving oral pyrethrins, placebo, or the untreated control. There was also no effect upon the virologic course of the infection as measured by the mean virus titers in vaginal secretions on days 1, 3, 5, and 7 after inoculation (Fig. 1B).

To investigate the effect of topical pyrethrins on genital herpes, 18 animals were inoculated intravaginally with HSV-2 and 8 h later were randomized to receive either intravaginal/topical pyrethrins (20%, 0.2 ml three times daily), intravaginal/topical mineral oil (0.2 ml three times daily), or no treatment. Animals received treatment for a total of 10 days. Topical pyrethrins had no effect on either the clinical course of genital herpes (Fig. 2A) or upon vaginal HSV-2 replication as indicated by the mean virus titers in vaginal secretions on days 1, 3, 5, and 7 (Fig. 2B).

To examine for the possible prophylactic efficacy of intravaginal/topical pyrethrins, 30 weanling guinea pigs received either no treatment (8 animals), intravaginal/topical mineral oil (8 animals), or intravaginal/topical pyrethrins (14 animals). Either 20%

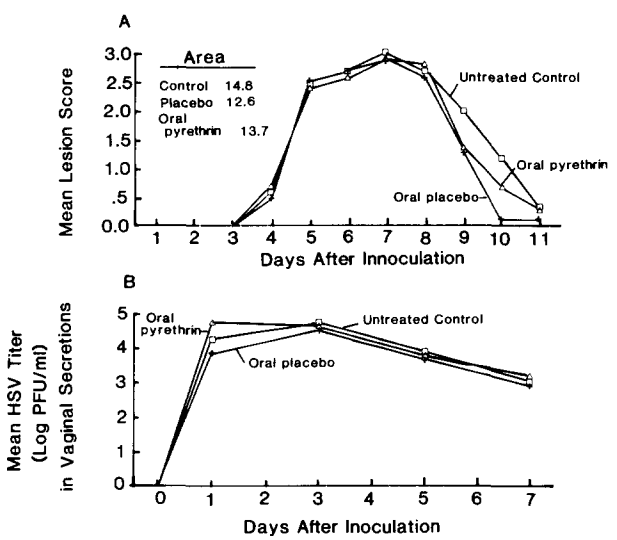

Fig. 1. Effect of oral pyrethrins on the genital skin disease (A) and the vaginal swab virus titers (B) of weanling Hartley guinea pigs intravaginally inoculated with herpes simplex virus (HSV) type 2. Area under the mean lesion score-day curve is shown in the inset. Therapy was initiated 8 h after virus inoculation and continued for 10 days.

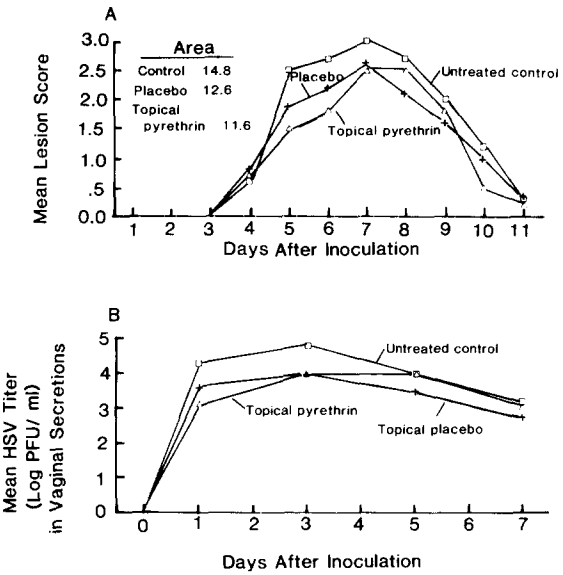

Fig. 2. Effect of intravaginal/topical pyrethrins on the genital skin disease (A) and the vaginal swab virus titers (B) of weanling Hartley guinea pigs intravaginally inoculated with herpes simplex virus (HSV) type 2. Area under the mean lesion score-day curve is shown in the inset. Therapy was initiated 8 h after virus inoculation and continued for 10 days.

pyrethrins or mineral oil was administered intravaginally (0.2 ml three times daily) beginning 24 h prior to and continuing for 72 h following intravaginal inoculation with HSV-2, MS strain. Neither the clinical course (Fig. 3A) nor vaginal virus replication

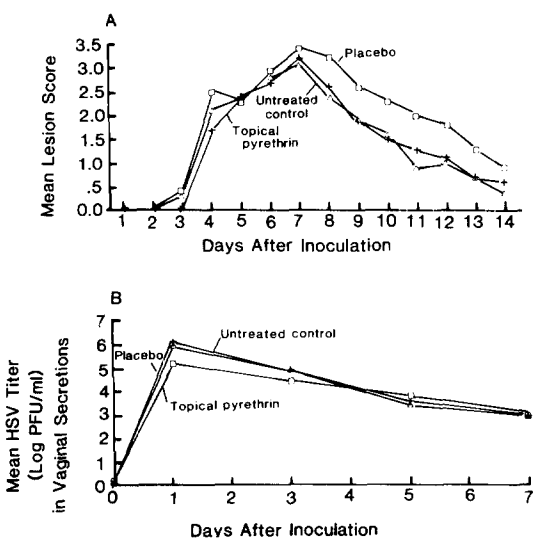

Fig. 3. Effect of prophylactic intravaginal/topical pyrethrins on the genital skin disease (A) and the vaginal swab titers (B) of weanling Hartley guinea pigs intravaginally inoculated with herpes simplex virus (HSV) type 2. The pyrethrin formulation was administered beginning 24 h prior to and continuing 72 h following virus inoculation.

TABLE 3

Effect of pyrethrins on the frequency of recurrent herpes simplex virus genital lesions[a]

Treatment[b]	Mean number of lesion days per animal[c]	Mean number of episodes per animal[d]	Mean number of days per episode
None (n =3)	10.0 ± 2.0[e]	7.0 ± 1.0	1.4 ± 0.8
Mineral oil (n = 4)	7.5 ± 4.4	4.8 ± 1.7[f]	1.6 ± 0.9
Pyrethrins (n = 3)	15.0 ± 6.1	8.3 ± 1.5	1.8 ± 1.0

[a] Both vesicular and erythematous recurrences were scored.

[b] 0.2 ml mineral oil or 20% pyrethrins in mineral oil was administered three times daily by vaginal douche with spreading of the leakage on the genital skin. Treatment was initiated 24 h prior to and continued for 72 h following intravaginal inoculation with 5×10^5 pfu HSV-2 MS strain.

[c] Animals were examined daily for six weeks beginning 17 days after virus inoculation.

[d] Episode = appearance of lesions with lesion-free days before and after.

[e] Values are mean ± S.D.

[f] Significantly less than the pyrethrin group ($P < 0.05$) by Student's t-test.

(Fig. 3B) were altered by the intravaginal/topical administration of pyrethrins. Additionally, intravaginal/topical pyrethrin administration during the primary infection did not reduce the frequency of subsequent episodes of recurrent genital HSV infection (Table 3).

Discussion

The reported usefulness of alcoholic extract of chrysanthemums as a remedy for genital HSV infections suggested that pyrethrins might possess antiherpes activity. The plaque reduction assays, however, demonstrated only minimal antiviral activity against HSV-2 in a single cell line. Employing the guinea pig model of genital HSV-2 infection, a system successfully utilized for the evaluation of antivirals in the treatment of initial and recurrent [5,6,12] genital infection, neither orally nor intravaginally administered pyrethrins were effective in altering the course of genital HSV infection. The prophylactic intravaginal administration of pyrethrins was ineffective at preventing infection or altering either initial or recurrent genital disease. Despite neurotoxic properties, which confer their effectiveness as insecticides, we found no evidence to suggest that pyrethrins may be useful in the prevention or treatment of infection by the neurotropic herpes simplex viruses.

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