J. Phys. Ther. Sci.
22: 395–404, 2010

Effects of Magnetic Infrared Laser Irradiation on Formalin-Induced Chronic Paw Inflammation of Mice

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Abstract. [Purpose] The purpose of the present study was to investigate the effect of magnetic infrared laser (MIL) irradiation on formalin-induced chronic inflammation. [Subjects] Male ICR mice. [Methods] Mice were subaponeurotically injected in the left hind paw with 0.02 ml of 3.75% formalin, then subjected to 1.33, 2.66 and 6.65 J/cm^2 of MIL irradiation, 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 and compared with those of dexamethasone, 15 mg/kg, intraperitoneally injected mice. [Results] After two formalin treatments, a marked increase in the paw thickness and volume was detected in the formalin-injected control as compared with the vehicle control. Also at the time of sacrifice the paw wet-weights, and paw TNF-α contents were also dramatically increased and confirmed by histopathological observations. However, these formalin-induced chronic inflammatory changes were significantly and dose-dependently decreased by MIL irradiation. [Conclusion] MIL radiation has favorable effects on formalin-induced chronic inflammation mediated by TNF-α suppression, and MIL therapy may represent an alternative approach for the treatment of chronic inflammatory diseases.
Key words: Chronic inflammation, Formalin, Magnetic Infrared laser, Mouse, Tumor Necrosis factor

(This article was submitted Apr. 2, 2010, and was accepted May 7, 2010)

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[1)]. Physical damage, chemical substances, micro-organisms and other agents are all possible causes of acute inflammation. The inflammatory responses to such

insults consist of changes in blood flow, increased permeability of blood vessels and the subsequent escape of cells from the blood into the tissues. The changes are essentially the same regardless of the cause or its location. 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[2,3].

Chronic inflammation induced by formalin injection of the hind paw of mice has 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. The drug effects are assessed by observation of the paw weight and volume, and histopathological changes, mainly of the paw and digit skin[2,4–7]. Recently, evidence has been provided of a widespread role of tumor necrosis factor-α (TNF-α) in mediating hyperalgesia at different levels[8], both facilitating neuronal excitability and triggering the release of other pro-inflammatory substances[9,10]. Therefore, TNF-α is treated as one of the key pro-inflammatory cytokines in acute and chronic inflammation models[11,12].

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[13]. 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[2,3,6].

Besides the traditional steroidal and non-steroidal anti-inflammatory drugs, many alternative physical techniques such as electric stimulation[14], short wave[15], ultrasound[16] and laser[17] have been satisfactorily used in the treatment of inflammatory diseases. Although magnetic infrared laser (MIL) therapy has been used in the treatment of various inflammatory diseases[18–20], most of the treatments were clinical approaches and the effects of MIL on pre-clinical inflammatory responses have seldom been studied except for its effect on xylene-induced acute inflammatory responses[21]. Accordingly, in the present study, the effects of the MIL therapy on formalin-induced chronic inflammation were investigated and compared with mice intraperitoneally injected with 15 mg/kg dexamethasone.

SUBJECTS AND METHODS

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 MIL irradiation 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).

A continuous MIL (Model: MOLTA-F-8-01, United Space Device Corporation, Moscow, Russia) with an output power 1, 2 or 5 mW and wavelength of 850 nm was used. The dosages (J/cm^2) are given with respect to spot size. The animals were allocated to six groups with 7 mice per group: vehicle control, formalin-treated control, 1.33, 2.66 and 6.65 J/cm^2 with the MIL irradiated groups, 15 mg/kg dexamethasone-treated groups. MIL was irradiated in contact with the treated side paw surface for 60s, once a day for 10 days, and 15 mg/kg of dexamethasone (Sigma, MO, USA) dissolved in saline was intraperitoneally administered, once a day for 10 days.

One hour before MIL irradiation or dexamethasone injection, a subaponeurotic injection of 0.02 ml 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[2].

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). In addition, body weight changes over the 10 days of the experimental period were calculated as body weight gains (g) = (Body weights at sacrifice –body weights at start of test substances or vehicle

treatments).

The thicknesses of the left hind paws were measured using an electronic 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 MIL 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[2]: paw volume (mm^3) = 1/2(length of long axis × length of short axis × thickness of paw).

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.

After wet-weight measurements of paws, for TNF-α evaluation, skin samples of the left hind paws were homogenized in 3 ml of phosphate-buffered saline (PBS) containing 10 mM EDTA and 20 KIU/ml 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[11]. 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). The anti-TNF capture monoclonal antibody (mAb) (5 Ag/ml) was absorbed on a polystyrene 96-well plate and the TNF-α present in the sample was bound to the antibody-coated wells. The biotinylated anti-TNF detecting mAb (0.25 Ag/ml) was added to bind TNF-α captured by the first antibody. After washing, streptavidin-peroxidase was added to the wells to detect the biotinylated detecting antibody and finally TMB substrate was added. A colored product was formed in proportion to the amount of TNF-α present in the sample, and was measured at an optical density 450 nm. The amount of cytokine in each supernatant was interpolated from the standard curves. The standard curves were recombinant cytokine curves generated by doubling dilutions from 2,500 to 39 pg/ml.

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. 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[2] with some modifications. The histopathologist was blinded to group distribution when this analysis was made.

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 6.1.3., SPSS Inc., USA).

RESULTS

No meaningful changes in body weight or body weight gain 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, which were detected from 5 days after administration as compared with the

Table 1. Changes in body weights of the mice

Group	Body weights		Gains
	At start of treatment	At sacrifice	
Controls			
Vehicle	31.49 ± 1.71	33.50 ± 1.71	2.09 ± 1.28
Formalin	31.70 ± 1.48	33.36 ± 1.14	1.66 ± 1.48
Dexamethasone	31.80 ± 1.71	32.57 ± 1.70 [a,b]	–2.41 ± 1.10 [a,b]
MIL irradiation			
6.65 J/cm^2	31.86 ± 1.85	33.63 ± 1.15	1.77 ± 1.60
2.66 J/cm^2	31.64 ± 2.49	33.11 ± 2.50	1.47 ± 2.58
1.33 J/cm^2	31.63 ± 1.27	33.40 ± 2.39	1.77 ± 2.05

Values are expressed as Mean ± SD of seven mice, g. Gains = Body weights at sacrifice - body weights at start of treatments. [a] $p<0.01$ as compared with vehicle control. [b] $p<0.01$ as compared with formalin control.

Table 2. Changes in paw thicknesses of the mice

Group	Paw thicknesses		Differences
	At start of treatment	At sacrifice	
Controls			
Vehicle	2.45 ± 0.16	2.57 ± 0.15	0.12 ± 0.19
Formalin	2.46 ± 0.17	4.46 ± 0.28 [a]	1.99 ± 0.30 [a]
Dexamethasone	2.46 ± 0.13	2.80 ± 0.30 [b]	0.33 ± 0.40 [b]
MIL irradiation			
6.65 J/cm^2	2.45 ± 0.10	3.55 ± 0.27 [a,b]	1.10 ± 0.23 [a,b]
2.66 J/cm^2	2.47 ± 0.10	3.89 ± 0.32 [a,b]	1.42 ± 0.33 [a,b]
1.33 J/cm^2	2.44 ± 0.09	4.07 ± 0.09 [a,b]	1.63 ± 0.15 [a,c]

Values are expressed as Mean ± SD of seven mice, mm. Differences = paw thicknesses at sacrifice-paw thicknesses at sacrifice at start of treatment. [a] $p<0.01$ as compared with vehicle control. [b] $p<0.01$ and [c] $p<0.05$ as compared with formalin control.

vehicle or formalin control. Accordingly, the body weight gain at the end of the 10-day experimental period of this group was also significantly ($p<0.01$) decreased. No meaningful changes in body weight or body weight gain were detected in any of the three MIL treatment groups in a comparison with the vehicle and formalin controls throughout the 10-day experimental period (Table 1).

A significant ($p<0.01$) increase of left hind paw thickness was detected in the formalin-injected 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 ($p<0.01$) decreased compared with the formalin-injected control from 4 days after the start of dexamethasone treatment, from 6 days after the start of MIL 6.65 J/cm^2 irradiation, and from 7 days after the start of MIL 2.66 and 1.33 J/cm^2 irradiation. The decreases in thickness were also significant ($p<0.01$ or $p<0.05$) for dexamethasone and all three different dosages of MIL compared with the formalin-injected control. A clear dose-dependent decrease in the paw thickness was found for the MIL irradiated groups (Table 2).

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 4 days after the start of dexamethasone administration, from 6 days after the start of MIL 6.65 J/cm^2 treatments, and from 7 days after start of 2.66 and 1.33 J/cm^2 irradiation.

Table 3. Changes in paw volumes of the mice

Group	Paw volumes		Differences
	At start of treatment	At sacrifice	
Controls			
Vehicle	58.57 ± 6.90	67.14 ± 4.88	8.57 ± 6.90
Formalin	55.71 ± 5.35	157.14 ± 13.80[a]	101.43 ± 13.45[a]
Dexamethasone	57.14 ± 4.88	80.00 ± 19.15[c]	22.86 ± 19.76[c]
MIL irradiation			
6.65 J/cm^2	58.57 ± 12.15	100.00 ± 24.49[a,c]	41.43 ± 32.37[b,c]
2.66 J/cm^2	57.14 ± 7.56	121.43 ± 10.69[a,c]	64.29 ± 12.72[a,c]
1.33 J/cm^2	58.57 ± 6.90	132.86 ± 16.04[a,d]	74.29 ± 18.13[a,d]

Values are expressed as Mean ± SD of seven 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. [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.

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

Group	Paw weights		Paw TNF-α contents (ng/g paw)
	Absolute (g)	Relative (%)	
Controls			
Vehicle	0.176 ± 0.012	0.527 ± 0.037	6.67 ± 1.59
Formalin	0.360 ± 0.021[a]	1.078 ± 0.052[a]	53.97 ± 12.00[a]
Dexamethasone	0.204 ± 0.023[b]	0.695 ± 0.082[a,b]	11.98 ± 4.56[a,b]
MIL irradiation			
6.65 J/cm^2	0.282 ± 0.013[a,b]	0.840 ± 0.043[a,b]	24.53 ± 6.17[a,b]
2.66 J/cm^2	0.297 ± 0.046[a,b]	0.899 ± 0.139[a,b]	31.73 ± 4.08[a,b]
1.33 J/cm^2	0.320 ± 0.022[a,b]	0.961 ± 0.072[a,b]	40.70 ± 4.14[a,c]

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

The decreases of volume were also significant ($p<0.01$ or $p<0.05$) for dexamethasone and all three different dosages of MIL as compared with the formalin-injected control. A clear dose-dependent decrease in the paw volume was found for the MIL irradiated mice (Table 3).

Significant ($p<0.01$) increases of left hind paw absolute and relative weights were detected in the formalin-injected control compared with the vehicle control. However, the paw weights were significantly ($p<0.01$) decreased in the dexamethasone- and MIL-treated groups compared with the formalin-injected control. A clear dose-dependent decreases in paw weights were found for the MIL treated groups (Table 4).

A significant ($p<0.01$) increase of left hind paw TNF-α contents was detected in the formalin-injected control compared with the vehicle control. However, the paw TNF-α contents were significantly ($p<0.01$ or $p<0.05$) decreased in the dexamethasone- and MIL-treated groups compared with formalin-injected control. A clear dose-dependent decrease in the paw TNF-α contents was found for the MIL irradiated mice (Table 4).

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-injected control. In the dexamethasone- and MIL-treated groups, these histopathological changes were dramatically decreased compared with the formalin-injected control. In addition, a clear dose-dependency treatment effect was also demonstrated for the MIL-treated groups (Fig 1 and 2).

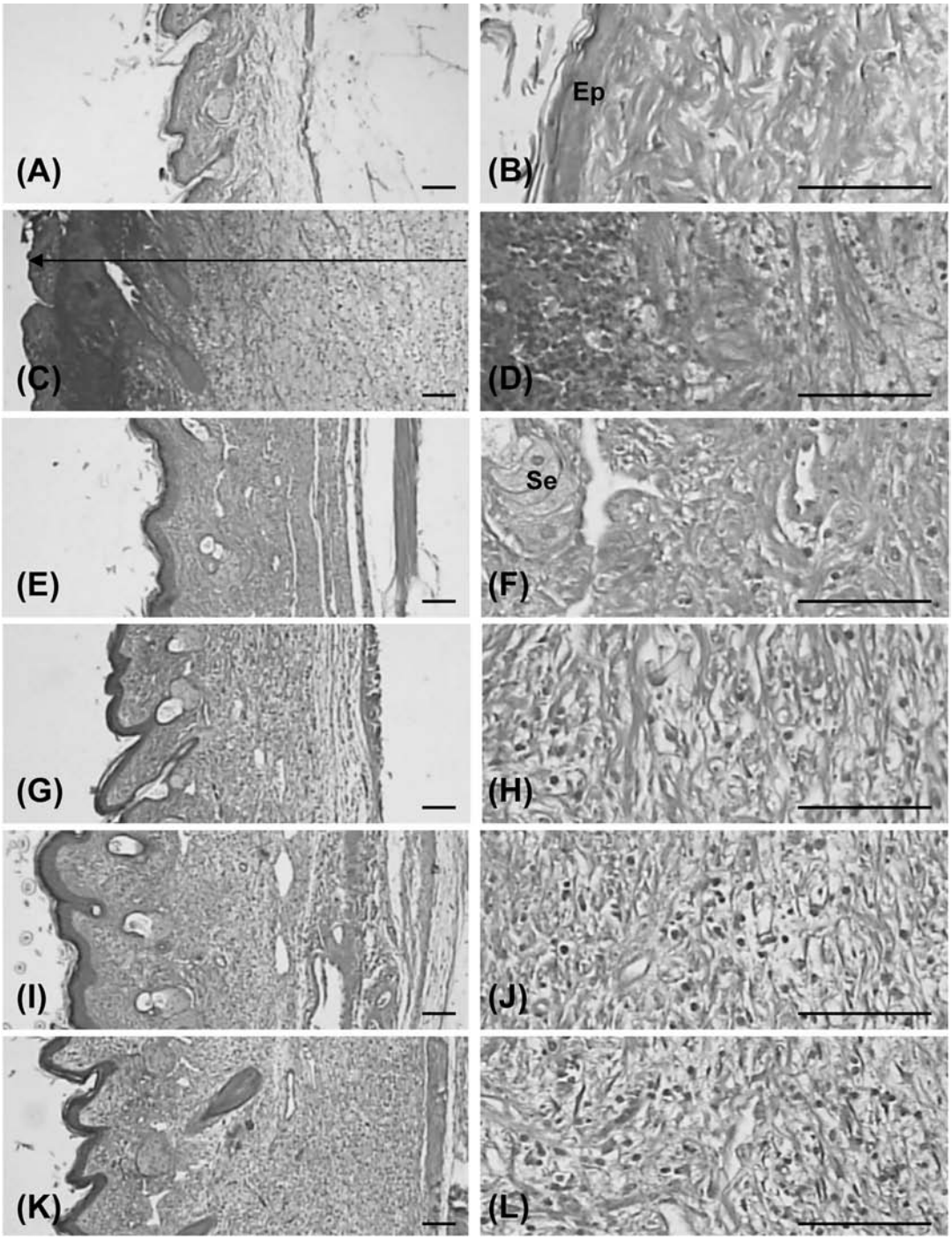

Fig. 1. The representative histological profiles of dorsum pedis skin: observed in vehicle control (A, B), formalin-injected control (C, D), and dexamethasone (E, F), MIL 6.65 (G, H), 2.66 (I, J), and 1.33 (K, L) J/cm^2 treated groups.
Note that histopathological changes related to chronic inflammation, 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 MIL 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.

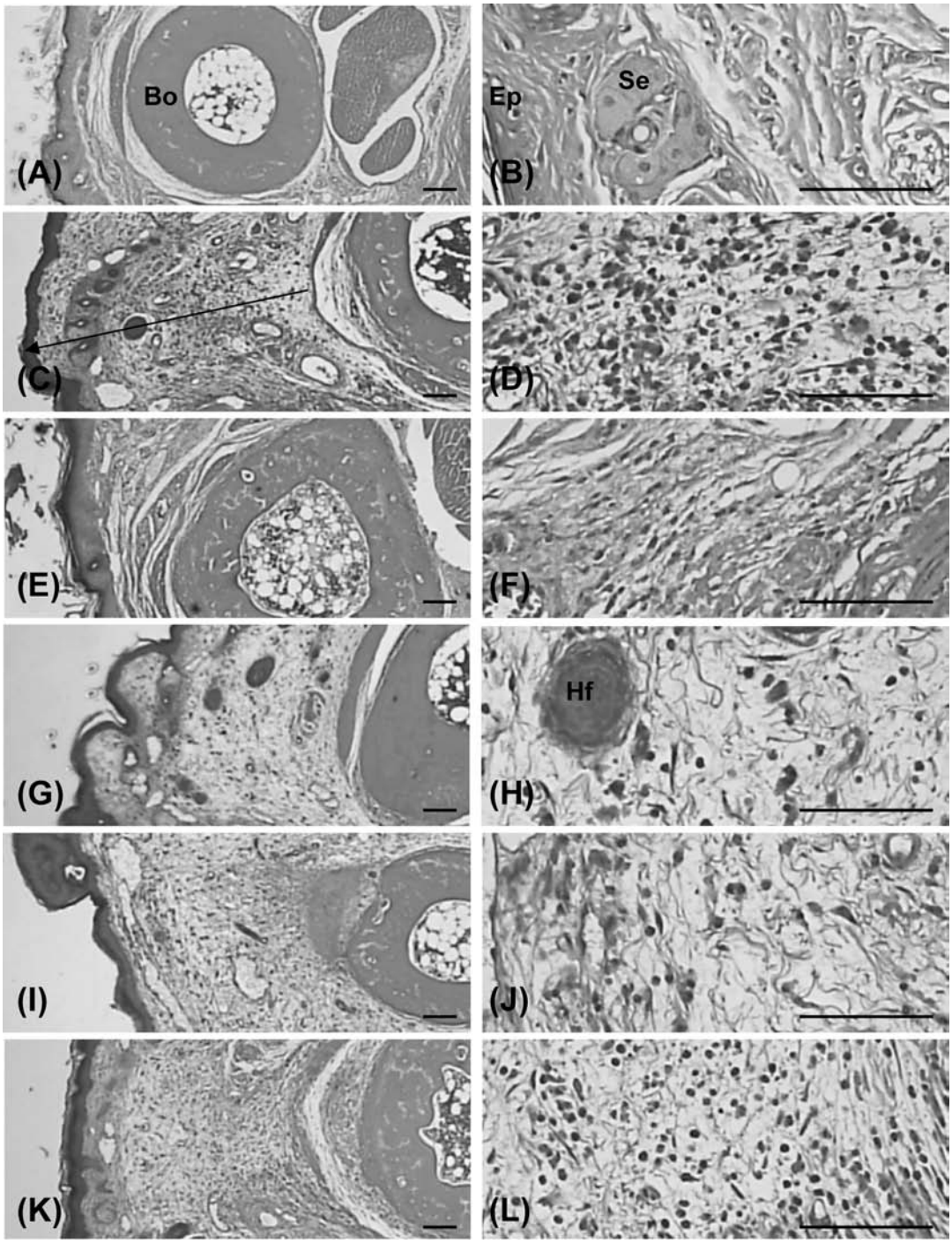

Fig. 2. The representative histological profiles of dorsum digit skin: observed in vehicle control (A, B), formalin-injected control (C, D), and dexamethasone (E, F), MIL 6.65 (G, H), 2.66 (I, J), and 1.33 (K, L) J/cm^2 treated groups.
Note that histopathological changes related to chronic inflammation, 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 MIL as compared with the formalin-injected control. The arrow indicates the thickness of dorsum pedis skin. Bo, metatarsal bones; Hf, hair follicle; Ep, epithelium – keratinized stratified squamous epithelium; Se, sebaceous gland; H&E stain; Scale bars = 160 μm.

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	0.92 ± 0.24	0.81 ± 0.18	9.86 ± 5.81	27.14 ± 7.43
Formalin	2.80 ± 0.27 [a]	2.58 ± 0.44 [a]	1293.86 ± 285.17 [a]	815.71 ± 213.82 [a]
Dexamethasone	1.20 ± 0.14 [b,c]	1.05 ± 0.22 [b,c]	91.29 ± 23.29 [a,c]	120.00 ± 33.68 [a,c]
MIL irradiation				
6.65 J/cm^2	1.79 ± 0.34 [a,c]	1.68 ± 0.15 [a,c]	186.00 ± 52.38 [a,c]	262.43 ± 62.53 [a,c]
2.66 J/cm^2	2.16 ± 0.27 [a,c]	1.93 ± 0.11 [a,c]	332.43 ± 74.90 [a,c]	341.71 ± 98.49 [a,c]
1.33 J/cm^2	2.43 ± 0.20 [a,c]	2.06 ± 0.16 [a,d]	542.14 ± 109.77 [a,c]	593.43 ± 88.58 [a,c]

Values are expressed as Mean ± SD of seven 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] $p<0.01$ and [d] $p<0.05$ as compared with formalin control.

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-injected control compared with the vehicle control. However, these increases of skin thickness and infiltrating inflammatory cells were significantly ($p<0.01$ or $p<0.05$) decreased in the dexamethasone- and MIL-treated groups when compared with the formalin-injected control. In addition, a clear dose-dependency treatment effect was also demonstrated for the MIL-irradiated mice (Table 5).

DISCUSSION

In the present study, the anti-inflammatory effects of MIL therapy were evaluated using formalin-induced chronic inflammation in mice paws, one of the simplest animal models for detecting chronic inflammation[2,4–7], 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 MIL. Therefore, these results are considered as direct evidence that MIL therapy improves the chronic inflammatory response induced by formalin, and correspond well with the clinical indications of MIL in the various inflammatory diseases[18–21].

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[13]. No meaningful changes in body weight or body weight gains were detected at any dosage of MIL 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[5,22]. In the present study, the increased paw thickness, volume, and weights were markedly inhibited by treatment with MIL at each of the three different dosages. Consequently, these inhibitions were considered to be direct evidence that MIL therapy has a favorable effect on reducing the chronic inflammatory response.

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[23,24]. Infiltration of inflammatory cells also results in a marked increase in the thickness of the skin (including hypodermis)[2]. However, in the present study, these histopathological changes were markedly and dose-dependently inhibited after treatment with MIL at each of the three different dosages. These inhibitions were considered as direct evidence that MIL has a relatively 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[25] and it is involved in various inflammations[11,26]. Recently, evidence has been provided of the widespread role of TNF-α in mediating hyperalgesia at different levels[8], both facilitating neuronal excitability and triggering the release of other pro-inflammatory substances[9,10]. In the present study, MIL treatment dose-dependently inhibited the elevations of TNF-α induced in mouse paws by subaponeurotic injection of formalin. Therefore, we consider that MIL showed favorable anti-inflammatory effects on chronic inflammations mediated by cytokineTNF-α suppression. However, we should not excluded the possibility that the previously reported antioxidative effect[27,28] and immune stimulation[29–32] of MIL therapy may also have been involved in the anti-inflammatory activity observed in the present study, because NO synthase inhibitors can reverse several classic inflammatory symptoms[33] and immunomodulatory agents can reduce inflammation[34].

Accordingly, based on the current results, we conclude that MIL therapy 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, MIL therapy may present an alternative approach for the treatment of chronic inflammatory diseases with relatively fewer side effects.

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