Effects of an acetone extract of *Boswellia carterii* Birdw. (Burseraceae) gum resin on adjuvant-induced arthritis in lewis rats


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Abstract

Ruxiang (*Gummi olibanum*), the dried gum resin of *Boswellia carterii* (BC), has been used in traditional Chinese medicine to alleviate pain and inflammation for thousands of years. In this random, blinded study, the anti-arthritic effects of a BC extract were observed and compared to vehicle control in a Lewis rat adjuvant arthritis model ($n=8$ group). Arthritis was induced by injecting CFA subcutaneously into the base of the tail, and the extract was administered orally (i.g.) for 10 consecutive days beginning on day 16 after the injection. Arthritic scores, paw edema, and the local tissue pro-inflammatory cytokines tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-1 beta (IL-1$\beta$) were assessed. Toxicity and adverse effects of the extract were evaluated. At 0.90 g/kg per day, BC significantly decreased arthritic scores between days 20 and 25 ($p<0.05$) and reduced paw edema on days 18, 20 and 22 compared to control ($p<0.05$). It also significantly suppressed local tissue TNF-$\alpha$ and IL-1$\beta$ ($p<0.05$). No major adverse effects were observed in animals during the repeated-dose treatment profile although mild fur discoloration was noted. The data show that BC extract has significant anti-arthritic and anti-inflammation effects and suggest that these effects may be mediated via the suppression of pro-inflammatory cytokines.

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1. Introduction

Rheumatoid arthritis (RA) is a common disease affecting 2–3% of the population in the U.S.A. Conventional medicine, including treatment with steroids, non-steroidal anti-inflammatory drugs (NSAIDs) and such biological agents as tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-1 beta (IL-1$\beta$) antagonists (Fleischmann et al., 1996; Dayer, 2003; Fleischmann et al., 2004), has shown only limited success against RA (American College of Rheumatology, 1996; Dayer, 2003; Fleischmann et al., 2004; Shin et al., 2003). Previous in vitro studies have shown that the boswellic acids derived from Ruxiang, or *Gummi olibanum*, the dried gum resin of *Boswellia carterii* Birdw. (BC), have potential immunomodulatory effects (Zhu, 1998; Jing et al., 1999; Liu and Qi, 2000). BC, commonly used in traditional Chinese medicine (TCM) to reduce swelling and alleviate the pain of acute RA, but their effects on chronic, prolonged RA are unsatisfactory. Moreover, the adverse effects of drug therapy are significant and include gastrointestinal disturbances, infections and cardiovascular risks (Scheiman, 2001; Mangee et al., 2003; Rubbert-Roth and Perniok, 2003; Ortiz, 2004). The inflammatory process of RA is reportedly associated with an increase of the pro-inflammatory cytokines TNF-$\alpha$ and IL-1$\beta$ (Dayer, 2003; Fleischmann et al., 2004; Shin et al., 2003).
inflammatory diseases or tumors (Bensky and Gamble, 1993; Zhu, 1998), may provide an effective alternative or adjunct to such conventional treatments.

Chinese herbs have been used in Asia for thousands of years to treat a variety of diseases and symptoms, including RA and related diseases (Bensky and Gamble, 1993); and in recent years, herbs have received increasing public interest in the West. In 1997 alone, U.S. consumers spent $5.1 billion on medicinal herbs (Eisenberg et al., 1998). In 2002, 19% of adults used herbs for reasons of health (Barnes et al., 2004), and some studies show that about 50% of RA patients report having used herbs (Taibi and Bourguignon, 2003; Soeken, 2004; Chandrashekar et al., 2002).

Commercial herbal products containing BC, one of 43 species in the genus Boswellia of the family Burseraceae (International Plant Name Index Query, 2004), are easily available in the U.S. as dietary supplements for patients with arthritis or other inflammation and pain related disorders (McGuffin et al., 1997). But scientific evaluation of its effects is lacking, its mechanisms of action are not well understood, and only a few studies of its analgesic, antihyperalgesic and anti-inflammatory effects on animal models have been reported (Zhen et al., 2003; Fan et al., 2005). The purpose of the present study was to investigate the BC extract for therapeutic effects, adverse effects or toxicity and cytokine-involved mechanisms of action in an animal model of adjuvant arthritis (AA) that mimics features of the clinical manifestations of human RA (Lathigra et al., 1988; Albani et al., 1992).

2. Materials and methods

2.1. Animal preparation and AA Induction

Male Lewis rats (Harlan Sprague Dawley, Indianapolis, IN), 5–6 weeks old, were kept under controlled environmental conditions (22 ± 0.5 °C relative humidity 40–60%, 7 a.m. to 7 p.m. alternate light–dark cycles, food and water ad libitum). The animals were purchased 1 week before the experiment and allowed to acclimate. They were housed in cages in which the floor was covered with sawdust to minimize the possibility of painful contact with a hard surface. The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine, and the ethical guidelines for the treatment of animals of the International Association for the Study of Pain were followed in all experiments (Zimmermann, 1983).

AA was induced in the Lewis rats by injecting 200 μl CFA solution, which is heat-killed M. tuberculosis (strain H37Ra, Difco Lab, Detroit, MI) suspended in mineral oil (Sigma, 5 mg/ml), subcutaneously at the base of the tail (cf. Moudgil et al., 1997). Rats developed signs of polyarthritis 8–10 days following the injection. The clinical features of AA manifested as erythema, induration and edema, and presented in multiple-joints as follows: (1) onset: clinical signs around days 8–10; (2) early phase: progressive severity of the clinical signs over the next 7–10 days; and (3) late phase: spontaneous regression during the next 10–14 days.

2.2. Arthritis assessments

Rats were assessed daily for signs of arthritis between days 7 and 25 post-CFA using a standard arthritic scoring system (Moudgil et al., 1997; Shin et al., 2003). The maximal arthritic score per rat was set at 16 (maximum of 4 points × 4 paws). All four paws were examined and graded for severity and loci of erythema, swelling and induration using a 5-point scale: 0 = no signs of disease; 1 = signs involving the ankle/wrist; 2 = signs involving the ankle plus tarsals (proximal part of the hind paw) and/or wrist plus carpals of the forepaw; 3 = signs extending to the metatarsals or metacarpals; and 4 = severe signs involving the entire hind or fore paw (Moudgil et al., 1997). Paw volume was measured every other day between days 16 and 24 with a plethysmometer (IITC Inc., Woodland Hills, CA), which consists of two vertical, interconnected, water-filled Perspex cells. The paw to be measured is dipped into the larger cell to the ankle line causing the water level in the smaller cell, which contains a transducer, to rise. The transducer converts detected paw volume into milliliters, then registers the exact volume electronically on a monitor (cf. Helges et al., 2004). Total paw volume, or the sum of the four paws, was calculated. To avoid potential bias, investigators performing all assessments or measurements were blinded to treatment group assignments.

2.3. Herbal preparation and extraction

Unprocessed BC gum resin was purchased from the Tong Ren Tang pharmaceutical company (Beijing, China) and the resin was identified according to the latest Pharmacopoeia of the People’s Republic of China (2000). It was ground into powder (∼80 mesh) and processed at room temperature with 70% aqueous acetone to completely extract both non-polar and polar components. Evaporation temperatures were kept below 55 °C to minimize the possible breakdown of thermolabile compounds that may be present in extracts. This initial crude extract was concentrated under reduced pressure, and the dried residue was coded and weighed for the study. The final extract was 40% the weight of the raw resin. The quality of the extract was monitored using high performance liquid chromatography (HPLC; Fan et al., 2005). The BC extract used in this study was from a single lot (lot number: 12012003).

2.4. Herbal administration

Rats were randomly divided into a BC treatment group (n = 8) and a vehicle control group (n = 8). The dosage of BC extract used in this study was 0.90 g/kg per day, which is the optimal dosage ascertained in our previous study (Fan et al., 2005).
2005). This is equivalent to using 100 g of raw BC resin in a human decoction (the rate of hot aqueous extract being 10%) as calculated by the method described in Yu (2000).

Between days 16 and 25 post-CFA injection, the rats received either BC or vehicle once a day for 10 consecutive days. Each dose of BC extract was dissolved in 2 ml of distilled water and sesame oil (2:1) and administered intragastrically (i.g.), using a 5 ml syringe with a 4 cm long gavage needle. Animals in the vehicle control group received daily i.g. doses of 2 ml distilled water and sesame oil (2:1).

2.5. Local tissue collection and ELISA

For cytokine assay, the local tissue was collected from the BC treatment and vehicle control animals at day 25 post-CFA following the arthritis assessments. Local tissue was also collected from a group of non-inflamed, normal animals treated with vehicle that served as a background control. After the rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.), soft tissue (1.0 g) was collected from the ankle, immediately placed on ice, and then homogenized using an ultrasonic processor (Cole-Parmer Instruments, Vernon Hills, IL). Tissue supernatant samples were frozen for storage (−80 °C) immediately after collection to prevent cytokine degradation.

IL-1β and TNF-α levels were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the procedure recommended by the manufacturer (BioSource International Inc., Camarillo, CA). In brief, IL-1β levels were measured by pipetting 50 μl of sample and 50 μl of standard diluent buffer into the wells of a microtiter plate coated with an antibody specific to rat IL-1β, then incubated for 3 h at 37°C. After two 10-min washings with PBS, biotinylated anti-rat IL-1β antibody was added and incubated at ambient temperature for 1 h. Streptavidin-peroxidase HRP was then added and incubated for 30 min to bind to the biotinylated antibody. After two more 10-min washings with PBS to remove unbound enzymes, color was developed by adding stabilized chromogen tetramethylbenzidine and a stop solution was added. Finally, the optical density was calculated at the ankle, immediately placed on ice, and then homogenized using an ultrasonic processor (Cole-Parmer Instruments, Vernon Hills, IL). Tissue supernatant samples were frozen for storage (−80 °C) immediately after collection to prevent cytokine degradation.

2.6. Toxicity/adverse effects assessment

Standard pharmacological categories of toxic and adverse behavioral reactions were used, following the methods described by Gad (2002). Animals were closely monitored for unusual behavioral changes and such symptoms as obvious temperature change (rectal temperature was taken with a digital thermometer before each BC or vehicle administration), diarrhea, weight loss (rats were weighed every other day), fur discoloration, lethargy, irritation and convulsion during the 10 days of treatment. After the observation period, all animals were euthanized and a gross necropsy was performed. All observations were performed by investigators who were blinded to treatment group assignments.

2.7. Statistical analysis

The number of animals used in each specific test was estimated via a power analysis based either on our own preliminary data or on published results employing similar experimental procedures and physiologic parameters (cf. Bausell and Li, 2002). The results are presented as mean ± standard error. For arthritic score and edema assessments, one-way repeated measure ANOVA was used for statistical comparison between the two groups; for cytokine testing, one-way ANOVA was used for statistical comparison. All post hoc comparisons were conducted using the Dunnett test. p < 0.05 was considered significant in all cases.

3. Results

3.1. BC decreases arthritic score and paw edema

The rats gradually developed multiple-joint AA beginning on day 8 after CFA was injected at the base of the tail. The manifestations peaked between days 18 and 20 (Figs. 1 and 2). There were no significant differences in arthritic score or edema between the two groups before BC or vehicle administration on day 16 (p > 0.05). BC extract at daily 0.90 g/kg significantly suppressed arthritic scores compared to vehicle control between days 20 and 25 post-CFA. (On day 20: 2.09 ± 0.55 versus 4.97 ± 0.82; on day 21: 1.90 ± 0.51, versus 5.03 ± 0.76; *p < 0.05, **p < 0.01, compared to vehicle control at the corresponding time points.

Values are mean ± S.E (n = 8/group).

Fig. 1. Comparison of arthritic scores of Lewis rats given Boswellia carterii extract (BC 0.90 g/kg per day, i.g.) to scores of those given vehicle control. Post-CFA injection, control group arthritic score increased gradually beginning on day 8 and peaked around day 20. BC significantly decreased the severity of arthritis and suppressed the peak arthritic score. *p < 0.05, **p < 0.01, compared to vehicle control at the corresponding time points.
on day 22: 1.81 ± 1.75 versus 5.19 ± 5.19; on day 23: 1.75 ± 0.50 versus 5.22 ± 0.76; on day 24: 1.62 ± 0.46 versus 5.19 ± 0.73; on day 25: 1.62 ± 0.52 versus 5.09 ± 0.71, respectively. \( F = 15.64, p < 0.001 \). Fig. 1. Paw edema was attenuated significantly compared to control on days 18, 20 and 22. (On day 18: 5.25 ± 0.23 ml versus 6.05 ± 0.44 ml; on day 20: 4.87 ± 0.23 ml versus 5.98 ± 0.43 ml; on day 22: 4.81 ± 0.11 versus 5.93 ± 0.46 ml, respectively. \( F = 5.21, p < 0.001 \). Fig. 2).

3.2. BC decreases cytokine levels in local arthritic tissue

On day 25 post-CFA injection, there were significant differences in local tissue TNF-α levels among BC treatment (9.56 ± 2.84 pg/mg protein), vehicle control (24.78 ± 4.18 pg/mg protein) and non-AA normal animal (7.27 ± 1.80 pg/mg protein) groups (\( F = 7.87, p = 0.005 \)). Levels in the vehicle control AA group were about three times higher than that of the normal animal group (\( q = 3.84, p < 0.01 \)). The BC extract significantly suppressed local TNF-α compared to the AA control group (\( q = 3.01, p < 0.05 \). Fig. 3).

Similar results were found in the local tissue IL-1 β assay (\( F = 7.30, p = 0.007 \)). The extract significantly suppressed IL-1β levels (53.39 ± 7.69 pg/mg protein) compared to vehicle control (117.29 ± 25.58 pg/mg protein, \( q = 2.39, p < 0.05 \). Fig. 4), while IL-1β levels were low in non-AA normal rats (12.45 ± 2.90, \( q = 4.08, p < 0.05 \) compared to control.

3.3. Toxicity or adverse effects of the BC extract

During the 10 days of daily i.g. administration, no significant behavioral changes or obvious symptoms except signs associated with the CFA-induced AA were observed in either vehicle control or the BC treatment groups. However, mild fur discoloration was observed in some animals (two of eight) treated with 0.90 g/kg BC. There was no difference in body weight between the treatment and vehicle control rats before BC administration on day 16 post-CFA injection. In this study, samples are as follows: in the non-arthritic normal group (no CFA injection × vehicle treatment, \( N \)), \( n = 4 \); in the BC group (CFA injection × BC extract treatment at 0.9 g/kg per day, BC), \( n = 7 \); and in control (CFA injection × vehicle treatment, \( C \)), \( n = 6 \). The data shows that the amounts of IL-1 β and TNF-α were significantly higher in control \( (C) \) than in the non-arthritic normal group \( (N) \). However after BC treatment, the levels of IL-1 β and TNF-α were significantly lower compared to control. \( p < 0.05, ** p < 0.01 \), compared to vehicle control. Data are expressed as the mean ± S.E.
4. Discussion

The present study demonstrated that the Chinese herb Ruxiang (BC) at a daily dosage of 0.90 g/kg significantly attenuated adjuvant-induced polyarthritis as shown by the decrease of both arthritic score and paw edema volume. The changes in arthritic score and paw edema volume were positively correlated. These findings are consistent with the results of previous studies, including ours that showed the anti-inflammatory, analgesic and anti-hyperalgesic effects of BC on different animal models (Zhen et al., 2003; Fan et al., 2005).

CFA-induced secondary inflammation mimics sub-acute RA (Lathigra et al., 1988; Albani et al., 1992; Moudgil et al., 1997). Because RA is characterized by excessive immunology in the synovium (Firestein and Zaffriner, 1997), the anti-polyarthritis effect of BC may be achieved by the potential immunomodulatory properties of BC. The main chemical constituents of the BC extract are boswellic acids (Fan et al., 2005; Zhou and Cui, 2002; Badria et al., 2003). It has been reported that boswellic acids selectively decrease the formation of leukotriene LTB4, a potent chemoattractant and activator of both granulocytes and macrophages (Safayhi et al., 1992) and reduce the infiltration of leukocytes into an inflammation site (Sharma et al., 1989). It is well known that leukocytes produce pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, which play important roles in both RA in humans (Dayer, 2003; Fleischmann et al., 2004; Shin et al., 2003; Firestein, 2004) and AA in rats (Smith-Oliver et al., 1999; Silva et al., 2000). Our data demonstrate that the BC extract significantly decreases the local tissue pro-inflammatory cytokines TNF-α and IL-1, which were induced by CFA inflammation. Previous studies have shown that the anti-arthritis effect of several other Chinese herbal products may also involve the local tissue cytokine pathway (Ho et al., 1999; Yeom et al., 2003; Shin et al., 2003). For example, PG201, an ethanol extract of an herbal formula, significantly inhibited the production of the pro-inflammatory mediators TNF-α and IL-1β in local arthritic tissue and suppressed the progression of collagen-induced arthritis in mice (Shin et al., 2003). Taking these data together, we conclude that the anti-arthritis effect of BC and other anti-arthritis Chinese herbs may result from the suppression of IL-1β and TNF-α and the interruption of the cytokine pathway during the process of local tissue inflammation.

In recent years, Chinese herbs have become a popular complementary therapy among patients, and a number of studies demonstrate their utility and explore their possible mechanisms (Ho et al., 1999; Mitakawa et al., 1998; Chen, 2001; Fang et al., 2001). This widespread usage has raised concerns in the medical community over the safety and possible side effects of herbs (Foster et al., 2000; White House, 2002). During the 10-day repeated-dose application of BC, we observed no significant adverse effects. However, the high dosage of BC used in this study may explain the fur discoloration observed in some of the animals, which may signify the potential for adverse effects. In any case, before BC is subjected to a clinical trial, an animal toxicity study equivalent to the length of the clinical trial, but at least 2 weeks long, must be performed (Food and Drug Administration, 2004).

It is worth noting that the effective dosage (0.9 g/kg) we used in the present study is equivalent to 100 g BC raw resin, or 10 times the amount (10 g) usually added to herbal formulas prescribed for human use. This suggests that, to achieve a therapeutic effect, a much higher dosage is required when an individual herb is used alone than when it is used in an herbal formula. According to Chinese herbal therapy, interactions among the different herbs in a formula may exert an additive or synergistic effects that maximize the therapeutic effects and neutralize the toxicities or side effects of the individual constituents (Pharmacopoeia, 2000). However, further investigation of the safety, effectiveness and mechanisms of BC and its interactions with other herbs in classical Chinese herbal formulas is warranted.

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References


