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Effect of DHU001, a Polyherbal Formula on Formalin-induced Paw Chronic Inflammation of Mice

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The effect of DHU001, a mixed herbal formula consisted of 7 types aqueous extracts for various respiratory disorders were evaluated on the formalin-induced paw chronic inflammation in mice after oral administration. Mice were subaponeurotically injected in the left hind paw with 0.02 m*l* of 3.75% formalin, then subjected to 500, 250 and 125 mg/kg of DHU001 oral administration, once a day for 10 days during which then the hind-paw thickness and volume were measured daily. The paw wet-weight, histological profiles, histomorphometrical analyses and paw tumor necrosis factor (TNF)-α contents were conducted at termination. After two formalin treatments, a marked increase in the paw thickness and volume was detected in the formalin-injected control as compared with that in the intact control, plus at the time of sacrifice the paw wet-weights, paw TNF-α contents were also dramatically increased with severe chronic inflammation signs at histopathological observations. However, these formalin-induced chronic inflammatory changes were dramatically decreased by treatment of dexamethasone and all three different dosages of DHU001. DHU001 has favorable effects on formalin-induced chronic inflammation mediated by TNF-α suppression, and DHU001 may represent an alternative approach for the treatment of chronic inflammatory diseases.

Key words: DHU001, Polyherbal Formula, Chronic Inflammation, Mouse, Tumor Necrosis Factor

INTRODUCTION

Inflammation is an essential protective process alerting organisms to physical, chemical and infective insults. However, the inflammatory response to several insults frequently leads to damage to normal tissues (Habashy *et al*. 2005). Chronic inflammation is an inflammatory response of prolonged duration, weeks, months, or even indefinitely, where the extended time course is provoked by the persistence of the causative stimulus of inflammation in the tissue (Kim *et al*., 2006, 2007).

Formalin-injected hind-paw chronic inflammatory mice have generally been used as a classic method to detect the efficacy of anti-inflammatory drugs, because marked chronic inflammation is evoked by aponeurotic formalin injection (Akindele and Adeyemi, 2007; Kim *et al*., 2010). Recently, evidence has been provided of a widespread role of tumor necrosis factor-α (TNF-α) in mediating hyperalgesia at different levels (Schäfers *et al*., 2003), both facilitating neuronal excitability and triggering the release of other pro-inflammatory substances (Watkins and Maier, 2002). Therefore, TNF-α is treated as one of the key pro-inflammatory cytokines in acute and chronic inflammation models (Bianchi *et al*., 2004).

Steroids have been a popular choice for treating various inflammatory disorders; however, the potential for significant local and systemic adverse events, like skin atrophy and hypothalamic-pituitary-adrenal axis suppression, has limited their use (Gupta and Chow, 2004). Dexamethasone is a well-known glucocorticoid, and it is the most widely used anti-inflammatory control drug in the development of new anti-inflammatory drugs (Kim *et al*., 2006).

DHU001 is a mixed herbal formula consisted of 7 types aqueous extracts; Ficis fructus, Liriopis tuber, Platycodi radix, Schisandrae fructus, Glycyrrhizae radix, Zingiberis rhizome and Menthae herba and being developed for various respiratory disorders (Lee *et al*., 2010). Most of seven herbal components of DHU001 have been used for treating or ameliorating various respiratory symptoms based on the anti-inflammatory and antioxidant effects (Sun and Pan, 2006; Aimbire *et al*., 2007; Minghetti *et al*., 2007; Lee *et al*., 2010). Recently,

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anti-inflammatory effects of DHU001 itself, was also demonstrated on the xylene-induced acute inflammation (Back *et al*., 2008) and dinitrofluorobenzene-induced contact dermatitis (Lee *et al*., 2010) with single mouse oral dose toxicity (Roh and Ku, 2010) and micronucleus test (Roh *et al*., 2009). An immunomodulatory agent can reduce the inflammation previously observed (Ramprasath *et al*., 2006). For example, nitric oxide (NO) plays an important role in inflammation, and NO synthase inhibitors can reverse several classic inflammatory symptoms (Amin *et al*., 1995) as related to anti-oxidative effects (Kim *et al*., 2006). Therefore, it can be postulated that DHU001 will have a favorable effect on reducing or speeding-up the recovery from local chronic inflammation induced by irritants. Accordingly, in the present study, the effects of the DHU001 on formalin-induced chronic inflammation were investigated and compared with mice intraperitoneally injected with 15 mg/kg dexamethasone.

MATERIALS AND METHODS

Experimental animals. Forty-eight male ICR mice (6-wks-old upon receipt; SLC, Japan) were used after acclimatization for 7 days. The three or four animals were allocated to polycarbonate cages in a temperature (20~25°C) and humidity (40~45%) controlled room. The light - dark cycle was 12 h : 12 h, while food (Samyang, Korea) and water were supplied *ad libitum*. The animals were fasted overnight before the start of DHU001 administration and before being sacrificed (about 18 h; water not restricted). This study was carried out with prior approval of the Animal Ethical Committee, The University of Daegu Haany University (Gyeongsan, Korea).

Preparation of DHU001. The herbal compositions of DHU001 were listed in Table 1. Each herbal component was purchased from Cho-Heung Pharmaceutical Ind. Co. (Daegu, Korea) after confirmation of the morphology under microscopy. Approximated amounts of each herbal component was mixed (317.5 g) and boiled in 2 *l* of distilled water for 2 hours and then filtrated. The filtrate was decompressed using a rotary vacuum evaporator (Lab. Camp, Daejeon, Korea) and lyophilized in a programmable freeze-dryer (IlShin Lab., Daejeon, Korea). Total acquired lyophilized extracts were 34.93 g (yield 11%). Powders of extracts were stored in a desiccator to protect against light and moisture. It was well dissolved up to 30 mg/m*l* concentration levels and appeared to be a deep brown solution.

Administration of drugs. The animals were allocated to six groups with 8 mice per group: vehicle control, formalin control, 500, 250 and 125 mg/kg with the DHU001 administered groups, 15 mg/kg of dexamethasone-water soluble (Sigma, MO, USA)-treated groups. Three different dosages of DHU001 were selected based on the results on the xylene-induced acute inflammation (Back *et al*., 2008), and 15 mg/kg of intraperitoneal treatment of dexamethasone was also selected from previous report (Kim *et al*., 2007). DHU001 aqueous extracts were orally administered once a day for 10 days, while the dexamethasone dissolved in saline were intraperitoneally administered at a volume of 10 m*l*/kg. In formalin-treated control and vehicle control, distilled water was orally administered instead of DHU001 as same methods.

Induction of chronic inflammation. One hour before DHU001 administration or dexamethasone injection, a subaponeurotic injection of 0.02 m*l* of 3.75% formalin (Sigma, MO, USA) was administered to the left hind paw (*Planta pedis*) on the first and third days of the experiment. In the case of the vehicle control, the same volume of saline as that used in the other dosing groups, including the formalin control, was administered in the same region using the same method as described previously (Kim *et al*., 2010).

Changes in body weights. Daily body weights of all experimental animals used in this study were measured from 1 day before the start of the experimental period to 10 days of treatment with an automatic electronic balance (Precisa Instrument, Switzland).

Paw thickness and volume measurements. The thicknesses of the left hind paws were measured using an elec-

Table 1. Herbal composition of DHU001 used in this study

Herbs	Scientific name	Amounts (g)
Ficis fructus	*Ficus carica* Linn.	140
Liriopis tuber	*Liriope spicata* Lour.	45
Platycodi radix	*Platycodon grandiflorum* Jacq.	60
Schisandrae fructus	*Schisandra chinensis* Baill	22.5
Glycyrrhizae radix	*Glycyrrhiza uralensis* Fisch	15
Zingiberis rhizoma recens	*Zingiber officinale* Roscoe	15
Menthae Herba	*Mentha arvensis* Linne var piperascens	20
Total	7 types	317.5

All herbs were purchase from from Cho-Heung Pharmaceutical Ind. Co. (Daegu, Korea) and yield 11% aqueous extracts were acquired.

tronic digital caliper (Mytutoyo, Japan) and recorded once a day for 10 days at 1 h before the first formalin injection, at 1 h before the second formalin injection or 2 h before DHU001 treatment. The lengths of the long axis (longitudinal; excluding dactyl region) and short axis of the left hind paws were measured using an electronic digital caliper and recorded once a day for 10 days. The paw volume was calculated as described in a previous report (Kim *et al*., 2010): paw volume (mm^3) = 1/2(length of long axis × length of short axis × thickness of paw).

Paw weight measurements. At sacrifice, the wet-weight of the left hind paws was measured, and to reduce any errors due to individual body weight differences, the relative weight (%) was calculated using the body weight at sacrifice and absolute weight: Relative paw weight (% of body weight) = (Absolute weight/Body weight at sacrifice) × 100.

Paw TNF-α content measurement. After wet-weight measurements of paws, for TNF-α evaluation, skin samples of the left hind paws were homogenized in 3 m*l* of phosphate-buffered saline (PBS) containing 10 mM EDTA and 20 KIU/m*l* aprotinin (Sigma, MO, USA). After centrifugation at 10,000 ×g, the supernatant was frozen at −70°C for TNF-α assay as described in a previous report (Bianchi *et al*., 2004). The levels of TNF-α in paw supernatants were measured by means of an enzyme-linked immunosorbent assay (ELISA) kit specific for mouse TNF-α (Santa Cruz Biotechnology, CA, USA).

Histopathology. The dorsum pedis (including the subcutaneous regions) skin was separated from the hind paw, and longitudinally trimmed, then were fixed in 10% neutral buffered formalin. In addition, the metatarsal region including the second metatarsal bones was cross-trimmed, and fixed in 10% neutral buffered formalin, then decalcified using decalcifying solution [24.4% formic acid, and 0.5 N sodium hydroxide] for 5 days (mixed decalcifying solution was exchanged once a day for 5 days). After that, the prepared dorsum pedis skin and the metatarsal region were embedded in paraffin, sectioned (3~4 μm) and stained with hematoxylin and eosin (H&E). The histological profiles of the hind paws were compared with those of the vehicle control.

Histomorphometry: To detect more detailed changes, the thicknesses from the epidermis to the hypodermis of the dorsum pedis and dorsum digit skin and the numbers of infiltrating inflammatory cells in the dorsum pedis and dorsum digit skin were measured as described previously (Kim *et al*., 2010) with some modifications. The histopathologist was blinded to group distribution when this analysis was made.

Statistical analyses. All data are expressed as the mean ± standard deviation (SD) of seven mice, and multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the data were analyzed with one way ANOVA followed by a least-significant differences multi-comparison test to determine which pairs of group comparison were significantly different. In cases of significant deviation from variance homogeneity as indicated by the Levene test, a non-parametric comparison test, the Kruskal-Wallis H test, was conducted. When a significant difference was observed with the Kruskal-Wallis H test, the Mann-Whitney U test was conducted to determine the specific pairs of groups which were significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, SPSS Inc., USA).

RESULTS

Changes on the body weights. No meaningful changes in body weights were detected between the formalin control and the vehicle control throughout the 10-day experimental period. However, dexamethasone-treated mice showed significant ($p < 0.01$ or $p < 0.05$) decreases in body weight,

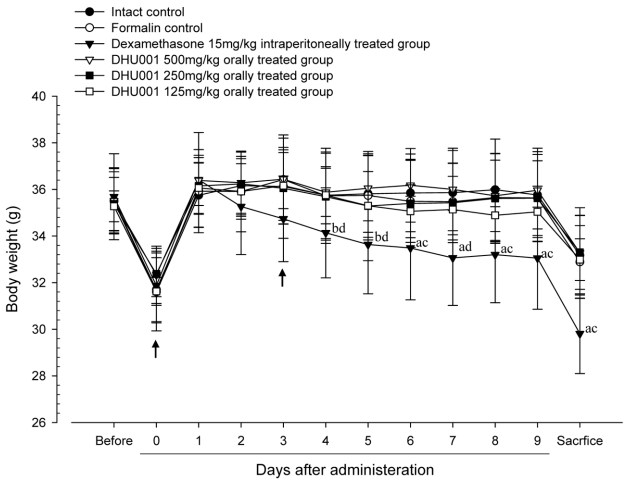

Fig. 1. Body weights detected during 10 days of continuous oral treatment periods of DHU001 in formalin-induced chronic inflammation mice. No meaningful changes on the body weights were detected in all administrated groups as compared with intact or formalin controls, respectively except for dexamethasone-treated mice in which significant decreases on the body weights were detected from 5 days after administration as compared with intact and formalin control. Values are expressed mean ± S.D. of eight mice. Before means 1 day before first formalin treatment or start of test material administration. Subaponeurotic injection of formalin was conducted at Day 0 and Day 3, respectively (arrows). All animals were overnight fasted at Day 0 and sacrifice. $^{a}p < 0.01$ and $^{b}p < 0.05$ as compared with intact control; $^{c}p < 0.01$ and $^{d}p < 0.05$ as compared with Formalin control.

which were detected from 5 days after administration as compared with the vehicle or formalin control. No meaningful changes in body weights were detected in any of the three DHU001 treatment groups in a comparison with the vehicle and formalin controls throughout the 10-day experimental period (Fig. 1).

Table 2. Changes in paw thicknesses of the mice

Group	Paw thicknesses		Differences [% changes*]
	At start of treatment	At sacrifice	
Controls			
Vehicle	2.45 ± 0.15	2.51 ± 0.07	0.05 ± 0.19
Formalin	2.46 ± 0.16	4.21 ± 0.26[a]	1.75 ± 0.41[a]
Dexamethasone	2.47 ± 0.12	2.67 ± 0.20[b]	0.21 ± 0.20[b] [−88.27]
DHU001 treatment			
500 mg/kg	2.45 ± 0.11	3.26 ± 0.28[ab]	0.81 ± 0.31[ab] [−53.75]
250 mg/kg	2.45 ± 0.19	3.45 ± 0.35[ab]	1.00 ± 0.43[ab] [−42.99]
125 mg/kg	2.45 ± 0.13	3.78 ± 0.17[ab]	1.33 ± 0.19[ac] [−23.82]

Values are expressed as Mean ± SD of eight mice, mm.
Differences = paw thicknesses at sacrifice − paw thicknesses at sacrifice at start of treatment.
* % changes between formalin control and treated group.
[a]$p < 0.01$ as compared with vehicle control.
[b]$p < 0.01$ and [c]$p < 0.05$ as compared with formalin control.

Table 3. Changes in paw volumes of the mice

Group	Paw thicknesses		Differences [% changes*]
	At start of treatment	At sacrifice	
Controls			
Vehicle	57.50 ± 7.07	66.25 ± 5.18	8.75 ± 6.41
Formalin	56.25 ± 5.18	146.25 ± 22.64[a]	90.00 ± 22.68[a]
Dexamethasone	56.25 ± 5.18	66.25 ± 10.61[b]	10.00 ± 13.09[b] [−88.89]
DHU001 treatment			
500 mg/kg	57.50 ± 7.07	83.75 ± 9.16[ab]	26.25 ± 13.02[ab] [−70.83]
250 mg/kg	57.50 ± 7.07	96.25 ± 10.61[ab]	38.75 ± 12.46[ab] [−56.94]
125 mg/kg	57.50 ± 4.63	120.00 ± 14.14[ac]	62.50 ± 16.69[ab] [−30.56]

Values are expressed as Mean ± SD of eight mice, mm^3.
Paw volume = 1/2(length of long axis × length of short axis × thickness of paw).
Differences = paw volumes at sacrifice − paw volumes at sacrifice at start of treatment.
* % changes between formalin control and treated group.
[a]$p < 0.01$ as compared with vehicle control.
[b]$p < 0.01$ and [c]$p < 0.05$ as compared with formalin control.

Table 4. Changes in paw weights and TNF-α contents of the mice

Group	Paw thicknesses		Paw TNF-α contents (ng/g paw) [% changes*]
	At start of treatment	At sacrifice	
Controls			
Vehicle	0.176 ± 0.011	0.532 ± 0.030	6.86 ± 1.56
Formalin	0.347 ± 0.018[a]	1.056 ± 0.061[a]	49.99 ± 13.18[a]
Dexamethasone	0.206 ± 0.022[bc]	0.693 ± 0.066[ac]	11.77 ± 4.26[ac] [−76.46]
DHU001 treatment			
500 mg/kg	0.271 ± 0.030[ac]	0.814 ± 0.087[ac]	19.01 ± 6.42[ac] [−61.97]
250 mg/kg	0.274 ± 0.022[ac]	0.824 ± 0.067[ac]	25.21 ± 7.45[ac] [−49.58]
125 mg/kg	0.307 ± 0.031[ac]	0.933 ± 0.114[ac]	34.95 ± 6.66[ad] [−30.10]

Values are expressed as Mean ± SD of eight mice.
Relative paw weights (% of body weight) = (Absolute weight/Body weight at sacrifice) × 100.
* % changes between formalin control and treated group.
[a]$p < 0.01$ and [b]$p < 0.05$ as compared with vehicle control.
[c]$p < 0.01$ and [d]$p < 0.05$ as compared with formalin control.

Changes on the paw thicknesses. A significant ($p < 0.01$) increase of left hind paw thickness was detected in the formalin control compared with that of vehicle control from 1 day after formalin injection. Consequently the increases of paw thickness during the 10-day experimental period were also significant ($p < 0.01$). However, left hind paw thicknesses were significantly ($p < 0.01$ or $p < 0.05$) decreased compared with the formalin control from 1 day after the start of dexamethasone treatment, from 2 days after the start of DHU001 500 mg/kg treatment, and from 3 days after the start of DHU001 250 and 125 mg/kg treatment. The changes of paw thickness during the 10-day experimental period were also significant ($p < 0.01$ or $p < 0.05$) for dexamethasone and all three different dosages of DHU001 compared with the formalin-injected control (Table 2).

Changes on the paw volumes. A significant ($p < 0.01$) increase of left hind paw volume was detected in the formalin-injected control compared with the vehicle control from 1 day after formalin injection. Consequently, the increases of paw volume during the 10-day experimental period were also significant ($p < 0.01$). However, paw volumes were significantly ($p < 0.01$ or $p < 0.05$) decreased compared with the formalin-injected control from 2 days after the start of dexamethasone, DHU001 500 and 250 mg/kg administration, and from 3 days after start of DHU001 125 mg/kg treatment. The decreases of volume during the 10-day experimental period were also significant ($p < 0.01$) for dexamethasone and all three different dosages of DHU001 as compared with the formalin control (Table 3).

Changes on the paw weights. Significant ($p < 0.01$) increases of left hind paw absolute and relative weights were detected in the formalin control compared with the vehicle control. However, the paw weights were significantly ($p < 0.01$) decreased in the dexamethasone- and DHU001-treated groups compared with the formalin control (Table 4).

Changes on the paw TNF-α contents. A significant ($p < 0.01$) increase of left hind paw TNF-α contents was detected in the formalin control compared with the vehicle control. However, the paw TNF-α contents were significantly ($p < 0.01$) decreased in the dexamethasone- and DHU001-treated groups compared with formalin control (Table 4).

Histopathological changes. Histopathological changes related to chronic inflammation, such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, leading to the hypertrophy of subcutaneous regions were observed in both the dorsum pedis and dorsum digit skins of the formalin control. In the dexamethasone- and DHU001-treated groups, these histopathological changes were dramatically decreased compared with the formalin control (Fig. 1 and 2).

Significant ($p < 0.01$) increases in the thickness and the numbers of infiltrating inflammatory cells in the dorsum pedis and dorsum digit skin were detected in the formalin control compared with the vehicle control. However, these increases of skin thickness and infiltrating inflammatory cells were significantly ($p < 0.01$) decreased in the dexamethasone- and DHU001-treated groups when compared with the formalin control (Table 5).

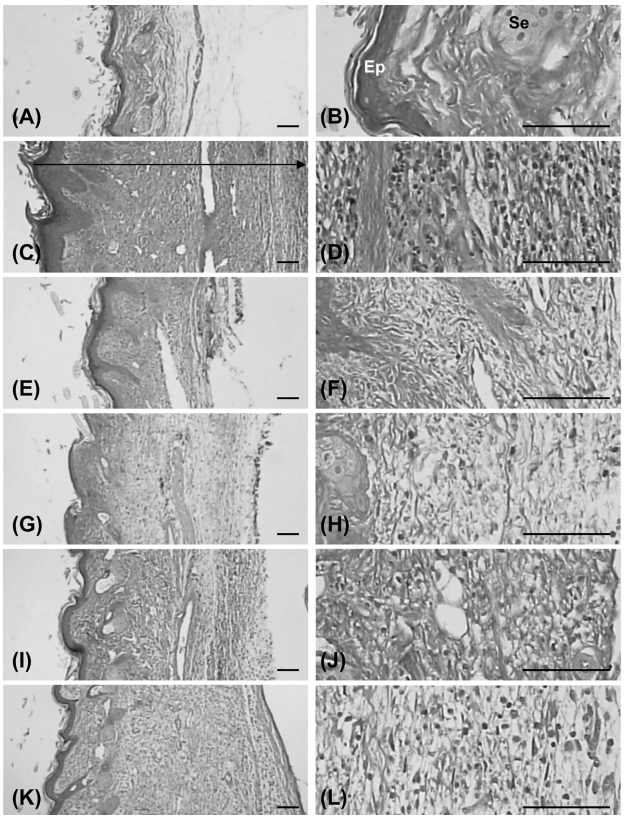

Fig. 2. The representative histological profiles of dorsum pedis skin: observed in vehicle control (A, B), formalin-injected control (C, D), and dexamethasone (E, F), DHU001 500 (G, H), 250 (I, J), and 125 (K, L) mg/kg treated groups. Note that histopathological changes related to chronic inflammation, such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, were observed in the dorsum pedis skins of the formalin-injected control, leading to the hypertrophy of subcutaneous regions. However these histopathological changes were dramatically decreased by treatment with dexamethasone and at all three different dosages of DHU001 as compared with the formalin-injected control. The arrow indicates the thickness of dorsum pedis skin. Ep, epithelium – keratinized stratified squamous epithelium; Se, sebaceous gland; H&E stain; Scale bars = 160 μm.

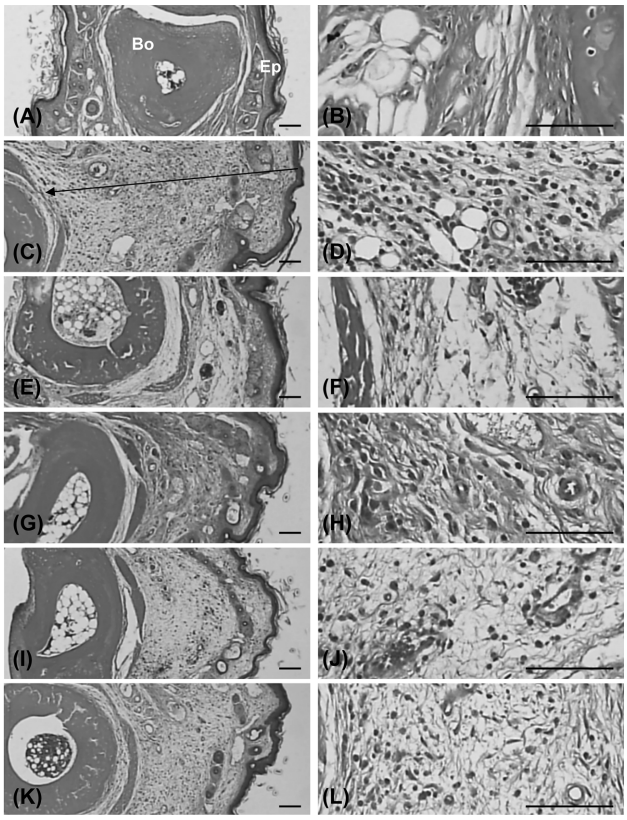

Fig. 3. The representative histological profiles of dorsum digit skin: observed in vehicle control (A, B), formalin-injected control (C, D), and dexamethasone (E, F), DHU001 500 (G, H), 250 (I, J), and 125 (K, L) mg/kg treated groups. Note that histopathological changes related to chronic inflammation, such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, were observed in the dorsum digit skins of the formalin-injected control, leading to the hypertrophy of subcutaneous regions. However these histopathological changes were dramatically decreased by treatment with dexamethasone and at all three different dosages of DHU001 as compared with the formalin-injected control. The arrow indicates the thickness of dorsum digit skin. Bo, metatarsal bones; Ep, epithelium – keratinized stratified squamous epithelium; H&E stain; Scale bars = 160 μm.

DISCUSSION

In the present study, the anti-inflammatory effects of DHU001, a polyherbal formula were evaluated using formalin-induced chronic inflammation in mice paws, one of the simplest animal models for detecting chronic inflammation (Kim *et al*., 2006; Akindele and Adeyemi, 2007), and compared with those of 15 mg/kg dexamethasone intraperitoneally injected. After two subaponeurotic formalin treatments, marked increases in the paw thickness and volume were detected in the formalin-injected control as compared with the vehicle control. In addition, at the time of sacrifice, the paw wet-weights and paw TNF-α contents had also dramatically increased. In histopathological observations, severe chronic inflammation signs such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, were detected in the formalin-injected control, and marked increases in the thickness of the skin of the dorsum pedis and of the dorsum digit were induced with increases in infiltrating inflammatory cells in the dorsum pedis and dorsum digits skin. However, these formalin-induced chronic inflammatory changes were dramatically decreased by treatment with dexamethasone or one of the three dosages of DHU001. Therefore, these results are considered as direct evidence that DHU001 treatment improves the chronic inflammatory response induced by formalin, and correspond well with the anti-inflammatory effects of DHU001 in the other inflammatory animal experiments (Back *et al*., 2008; Lee *et al*., 2010).

The body weight decreases detected in the dexamethasone-treated group were considered to be due to the direct toxicity of glucocorticoid. Steroids have been a popular choice for treating various cutaneous disorders; however, the potential for significant local and systemic adverse events, like skin atrophy and HPA axis suppression, has limited their use (Gupta and Chow, 2004). No meaningful changes in body weight or body weight gains were detected at any dosage of DHU001 as compared with the formalin control in this study.

After a local injection of formalin, marked increases in the paw thickness, volume, and weight are detected as a general chronic inflammation response, and these increases are used as valuable markers for testing anti-inflammatory effects (Pillai *et al*., 2003). In the present study, the increased paw thickness, volume, and weights were markedly inhibited by treatment with DHU001 at each of the three different dosages. Consequently, these inhibitions were considered to be direct evidence that DHU001 treatment has a favorable effect on reducing the chronic inflammatory response.

TNF-α, a 17-kDa protein, which was first identified as a product of activated macrophages, is a well-known proinflammatory cytokine (Whittle *et al*., 2008) and it is involved in various inflammations (Schottelius *et al*., 2010). Recently, evidence has been provided of the widespread role of TNF-α in mediating hyperalgesia at different levels (Schäfers *et al*., 2003), both facilitating neuronal excitability and triggering the release of other pro-inflammatory substances (Watkins and Maier, 2002). In the present study, DHU001 treatment dose-dependently inhibited the elevations of TNF-α induced in mouse paws by subaponeurotic injection of formalin. Therefore, we consider that DHU001 showed favorable anti-inflammatory effects on chronic inflammations mediated by cytokine TNF-α suppression. However, we should not excluded the possibility that the previously reported antioxidative effects and immune stimulation of treatment DHU001 components (Sun and Pan, 2006; Aimbire *et al*., 2007; Minghetti *et al*., 2007; Lee *et al*., 2010) may also have been

Table 5. Changes in histomorphometry analyses of the mice

Group	Thicknesses of skin (mm)		Number of inflammatory cells (cells/mm^2)	
	Dorsum pedis (mm)	Dorsum digitalis	Infiltrating dorsum pedis	Infiltrating dorsum digit
Controls				
Vehicle	0.91 ± 0.23	0.84 ± 0.19	10.50 ± 5.68	29.88 ± 10.34
Formalin	2.85 ± 0.29[a]	2.62 ± 0.43[a]	1369.25 ± 339.38[a]	803.38 ± 232.27[a]
Dexamethasone	1.23 ± 0.15[ac]	1.09 ± 0.23[bc]	93.00 ± 22.10[ac]	128.25 ± 38.95[ac]
DHU001 treatment				
500 mg/kg	1.60 ± 0.18[ac]	1.22 ± 0.18[ac]	115.38 ± 17.43[ac]	285.88 ± 54.40[ac]
250 mg/kg	1.77 ± 0.17[ac]	1.60 ± 0.17[ac]	257.50 ± 74.44[ac]	318.00 ± 98.11[ac]
125 mg/kg	2.10 ± 0.27[ac]	1.91 ± 0.29[ac]	819.75 ± 182.58[ac]	424.38 ± 140.30[ac]

Values are expressed as Mean ± SD of eight mice.
Thickness of skin = the thicknesses from epidermis to hypodermis>
[a]$p < 0.01$ and [b]$p < 0.05$ as compared with vehicle control.
[c]$p < 0.01$ as compared with formalin control.

involved in the anti-inflammatory activity observed in the present study, because NO synthase inhibitors can reverse several classic inflammatory symptoms (Ramprasath *et al*., 2006) and immunomodulatory agents can reduce inflammation (Amin *et al*., 1995).

Histopathologically, severe fibrosis, the formation of necrotic debris, infiltration of inflammatory cells (mainly lymphocytes) and hypertrophy of the subcutaneous regions are used as signs of chronic inflammation after a local injection of formalin (Kenjo *et al*., 2002). Infiltration of inflammatory cells also results in a marked increase in the thickness of the skin (including hypodermis) (Kim *et al*., 2006, 2010). However, in the present study, these histopathological changes were markedly and dose-dependently inhibited after treatment with DHU001 at each of the three different dosages. These inhibitions were considered as direct evidence that DHU001 has a relatively favorable effect on reducing the chronic inflammatory response.

Accordingly, based on the current results, we conclude that DHU001 treatment had a favorable effect, mediated by TNF-α suppression, on reducing the chronic inflammatory response induced in mouse paws by formalin injection. Although the anti-inflammatory efficacies were low compared with dexamethasone, DHU001 may present an alternative approach for the treatment of chronic inflammatory diseases with relatively fewer side effects.

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