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Topical dermal application of essential oils attenuates the severity of adjuvant arthritis in Lewis rats

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Abstract

This study was aimed at examining the effect of an ointment containing essential oils (EO) on the severity of adjuvant arthritis (AA), an experimental model of human rheumatoid arthritis (RA), in Lewis rats and to define the underlying mechanisms. At the onset of AA, rats received topical application twice daily of ointment containing 20% EO or placebo ointment. The synovial fluid (SF) and synovium-infiltrating cells (SIC) of rats were tested for pro-inflammatory cytokines TNF- α and IL-1 β . The hind paws and skin were examined histologically. The activity/level of matrix metalloproteinases (MMPs) and anti-mycobacterial heat-shock protein 65 (Bhsp65) antibodies was tested. Arthritic rats treated with ointment containing EO developed less severe clinical arthritis compared to the controls, and this activity was attributable to EO and not the carrier oil. The levels of TNF- α and IL-1 β , and the activity of MMPs in SF and SIC-lysate were significantly ($p < 0.05$) reduced in EO-treated arthritic rats compared to the controls. However, the levels of anti-Bhsp65 antibodies were unaffected by treatment. Thus, topical dermal delivery of EO-containing ointment downmodulates the severity of AA in Lewis rats by inhibiting defined mediators of inflammation. Such ointments should be tested in patients with RA and other arthritic conditions.

Keywords

Essential oils; Arthritis; Cytokines; Topical application; MMPs; Inflammation

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease of high prevalence rate in the United States (US) (Kim et al., 2008) and several other countries. RA is a leading cause of disability, and it adversely affects the quality of life (2009; das Chagas Medeiros et al., 2000; Kvien, 2004). The drugs that are currently prescribed to treat RA are expensive and often cause adverse health effects (Bernatsky and Ehrmann Feldman, 2008; Hougardy et al., 2000). Therefore, safer and less expensive alternative therapeutic products are increasingly being sought (Barnes et al., 2008; Fennell et al., 2009). In this regard, a variety of natural plant

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products are being explored for their anti-arthritic activity in animal models of RA, including adjuvant-induced arthritis (AA) (Khanna et al., 2007; Venkatesha et al., 2011).

Several reports suggest the efficacy of topical application of medicinal products for different inflammatory conditions. Singh et al, reported the anti-inflammatory activity of Boswellic acids through topical application using different models of acute and chronic inflammation, e.g., arachidonic acid- and croton oil-induced mouse ear edema, carrageenan-induced rats paw edema and rat AA (Singh et al., 2008). In another study, topical application of ginkgetin and biflavonoid mixture was reported to dose-dependently inhibited skin inflammation in mice with croton oil-induced ear edema (Kwak et al., 2002).

AA serves as an excellent experimental model of human RA (Kim et al., 2008; Kim et al., 1997; Satpute et al., 2007; Tanaka et al., 1998). AA can be induced in the Lewis rat (RT.1^l) by subcutaneous injection of heat-killed *M. tuberculosis* H37Ra (Mtb) at the base of the tail. Mtb-primed leukocytes migrate from the draining lymph nodes into the target organ, the joints, and induce inflammatory arthritis. Cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) produced by the immune cells play critical roles in the arthritogenic process. Among the biochemical mediators of inflammation, matrix metalloproteinases (MMPs) contribute to the tissue damage observed in arthritis.

Essential oils are natural plant products that possess anti-oxidant, anti-bacterial, anti-fungal and anti-inflammatory properties (Agarwal and Rangari, 2003; Burt, 2004; Kordali et al., 2005). Essential oils from ginger, orange and black cumin have been shown to hold promise for the relief of symptoms of arthritis (Osborn et al., 2001; Tekeoglu et al., 2007; Yip and Tam, 2008). Essential oils and their components are rapidly absorbed through the skin and are detectable in plasma after topical application (Jaeger, 1992). Therefore, topical dermal delivery of EO represents an effective method to directly deliver a natural, anti-inflammatory agent to the site of inflammation. We report here that topical application of an ointment containing EO (20%, v/v) to the arthritic hind paws of Lewis rat downmodulates the severity of AA. Furthermore this suppression of AA involves changes in the disease-related immunological and biochemical mediators of inflammation.

MATERIALS AND METHODS

Rats

Four to six week old inbred, male Lewis rats (125 g –140 g) were purchased from Harlan Sprague Dawley (Indianapolis, Indiana) and maintained in the vivarium facility of the University of Maryland School of Medicine, Baltimore, Maryland. The guidelines of the Institutional Animal Care and Use Committee (IACUC) were adhered to in all procedures performed on animals in this study.

Induction and monitoring of adjuvant arthritis (AA)

Mtb (DIFCO Laboratories, Detroit, MI) was ground with mortar and pestle and then mineral oil (Sigma-Aldrich Inc. St. Louis, MO) was added to it. This suspension (10 mg/ml) was thoroughly mixed prior to use. Arthritis was induced in rats by injecting 200 μ l of Mtb in oil (2 mg/rat) at the base of the tail (Tong and Moudgil, 2007). Rats were observed regularly for erythema, induration and swelling of the paws. The severity of AA in the hind paws was graded on a scale of 0–4 per paw as described elsewhere (Moudgil et al., 1997). Sum of scores of hind paws yielded the arthritic score per rat in this study.

Histological examination of hind paws of rats

The hind paws were harvested and immersed for 9 days in Cal-Ex Decalcifying solution CS510-1D (Fisher Scientific, Fair Lawn, NJ). The paws were then embedded in paraffin, sectioned serially using a microtome, mounted on microscope slides, and stained with hematoxylin and eosin (H&E) (Histology Core, UMB) (Murphy et al., 2003). Histopathological changes in the joints (synovial hyperplasia, pannus formation, and cartilage and bone damage) were observed under a microscope (Nikon Eclips E800 Microscope, Nikon Industries Inc. Melville, NY 11747) using the Spot Imaging Software, (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and digital images were acquired (Tong and Moudgil, 2007).

Preparation of ointment containing essential oils (EO)

Essential oils (EO) and bees wax (BW) were purchased from Camden-Grey Essential Oils Inc. (Doral, Florida), and corn Oil (CO) was purchased from a local supermarket. CO was used as the carrier oil for EO, whereas BW was used as the base to prepare a semi-solid ointment of EO and CO. BW and carrier oil were heated together until thoroughly combined and then removed from the heat source. The blend of anti-inflammatory and analgesic essential oils mixed in specific proportions (Table 1) was added to the carrier oil-BW mixture, covered and mixed thoroughly by vortexing. The resulting ointment was allowed to cool. Experimental ointments were prepared using the desired concentrations of EO (10–40%, v/v). Placebo ointment contained CO and BW but no EO.

Assessing the anti-arthritic activity of EO

The study involved three groups of Lewis rats: the experimental group treated with EO, the placebo ointment-treated control group, and an additional control group of rats that was immunized with Mtb but received no treatment with any ointment. The optimal dose of EO (20%) as well as the duration of treatment (7 d) was based on the results of preliminary titration experiments. Twice daily, the test/placebo ointment containing EO was pressed between the fingers until soft and warm, and then gently massaged into the arthritic paws. The treatment was begun at the onset of AA. Rats were scored every other day for the severity of arthritis and were euthanized approximately 25 d after Mtb immunization. Hind paws of rats harvested at the terminal step were subjected to histopathological examination.

Determining the skin toxicity of EO

A 1-inch wide area of the back from the nape of the neck to the base of the tail of naïve rats was shaved. Thereafter, an ointment containing EO (20–40%) was applied to the shaved area twice daily. The test area was observed regularly for signs of skin inflammation and re-growth of fur. Further, the skin sections were subjected to histopathological examination for integrity of the epidermis, damage to the epidermal and dermal areas, and the state of hair and hair follicles.

Measurement of cytokines and matrix metalloproteinase (MMP) activity

Rats were treated with ointments twice daily as described above, and were euthanized at the peak phase of the disease. Synovial fluid was collected from inflamed joints of rats, divided into aliquots, and stored at -80°C until tested. The SIC were collected from excised joints, suspended in Dulbecco's Modified Eagle Medium (DMEM; from GIBCO/Invitrogen, Auckland, New Zealand) and sonicated to obtain cell lysate. Protein content of the lysate was estimated using Bio Rad Protein Assay and Beckman DU 70 Spectrophotometer. The levels of inflammatory cytokines (IL-1 β and TNF- α) were tested using ELISA (Cytokine Core, UMB). A gelatin zymogram was run to determine the level of MMP activity. Different concentrations of the total protein sample obtained from synovial fluid and SIC lysate were

loaded on to a gelatin-coated pre-casted polyacrylamide gel (Bio Rad). Electrophoresis was carried out under sodium dodecyl sulfate (SDS) non-reducing condition at constant voltage. Gel was incubated with 2.5 % Triton X-100 at room temperature for 1–2 h to remove SDS. Then the gel was washed 3–4 times to remove Triton X-100 and incubated at 37 °C in a developing buffer (Tris-HCl, pH 7.4) containing 10mM CaCl₂. After 24 h, the gel was stained with Coomassie Brilliant Blue R250. The metalloprotease activity was visualized after de-staining and then photographed. The intensity of the bands was quantitated by densitometry.

Measurements of serum anti-mycobacterial heat-shock protein 65 (Bhsp65) antibody levels

Blood samples of arthritic Lewis rats were collected on d 25 after Mtb immunization and processed to obtain serum. ELISA plates were coated with recombinant Bhsp65 (100 ng/well). Diluted serum (1:100 and 1:500) was tested for antibodies against Bhsp65 using ELISA (Yang et al., 2011). Horseradish peroxidase (HRP)-conjugated mouse anti-rat IgG (1:1500) was used as a secondary antibody. The results were expressed as optical density (OD) at 450 nm.

Statistical analysis

Student t-test and ANOVA were used to analyze mean differences between groups of rats. P-values of <0.05 were considered significant.

RESULTS

Arthritic rats were treated daily by local application of the ointment containing 20% EO (Table 1). The treatment was begun at the onset of AA and continued for a total of 7 d. Throughout the disease process, the severity of arthritis was graded regularly. Upon termination of the study the hind paws of rats were subjected to histopathology. Rats treated with 20% EO showed a significantly reduced severity of AA compared to rats treated with ointment lacking EO (Fig. 1a). Furthermore, histological examination of the paws of arthritic rats showed that synovial inflammation, pannus formation and bone damage were much less severe in animals that were treated with ointment containing 20% EO than that of untreated controls or placebo ointment-treated rats (Fig. 1b). Furthermore, neither <20% EO nor higher concentrations (30% and 40%) applied to shaved area of the skin of naïve rats twice daily for 14 d caused any significant adverse reaction to the skin as assessed by re-growth of fur and histological examination of the test area (Fig. 2).

Cytokine analysis of the synovial fluid and SIC lysate revealed significant ($p < 0.05$) suppression of TNF- α (Fig. 3a) and IL-1 β (Fig. 3b) in animals treated with ointment containing 20% EO, compared to that of animals treated with ointment lacking EO. Similarly, a gelatin zymogram of the synovial fluid and SIC lysate showed that MMP activity was also suppressed in EO-treated rats compared to placebo-treated rats (Fig. 4).

To determine the influence of topical treatment of rats with EO on humoral immune response of arthritic Lewis rats, we tested the serum levels of antibodies to the disease-related antigen, Bhsp65 on d 25 after Mtb injection (Fig. 3c). The level of anti-Bhsp65 antibodies in rats treated with the control ointment lacking EO was comparable to that of rats treated with ointment containing EO.

DISCUSSION

We report here the topical application of EO as an anti-arthritic intervention in Lewis rats having AA. Our results show that arthritic rats that were treated for 7 d with 20% EO

experienced optimal reduction in arthritic inflammation of hind paws, and bone and cartilage damage of the paw joints. CO, the vehicle used in our study, when tested alone without EO, had no effect on the incidence or severity of AA. These results show that EO is the active anti-arthritic component of the ointment tested in this study. Interestingly, rats treated with 20% EO continued to improve for the duration of the study even when treatment was stopped after 7 d. Based on these results, we suggest that a practical regimen for topical application of EO for the treatment of patients with arthritis would be to apply the ointment until optimal effect is achieved and then apply the ointment intermittently thereafter.

For therapeutic purposes, EO can be administered by 3 methods, absorption through the skin, inhalation and ingestion. Although we have attributed the beneficial anti-arthritic effect of EO to topical application, grouping animals in separate cages by treatment regimen was insufficient to prevent rats in the control and placebo groups from inhaling the volatile essential oils. Occasionally, we observed animals licking the ointment from their paws; however, benefits of ingesting or inhaling essential oils were not investigated here. Nevertheless no adverse reactions from ingestion of ointment were observed.

TNF- α and IL-1 β are pro-inflammatory cytokines produced by macrophages, monocytes, dendritic cells and a variety of other cell types (Dinarello, 2009; Feldmann and Maini, 2008). These cytokines are products of innate immune response to bacterial products. Moreover, activation of macrophages by activated antigen-specific T cells enhances the secretion of these cytokines. Thereby, the production of TNF- α and IL-1 β is associated with inflammation induced in response to foreign or self antigens (Dinarello, 2009; Feldmann and Maini, 2008). Increased production of TNF- α and IL-1 β is a characteristic feature of human RA (Feldmann and Maini, 2008). These cytokines in turn, act on other immune cells and stromal cells resulting in changes in endothelial cell function, expression of adhesion molecules and chemokines, leukocyte migration into the joints and production of other cytokines and biochemical mediators of inflammation such as cyclooxygenase-2 (COX-2), leukotriene B4 (LTB4), and prostaglandin E2 (PGE2). Therefore, TNF- α and IL-1 β are targets for therapeutic products aimed at suppressing inflammation in arthritis (Feldmann and Maini, 2008). In this regard, our results showing that arthritic rats treated with topical dermal application of EO exhibited reduced levels of TNF- α and IL-1 β in synovial fluid and SIC lysate compared to control rats are of significance (Fig. 3a,b). Our results highlight the inhibition of pro-inflammatory cytokines as one of the mechanisms by which EO mediate their anti-arthritic activity.

MMPs are extracellular protein-degrading proteases that damage bone and cartilage in arthritic joints (Fietz et al., 2008; Francoz et al., 2008; Murphy and Lee, 2005). Increased levels of MMPs in the synovial fluid correlate with the release of matrix proteins like fibronectin (Sandya and Sudhakaran, 2007). Suppression of MMPs and pro-inflammatory cytokines in synovial fluid and SIC helps in controlling bone damage and inflammation during active arthritis. Taken together, in our study, direct measurements of the levels of proinflammatory cytokines and the activity of MMPs, as well as histological examination of joints support the conclusion that tissue damage (indirectly reflecting the outcome of MMP activity) was suppressed in the EO -treatment group compared to the controls.

Arthritis is a complex immunological disease. Both T cells and antibodies have been invoked in the pathogenesis of autoimmune arthritis in different animal models of RA, for example, collagen-induced arthritis (Cremer et al., 1984). Although the role of antibodies in the induction of AA is not yet clear, a couple of reports have shown a protective effect of antibodies in AA (Astin, 2002; Ulmansky et al., 2002). In this context, we tested whether antibodies to Bhsp65 contribute to the protective effect of EO-treatment of in Lewis rats with AA. Our results showed no significant difference between antibody levels in serum

from animals treated with 20% EO compared to those treated with the vehicle alone. These results suggest that apparently, antibodies are not involved in mediating the protective effect of dermally-delivered EO. Because the rats were subjected to a topical application of the ointment on their paws, the effects of the treatment might be limited to the joint without influencing the systemic antibody response.

CONCLUSION

Local application of an ointment containing 20% EO was effective in reducing the severity of AA in Lewis rats. The observed suppression of clinical and histological features of arthritis was further supported by a significant reduction in the immunological and biochemical mediators of inflammation in the target organ, the joints. Thus, topical delivery of EO holds promise as a noninvasive and efficacious intervention for the treatment of inflammatory arthritis, and it merits further study in patients with RA and possibly other types of arthritis that have a component of inflammation.

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Fig. 1.

(a) Comparison of the severity of arthritis in rats treated with ointment containing 20% EO versus those treated with ointment lacking EO. (b) H&E-stained histopathological sections of the joint showing the joint space, the cartilage surface, pannus formation, and mononuclear cell infiltration of the synovial tissue in arthritic rats treated with local application of 20% EO. Also shown are paw sections of a control (untreated) rat and a naive non-arthritic rat

Fig. 2. Photographs (**a**) and H&E-stained histopathological sections (**b**) of the skin exposed to topical application of ointment containing 20–40% EO

Fig. 3.

(a) The synovial fluid (SF) and synovium-infiltrating cell (SIC) lysate harvested from the joints of rats treated with ointment containing 20% EO were tested in an ELISA for the pro-inflammatory cytokines TNF- α (i) and IL-1 β (ii) (n= 3 each) (b) Comparison of antibody levels of rats (n= 6 each) treated with ointment containing 20% EO or ointment lacking EO

Fig. 4. MMP activity in synovial fluid (SF) and synovium-infiltrating cell (SIC) lysate of EO-treated rats compared to placebo-treated rats. The intensity of the bands in the zymogram (a) were quantitated using densitometry (b, SF and c, SIC lysate)

Table 1

The identity and relative proportion of different essential oils constituting the test ointment.

	Essential oils	Percent by volume
1	<i>Ocimum basilicum</i> (basil)	8.9
2	<i>Citrus aurantium</i> (bitter orange)	8.9
3	<i>Piper nigrum</i> (black pepper)	8.9
4	<i>Salvia sclarea</i> (clary sage)	8.9
5	<i>Juniperus virginiana</i> (cedarwood)	2.2
6	<i>Eugenia caryophyllata</i> (clove bud)	8.9
7	<i>Eucalyptus globulublus</i> (eucalyptus)	4.4
8	<i>Calophyllum inophyllum</i> (foraha)	4.4
9	<i>Foeniculum vulgare</i> (fennel)	4.4
10	<i>Zingiber officinale</i> (ginger)	8.9
11	<i>Helichrysum angustifolia</i> (immortel)	4.4
12	<i>Lavendula angustifolia officinalis</i> (lavender)	4.4
13	<i>Muristica fragrans</i> (nutmeg)	8.9
14	<i>Pinus sylvestrius</i> (pine needle)	4.4
15	<i>Rosemarinus officinalis</i> (rosemary)	4.4
16	<i>Salvia officinalis lamiacae</i> (sage)	4.4

The ointment contains 20–40% essential oil mixture (above), 70–50% carrier oil (corn oil) and 10 % stabilizer (bees wax) by volume.