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Research Communication

Suppression of Carrageenan- and Collagen II-Induced Inflammation in Mice by Geranium Oil

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To obtain experimental evidence on the therapeutic efficacy of essential oils in aromatherapy for inflammatory diseases, we examined the effects of geranium oil on carrageenan-induced and collagen II-induced inflammation in mice, to assess acute and chronic anti-inflammatory activities of the oil. Single intraperitoneal injection of 5 μL of geranium oil clearly suppressed the carrageenan-induced footpaw edema and increase in tissue myeloperoxidase activity, and repeated administration of the oil suppressed collagen-induced arthritis. These results revealed that geranium oil suppressed both acute and chronic inflammatory responses in mice

INTRODUCTION

Aromatherapy is one of the alternative medicines using essential oils and has long been used as an herbal medicine. Recently essential oils have been empirically used worldwide for clinical conditions including various kinds of inflammatory diseases, such as allergy, rheumatism, and arthritis. These activities have mainly been recognized through clinical experience, but there has been relatively little evidence about the pharmacological actions of these oils.

Several investigators have suggested that tea tree [1, 2] and lavender [3] oils suppressed allergic symptoms through the suppression of histamine release [4, 5] and cytokine production [6] in vitro and in vivo. Several essential oils such as eucalyptus [7] and lavender [8] oils inhibited carrageenan-induced paw edema. Moreover, in human, skin application of tea tree oil was reported to suppress the edema induced by intradermal injection of histamine [9]. However, the chronic effects of essential oils using inflammatory mice model have hardly been investigated.

Previously we reported that the essential oils such as geranium oil suppressed the adherence response of neutrophils in vitro [10], and that the intraperitoneal administration of geranium oil lowered neutrophil recruitment into the peritoneal cavity induced by injection of a chemotactic agent, casein in vivo [11]. We also reported that both intraperitoneal and cutaneous applications of the oil suppressed cellular inflammation and neutrophil accumulation to the inflammatory sites which were induced by curdlan, a linear (1 → 3)-β-D-glucan known as an immunostimulating substance in fungi [12]. These results suggested the possibility that geranium oil might effectively suppress symptoms in inflammatory disease associated with neutrophil activities.

In the present study, we investigated the effects of geranium oil on carrageenan-induced foot edema and collagen-induced arthritis, which are models for acute and chronic inflammation accompanied by neutrophil accumulation.

MATERIALS AND METHODS

Essential oils

Geranium oil was provided by Pranarom (Kenso-igakusha Ltd, Tokyo, Japan). The oil was diluted to 0.625, 1.25, 2.5% solution by 2.5% dimethyl sulfoxide (DMSO), and 25 μL of Tween 20 was added to 2 mL of the essential oil solution. Main constituents of the oil based on the company's data were citronellol (22.42%), geraniol (18.25%), linalool (5.59%), citronellyl formate (10.24%), geranyl formate (7.36%), guaiadiene (6.88%), and isomenthone (7.58%).

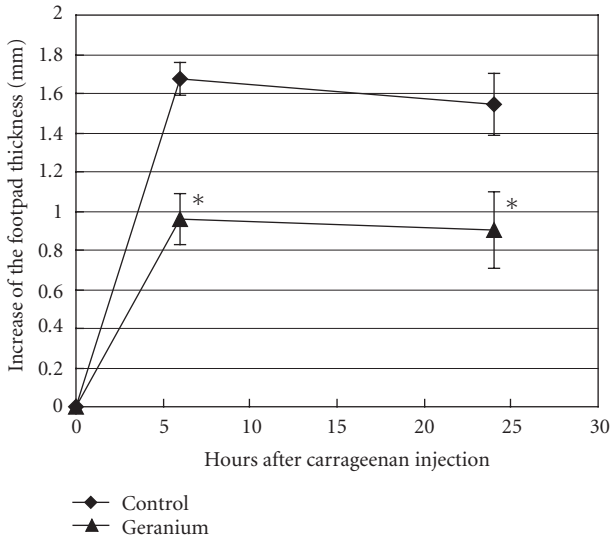


Figure 1: Effects of intraperitoneal administration of geranium oil on foot swelling induced by carrageenan injection. Carrageenan was injected to left footpad of mice, and 10 minutes after the injection, geranium oil or DMSO was given intraperitoneaouly. Six and 24 hours later, the increase of the foot thickness was measured. Each value represents an average from 5 mice and the standard error. $^*P < .05$ difference from control.

Agents

Polyoxyethylene (20) sorbitan monolaurate (Tween 20) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Bovine collagen II was from Cosmo Bio (Tokyo, Japan), complete Freund's adjuvant (CFA) was from DIFCO (Michigan, USA). Carrageenan λ, Hexadecyltrimethylammonium bromide (HTAB), human myeloperoxidase (MPO), and tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Tokyo, Japan).

Animals

All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. Six-week-old male DBA mice (Charles River Japan, Inc, Kanagawa, Japan) were used for all animal experiments. The photoperiods were adjusted to 12 hours of light and 12 hours of darkness daily, and the environmental temperature was constantly maintained at 21°C. The mice were kept in cages housing 4–6 animals and were given ad libitum access to food and water.

Carrageenan-induced edema: footpad reaction

Footpad reaction was based on the method of Abe et al [13] and partly modified. Ten mg of carrageenan were dissolved in 1 mL of saline and 0.05 mL of the solution was injected to the mouse left footpad to induce edema. The photos of feet from a lateral view were taken before and 6 and 24 hours after carrageenan injection using a digital camera, and foot thickness was measured from the photo (Figure 1). The edema was calculated by the difference of thickness between 0 and 6 or 24 hours. Ten minutes after carrageenan injection, the mice were intraperitoneally given 0.2 mL of 2.5% geranium solution. A dose of 2.5% solution corresponds to 5 μL of pure oil. Control mice received 0.2 mL of 2.5% DMSO solution. Mice were sacrificed by carbon dioxide 24 hours after carrageenan injection. The feet were resected 5 mm above their heels, soaked in 2 mL of 80 mM sodium phosphate buffer, pH 5.4, containing 0.5% HTAB (0.5% HTAB solution), weighed and kept at −20°C until the MPO assay. We used a nontreated right foot of the same mice as a reference.

Myeloperoxidase (MPO) assay

The MPO assay was based on the method of De Young et al [14] and partly modified. Frozen samples were thawed at room temperature and homogenized at 0°C using a Polytron (Kinematica AG, Lucerne, Switzerland). The homogenates were poured into sampling tubes and centrifuged at 12000× g at 4°C for 15 minutes.

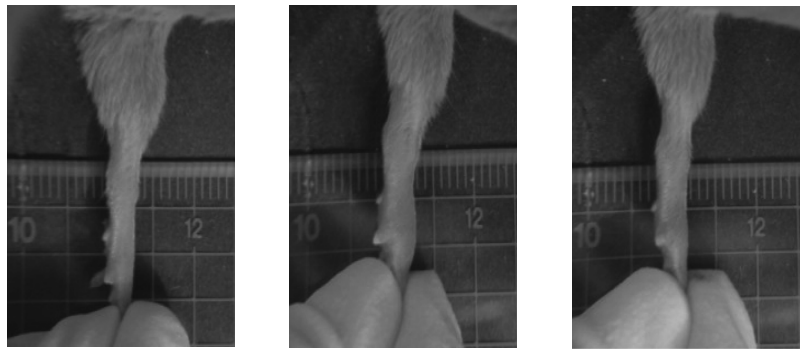
Triplicate 30 μL samples of resulting supernatant were poured into 96 well microtiter plates. For assay, 200 μL of a mixture containing 100 μL phosphate buffered saline, 85 μL of 0.22 M sodium phosphate buffer, pH 5.4, and 15 μL of 0.017% hydrogen peroxide were added to the wells. The reaction was started by the addition of 20 μL of 18.4 mM TMB·2HCl in 8% aqueous dimethylformamide. Plates were stirred and incubated at 37°C for 3 minutes and then placed on ice where the reaction in each well was stopped by addition of 30 μL of 1.46 M sodium acetate buffer, pH 3.0. The MPO value was calculated by measuring the absorbance of samples at 620 nm (OD value) followed by its conversion into MPO values per foot.

Collagen-induced arthritis

Induction of type II collagen-induced arthritis was based on the method of Ochi et al [15] and partly modified.

Collagen II from bovine articular cartilage was dissolved overnight at 4°C in 0.1 M acetic acid at a concentration of 2.5 mg/mL. The solution was emulsified with 1.2 times volume of CFA, and 100 μL of the emulsion were administered subcutaneously at the base of the tail of the mice for immunization on day −21. Booster injection of 100 μL of the emulsion was given on day 0. Mice were intraperitoneally given 0.2 mL of geranium oil solutions from day 0 to 21, 5 days per week (injection period). Control mice were given 0.2 mL of 2.5% DMSO solution. Their weight and paws were measured 2 days each week from day 0 to 39.

Mouse paws were scored for arthritis based on the method of Kim et al [16] using a macroscopic scoring system ranging from 0 to 4 (0, no swelling; 1, swelling of one joint; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis over the entire paw and joints). The arthritic score for each mouse was the sum of the scores of all four paws.



(a) (b) (c)

Figure 2: Typical swelling of control mice and that of geranium-treated mice. Nontreated foot (a), carrageenan injected control (b), and geranium-treated (c) feet 24 hours after carrageenan injection were shown. The mouse was administered with geranium oil 10 minutes after carrageenan injection.

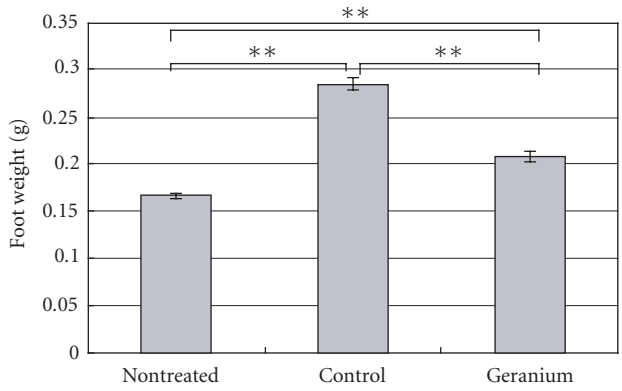


Figure 3: Effects of intraperitoneal administration of geranium oil on foot swelling estimated by their weight. Carrageenan was injected to left footpad of mice, and 10 minutes after the injection, geranium oil or DMSO was given intraperitoneaouly. Twenty four hours later, their feet were resected to measure their weight. Each value represents an average from 5 mice and the standard error. ** $P < .01$ difference from control.

Statistical analysis

The results were expressed by the mean ± standard error. The data were statistically compared using the Student's t-test and the χ-square test.

RESULTS

Effects of geranium oil on carrageenan-induced edema in the hindpaws of mice

Carrageenan injection to the footpad increased the foot thickness of control mice by 1.68 ± 0.08 mm after 6 hours and the swelling continued for 24 hours (1.55 ± 0.16 mm) (Figure 1). Intraperitoneal injection of geranium oil significantly suppressed the increase in foot thickness both 6 and 24 hours after carageenan injection (0.96 ± 0.13 mm and 0.91 ± 0.19 mm, resp). Figure 2 shows photos of the typical swelling of control mice and that of geranium-treated mice 24 hours after carageenan injection. These photos clearly indicated that the foot treated with geranium oil was less swollen than the control foot.

In order to confirm the inflammatory response, foot weights and MPO activity in foot homogenates were measured.

The weight of the carrageenan-injected control foot was significantly increased compared with the nontreated foot (0.28 ± 0.01g and 0.17 ± 0.00g, resp), as shown in Figure 3. This figure also shows that intraperitoneal injection of geranium oil significantly lowered the weight gain (0.21 ± 0.01g).

The same feet were used for measurement of MPO activity which represented the number of neutrophils. Carrageenan injection to the footpad induced a marked increase of the MPO value of the foot compared with the nontreated foot (61.51 ± 16.84 units/foot and 4.02 ± 1.96 units/foot, resp) (Figure 4). Geranium oils suppressed the increase of MPO value significantly (44.38 ± 6.30 units/foot). This suggested that intraperitoneal injection of geranium oil lowered neutrophil accumulation to the carrageenan-injected foot.

Effects of geranium oil on collagen-induced arthritis in mice

Next, we examined the effects of the oil against the collagen-induced arthritis in mice as a chronic inflammation model.

One of control mice immunized with collagen II (on days −21 and 0) developed an edema (arthritis) from day 7, and then most of them elicited edema, 6 of 10 on day 21 and 7 of 10 on day 39 (Figure 5). Their symptoms were aggravated gradually after the second collagen II injection. In mice given 5 μL of geranium oil, edema of the feet was observed only on one animal with slight swelling. There were statistical differences between control and the 5 μL geranium oil group, on day 10 and after day 17 using χ-square test. No aggravation of symptoms was observed even after completion of geranium oil injection.

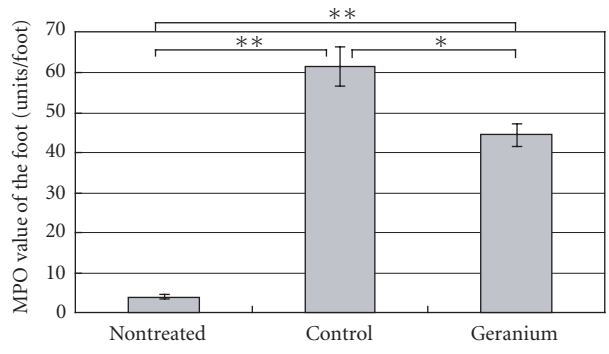


Figure 4: Effects of intraperitoneal administration of geranium oil on MPO activity in foot homogenates. Carrageenan was injected to left footpad of mice, and 10 minutes after the injection, geranium oil or DMSO was given intraperitoneaouly. Twenty four hours later, their feet were resected to measure their MPO activities. Each value represents an average from 5 mice and the standard error. $^{*}P < .05$, $^{**}P < .01$ difference from control.

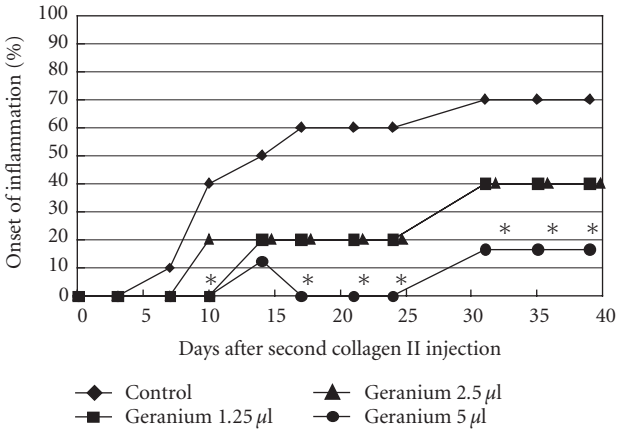


Figure 5: Effects of intraperitoneal administration of geranium oil on the ratio of the mice which revealed feet swelling by collagen II induction. Collagen II with CFA was subcutaneously injected to the base of the tail of the mice on days −21 and 0. Geranium oils were given from day 0 to 21, 5 days/week. Each value represents percentages of mice with foot swelling. $^{*}P < .05$ difference from control using χ-square test.

Oral administration of indomethacin used as a reference suppressed the edema during the injection period, but 1 week after completion of injections the feet appeared to be swollen (data not shown).

Time course of the score of inflammatory symptoms is depicted in Figure 6. The symptoms of the control mice were exacerbated time-dependently. The score of the mice injected with 5 μL of geranium oil was clearly lower than that of control mice with statistical significance on days 24, 35, and 39. On the other hand, 1.25 and 2.5 μL oils seemingly lowered the scores, but the differences were not statistically significant.

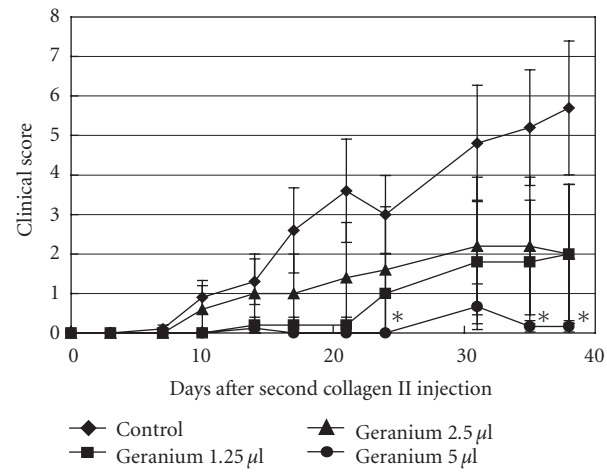


Figure 6: Effects of intraperitoneal administration of geranium oil on the inflammatory score. Collagen II with CFA was subcutaneously injected to the base of the tail of the mice on days −21 and 0. Geranium oils were given from day 0 to 21, 5 days/week. The arthritis was scored as described in material and methods. Each value represents an average from 5–10 mice and the standard error. $^{*}P < .05$ difference from control.

Figure 7 shows typical pictures of the feet of a control and a 5 μL geranium injected mouse.

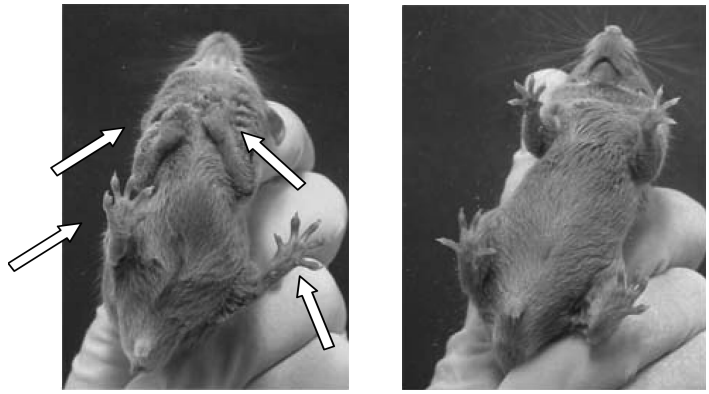
Figure 8 indicates the change of body weight during treatment. The weight of mice treated with geranium oil decreased immediately after oil injection. The reduction was largest in the group injected with 5 μL of geranium oil. Their weight loss was gradually recovered, but the recovery was slow in the groups injected with 2.5 and 5 μL of geranium oil. In the group injected with 5 μL of the oil, 2 mice died on days 18 and 21.

DISCUSSION

In the present study, we showed that intraperitoneal administration of geranium oil suppressed two types of inflammatory responses, carrageenan-induced edema and collagen-induced arthritis.

When mice received intraperitoneal injection of 5 μL of geranium oil 10 minutes after carrageenan injection, carrageenan-induced edema was significantly suppressed at 6 and 24 hours. This indicates that the suppressive effect of the oil on the acute inflammation continued at least for 24 hours. We also measured the weight and MPO activity as parameters of neutrophil accumulation at 24 hours, and the results suggest that the oil suppressed the acute inflammation accompanied by neutrophil accumulation (Figures 3 and 4).

We previously reported that intraperitoneal administration of geranium oil suppressed the casein-induced accumulation of neutrophils in the peritoneal cavity [11], and both intraperitoneal and cutaneous applications of the oil suppressed cellular inflammation and neutrophil accumulation



(a) (b)

Figure 7: Macroscopic arthritis of control mice and that of geranium-treated mice. The mice were treated as represented in the legend to Figure 5 and their feet were observed on day 39. Arrows indicate the swelling of foot. (a) Control mouse; (b) mouse administered with 5 μL geranium oil.

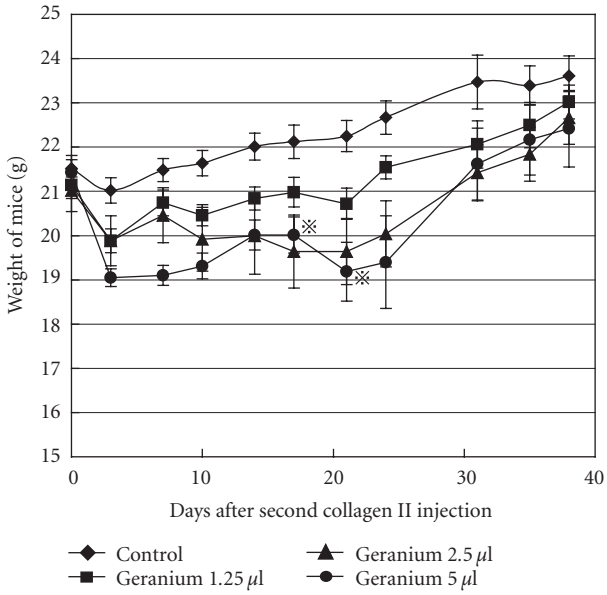


Figure 8: Changes in body weight during and after geranium oil injection to mice. Collagen II with CFA was subcutaneously injected to the base of the tail of the mice on days −21 and 0. Geranium oils were injected from day 0 to 21, 5 days/week. Each value represents an average of 5–10 mice and the standard error. ※ : death of mouse.

to the inflammatory sites which were induced by a (1 → 3)-β-D-glucan, curdlan [12]. In this study, geranium oil inhibited the carrageenan-induced edema from 6 hours and neutrophil accumulation for 24 hours. These indicate that geranium oil suppresses the neutrophil accumulation in various inflammatory responses from an early stage.

The cellular mechanism of the suppression of edema by geranium oil remains to be clarified. It is reported that carrageenan injection induced the production of histamine and cytokine such as TNF-α [17, 18]. TNF-α is one of the major inflammatory cytokines with the capacity to prime activation of the neutrophils for their various functions [19]. In our previous study, we showed that geranium oil inhibited TNF-α-induced neutrophil adherence to a plastic plate at very low concentrations (0.00625%) in vitro [10], so we can assume that this suppression might be partially caused by inhibiting neutrophil response to TNF-α in vivo.

We also evaluated the long-term effect of the oil using collagen-induced arthritis mouse as the chronic anti-inflammatory disorder model. Collagen-induced arthritis in mice has many characteristics in common with human rheumatoid arthritis such as foot swelling and has been the most used animal model for the disease [15, 20]. Five μL of geranium oil suppressed the swelling and the effect continued even after cessation of oil injection. As far as we know, this is the first report indicating that geranium oil suppressed the later phase of inflammatory response as well as the earlier phase in in vivo experiments.

Rheumatoid arthritis is considered an autoimmune disease involving joint inflammation associated with TNF-α production. Various cells such as Th1 cells, neutrophils and macrophages, and their cytokines such as IL1 and TNF infiltrate the synovial tissues to destroy joints [21]. In our experiments, geranium oil was administered to the mice after a booster injection of collagen II. We can therefore assume that the oil suppresses the later phase of autoimmune reaction, or the onset of symptoms after autoimmune reaction, not the early stage of the reaction.

Previous pathological studies [20, 22] on experimental murine arthritis showed that there were marked edema of synovium and infiltration of many polymorphonuclear cells such as neutrophils in the early phase of arthritis onset, followed by the chronic destructive phase in which pronounced proliferation of synovium containing mononuclear cells was observed. From these findings and our previous data, we can speculate that geranium oil may suppress the onset of the symptoms at least partially through inhibition of neutrophil

infiltration. To check this possibility, we wish to evaluate the MPO value in further study.

It was noted that 5 μL of geranium oil suppressed the foot swelling during and after the oil injection period, suggesting a long-lasting effect of this oil. Preliminary study showed that indomethacin inhibited the swelling only during its administration, and 1 week after completion of the injection the feet gradually swelled (data not shown). This indicates that repetitive administration of the oil may elicit a long-lasting effect.

In aromatherapy, several essential oils can be applied as a help in therapeutic treatments for inflammatory symptoms with lesional neutrophil accumulation, such as arthritis, aphthous stomatitis, lesional bacterial or fungal infections. Their effectiveness is postulated clinically, but little experimental evidence has been obtained. Our two results give basic evidence about the activity of geranium oil for both acute and chronic inflammatory disorders.

In relation to the application of the essential oil, we must mention its toxicity. In our later experiment, 2 mice of the group which were intraperitoneally given 5 μL of geranium oil died during the experiment. The body weight of this group was greatly reduced, so the administration protocol might have been too severe for them. In order to develop a less toxic administration procedure, it is our opinion that the selection of administration routes of the essential oil must be critical, since cutaneous application of geranium oil suppressed the curdlan-induced skin inflammation without apparent toxic response [12]. By optimizing the dosage and administration route, we hope to propose safer and more effective treatment protocol using essential oil for inflammatory diseases.

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