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Inducible Expression of NOS and COX-2 in Evaluating the Effects of Ruyi-Jinhuang Gao on Somatic Pain – Associated Inflammation

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ABSTRACT

Ruyi-Jinhuang Gao (RJG) is an ointment prepared from Ruyi-Jinhuang San (RJS), a traditional formula of powder-type Chinese medicine, usually used in anti-inflammatory analgesic care in China. The present study is aimed to detect the anti-inflammatory signaling of RJG exposed by an experimental model of lipopolysaccharides (LPS)-induced pro-inflammatory expression of inducible nitric oxide synthase (iNOS) and prostaglandin endoperoxide synthase (PGH synthase-2, COX-2). LPS-induced activation of iNOS and COX-2 has been recognized to increase cytokines and nitric oxide (NO), which play predominant roles in inflammation. In the culture of RAW264.7 cells, RJG concentration-dependently inhibited LPS-induced iNOS and COX-2 expression. However, the herbal components of RJG displayed different results, including increase and decrease of both protein expressions. Among them, inhibition by Curcuma Zedorarica Rhizoma (Cu), Atractylodis Lancea Rhizoma (At), and Glycyrrhizae Radix (Gl) are more potent than that by others. Inhibition by Cu and At are associated with their cytotoxicity. Glycyrrhizae Radix (Gl), with lower cytotoxicity in comparison with Cu and At, is the most potent component of RJG on inhibiting LPS-induced iNOS and COX-2 expression.

In comparison with LPS-induced tumor necrosis factor-α (TNF-α), croton oil-induced formation of TNF-α and interleukin-4 (IL-4), RJG did not increase the pro-inflammatory cytokines. These facts indicated that RJG could not significantly sensitize an immunologic response during the treatment of non-wounded inflammation on body surface. Ruyi-Jinhuang Patch (RJP), a pharmaceutical preparation of RJG, has proven to have significant relief of cutaneous inflammation-associated somatic pain in clinical use.

Key words: RJG, LPS, iNOS, COX-2, cytokines, anti-inflammation

INTRODUCTION

Ruyi-Jinhuang Gao (RJG) is an ointment prepared from water and alcohol extracts of Ruyi-Jinhuang San (RJS), a traditional formula of yellowish powder-type Chinese medicine described in I-Tzong-Jin-Jiann. Its usage is currently re-printed in the Pharmacopoeia of China (Peijing, China, 2000). RJS has been used in local anti-inflammatory analgesic care for patients with myofascial pain, arthritis, trauma, frozen shoulder, tendonitis, and neuropathic and various somatic pains. It is a mixture of 10 herbs, including Trichosanthes Root (Tr, 320 parts), Angelicae Dahuricae Radix (An, 160 parts), Phellodenri Cortex (Ph, 160 parts), Rhei Rhizoma (Rh, 160 parts), Curcuma Zedorarica Rhizoma (Cu, 160 parts), Glycyrrhizae Radix (Gl, 64 parts), Magnoliae Cortex (Ma, 64 parts), Atractylodis Lancea Rhizoma (At, 64 parts), Arisaematis Rhizoma (Ar, 64 parts), and Auranti Nobilis Pericarpium (Au, 64 parts).

Lipopolysaccharides (LPS), the structural component of the outer membranes of gram-negative bacteria[(1)], play a pivotal role in the inflammatory response in *in vivo* and have been used to induce inflammatory response mediated by action on macrophage in *in vitro*. Croton oil, an immunological sensitizer on skin, was described to initiate cutaneous inflammation-like contact dermatitis and cytokine release in mice plasma[(2,3)]. In addition, it is suggested that anti-inflammatory agents may inhibit the immunologic response induced by croton oil during their contact with dermis. However, unsuitable use of glucocorticoids may modulate the immune function in the skin through aberrant production of tachykinin, such as

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substance P or other epidermal cell derived cytokines[4]. Chinese medicine is an useful alternative to prevent non-wounded inflammatory responses without sensitization activity on body surface of patients. Animal studies have reported that toxic actions of LPS and croton oil resulted in the release of cytokines, especially tumor necrosis factor-α (TNF-α, interleukin 1 (IL-1), interleukin 8 (IL-8) and interleukin 4 (IL-4). These cytokines drive a cascade of mediators and lead to increase of NO[5,2]. However, both LPS-induced pro-inflammatory signaling in macrophage and croton oil-induced dermatitis may provide useful information for patients desiring pain relief.

Macrophages have a central role in controlling inflammation because they can develop into cells that cause further injury or facilitate tissue repair[6]. When activated by LPS, macrophages inhibit the growth of a wide variety of invasive microorganisms through releasing nitric oxide (NO), cytokines, TNF-α, and eicosanoid mediators of the immune response[7,8,9]. Many cytokines are involved in inflammation. Under normal circumstances, local production of these mediators is precisely regulated by inducible activation of iNOS and COX-2 and thus serves to orchestrate the inflammation pathway, including causing somatic pain. Cutaneous hyperalgesia at the site of an injury can be explained by sensitization of nociceptors. This sensitization is likely to be due to local release of chemical mediators in the inflamed area[10].

The aim of this study was to investigate whether RJG can inhibit LPS-induced expression of nitric oxide synthase (iNOS) and prostaglandin endoperoxide synthase (PGH synthase-2, COX-2) in macrophage. We also hoped to provide a model of pro-inflammatory signaling to explain the anti-inflammatory benefits of RJS and RJG on unwounded inflammatory human body surface for pain relief. The inhibition intensities of each plant components in RJG on expression of iNOS and COX-2 were also compared. Those results might provide the way to recreate a more potent formula of RJG in the future.

MATERIALS AND METHODS

I. *Animals*

BALB/c derived male mice, weighing 22 ± 2 g were provided by National Laboratory Animals Breeding and Research Center (NLABRC, Taiwan). The animals were housed in individually ventilated cages (IVC Racks, 36 Mini Isolator systems) under Specific Pathogen-Free (SPF) conditions throughout the experiment. Each APEC® cage was autoclave sterilized and contained 5 mice (in cm, 26.7 length × 20.7 width × 14.0 height), and then maintained in a hygienic environment under controlled temperature (22-24˚C) and humidity (60-80%) with 12 hr light/dark cycle. The animals were given free access to sterilized lab. Chow and sterilized disposal of animals were performed according to the guide for the care and use of Laboratory Animals (National Academy Press, Washington, DC, USA, 1996)[11].

II. *Chemicals*

LPS (*E. coli* serotype 026:B6 and *E. coli* 026:B6), albumin bovine (BSA), aprotinin, bromophenol blue, brilliant blue G, ethylene glycol-bis-tetraacetic acid (EGTA), glycerol, leupeptin, phenylmethylsulfonyl fluoride (PMSF), DMEM, FBS, penicillin G and streptomycin were obtained from GIBCO/BRL Life technologics, Grand Island, NY, USA. Anti-iNOS mAb and goat anti-mouse IgG-HRP were obtained from BD Transduction Laboratories, USA. Anti-COX-2 goat polyclonal IgG, anti-actin goat polyclonal IgG and Donkey anti-goat IgG horseradish peroxide conjugate were obtained from Santa Cruz Biotechnology (USA). Acetone was from Wako, Japan, Croton Oil, Tween-20 and phosphate-buffer saline were from Sigma (USA). Ethanol was from Merck, Germany. Mouse IL-4 kit was from R & D System, USA. Mouse TNF-α kit was from R & D System, USA. Test substance RJG (6 mg/100 μL acetone/mouse), vehicle (100 μL acetone/mouse) and croton oil (2%/100 μL acetone/mouse) as positive control were applied topically to mice on preshaved back region and posterior surface of ear. The dosing volume was 100 μL/mouse. RJG was extracted with a solvent system (ethanol/distill water, 7/3, 200 mL) at room temperature from dry RJS powder (100 g) for 1 week. Obtained extracts were diluted to make up the expected working solution of 0.30 mg/100 μL for RJG and 1.0 mg/100 μL for each plant component. LPS (10 mg) was dissolved in distilled water (1.0 mL) and then diluted to 100 μg/mL with culture medium as stock solution. Extract was placed on an aluminum foil chip over an electronic heater to evaporate the solvent under a constant temperature (75 ± 5˚C) and RJG was weighed by an electronic balance. RJG (0.30 mg/100 μL) was then further diluted with extraction solvent into 0.06, 0.12, 0.18, 0.24 mg/100 μL by a micropipette (Eppendorf®). All these dilutions of RJG (100 μL) were then mixed with cell culture (9.8 mL) and LPS (10 μg/100 μL stock solution) to make up 10 mL in each of 100 mm culture dishes for incubation before Westen blot experiments. The final concentration of LPS during incubation was 1.0 μg/mL. In test on cell survival, extract of plant component (100 μL), LPS (100 μL), and cell (800 μL) culture were mixed in each well (1.0 mL) of the plates for incubation. RJS was purchased from Kaiser Pharmaceutical Co., Ltd. (Tainan, Taiwan). Species examination for each of the plant components in RJS (Lot number: 363936; effective until 2007, Kaiser Pharmaceutical Co., Ltd.) was confirmed by one of authors, Dr. Ming-Hong Yen.

III. *Cell Culture*

As shown in a previous report[12], mouse macrophage cell line RAW 264.7 was obtained from the American Type

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Culture Collection. Cells were cultured in DMEM medium (10 mL), supplemented with 2 mM L-glutamine, antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (GIBCO/BRL) and maintained at 37˚C in a humidified incubator containing 5% CO_2. Cells in the 2nd-7th passage were used for the experiments.

IV. *Western Blot Analysis*

According to the previous report[12], RAW 264.7 cells were cultured in a 100-mm plate in the presence of LPS (1.0 μg/mL) and RJG for 6 hr and 15 hr, respectively. Cells were washed, harvested, and homogenized. The lysate was centrifuged at 15,000×g for 30 min, and the supernatant was freeze-dried. Using 8% SDS-polyacrylamide minigels and transferring to immobilon polyvinylidene difluoride membranes (Millipore, Germany), the membrane was incubated overnight at 4˚C with 1% BSA and then incubated with anti-iNOS (N32030, Transduction Laboratory, USA), anti-COX-2 and anti-β-actin antibodies (Santa Cruz Biotechnology Inc, USA). Expression of protein was detected by enhanced chemiluminescence (ECL) using Hyperfilm and ECL reagent (Amersham, UK).

V. *Cytotoxicity and Cell Viability*

According to the method of Mosmann[13], RAW 264.7 cells were plated at a density of 1×10^5 cells/well into 24-well plates. After overnight growth, cells were treated with a different concentration of RJG for 6 and 15 hr. The cytotoxic effect of the mentioned agents was evaluated with the MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in PBS at a concentration of 5 mg/mL and filtered with Millipore. From the stock solution, 100 μL per 1 mL of medium was added to each well, and plates were gently shaken and incubated at 37˚C for 4 hr. Treatment of living cells with MTT produced dark blue formazan product, whereas no such staining was observed in dead cells. After loading with MTT, the medium was replaced with 1 mL of acidified β-isopropanol and shaken for 20-30 min at room temperature for color development. The 96-well plate was then read with an enzyme-linked immunosorbent assay reader (570 nm) to obtain the absorbance density values.

VI. *Serum Immunoreactivity*

In order to determine whether RJG possesses sensitization activity, serum IL-4 and TNF-α levels were measured in BALB/c mice after application of RJG (6 mg/100 μL acetone/mouse). In contrast, croton oil, as a reference sensitizer, was also administered topically at a dose of 6 mg/mouse onto the pre-shaved back region and posterior ear surface of test mice. Serum IL-4 and TNF-α levels were measured at 5 and 24 hr after application.

VII. *Patients for Pain Relief*

A total of 45 patients suffering from various somatic pains were enrolled in the clinical evaluation of Ruyi-Jinhuang Patch (RJP, Sinlix Ruyi® Patch, Sinlix Technologic Co., Kaohsiung, Taiwan), a preparation of RJG for pain relief. Those patients with somatic pain received RJP patch on the pain site. The following observations were recorded: pain pattern, pain sites, pain intensity shown by visual analog scale (VAS) before and after treatment, subjective feeling after patching, and side effect.

A patch of regular size (4 × 6 inches) was used for this study. Each patch was fixed at the pain site for 2 days. The maximum number of sites to be patched was 3. If exceeding 3 pain sites, patients would be excluded from the study. Patients were requested to fill the form for the evaluation of pain relief at 0, 0.5, 1, 4, 8, 16, 24, and 48 hr after patching.

VIII. *Statistics*

All values were expressed as mean ± SEM. Student's *t*-test was applied to paired or unpaired observations. A probability value (P) of less than 0.05 was considered to be significant. In some figures the error bars fell within the symbol size. Whenever a control group was compared with more than one treated group, one-way ANOVA or two-way repeated measures ANOVA was used. Statistical analysis was performed by ANOVA followed by the Bonferroni correction for comparison of multiple means. Analysis of the data and plotting of the figures were done with the aid of software (SigmaStat and SigmaPlot, Version 5.0, San Rafael, CA, USA; GraphPad PRISM™, Version 2.0, San Diego, CA, USA) run on an IBM compatible computer.

RESULTS

I. *iNOS Expression*

After LPS (1.0 μg/mL) treatment on RAW 264.7 cells for 6 hr, iNOS protein considerably increased in these cells, in comparison with vehicle control group (CTL). Co-treatment of cells with LPS (1.0 μg/mL) and RJG (0.06, 0.12, 0.18, 0.24, and 0.30 mg/mL) for 6 hr significantly inhibited iNOS protein induction in RAW 264.7 cells (Figure 1). Extracts of each herbal component of RJG displayed various inhibition activities on LPS-induced iNOS expression at 6 hr and 15 hr, respectively. Ph, Ma, Rh, Gl, Cu, and At effectively inhibited LPS-induced iNOS expression at 6 hr. However, at 15 hr, Ph, Gl, Cu, and At were more potent in inhibiting the expression (Figure 4).

II. *COX-2 Expression*

RAW 264.7 macrophages cells did not express detectable COX-2 protein when they were incubated with

medium without LPS for 6 and 15 hr. After LPS (1.0 μg/mL) treatment for 6 and 15 hr, COX-2 protein substantially increased in these cells. Co-treatment of cells with LPS (1.0 μg/mL) and RJG (0.24 and 0.30 mg/mL) for 15 hr significantly inhibited COX-2 protein induction in RAW 264.7 macrophages; all concentrations of RJG treatment for 6 hr ineffectively inhibited COX-2 (Figure 2). At 6 hr, Tr, Ar, Gl, Cu, and At were more potent in inhibition; however, at 15 hr, Cu, At and Gl, were more potent in inhibiting LPS-induced-COX-2 expression (Figure 5).

III. *Cell Viability or Cytotoxicity*

Examination of cell viability or cytotoxicity in RAW 264.7 macrophages by MTT assay indicated that RJG at 0.12-0.30 mg/mL significantly affected the viability of RAW 264.7 cells cultured at 6 hr. On the other hand, RJG decreased the cell viability to about 80% at 0.30 mg/mL. After incubation with cells for 15 hr, RJG concentration-dependently decreased the cell viability and at 0.30 mg/mL up to about 50% (Figure 3). In contrast, each herbal extract (1.0 mg) exerted different effects on viability in cell culture (1.0 mL) at 6 hr and 15 hr, respectively. The components Tr, Ar, and Au moderately increased the survival rate; Ph, Cu, At and Gl, decreased the survival rate at 15 hr. Moreover, Cu and At significantly decreased the survival rate not only at 6 hr, but also at 15 hr (Figure 6). Gl possessed lower cytotoxicity than Cu and At.

IV. *Immunologic Safety*

As shown in Figure 5A and B, no significant effects on serum IL-4 or TNF-α levels were observed for RJG (6

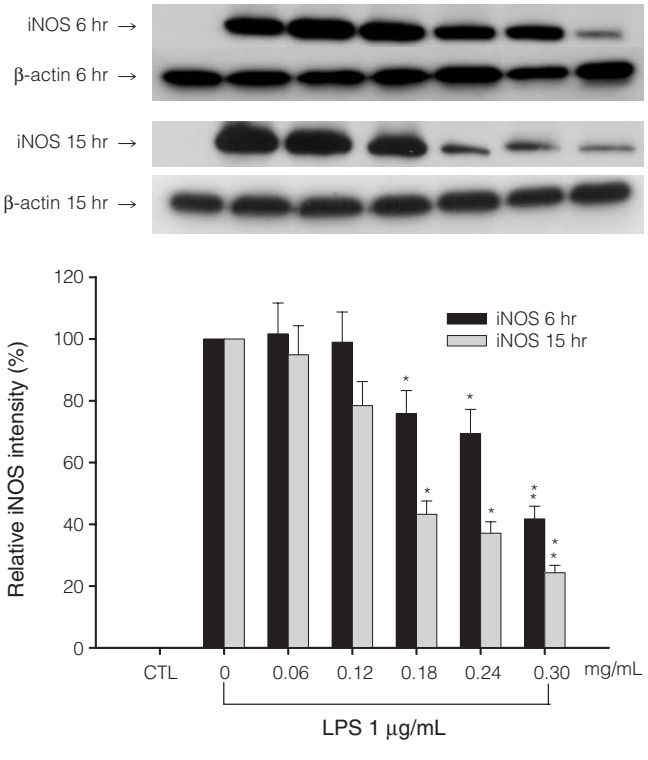

Figure 1. Representative Western blots data depicting the protein expression of iNOS at 6 and 15 hr in the RAW 264.7 cell culture, in the presence of vehicle control (CTL) or RJG and LPS 30 min prior to them. Densitometry analyses are presented as the relative ratio of iNOS protein/β-actin protein. Data are expressed as means ± SEM. * $p < 0.05$ and ** $p < 0.01$ (n = 3) were compared with LPS group, ANOVA was followed by Dunnett's test.

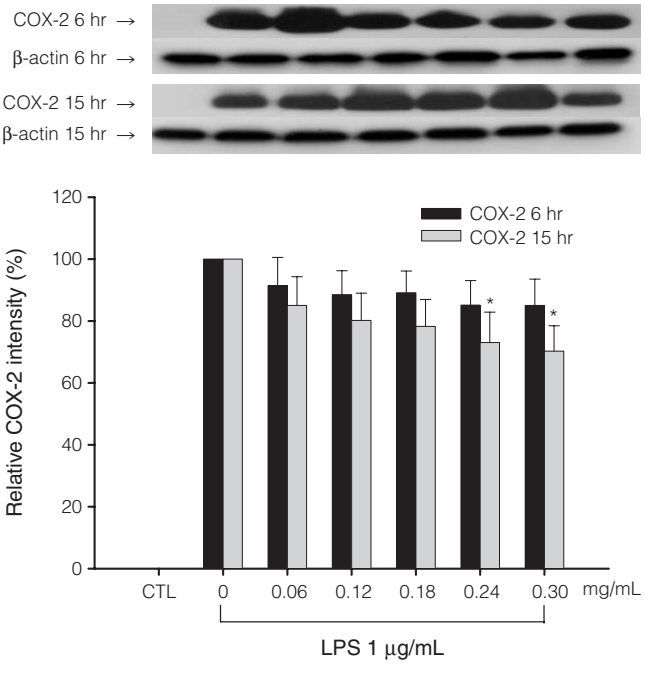

Figure 2. Representative Western blots data depicting protein expression of COX-2 at 6 and 15 hr in the RAW 264.7 cell culture, in the presence of vehicle control (CTL) or RJG and LPS 30 min prior to them. Densitometry analyses are presented as the relative ratio of COX-2 protein/β-actin protein. Data are expressed as means ± SEM. * $p < 0.05$ (n = 3) were compared with LPS group, ANOVA followed by Dunnett's test.

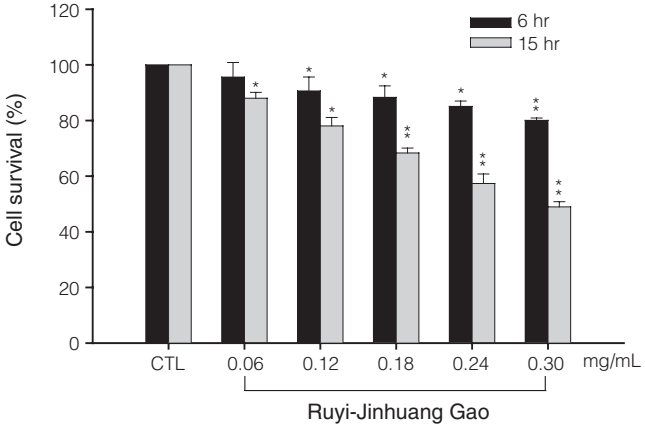

Figure 3. Cell survival rate caused by RJG. RAW 264.7 cells were incubated with vehicle control (CTL) or RJG for 6 and 15 hr and followed by MTT test. Data are expressed as means ± SEM. * $p < 0.05$ and ** $p < 0.01$ (n = 3) were compared with CTL group. ANOVA was followed by Dunnett's test.

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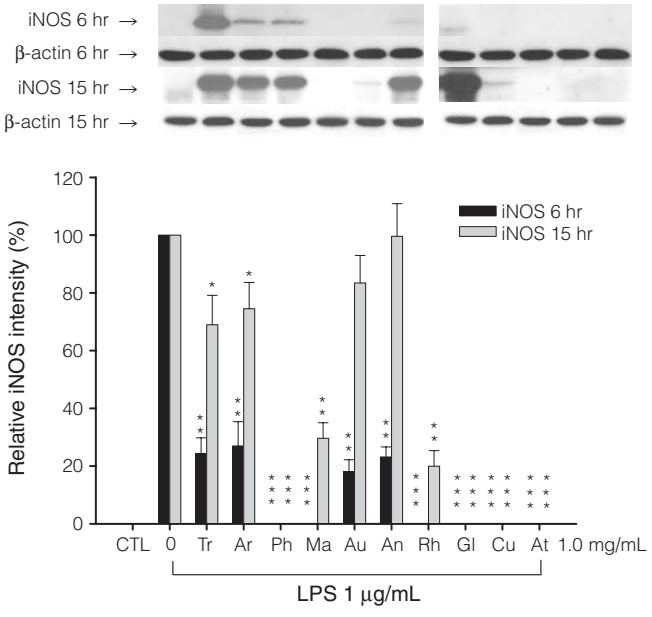

Figure 4. Representative Western blots data depicting protein expression of iNOS at 6 and 15 hr. Herbal components were incubated with RAW 264.7 cells, in the presence of vehicle control (CTL) or each herbal extract and LPS 30 min prior to them. Densitometry analyses are presented as the relative ratio of iNOS protein/β-actin protein. Data were obtained from three independent experiments and expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (n = 3) were compared with LPS group. ANOVA was followed by Dunnett's test.

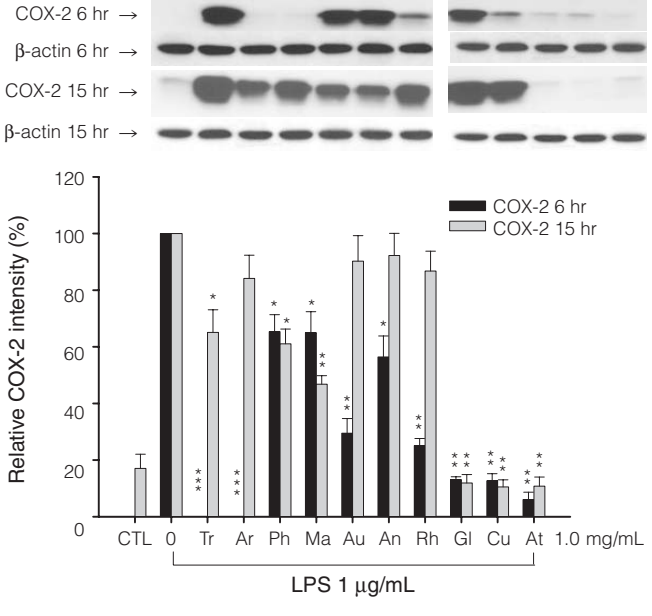

Figure 5. Representative Western blots data depicting protein expression of COX-2 at 6 and 15 hr in the culture of macrophage cell line RAW 264.7, in the presence of vehicle control (CTL) or each herbal extract (abbreviated in text) and LPS 30 min prior to them. Densitometry analyses are presented as the relative ratio of COX-2 protein/β-actin protein. Data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (n = 3) were compared with that of LPS group. ANOVA was followed by Dunnett's test.

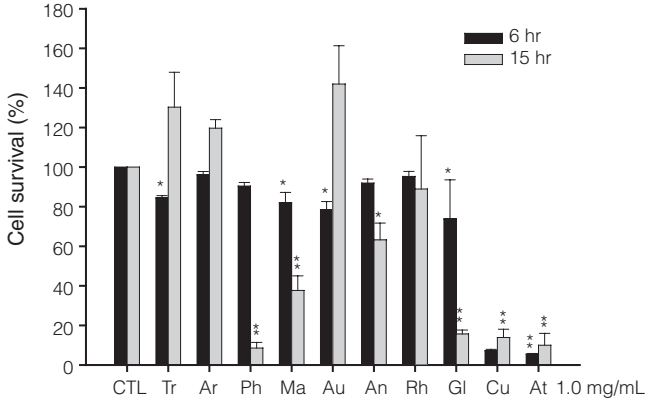

Figure 6. Cell survival rate caused by herbal extracts. RAW 264.7 cells were incubated for 6 and 15 hr in the presence of vehicle control (CTL) or each herbal extract and then measured by MTT assay. Abbreviation of plant components are the same as in Figure 5. Data are expressed as means ± SEM. $*p < 0.05$ and $**p < 0.01$ (n = 3) were compared with control group. ANOVA was followed by Dunnett's test.

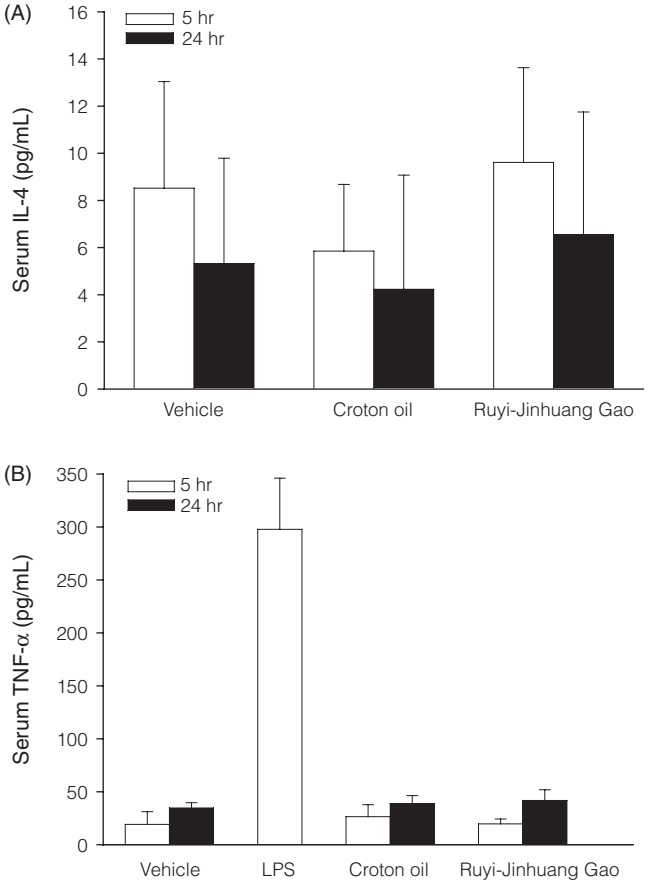

Figure 7. Immunologic sensitization response to RJG, croton oil, and vehicle, applied topically on each test animal. LPS was administered intravenously as positive control. Blood sample of each animal was collected at 5 and 24 hr after the treatment. Plasma IL-4 (A) and TNF-α. (B) levels were determined by ELISA assay. Unpaired Student's *t* test is applied for comparison between treated and vehicle groups. Differences are considered insignificant (n = 3).

mg/mouse) and croton oil (6 mg/mouse), compared to the vehicle control group at 5 and 24 hr post-treatment. Contact of croton oil with mouse ear dermis induced an increase of cutaneous inflammation but could not increase serum IL-4 and TNF-α (Figure 7). Intravenous LPS at 60 mg/kg certainly induced a significant ($p < 0.05$) increase in serum TNF-α relative to the vehicle control group (Figure 8).

V. *Clinical Finding*

The demographic distribution of these patients, including sex (male/female = 18/27) and age (49.5 ± 13.8 year), were insignificantly different. All the cases received RJP without significant difference among them. The pain pattern of the patients including pain chronicity, tentative cause of pain, number of pain sites, and location of pain sites were as follows: upper limb (36%); back (27%); lower limb (22%); and neck (18%). The pain relief effect of patients was found to be significantly different from that at start time ($p < 0.05$, paired *t*-test) after 4 hr of patching and this trend continued up to 12 hr (Table 1). The presence (89%) of warming effect after patching was noted in all patients. The onset time of feeling warmth after patching is 16.8 ± 7.8 min; the duration of the warming effect lasted for 155.7 ± 6.2 min. The side effects caused by patching of RJG were minimally found with localized purities and skin rash (2%). Finally, the degree of satisfaction (91%) was noted to be significant.

Table 1. Pain relief effects of Ruyi-Jinhuang Gao after patching

Time after patching (hr)	VAS (pain score)
0	7.3 ± 2.1
0.5	7.1 ± 1.8
1	5.3 ± 2.4
4	3.5 ± 1.4*
8	3.8 ± 1.7*
12	4.3 ± 1.5*
16	5.8 ± 2.4

*Significantly different from that at 0 time ($p < 0.05$, paired *t*-test).

DISCUSSION

Local myofascial, arthritic, traumatic, shoulder, tendonitis, and neuropathic pains are un-wounded inflammatory somatic pain responses felt by patients near the body surface. The hydrophilic ointment RJG, dressed on a semi-plastic cloth, combines herbal extracts of RJS with pharmaceutical recipients, such as vegetable oil ester, fatty acid, water, and various natural and semi-synthetic cellulose. In contrast, in the practice of clinical therapy performed by Chinese Medicine practitioners, RJS is traditionally mixed with cold water, dressed on a gauze and applied together to the somatic surface of the painful muscle of patients. Newly formulated RJP has proven to be clinically useful in easing pains without significant itchy response on the skin. Our clinical observation showed that RJP was with long-lasting anti-inflammatory analgesic benefits for 12-24 hr.

RJS and RJG, which inhibit iNOS expression resulting in decrease of NO generation, may have beneficial therapeutic effects in the treatment of diseases due to overproduction of NO[7,8,12]. Our results indicated RJG significantly inhibits iNOS and moderately inhibits COX-2 expression in LPS-treated macrophages in a concentration- and time-dependent manner (Figures 1 and 2). However, RJG did not significantly induce dermal inflammation associated cytokine (IL-4 and TNF-α). formation (Figure 7). Thus, RJG may be safe and useful in the prevention and treatment of inflammation-associated expression of iNOS and COX-2 in macrophage existing within a non-wounded body surface (Figures 1 and 2).

All components of RJG displayed inhibition activities on the expression of iNOS and COX-2 at 6 and 15 hr (Figures 4 and 5). Among these, Ph, Ma, Rh, Gl, Cu, and At were more potent in inhibiting iNOS expression (Figure 4), Tr and Ar were more potent in inhibiting COX-2 expression at 6 hr (Figure 5). At 15 hr, Ph, Ma, Rh, Gl, Cu, and At were more potent in inhibition of iNOS expression; Tr, Ar, Ph, Ma, Gl, Cu, and At were more potent in inhibiting COX-2 expression (Figures 4 and 5). Gl, Cu, and At significantly inhibited the expression of iNOS and COX-2 at 15 hr, indicating they were more potent than other herbal plants in protecting against LPS-induced inflammatory signalling. Since Cu and At also reduced the survival rate of macrophage, implying that inhibition of iNOS and COX-2 caused by Cu and At was partly dependent on cytotoxicity. Thus, inhibition on LPS-induced iNOS expression by RJG was also partly dependent on cytotoxicity of Cu and At (Figure 6). Gl inhibited both iNOS and COX-2 expression without inducing cytotoxicity at 6 hr and so was obviously the most promising and important anti-inflammatory herbs in RJG (Figures 4 and 5). Inflammation was mediated by multiple mediators, which caused a series of pathologic events, including local edema formation, increased vascular permeability, and feeling pain. It was suggested that different herbs in RJG may displayed various anti-inflammatory activities at different stages of processing inflammation.

MTT test indicated that RJG caused less cytotoxicity at 6 hr and higher cytotoxicity at 15 hr (Figure 6). The components Cu and At at 6 hr and Ph, Ma, An, Gl, At at 15 hr decreased the survival rate of macrophage cell culture. Among these components, Ph, Gl, Cu, and At were more obviously potent than others in terms of cytotoxicity. In contrast, Tr, Ar, and Au insignificantly increase the survival rate indicating that they are not cytotoxic (Figure 6).

RJG is not only cytotoxic, but also anti-inflammatory, evidenced from inhibition of iNOS and COX-2 expression after incubation with LPS in macrophages cell line. The anti-inflammatory outcome of RJG may be explained as follows: (1) the presence of anti-inflammatory herbal components Gl, Cu, and At suppress the pro-inflammatory signaling, iNOS and COX-2; (2) Tr, Ar, and Au protect

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against cell death; and (3) the cytotoxic effect was suppressed by other herbal components.

We demonstrate that RJG is an immune modulator that reduces pro-inflammatory iNOS and COX-2 in LPS-treated macrophages. RJG did insignificantly increase TNF-α and IL-4 after contact with the dermis, indicating it did not sensitize an immunologic response. Croton oil was used as control in mice to cause ear edema, but it also could not significantly increase TNF-α and IL-4[3,14,15]. The feeling of warmth induced by RJG was suggested to be due to sensory neuron activation on skin caused by pungency of Cu.

Clinical evidence show that patients feel satisfaction after patching of RJG. Although we can not test directly on macrophages of patients, our *in vitro* results from cell culture implicate the pro-inflammatory response *in vivo*. Further investigation on transdermal absorption of RJG would be our next challenge.

CONCLUSIONS

Inhibiting activities on pro-inflammatory iNOS and COX-2 expression in macrophages and lack of cytokine formation in plasma by RJG may indicate its utility in painful musculoskeletal conditions to relieve somatic pain.

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