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**Fosphenytoin alleviates herpes simplex virus infection-induced provoked and spontaneous pain-like behaviors in mice**

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Summary

In this study, we investigated the effects of fosphenytoin (fPHT) a water-soluble prodrug of phenytoin, on the pain responses of a mouse herpes zoster (HZ) pain model. Transdermal herpes simplex virus type 1 (HSV-1) inoculation induced mechanical allodynia and hyperalgesia of the hind paw and spontaneous pain-like behaviors, such as licking the affected skin. Intravenous injection of fPHT (15 and 30 mg/kg) alleviated HSV-1-induced provoked pain (allodynia and hyperalgesia). The suppressive effects of fPHT on provoked pain were weaker than those of diclofenac and pregabalin which were used as positive controls. fPHT, diclofenac, and pregabalin significantly suppressed HSV-1-induced spontaneous pain-like behaviors. Among them, high-dose fPHT (30 mg/kg) showed the strongest suppression. Intravenous fPHT may become a viable option for an acute HZ pain, especially for spontaneous pain.

Keywords

Fosphenytoin; acute herpes zoster-associated pain; spontaneous pain; allodynia; hyperalgesia
INTRODUCTION

Herpes zoster (HZ) is caused by the reactivation of human herpesvirus 3 (varicella zoster virus, VZV) in the sensory ganglion in human subjects and is characterized by clustered vesicles and severe pain. Patients with HZ complain of continuous severe spontaneous pain, such as burning pain, and allodynia, which refers to pain caused by innocuous stimuli. Although HZ usually resolves in two to four weeks, some patients experience pain for a long time even after the resolution of HZ. Acute HZ pain is thought to be a risk factor for postherpetic neuralgia (PHN). Therefore, it is important to control acute HZ pain effectively in order to prevent PHN.

Fosphenytoin (fPHT), a water-soluble prodrug of phenytoin, is an anti-epileptic drug recommended as the second-line therapy for patients with status epilepticus. After intravenous injection, fPHT is converted to phenytoin by serum phosphatases with a conversion half-life of approximately 10 minutes. Advantages of fPHT include more convenient and rapid intravenous administration, availability for intramuscular injection, and low toxicity at injection sites. Several lines of evidence suggest that fPHT is effective in the treatment of trigeminal neuralgia. Although phenytoin has been reported to be effective for PHN, there are no reports for the effectiveness of fPHT on acute HZ pain and PHN.

Previously, we have established mouse models of acute HZ pain and postherpetic
neuralgia. As a first step to clarify the efficacy of fPHT for HZ-associated pain, in this study, we examined the effects of fPHT on acute HZ pain.
MATERIALS AND METHODS

Animals  Female C57BL/6J mice weighing 18–20 g (6 weeks old at the start of experiments; Japan SLC, Shizuoka, Japan) were used. They were housed in standard polycarbonate cages (four mice per cage) under controlled temperature (22°C ± 1°C) and humidity (55% ± 10%) with a 12-h light–dark cycle (lights on at 07:00) and were allowed to access food and water freely. The animal experiments were approved by the Animal Care Committee of the University of Toyama.

Virus inoculation  The mice were inoculated with HSV-1 as described previously. Briefly, the depilated shin skin of the right hind paw (5 mm × 5 mm) was scarified and inoculated with HSV-1 (7401H strain; 1×10^6 plaque-forming units). The contralateral hind paw was not inoculated.

Drug administration  Fosphenytoin (Fostoin®, Nobelpharma Co., Ltd., Tokyo, Japan) was diluted with physiological saline, and administered intravenously (i.v.). Diclofenac (Sigma-Aldrich, St. Louis, MO, USA) and pregabalin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were dissolved in distilled water and administered orally. All mice were injected with i.v. fPHT or saline (control), and were then administered oral (p.o.) diclofenac, pregabalin, or water (control) within 5 min after the i.v. injection.

Behavioral tests  Mechanical allodynia and hyperalgesia were assessed using von Frey
filament with a bending force of 0.17 or 1.20 g (North Coast Medical, Inc., Morgan Hill, CA, USA), respectively. After at least 30 minutes acclimation, von Frey filament was pressed perpendicularly against the plantar skin and held for one to three seconds with it slightly buckled. Responses to the punctate stimulation were ranked as follows: 0 = no response; 1 = move away from von Frey filament; 2 = immediate flinching or licking of the hind paw. The stimulation of the same intensity was applied six times at intervals of several seconds and the average score served as pain-related score.

In measuring spontaneous pain, the behaviors of the mice were videotaped for 30 min to determine the duration of licking (an index of pain).

**Statistical Analysis** Statistical analysis was performed using the SigmaPlot Software version 14 (Systat Software, Ltd., Chicago, IL). Data are presented as mean ± standard errors of mean (SEM). Statistical significance between groups was analyzed using one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA followed by a post hoc multiple comparison test of Dunnett’s Method or Holm-Sidak method; a P value of < 0.05 was considered significant.
RESULTS

HSV-1 inoculation on the hind paw produced HZ-like skin lesions in the same dermatome from day 5 after inoculation; the skin lesion score peaked around day 7 following inoculation, which is consistent with previous reports.\textsuperscript{9,10} HSV-1 inoculation induced mechanical allodynia (increased responses to 0.17-g stimuli) and hyperalgesia (increased responses to 1.20-g stimuli) of the hind paw (Fig. 1 and 2), and also induced spontaneous pain-like behavior, such as licking the affected skin (Fig. 3).

The effects of fPHT (15 and 30 mg/kg, i.v.), diclofenac (30 mg/kg, p.o.), and pregabalin (3 mg/kg, p.o.) on mechanical allodynia and hyperalgesia observed on day 6 following inoculation were examined. A dose 30 mg/kg of fPHT showed a tendency to suppress allodynia from one hour after administration, but it was not significant (Fig. 1A, Table 1). In the 15 mg/kg fPHT group, significant suppression was observed at three hours after administration compared with the vehicle control group (Fig. 1A, Table 1). For hyperalgesia, both doses of fPHT showed a significant inhibitory effect around two to four hours after administration (Fig. 1B, Table 1). Diclofenac showed a tendency to suppress allodynia and significantly reduced hyperalgesia (Fig. 2A and B, Table 2). Pregabalin showed potent and significant analgesic effects on both allodynia and hyperalgesia (Fig. 2C and D, Table 2). No significant change was observed in the response of contralateral hind paw with any of the drug administrations (Fig. 1C and D).
Next, the effects of fPHT, diclofenac, and pregabalin on spontaneous pain-like behaviors were examined. The behavior was measured for 30 minutes starting 1 hour after drug administration. On day 6 following inoculation, HSV-1-inoculated mice showed spontaneous pain-like behaviors such as licking of the affected skin (Fig. 3). All medications significantly decreased the time spent in licking behaviors. Notably, fPHT (15 and 30 mg/kg, i.v.) suppressed spontaneous pain-like behavior in a dose-dependent manner, and although statistically not significant, the 30 mg/kg dose suppressed the behavior most strongly compared with all trial drugs.
DISCUSSION

In the present study, we demonstrated for the first time that intravenous injection of fPHT alleviates the HSV-1-induced mechanical hyperalgesia and spontaneous pain-like behaviors. Although the effects of fPHT on provoked pain (alldynia and hyperalgesia) were slightly weaker than those of diclofenac and pregabalin, 30 mg/kg fPHT suppressed spontaneous pain-related behavior more potently than diclofenac or gabapentin. “Spontaneous pain” in HZ is severe, autonomous and inescapable, which makes the patient very uncomfortable, whereas “provoked pain” can be avoided by not touching or moving the affected area. Patients with HZ often complain of spontaneous pain such as burning, tingling, or numbness of the skin. In the treatment of acute HZ pain, suppression of spontaneous pain is an important issue. Therefore, it is considered to be clinically meaningful that fPHT potently suppressed spontaneous pain-related behaviors and its effect was potent than that of diclofenac or pregabalin.

The mechanism of action of fPHT is related to the blockade of voltage-operated sodium channels.\(^4\) Nerve blockade with local anesthetics (e.g., bupivacaine) provides pain relief from HZ.\(^1,14,15\) It has been reported that HSV-1-infected rat dorsal root ganglion (DRG) neurons generate spontaneous activity, and the VZV infection results in increased protein expression of sodium channels Nav1.3 and Nav1.8 in the rat DRG.\(^16,17\) Taking into consideration that spontaneous pain is thought to be the result of ectopic firing of sodium channels in the
nervous system, the involvement of sodium channels in acute HZ pain, especially spontaneous pain, is strongly suggested. Further studies are required to clarify the subtype of sodium channel (i.e., Nav1.3, 1.7, 1.8 etc.) and the location where it is expressed in HSV-1-inoculated mice.

It has been reported that intravenous fPHT results in almost complete and long-lasting pain relief of patients with trigeminal neuralgia refractory to various medications such as carbamazepine, lamotrigine, or gabapentin. In the present study, fPHT potently inhibited spontaneous pain-like behaviors. In the treatment of severe HZ pain, intravenous fPHT may become a viable option for an acute HZ pain, especially for spontaneous pain.

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Conflict of Interest

The authors declare that this study received funding from Nobelpharma Co., Ltd. Drs. Yoshimi Kitada and Saori Arai are full-time employees of Nobelpharma Co., Ltd. The other
authors declare no conflict of interest.
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Table 1. Two Way Repeated Measures ANOVA (One Factor Repetition) for fPHT (15 and 30 mg/kg, i.v.)

| Drug Administration | Factor                | DF | Sum of Squares | Mean Square | F-Value | P-Value |
|---------------------|-----------------------|----|----------------|-------------|---------|---------|
| 0.17 g lpsi         | Treatment             | 2  | 0.269          | 0.134       | 3.180   | 0.044   |
|                     | Subject(Treatment)    | 45 | 2.509          | 0.0557      |         |         |
|                     | Time                  | 6  | 0.871          | 0.145       | 3.874   | 0.001   |
|                     | Treatment x Time      | 12 | 0.644          | 0.0536      | 1.431   | 0.160   |
|                     | Residual              | 126| 4.722          | 0.0375      |         |         |
|                     | Total                 | 191| 8.281          | 0.0434      |         |         |
| **Comparison for Treatment** | | | | | | |
| VEH vs. 15 mg/kg    | Diff of Mean          | 0.180 | t      | 2.159 | 0.071 | No |
| VEH vs. 30 mg/kg    | Diff of Mean          | 0.119 | t      | 1.428 | 0.160 | No |

| 1.20 g lpsi         | Treatment             | 2  | 1.410          | 0.705       | 6.147   | 0.003   |
|                     | Subject(Treatment)    | 45 | 10.217         | 0.227       |         |         |
|                     | Time                  | 6  | 1.250          | 0.208       | 2.793   | 0.014   |
|                     | Treatment x Time      | 12 | 0.908          | 0.0757      | 1.014   | 0.440   |
|                     | Residual              | 126| 9.402          | 0.0746      |         |         |
|                     | Total                 | 191| 23.326         | 0.122       |         |         |
| **Comparison for Treatment** | | | | | | |
| VEH vs. 15 mg/kg    | Diff of Mean          | 0.405 | t      | 2.402 | 0.041 | Yes |
| VEH vs. 30 mg/kg    | Diff of Mean          | 0.299 | t      | 1.777 | 0.082 | No  |

*Multiple Comparisons versus Control Group (Holm-Sidak method) : Overall significance level = 0.05
Table 2. Two Way Repeated Measures ANOVA (One Factor Repetition) for diclofenac (30 mg/kg, p.o.), and pregabalin (3 mg/kg, p.o.)

| Drug Administration | Factor          | DF | Sum of Squares | Mean Square | F-Value | P-Value |
|---------------------|-----------------|----|----------------|-------------|---------|---------|
| Diclofenac          | Treatment       | 1  | 0.0627         | 0.0627      | 1.445   | 0.232   |
| 0.17 g lpsi         | Subject(Treatment) | 30 | 1.305          | 0.0435      |         |         |
|                     | Time            | 6  | 0.267          | 0.0445      | 1.026   | 0.414   |
|                     | Treatment x Time| 6  | 0.0653         | 0.0109      | 0.251   | 0.958   |
|                     | Residual        | 84 | 3.642          | 0.0434      |         |         |
|                     | Total           | 127| 5.873          | 0.0462      |         |         |
| Diclofenac          | Treatment       | 1  | 0.818          | 0.818       | 8.291   | 0.005   |
| 1.20 g lpsi         | Subject(Treatment) | 30 | 3.340          | 0.111       |         |         |
|                     | Time            | 6  | 1.369          | 0.228       | 2.423   | 0.033   |
|                     | Treatment x Time| 6  | 1.252          | 0.209       | 2.216   | 0.049   |
|                     | Residual        | 84 | 7.909          | 0.0942      |         |         |
|                     | Total           | 127| 17.649         | 0.139       |         |         |
| Pregabalin          | Treatment       | 1  | 0.215          | 0.215       | 7.366   | 0.008   |
| 0.17 g lpsi         | Subject(Treatment) | 29 | 0.930          | 0.0311      |         |         |
|                     | Time            | 6  | 0.479          | 0.0798      | 2.812   | 0.015   |
|                     | Treatment x Time| 6  | 0.257          | 0.0428      | 1.508   | 0.186   |
|                     | Residual        | 81 | 2.300          | 0.0284      |         |         |
|                     | Total           | 123| 4.761          | 0.0657      |         |         |
| Pregabalin          | Treatment       | 1  | 2.103          | 2.103       | 24.203  | <0.001  |
| 1.20 g lpsi         | Subject(Treatment) | 29 | 4.141          | 0.143       |         |         |
|                     | Time            | 6  | 1.669          | 0.278       | 4.234   | <0.001  |
|                     | Treatment x Time| 6  | 1.037          | 0.173       | 2.630   | 0.022   |
|                     | Residual        | 81 | 5.323          | 0.0657      |         |         |
|                     | Total           | 123| 18.458         | 0.150       |         |         |
Fig. 1  Effects of fosphenytoin (fPHT) on mechanical allodynia and hyperalgesia induced by transdermal HSV-1 inoculation in mice. The unilateral hind paw was inoculated with HSV-1 (A and B) and the contralateral hind paw was without inoculation (C and D). A and C; pain score to 0.17-g stimuli. B and D; pain score to 1.20-g stimuli. fPHT (15 and 30 mg/kg) was injected intravenously at day 6 after inoculation. The data presented are means and SEM. (n = 8-16). *P < 0.05 vs VEH (SLN + WTR) no adjustment for multiple time points comparison.
Fig. 2  Effects of Diclofenac (DIC) and pregabalin (PGB) on mechanical allodynia and hyperalgesia induced by transdermal HSV-1 inoculation in mice. DIC (30 mg/kg) and PGB (3 mg/kg) were administered orally at day 6 after inoculation. The data presented are means and SEM. (n = 8-16). *P < 0.05 vs VEH (SLN + WTR) no adjustment for multiple time points comparison.
Fig. 3  Effects of fosphenytoin (fPHT), diclofenac (DIC), and pregabalin (PGB) on HSV-1 inoculation-induced spontaneous pain-like behaviors in mice. The time spent in licking behavior was measured for 30 min from 1 h after administration. The data presented are means and SEM. (n = 8-16). *P < 0.05 vs VEH (SLN + WTR) when analyzed using one way ANOVA followed by Dunnett’s Method for multiple comparisons. One way ANOVA results for factor of Between Groups; DF = 4, Sum of Squares = 389121.7, Mean Square = 97280.4, F-value = 5.467, P = 0.002.