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Topical cream-based oxyresveratrol in the treatment of cutaneous HSV-1 infection in mice

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ABSTRACT

Anti-herpes simplex virus (HSV) activities of oxyresveratrol in vitro and topical administration in cutaneous HSV-1 infection in mice were examined. The inhibitory concentrations for 50% plaque formation (IC_{50}) of oxyresveratrol against HSV-1 clinical isolates and HSV-2 clinical isolates were 20.9–29.5 and 22.2–27.5 μg/ml, respectively. In topical administration in cutaneous HSV-1 infection in mice, 2.5%, 5%, 10% and 20% oxyresveratrol in cream vehicle applied three times daily for 7 days after infection were evaluated and 10% and 20% oxyresveratrol cream were significantly effective in delaying the development of skin lesions and protection from death ($P < 0.01$). The concentration of 10% oxyresveratrol in cream was significantly more effective than that of 30% oxyresveratrol in vaseline applied three times daily ($P < 0.01$). Oxyresveratrol cream at 20% was as effective as 5% ACV cream applied three times daily ($P < 0.01$). Both 10% and 20% oxyresveratrol cream were as effective as that of 5% ACV cream applied two times daily ($P > 0.05$). Therapeutic efficacy of oxyresveratrol in cream vehicle was dose-dependent and the maximum efficacy observed on day 6 after infection was shown at 10% oxyresveratrol in cream applied three times daily. The frequency of application of 10% oxyresveratrol cream at three, four and five times daily was as effective as that of 5% ACV cream applied five times daily ($P > 0.05$). These results demonstrated that topical administration of oxyresveratrol in novel cream vehicle reduced the concentration of oxyresveratrol to 10% and was suitable for cutaneous HSV infection.

1. Introduction

Infections with herpes simplex virus (HSV) type 1 (HSV-1) and HSV type 2 (HSV-2) are one of the most common communicable diseases in humans. Although infections are often subclinical, HSV can cause mild to severe diseases, especially in immunocompromised patients. HSV establishes latency in the nuclei of neuronal cells and may reactivate, with or without symptoms, throughout the host's lifetime. Most of the targets for antiviral agents are the viral DNA polymerase, and indeed acyclovir (ACV), penciclovir, famciclovir and valaciclovir remain the reference treatment (Galasso et al., 1997; De Clercq, 2001, 2004; Greco et al., 2007). Currently, topical preparations such as ACV, penciclovir, foscarnet, docosanol, heparin as well as zinc are available (Tennican et al., 1980; Spurance and Freeman, 1990; Herold et al., 1995; De Clercq 2004). Topical antiviral chemotherapy has potential advantages over systemic drug for the treatment of cutaneous herpes simplex virus infections with higher target tissue drug levels and greater efficacy including convenience, especially for recurrent diseases. The development of new anti-herpes drugs especially for topical treatment that can inhibit the development of skin lesion by both wild-type viruses and drug-resistant strains is the crucial needs.

Anti-herpetic activities of plant extracts have given interesting results for the search of new antiviral agents (Vanden Berghe et al., 1993; Kurokawa et al., 1998, 1999; Lipipun et al., 2003). *Clinacanthus nutans* extract has been traditionally used in Thailand for the topical treatment of HSV and varicella-zoster virus infections (Sangkitporn et al., 1993, 1995). Identification of

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bioactive anti-HSV molecules and structure–activity relationship studies were reviewed (Khan et al., 2005). Oxyresveratrol (*trans*-2,3′,4,5′-tetrahydroxystilbene), the active principle was purified from *Artocarpus lakoocha*, a Thai medicinal plant (Sritularak et al., 1998; Likhitwitayawuid et al., 2005). Oxyresveratrol was effective against both wild type HSV-1 (7401H and KOS) and HSV-2 (Baylor186), clinical isolates of HSV-1, TK-deficient HSV-1 strain and PAA-resistant HSV-1 strain. There were no significant differences among the IC_{50} ($P > 0.05$) of various strains of HSV-1. ACV-resistant HSV-1 strains were similarly susceptible to oxyresveratrol, indicating that the mode of anti-HSV action of oxyresveratrol was different from that of ACV (Chuanasa et al., 2008). Oxyresveratrol also effective against wild type varicella-zoster virus (VZV), ACV resistant thymidine kinase deficient VZV and DNA polymerase VZV mutants (Sasivimolphan et al., 2009). The mechanism of its anti-HSV action in inhibition of protein synthesis was analyzed by immunoprecipitation and SDS–PAGE, and the data indicated a similar pattern of inhibition in late protein (gC, gD) synthesis compared with phosphonoacetic acid (PAA). Oxyresveratrol has been shown in vitro to target both early and late events in HSV replication (Chuanasa et al., 2008). It is unlikely that this would be the sole source of inhibitory properties on HSV replication.

Oxyresveratrol was more effective in topical administration than oral administration in HSV-1 cutaneous mouse model. The survival time and percent mortality observed in oxyresveratrol orally administered in mice at dose 500 mg/kg/dose, three times daily were not significantly different ($P > 0.05$) compared with the untreated control group. This may be due to inactive metabolites of oxyresveratrol. Topical application of 30% oxyresveratrol ointment five times daily significantly delayed the development of skin lesions and protected mice from death ($P < 0.01$) (Chuanasa et al., 2008). In order to investigate for the efficacy of oxyresveratrol cream in this present paper, the inhibitory activity of oxyresveratrol against HSV clinical isolates were examined and the effective concentration of oxyresveratrol in cream vehicle was evaluated for the topical treatment of cutaneous HSV-1 infection in mice. The results suggested that oxyresveratrol inhibited replication of all HSV clinical isolates tested. The concentration of oxyresveratrol could be decreased to 10% and the frequency of application was three times daily. Further investigation is needed for toxicological studies as well as clinical studies.

2. Materials and methods

2.1. Viruses and cells

HSV strains used were HSV-1 (7401H), HSV-1 (KOS) and HSV-2 (Baylor186). Virus stocks were prepared from infected-cultured cells as reported previously (Shiraki and Rapp, 1988; Kurokawa et al., 1993; Lipipun et al., 2003). Twenty clinical viral isolates of HSV-1 and HSV-2 were kindly provided by Division of Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand. African green monkey kidney cells (Vero cells) were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% and 2% fetal bovine serum (FBS), respectively.

2.2. Drugs and chemicals for cream preparation

ACV was purchased as powder from Sigma product. Vilerm (5% ACV cream) was purchased from Siam Bheasach Co. Ltd., Thailand. The chemicals (pharmaceutical grade) used for cream preparation were cetyl alcohol, isopropyl myristate, span80, tween80, propylene glycol, methyl paraben and propyl paraben.

2.3. Purification of oxyresveratrol from A. lakoocha

Oxyresveratrol was purified from the heartwood of *A. lakoocha* Roxburgh (Moraceae) and the method was previously reported (Sritularak et al., 1998; Likhitwitayawuid et al., 2005). Briefly, heartwood of *A. lakoocha* was extracted in methanol. The active fraction was isolated from the methanol extract using vacuum liquid chromatography. The purified compound was analyzed as *trans*-2,4,3′,5′-tetrahydroxystilbene (oxyresveratrol) by spectroscopy. The chemical was assayed by HPLC and found to be greater than 99% pure. Oxyresveratrol at 2.5%, 5%, 10% and 20% w/w was incorporated in cream base and was used in animal experiments.

2.4. Effect of oxyresveratrol against clinical herpes simplex virus isolates by plaque reduction assay

Oxyresveratrol was examined for its inhibitory activity on plaque formation against clinical isolates. Duplicate cultures of Vero cells in 24 well tissue culture plates were infected with 50 plaque forming units (PFU)/0.1 ml of wild-type HSV-1 (KOS), HSV-1 (7401H), HSV-2 (Baylor186), ten clinical isolates of HSV-1, and ten clinical isolates of HSV-2 for 1 h at room temperature. Cells were added with overlay medium containing various concentrations of ACV or oxyresveratrol and then incubated at 37 °C for 2 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a microscope. The 50% inhibitory concentration for plaque formation (IC_{50}) was determined (Shiraki et al., 1991; Lipipun et al., 2003 and Suzuki et al., 2006).

2.5. Anti-HSV activity of oxyresveratrol in cream preparation by plaque reduction assay

To evaluate the anti-HSV activity of oxyresveratrol in cream preparation, 20% oxyresveratrol in cream was diluted in completed media and its anti-HSV activity was assessed in Vero cells against HSV-1 (KOS) and HSV-2 (Baylor186) by the plaque reduction assay. In post treatment assay, triplicate cultures of Vero cells in 24 well tissue culture plates were infected with 50 plaque forming units (PFU)/0.1 ml of HSV-1 or HSV-2 strains for 1 h at room temperature. Cells were added with overlay medium containing various concentrations of oxyresveratrol cream and then incubated at 37 °C for 2 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a microscope and the 50% inhibitory concentration for plaque formation (IC_{50}) was determined (Shiraki et al., 1991; Lipipun et al., 2003 and Suzuki et al., 2006). In the inactivation plaque reduction assay, the virus was incubated with each dilution of oxyresveratrol in cream vehicle for 1 h and the mixture was added into the cells for 1 h. Then the cells were overlaid and further performed as post treatment assay. Cream base without oxyresveratrol was diluted in the same manner as oxyresveratrol in cream vehicle and was subjected to the plaque reduction assay as above.

2.6. Effect of cream formulation on non-infected Vero cells viability determined by the MTT reduction assay

Effect of cream formulation on non-infected Vero cells viability was evaluated by the MTT reduction assay. Vero cells were seeded at a concentration of 2×10^5 cells/ml in 96-well tissue culture plates and grown at 37 °C for 1 day. The culture medium was replaced by fresh medium containing oxyresveratrol or oxyresveratrol in cream preparation at various concentrations, 25–400 μg/ml and cells were further grown for 2 days. After incubation the media was replaced with 50 μl of a 1 mg/ml

solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Co.) in media. Cells were incubated at 37 °C for 3 h, the untransformed MTT was removed and 50 μl of acid–isopropanol (0.04 N HCl in isopropanol) was added to each well. After a few minutes at room temperature for dissolving the crystals, the plates were read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 620 nm. The concentration of oxyresveratrol reducing cell viability by 50% (CC_{50}) was determined. The cream base was diluted in the same manner as oxyresveratrol in cream vehicle and was subjected to the MTT reduction assay as described above.

2.7. Therapeutic efficacy of topical oxyresveratrol in cutaneous HSV-1 infection in mice

Female BALB/c mice (6 weeks old) were purchased from Sankyo Labo Service Co. Ltd., Tokyo, Japan. The right midflank of each mouse was clipped and depilated with a chemical depilatory, hair remover. Two or three days later, the naked skin was scratched using 27-guage needles and 5 μl of HSV-1 (7401H) suspension of 1×10^6 PFU was applied to the scarified area (Kurokawa et al., 1993; Lipipun et al., 2003; Chuanasa et al. 2008). Since the application of five times daily completely inhibited the development of skin lesion, the application of three times daily was suitable to compare the efficacy among various preparations. Oxyresveratrol was administered to mice (9–10 mice per group) as follows: Experiment 1 (topical treatment, the effect of formulation), oxyresveratrol prepared in cream formulation at 10% and 20% w/w and oxyresveratrol prepared in petroleum jelly (Vaseline™) base at 30% w/w were applied topically on the scratched area (5 mg/cm^2/dose) 1 h after HSV-1 infection and three times daily for 7 days. Acyclovir cream applied three times daily and mice without treatment were used as control. Experiment 2 (topical treatment, the effect of concentration), oxyresveratrol prepared in cream formulation at 2.5%, 5%, 10% and 20% concentrations w/w were applied topically on the scratched area (5 mg/cm^2/dose) 1 h after HSV-1 infection and three times daily for 7 days. The cream base applied three times daily, acyclovir cream applied once and twice daily and mice without treatment were used as control. Experiment 3 (topical treatment, the effect of the frequency of application, oxyresveratrol prepared in cream formulation at 10% w/w was applied topically on the scratched area (5 mg/cm^2/dose) 1 h after HSV-1 infection once, twice, three, four or five times daily for 7 days in each group. ACV applied five times daily and mice without treatment were used as control. In all experiments the development of skin lesions and mortality were continuously observed three times or five times daily for 7–10 days after HSV-1 infection and scored as follows: 0, no lesion; 2, vesicles in local region; 4, erosion and/or ulceration in local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; and 10, severe zosteriform lesion and death. We conducted procedures in conforming to the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of the Animal Care Committee at University of Toyama.

2.8. Statistical analysis

The repeated measure ANOVA was used to analyze the difference between oxyresveratrol-treated mice and control mice in mean skin lesions for 7–10 days after infection. The Student's *t*-test (one tailed test) was used to evaluate the significance of the differences in the mean weight between control and oxyresveratrol-treated mice. A *P*-value of less than 0.05 or 0.01 was defined as statistically significant.

3. Results

3.1. Effect of oxyresveratrol against clinical herpes simplex virus isolates by plaque reduction assay

The antiviral activities of oxyresveratrol against wild type HSV-1 (KOS), wild type HSV-2 (Baylor186), ten clinical HSV-1 isolates and ten clinical HSV-2 isolates were examined by plaque reduction assays. As shown in Table 1, the values of IC_{50} of oxyresveratrol for wild-type HSV-1 (KOS), HSV-1 (7401H) and HSV-2 (Baylor186) were 24.3 ± 2.6, 22.3 ± 3.9 and 22.3 ± 3.8 μg/ml, respectively. The IC_{50} of oxyresveratrol for HSV-1 clinical isolates and HSV-2 clinical isolates were 20.9–29.5 and 22.2–27.5 μg/ml, respectively. The values of IC_{50} of ACV for wild-type HSV-1 (KOS), HSV-1 (7401H) and HSV-2 (Baylor186) were 0.09 ± 0.02, 0.31 ± 0.04 and 0.43 ± 0.04 μg/ml, respectively. The values of IC_{50} of ACV for HSV-1 clinical isolates and HSV-2 clinical isolates were 0.07–0.19 and 0.11–0.36 μg/ml, respectively. There were no significant differences among the IC_{50} ($P > 0.05$) of various isolates of HSV-1. The clinical isolates were similarly susceptible to oxyresveratrol.

3.2. Anti-HSV activity of oxyresveratrol in cream by plaque reduction assay

The anti-HSV activities of oxyresveratrol in cream formulation against HSV-1 (KOS) and HSV-2 (Baylor186) were examined by plaque reduction assays. As shown in Fig. 1, the values of IC_{50} of oxyresveratrol against HSV-1 (KOS) and HSV-2 (Baylor186) were 22.5 ± 3.4 and 25.0 ± 2.5 μg/ml in post-treatment assay (Fig. 1A) and 22.5 ± 2.5 and 25.0 ± 3.0 μg/ml in the inactivation-treatment assay (Fig. 1B), respectively.

3.3. Effect of cream formulation on non-infected Vero cells viability determined by MTT reduction assay

No cytotoxic effects of oxyresveratrol cream at concentration of 50 μg/ml and cream base were observed (Fig. 2). The CC_{50} of

Table 1
The 50% inhibitory concentration (IC_{50}) of oxyresveratrol against HSV-1 and HSV-2 clinical isolates determined by plaque reduction assay.[a]

HSV	IC_{50} (mean ± SD, μg/ml)	
	Oxyresveratrol	Acyclovir
HSV-1		
KOS strain	24.3 ± 2.6	0.09 ± 0.02
7401H strain	22.3 ± 3.9	0.31 ± 0.04
Clinical isolate 1	24.1 ± 1.4	0.19 ± 0.05
Clinical isolate 2	28.8 ± 1.8	0.11 ± 0.02
Clinical isolate 3	23.2 ± 2.6	0.11 ± 0.03
Clinical isolate 4	26.3 ± 1.8	0.13 ± 0.05
Clinical isolate 5	29.5 ± 4.3	0.11 ± 0.03
Clinical isolate 6	27.2 ± 4.0	0.08 ± 0.02
Clinical isolate 7	23.8 ± 1.8	0.07 ± 0.03
Clinical isolate 8	27.5 ± 3.5	0.18 ± 0.04
Clinical isolate 9	23.3 ± 2.5	0.07 ± 0.03
Clinical isolate 10	20.9 ± 2.9	0.15 ± 0.04
HSV-2		
Baylor186 strain	22.3 ± 3.8	0.43 ± 0.04
Clinical isolate 1	26.5 ± 4.2	0.11 ± 0.02
Clinical isolate 2	27.1 ± 3.8	0.18 ± 0.03
Clinical isolate 3	27.5 ± 3.5	0.15 ± 0.04
Clinical isolate 4	26.9 ± 4.4	0.22 ± 0.05
Clinical isolate 5	26.2 ± 5.3	0.19 ± 0.05
Clinical isolate 6	26.5 ± 4.9	0.31 ± 0.04
Clinical isolate 7	23.4 ± 2.7	0.22 ± 0.04
Clinical isolate 8	26.3 ± 4.4	0.36 ± 0.07
Clinical isolate 9	22.2 ± 3.2	0.19 ± 0.04
Clinical isolate 10	26.6 ± 4.2	0.23 ± 0.03

[a] Mean and SD of each IC_{50} were determined by three independent experiments.

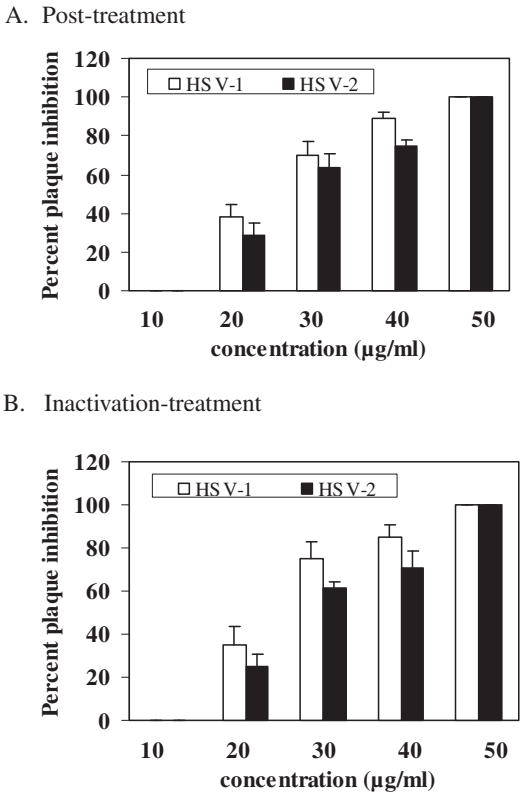

Fig. 1. Anti-HSV activity of oxyresveratrol cream against HSV by the plaque reduction assay. (A) Post treatment, (B) inactivation treatment. No antiviral activity of cream base were observed against HSV-1 and HSV-2 (the IC_{50} values of oxyresveratrol in formulation were determined from three independent experiments and mean ± S.D were 22.5 ± 3.4 and 25.0 ± 2.5 µg/ml in A; 22.5 ± 2.5 and 25.0 ± 3.0 µg/ml in B, for HSV-1 and HSV-2, respectively).

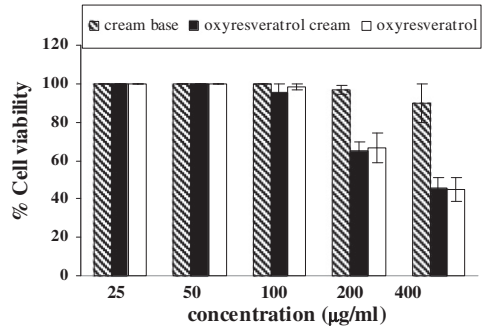

Fig. 2. Effect of cream formulation on non-infected Vero cells viability determined by the MTT reduction assay. Each point represents the mean ± S.D. ($n = 3$).

oxyresveratrol was 250 ± 27.8 µg/ml determined by the MTT reduction assay.

3.4. Therapeutic efficacy of topical oxyresveratrol on cutaneous HSV-1 infection in mice

To evaluate anti-HSV-1 efficacy of oxyresveratrol for cutaneous treatment, oxyresveratrol formulation was prepared in cream or in petroleum jelly (Vaseline™) base. Oxyresveratrol in cream vehicle at 10% or 20% applied three times a day for 7 days were compared with control groups including untreated control. Oxyresveratrol cream at 10% and 20% applied three times a day for 7 days showed significant delay of development and progression of skin lesion and reduction of the number of mice with skin lesions compared with untreated control ($P < 0.01$). The therapeutic efficacy of 10% and 20% oxyresveratrol in cream vehicle were as effective as acyclovir applied three times daily ($P > 0.05$). They exhibited significantly greater effectiveness ($P < 0.01$) than 30% oxyresveratrol in vaseline applied three times a day. The end point of treatment was the improvement of skin lesion. As showed in Fig. 3, the average score of skin lesions were 6.22, 0.44, 0, 0 and 5.56 at day 7 for the untreated control, 10% oxyresveratrol cream three times daily, 20% oxyresveratrol cream three times daily, ACV cream three times daily and 30% oxyresveratrol in vaseline three times daily groups, respectively. The percent death of mice observed at day 7 after infection were 44.44%, 0%, 0%, 0% and 22.22% for the untreated control, 10% oxyresveratrol cream three times daily, 20% oxyresveratrol cream three times daily, ACV cream three times daily and 30% oxyresveratrol in vaseline three times daily groups, respectively. The results showed that the animal lesion scores of no treatment group and cream base-treated mice were not significantly different ($P > 0.05$) (Fig. 4). This indicated that the cream base used to prepare oxyresveratrol cream did not possess antiviral activity. To determine the optimal concentration of oxyresveratrol in cream vehicle for treatment, it was shown that oxyresveratrol cream acquired concentration-dependent efficacy, the maximum efficacy observed on day 6 after infection was the concentration of 10% oxyresveratrol, topical applied three times daily (Fig. 5). Oxyresveratrol cream at 10% and 20% applied three times a day for 7 days showed significant delay of development and progression of skin lesion and reduction of the number of mice with skin lesions compared with untreated control ($P < 0.01$) (Fig. 4). The percent death of mice observed on day 9 after infection were 100%, 100%, 80%, 70%, 20%, 20%, 40% and 0% for the untreated control, cream base, 2.5%, 5%, 10% and 20% oxyresveratrol cream three times daily, ACV cream once daily and ACV cream twice daily group, respectively. Therefore, it is suggested that the therapeutic efficacy could be obtained by reducing oxyresveratrol concentration of 30% in vaseline base to 10% in cream base.

To determine time interval or optimal frequency of the treatment of oxyresveratrol in cream vehicle, it was shown that oxyresveratrol cream acquired frequency-dependent efficacy and five, four and three applications a day provided the best therapeutic efficacy with a significant difference compared with untreated control ($P < 0.01$) (Fig. 6). These topical applications also were as effective as application of ACV cream applied five times daily ($P < 0.01$). The infected mice applied with ACV cream as positive control showed significant retardation ($P < 0.01$) in the development of lesion score and time survival and all mice survived. The percent death of mice observed at day 10 after infection were 100%,

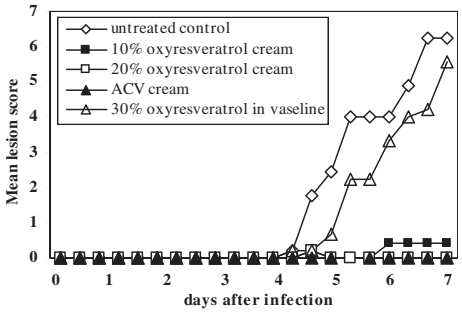

Fig. 3. Effect of oxyresveratrol cream on mice cutaneously infected with HSV-1. Oxyresveratrol in cream at 10% and 20% w/w and oxyresveratrol in vaseline base at 30% w/w were topically applied 1 h after HSV-1 infection and three times daily for 7 days, mice without treatment, and ACV cream applied three times daily were used as control (10% and 20% oxyresveratrol cream were significantly effective ($P < 0.01$) compared with mice without treatment control by the repeated measure ANOVA).

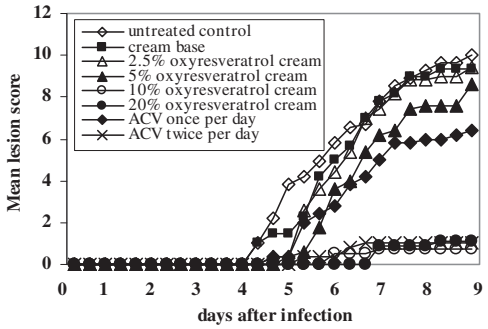

Fig. 4. Effect of concentration of oxyresveratrol cream on mice cutaneously infected with HSV-1. Oxyresveratrol in cream at various concentrations, 2.5%, 5%, 10% and 20% were topically applied 1 h after HSV-1 infection and three times daily for 7 days, mice without treatment, cream base applied three times daily or ACV cream applied once and twice daily were used as control (10% and 20% oxyresveratrol cream were significantly effective ($P < 0.01$) compared with mice without treatment control and cream base and significantly greater than 2.5% and 5% by the repeated measure ANOVA).

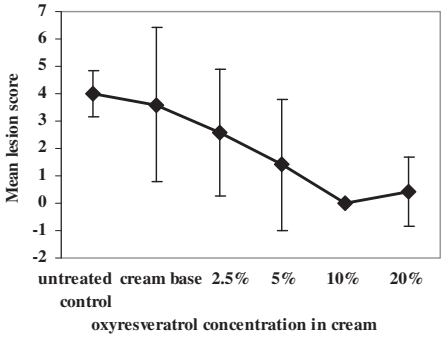

Fig. 5. Effect of concentration of oxyresveratrol cream on mice cutaneously infected with HSV-1 observed at day 6 after infection. Oxyresveratrol in cream at various concentrations, 2.5%, 5%, 10% and 20% were topically applied 1 h after HSV-1 infection and three times daily for 7 days, mice without treatment, cream base applied three times daily and ACV cream applied once and twice daily were used as control.

66.66%, 40%, 30%, 10%, 10%, 0% and 30% for the untreated control, 10% oxyresveratrol cream once daily, 10% oxyresveratrol cream twice daily, 10% oxyresveratrol cream three times daily, 10% oxyresveratrol cream four times daily, 10% oxyresveratrol cream five times daily, ACV cream three times daily and 30% oxyresveratrol in vaseline five times daily, respectively. Mortality rate of 10% oxyresveratrol cream topically-treated (four to five times daily for 7 days) group (10%) was lower than that of 30% oxyresveratrol in vaseline topically-treated (five times daily for 7 days) group (30%). It is suggested that oxyresveratrol concentration in cream could be decreased to 10% for topical application to treat cutaneous mouse HSV-1 infection. To evaluate the toxicity of oxyresveratrol, the mean of body weight of mice in each group was statistically analyzed. The mean weights of mice administered at the 2.5%, 5%, 10% and 20% of oxyresveratrol in cream vehicle topically applied three times daily for 7 days were not significantly different compared with the control mice on day 0 and day 7, showing no toxicity of the oxyresveratrol in cream base ($P > 0.05$, Student's *t*-test). No skin damage or irritation was observed in all animal used.

4. Discussion

In this study oxyresveratrol was shown to exhibit anti-HSV activity in the plaque reduction assay against HSV-1 clinical isolates and HSV-2 clinical isolates as well as wild-type HSV-1 and HSV-2 in vitro. It was reported in our work that oxyresveratrol had efficacy on ACV-resistant mutant strains and suggested that its antiviral activity may involve a mode of action different from that of ACV (Chuanasa et al., 2008). It might be useful for use against ACV-resistant mutant virus strains. The anti-HSV activity of oxyresveratrol in cream vehicle was evaluated. In plaque reduction assay, the values of IC_{50} of oxyresveratrol in post treatment were 22.5 ± 3.4 and 25.0 ± 2.5 μg/ml for HSV-1 (KOS) and HSV-2 (Baylor186), respectively. The anti-HSV activity of oxyresveratrol in cream vehicle was also examined by incubation virus with oxyresveratrol cream at various concentrations by inactivation assay. The IC_{50} of oxyresveratrol against both HSV-1 and HSV-2 were not different from that obtained by post-treatment. The anti-HSV activities of ingredients in cream formulation against HSV-1 (KOS) and HSV-2 (Baylor186) were examined by plaque reduction assays. The cream base showed no anti-HSV activity. All ingredients of oil and surfactants used in cream formulation showed no anti-HSV activity by the plaque reduction assay at concentrations of 1000 and 500 μg/ml, respectively which were higher amount of those presented in the formulation. The two preservatives, methyl paraben (0.5%) and propyl paraben (0.5%) were used in

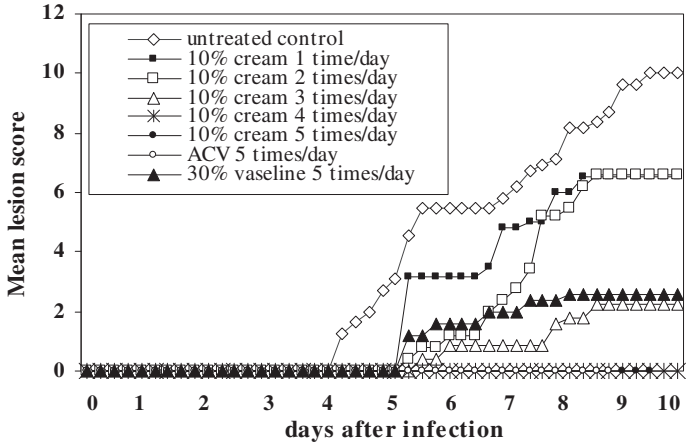

Fig. 6. Effect of frequency of application of oxyresveratrol cream on mice cutaneously infected with HSV-1. Oxyresveratrol in cream at 10% was topically applied 1 h after HSV-1 infection once, twice, three, four, and five times daily for 7 days, mice without treatment and ACV cream applied five times daily were used as control (10% oxyresveratrol cream applied three, four and five times daily were significantly effective ($P < 0.01$) compared with mice without treatment control and significantly greater than 10% oxyresveratrol cream applied once or twice daily by the repeated measure ANOVA).

formulation. Methyl paraben showed no anti-HSV activity at the concentration of 500 μg/ml. The IC_{50} values of propyl paraben against HSV-1 and HSV-2 were 50 ± 4.6 μg/ml and 50.0 ± 3.0 μg/ml respectively and propyl paraben presented in the formulation was lower than the IC_{50} (data not shown). This result indicated that there was no effect of the ingredients in cream on the anti-HSV activity of oxyresveratrol. Therefore, the anti-HSV activity of oxyresveratrol in cream was stable and anti-HSV activity observed was due to the anti-HSV activity of oxyresveratrol. The CC_{50} of oxyresveratrol was 250 ± 27.8 μg/ml determined by the MTT reduction assay as indicated in Fig. 2. To further assess the lack of the cytotoxicity of this compound, the cytotoxicity were also assessed in other two conditions of cells, (1) the effect on cell proliferation of actively growing cells, adding oxyresveratrol to the cells at 3 h of cell seeding and cells were incubated further for 72 h and (2) the effect on confluent cells, adding oxyresveratrol to the cells at 24 h of cell seeding and cells were incubated further for 72 h. The CC_{50} of oxyresveratrol in proliferating cells and confluent cells were 243.22 ± 37.08 and 224.10 ± 23.11 μg/ml, respectively. There was no significant difference ($P > 0.05$) in CC_{50} values of oxyresveratrol to the proliferating cells and confluent cells.

In cutaneous HSV-1 infection in mice, oxyresveratrol in the cream vehicle topically applied was compared with 5%ACV cream and oxyresveratrol in vaseline. The results showed that efficacy of oxyresveratrol cream were dose- and frequency-dependent. Oxyresveratrol in cream base at 10% applied three to five times daily was the most significantly effective in delaying the skin lesion development and in death protection. The schedule of administration of 10% oxyresveratrol applied once and twice daily were not significantly effective ($P > 0.05$, Fig. 4). Our studies provided evidence that cream was valuable as a topical delivery carrier to enhance the penetration of oxyresveratrol and could deliver an adequate amount of oxyresveratrol to the site(s) of infection.

Topical formulations for the treatment of herpetic mucocutaneous infections have several potential benefits over oral and intravenous administration, including targeting of drug to the specific sites of infection, higher tissue drug levels, reduced side effects, lower treatment costs, and better patient compliance and convenience. Percutaneous penetration involves the dissolution of a drug in a vehicle, diffusion of the solubilized drug from the vehicle to the surface of the skin, and the penetration of the drug through the layers of the skin. This penetration may be improved by selecting the appropriate vehicle. It was shown that the topical ACV in cream vehicle was significantly more effective than ACV ointment tested by in vitro penetration through excised guinea pig skin and by the dorsal cutaneous guinea pig model of herpes simplex virus type 1 infection (Spruance et al., 1986). It was reported in the in vivo percutaneous absorption of 3% ketoprofen in protection the inflammation in carrageenan-induced paw edema in mice, the percent edema inhibition of ketoprofen in cold cream was significantly higher than that of ketoprofen in white petrolatum (Vaseline) (Giirol et al., 1996). The efficacy of anti-herpes agents in the topical treatment of cutaneous HSV infection may vary considerably, depending on a number of factors such as the intrinsic antiviral potency of the compound, the virus type and strain, the time of treatment, the concentration of the drug, the number of applications, and the delivery form (vehicle) for the drug (De Clercq, 1984). In this study, the result showed that the efficacy of the oxyresveratrol in cream formulation was higher than that of oxyresveratrol in vaseline in cutaneous HSV-1 infection in mice.

In conclusion, this study described the therapeutic efficacy of oxyresveratrol. Oxyresveratrol was effective against the HSV clinical isolates tested. Oxyresveratrol cream with the suitable viscosity was used to deliver oxyresveratrol for topical administration. Compared with the oxyresveratrol in vaseline, cream could significantly enhance the permeation of oxyresveratrol in vivo. These results suggested that the therapeutic anti-HSV activity as effective as 5% ACV cream applied five times daily could be obtained by the concentration of 10% oxyresveratrol in cream vehicle topically applied three times daily. Furthermore, the oxyresveratrol cream presented the excellent efficacy which provides a probability for clinical application.

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