Antagonism of Croton Oil Inflammation by Topical Emu Oil in CD-1 Mice

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ABSTRACT: Emu oil is derived from the emu (*Dromaius novaehollandiae*), which originated in Australia, and has been reported to have anti-inflammatory properties. Inflammation was induced in anesthetized CD-1 mice by applying 50 μL of 2% croton oil to the inner surface of the left ear. After 2 h, the area was treated with 5 μL of emu, fish, flaxseed, olive, or liquified chicken fat, or left untreated. Animals were euthanized at 6 h postapplication of different oils, and earplugs (EP) and plasma samples were collected. Inflammation was evaluated by change in earlobe thickness, increase in weight of EP tissue (compared to the untreated ear), and induction in cytokines interleukin (IL)-1α and tumor necrosis factor-α (TNF-α) in EP homogenates. Although reductions relative to control (croton oil) were noted for all treatments, auricular thickness and EP weights were significantly reduced (–72 and –71%, respectively) only in the emu oil-treated group. IL-1α levels in homogenates of auricular tissue were significantly reduced in the fish oil (–57%) and emu oil (–70%) groups relative to the control group. The cytokine TNF-α from auricular homogenates was significantly reduced in the olive oil (–52%) and emu oil (–60%) treatment groups relative to the control group. Plasma cytokine levels were not changed by croton oil treatment. Although auricular thickness and weight were significantly correlated with each other (r = 0.780, $P < 0.003$), auricular thickness but not weight was significantly correlated with cytokine IL-1α (r = 0.750, $P < 0.006$) and TNF-α (r = 0.690, $P < 0.02$). These studies indicate that topical emu oil has anti-inflammatory properties in the CD-1 mouse that are associated with decreased auricular thickness and weight, and with the cytokines IL-1α and TNF-α.

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Although several patents have been issued based on the various biological properties of emu oil, only two studies using rodents as experimental models have reported on the anti-inflammatory properties of emu oil in peer-reviewed journals (1,2). Neither study evaluated tissue cytokine levels as biomarkers of inflammation. Two other studies looked at the anti-inflammatory and wound-healing properties of emu oil when applied topically (3,4).

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Abbreviations: EP, ear plug; IL, interleukin; TNF-α, tumor necrosis factor-α.

Emu oil studies. Emu oil is derived from both retroperitoneal and subcutaneous adipose tissue sites. One study in CD-1 mice found that the auricular swelling induced by applying 50 μL of 2% croton oil was significantly reduced 6 h after the application of 5 μL of emu oil, when compared to the control and the porcine oil groups (1). In this study, inflammation was measured by the weight difference between left (inflamed) and right (noninflamed) earplugs (EP). A second study, using both female out-bred Wistar and Dark Agouti rats with adjuvant-induced polyarthritis, revealed significant reductions in paw swelling (up to 84%) and arthritis score (up to 70%) upon exposure to emu oil (2). Although this anti-inflammatory effect of emu oil has now been reported in these two separate studies, the role of proinflammatory cytokines such as interleukin (IL)-1α and tumor necrosis factor-α (TNF-α) has not been investigated. Since it is well established that acute and/or chronic inflammation is mediated by expression of a host of proteins including the cytokines, it is hypothesized that the anti-inflammatory effect of emu oil may be associated with decreased levels of certain proinflammatory cytokines (5–7).

Role of FA in inflammatory responses. Particularly germane to the present communication are the reported findings that exposure to oils enriched in the n-9 FA, oleic acid, and the n-3 FA, such as α-linolenic acid, EPA, and DHA, is associated with anti-inflammatory activity when compared to the proinflammatory actions of the n-6 FA, linoleic acid (8,9). Moreover, both of these review articles describe several studies indicating that the anti-inflammatory activities of the n-9 and, in particular, the n-3 FA, are associated with reductions in levels of cytokines IL-1α, TNF-α, IL-6, and IL-1β. The FA composition of emu oil was unremarkable, especially when

TABLE 1
Content[a] of Major FA (%)

	16:0	18:0	18:1	18:2	18:3	20:5	22:6	Other
Emu oil	20	11	49	15	ND	ND	ND	5
Fish oil	16	3	21	3	ND	18	12	27
Flaxseed oil	5	4	20	13	58	ND	ND	0
Olive oil	8	3	84	4	ND	ND	ND	1
Liquified chicken fat	22	6	37	21	ND	ND	ND	14

[a]Values represent means of FA analyses performed in triplicate. ND, not detected (limits of detection <0.05%).

compared to other oils reported to have anti-inflammatory activity (Table 1). For example, emu oil contains significantly less of the reported anti-inflammatory FA oleic acid, α-linolenic acid, EPA, and DHA, than are found in olive oil, flaxseed oil and fish oil, respectively. Thus, it would appear that the anti-inflammatory properties of emu oil are probably not fully explained by the FA profile.

Model justification. Compared to other animal models, CD-1 mice were found to be highly susceptible to induction of cutaneous inflammation following exposure to croton oil (4,10), lipopolysaccharide (11), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (12), and petroleum distillates (13). High levels of inflammation were found when compounds were applied topically or administered as dietary component (14–17). Studies done with other mouse strains such as Balb/C or C57BL6/J did not produce as significant an inflammatory reaction to croton oil, as with the CD-1 mice (18–20). In addition, the CD-1 mouse strain lacks T4 cells, part of the natural anti-inflammatory defense mechanisms of the body to causative agents. Because of this, the inflammatory response to exogenous agents was more pronounced in the CD-1 strain than in other strains of mice.

Goals of the study. (i) Compare the anti-inflammatory properties of emu oil with other oils also reported to reduce inflammation (21–23); (ii) determine whether reported anti-inflammatory properties of these oils were associated with effects on proinflammatory cytokines. The role of various cytokines in the inflammatory process is well established (5,6,24). Cytokines are large groups of locally acting proteins involved in cell signaling during immune responses. The cytokines IL-1α, IL-1β, TNF-α, and IL-6 are often designated as proinflammatory cytokines. They are predominantly produced by circulating monocytes and tissue macrophages and mediate the host response to inflammatory stimuli.

EXPERIMENTAL PROCEDURES

Animals. Seven-week-old male CD-1 mice (Charles River Laboratories, Wilmington, MA) with a mean body weight of 25 to 28 g were housed in standard polycarbonate cages (33 × 23 × 12 cm) under controlled conditions of temperature (22 ± 0.5°C), RH (50%), and 12:12 light/dark cycle. Mice had free access to water and rodent chow (Ralston Purina, St. Louis, MO) and were allowed to adapt to laboratory housing for 1 wk before the commencement of the study. The care and treatment of the experimental animals conformed to the guidelines of the Institutional Animal Care and Use Committee of University of Massachusetts Lowell and *Guide for Care and Use of Laboratory Animals* (25).

Different treatment protocols. In this study, 60 mice were randomly assigned to six groups of 10, with the following designation: Group 1 = control (croton oil), Group 2 = emu oil, Group 3 = fish oil, Group 4 = flaxseed oil, Group 5 = olive oil, Group 6 = liquified chicken fat. Emu oil was obtained from LB Processors, LLC (Chapmansboro, TN) and the remaining oils were provided by ACH (Memphis, TN). These choices of oils for study were based on previous reports indicating that these oils possessed anti-inflammatory properties (23,26). Animals were anesthetized at the beginning of the study, with a combination of ketamine (100 mg/mL) (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/mL) (Bayer, Shawnee Mission, KS) at a dosage of ketamine/xylazine, 87 mg/kg/13 mg/kg body wt, delivered intramuscularly (27)

Induction and evaluation of inflammation. Croton oil (2%) was prepared for topical administration by addition of 20 μL of croton oil (catalog no. 6719; Sigma, St. Louis, MO) to 1-mL acetone. Fifty microliters of this preparation was slowly applied to the inner surface of the left pinna of the anesthetized mouse. Two hours later, 5 μL of emu, fish, flaxseed, or olive oil or liquified chicken fat was applied to the inflamed site with a Hamilton microliter syringe. The control group was left untreated. Six hours after application of oils to the site, blood was collected from anesthetized mice and plasma samples were prepared. Animals were then sacrificed using carbon dioxide gas for determination of auricular thickness, EP weight, and cytokine levels in auricular homogenates. Pilot experiments indicated that this schedule was optimal for determining the effect of different anti-inflammatory treatments. Thickness measurements of the left auricles were taken using a micrometer caliper (Control Co., Friendswood, TX) as reported by Lopez *et al.* (1). The final measurement was taken at 6 h posttreatment with emu, fish, flaxseed, or olive oil or liquified chicken fat. At the 6-h time point, the animals were euthanized using carbon dioxide. Uniform-sized auricular tissue EP were punched from both the auricles using a biopsy punch and weighed. The weight difference between the untreated right and the treated left EP indicated the magnitude of swelling posttreatment, as well as the reduction response.

Evaluation of cytokines. Exposed and unexposed EP were harvested and weighed at time of sacrifice, immersed immediately in liquid nitrogen, and stored at −80°C for further analysis. Levels of IL-1α and TNF-α mouse cytokines from plasma and tissue samples were measured by ELISA. Frozen ears were prepared for cytokine analyses as described by Wang and Stashenko (7). Frozen ear tissue samples were ground using a pre-cooled sterile mortar and pestle and the tissue fragments dispersed in 800 μL lysis buffer consisting of 100 μg/mL BSA (fraction V; Sigma), 100 μg/mL Zwittergent-12 (Boehringer Mannheim, Indianapolis, IN), 50 μg/mL gentamycin (Life Technologies, Rockville, MD), 10 mM HEPES buffer (Life Technologies), 1 μg/mL aprotinin (Sigma), 1 μg/mL leupeptin (Sigma), and 0.1 μM EDTA (Sigma) in RPMI 1640 (Mediatech, Herndon, VA). This process releases the cytokines from the cells. The supernatant containing the cytokines was collected after centrifugation and stored at −80°C until assayed. Mouse cytokine assays were carried out using commercially available ELISA kits obtained from the following sources: IL-1α (Endogen, Cambridge, MA; sensitivity 6 pg/mL) and TNF-α (3 pg/mL) from BioSource International (Camarillo, CA). Results were expressed as pg cytokine/mg tissue.

FA analyses of oils. For FA analyses of the various oils, a 300-μL aliquot of oil was mixed with 5 mL of methanol con-

taining 0.2% BHT. Ten milliliters of chloroform was added, and the sample vortexed for 30 s. After addition of 1.0 mL of 0.15 M NaCl, the mixture was vortexed again and centrifuged at 500 × *g* for 10 min. The top aqueous layer was discarded, and the bottom organic layer was stored at −80°C under N_2 in a glass vial with a Teflon-lined cap. Prior to analyses, samples were evaporated to dryness under N_2 and esterified as previously described (28) with Instant Methanolic HCl kit (Alltech-Applied Science, Deerfield, IL). The FAME profile was determined on a Hewlett-Packard model 5890 gas–liquid chromatograph, with a DB-23 column (30 m column length, 0.25 μm film thickness, helium carrier gas) (J&W Scientific, Folsom, CA).

Statistical analyses. Sigma Stat software (Jandel Scientific, San Rafael, CA) was used for all statistical evaluations. A one-way ANOVA was used to analyze all data. When statistical significance was found by ANOVA, the Student–Newman–Keuls separation of means was used to determine group differences. Correlations (*r*) between auricular thickness, EP weights, and IL-1α and TNF-α were performed using Pearson's product-moment correlation coefficient. To determine whether auricular thickness was correlated with levels of cytokines, the different oil treatments were combined to provide a sufficient sample size and greater range of values, as previously described (29). All values were expressed as mean ± SD, and statistical significance was set at the minimum $P < 0.05$ (30).

RESULTS

To optimize the time of inflammatory induction, treatment duration, and cytokine responses, a preliminary study was conducted in 10 CD-1 animals. From this preliminary study it was found that the inflammatory reaction to 2% croton oil application reached its peak at 2 h posttreatment, as reported by Lopez *et al.* (1). From other studies (21–23) it has been demonstrated that fish, flaxseed, and olive oils can elicit an anti-inflammatory response. The maximal anti-inflammatory responses for different oils were observed at 6 h following topical application of the oils. Although previous studies of inflammation have been reported in other strains such as Balb/c or C57BL6/J, these strains do not produce as significant an inflammatory response to croton oil as compared to the CD-1 animals (18).

The thickness and weight differences seen in this present study between the various groups are shown in Table 2. Compared to the untreated control group, auricular thickness reductions were statistically significant ($P < 0.05$) only in the emu group (−72%), although nonsignificant reductions occurred for fish oil (−50%), flaxseed oil (−50%), olive oil (−40%), and liquified chicken fat (−28%) groups.

Auricular weight reductions were maximum with the emu oil group (−70%), which again were statistically significant ($P < 0.05$), although reductions were also noted for fish oil (−54%), flaxseed oil (−46%), olive oil (−44%), and liquified chicken fat (−25%) that were not statistically significant (Table 2).

EP tissue concentrations of the two different cytokines IL-1α and TNF-α as measured by ELISA method are shown in Table 3. For IL-1α, statistically significant reductions were noted for the emu oil (−70%), and fish oil (−57%) groups ($P < 0.05$), with lesser reductions observed for olive oil (−49%), flaxseed oil (−43%), and liquified chicken fat (−24%) that were not statistically significant (Table 3). EP tissue levels of TNF-α were significantly reduced in emu (−60%) and olive oil (−52%) ($P < 0.05$)-treated groups, with lesser reductions observed for fish oil (−36%), flaxseed oil (−34%), and liquified chicken fat (−28%) (Table 3).

Plasma levels of both IL-1α and TNF-α showed no consistent pattern of response to any of the various treatments (data not shown). Whereas there was a significant association between EP thickness and weight ($r = 0.780$, $P < 0.003$) (Fig. 1), only EP thickness was significantly correlated with TNF-α ($r = 0.690$, $P < 0.02$) (Fig. 2) and IL-1α ($r = 0.750$, $P < 0.006$) (Fig. 3).

TABLE 2
Thickness and Weight Differences[a] of Ears in Mice Treated with Various Oils 2 h After Croton Oil Application and 6 h After Oil Treatment

Treatment	Thickness (mm)	Weight (mg)
Control	0.285 ± 0.023[a]	24.44 ± 6.45[a]
Emu oil	0.081 ± 0.009[b]	7.22 ± 1.45[b]
Fish oil	0.143 ± 0.013[a,b]	11.22 ± 1.84[a,b]
Flax oil	0.143 ± 0.025[a,b]	13.27 ± 4.13[a,b]
Olive oil	0.171 ± 0.026[a,b]	13.78 ± 1.76[a,b]
Liquified chicken fat	0.205 ± 0.022[a,b]	18.08 ± 2.00[a,b]

[a]Values are mean ± SD, $n = 10$. Values in a column not sharing a common superscript roman letter are significantly different at $P < 0.05$.

DISCUSSION

Application of emu oil at 2 h after croton oil exposure significantly reduced the degree of inflammation in the auricles of CD-1 mice, in agreement with previous work (1). In the present study, in addition to the comparison of auricular thickness and weights, the possible relationship between the cytokines IL-1α and TNF-α and the anti-inflammatory process in response to various treatments was investigated.

On the basis of the preliminary study and the current study conducted in CD-1 mice, it was demonstrated that auricular tissue levels of both IL-1α and TNF-α were the predominant

TABLE 3
Ear Plug Tissue Concentrations[a] of IL-1α and TNF-α in Mice Treated with Various Oils 2 h After Croton Oil Application and 6 h After Oil Treatment

Treatment	IL-1α (pg/mg)	TNF-α (pg/mg)
Control	307.2 ± 35.02[a]	79.25 ± 15.53[a]
Emu oil	92.3 ± 12.18[b]	31.74 ± 3.62[b]
Fish oil	132.2 ± 19.65[b]	50.67 ± 10.17[a,b]
Flax oil	173.9 ± 40.95[a,b]	52.61 ± 7.14[a,b]
Olive oil	155.9 ± 27.38[a,b]	38.27 ± 5.23[b]
Liquified chicken fat	227.7 ± 23.13[a,b]	56.85 ± 6.19[a,b]

[a]Values are mean ± SD, $n = 10$. Values in a column not sharing a common superscript roman letter are significantly different at $P < 0.05$. IL, interleukin; TNF-α, tumor necrosis factor-alpha.

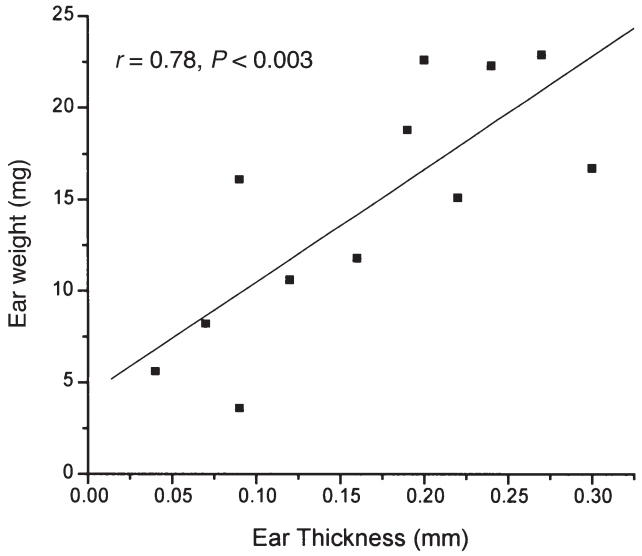

FIG. 1. Correlation between ear plug thickness and weight for all treatments.

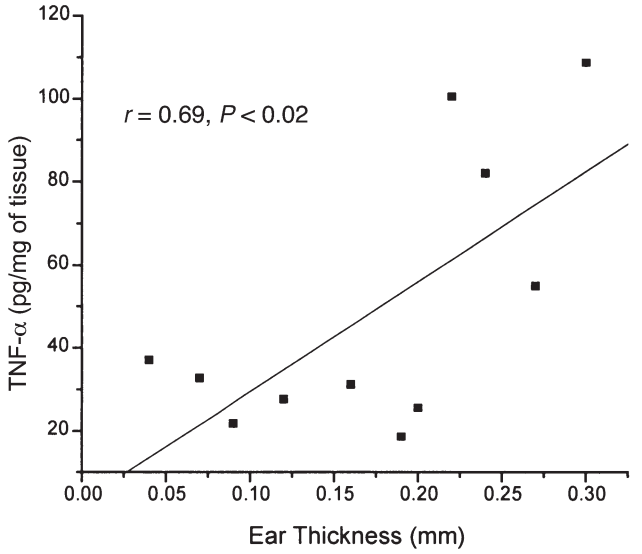

FIG. 2. Correlation between ear plug thickness and tumor necrosis factor-α (TNF-α) for all treatments.

cytokines responsible for this inflammatory induction, and the anti-inflammatory action of emu oil was directed toward these cytokines. The specific pathway for this action is not clearly identified, and it may depend on the levels and time of release.

It is noteworthy to look at this anti-inflammatory phenomenon from the viewpoint of the FA constituents of the different oils. The different oils used in this study contained either n-3 or n-9 PUFA. It has been reported that fish oil and other oils rich in n-3 or n-9 FA inhibit macrophage migration, an anti-inflammatory effect mediated through restriction of cytokine production (8,9). The n-3 FA also are known to decrease the levels of proinflammatory cytokines IL-1α, IL-6, and TNF-α and to increase the anti-inflammatory cytokine

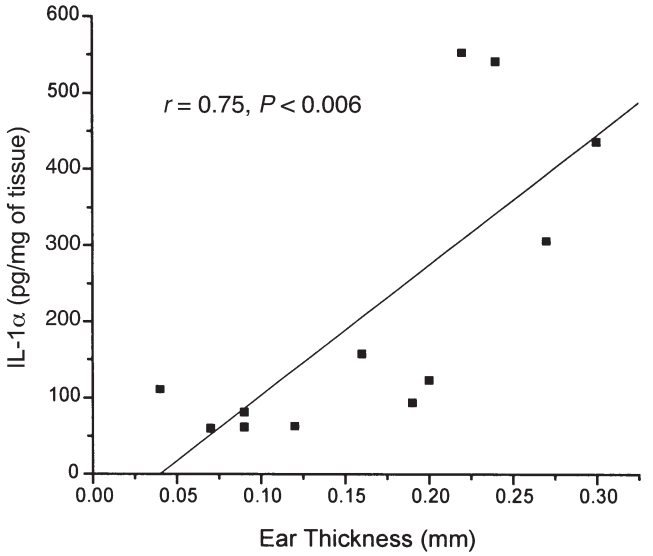

FIG. 3. Correlation between ear plug thickness and interleukin (IL)-1α for all treatments.

IL-2 (21). *In vitro* studies with n-3 FA reveal that one potential mechanism for the beneficial effects of these oils is associated with the suppression of cell-mediated immune responses (22).

Other dietary studies, which investigated the inflammatory response in the ears of mice sensitized with 2,4-dinitro-1-fluorobenzene, revealed immunosuppressive effects as measured by the degree of ear swelling in mice consuming a diet rich in DHA but not EPA. The effect of DHA but not EPA was also associated with reductions in the expression of IL-6, IL-β, and IL-2 mRNA. Fish oil contains variable amounts of EPA and DHA along with other FA, and thus it is not clear whether the reduced inflammatory response with fish oil noted in the present study was due to EPA, DHA, or both.

Although not statistically significant, the reduction in inflammation by the flaxseed oil-treated mice demonstrated in this study is in agreement with other studies of oils enriched in α-linolenic acid (21–23). But, in contrast to these dietary studies, in this study the different oils were applied topically. Further application of these findings will depend on studies done in human subjects.

Whereas it was possible from this study to demonstrate that emu oil's anti-inflammatory properties were associated with reductions in proinflammatory cytokines, it does not appear to be the result of its FA composition. Emu oil is enriched in monounsaturated FA but not to the extent of olive oil, and although it has low levels of the proinflammatory n-6 FA, linoleic acid (8,9), it is essentially devoid of the anti-inflammatory n-3 FA.

One report (3) does suggest that emu oil's anti-inflammatory activity may reside in its non-TG component, sometimes called the unsaponifiable fraction. However, the nature of the active ingredient(s) remains unresolved.

Results from these studies lead us to conclude that in CD-1 mice, topical emu oil is relatively more anti-inflammatory

than other oils or chicken fat. Direct measurements of decreases in thickness and weights of ear tissue treated with emu oil support this finding. In addition, the cytokine evaluations suggest one of the many pathways of the anti-inflammatory action.

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