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Mechanisms of anti-inflammatory effect of an active ingredients group from Jinxuan Zhike Xunxi San

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ABSTRACT

Aim of the study: JZXS has been used for inflamed hemorrhoids as a chinese prescription for several decades. The present study was designed to investigate the anti-inflammatory activities, as well as the mechanisms, of an active ingredients group (AIG) obtained from Jinxuan Zhike Xunxi San (JZXS).
Materials and methods: The anti-inflammatory activities and mechanisms of AIG were evaluated by xylene-induced ear edema experiments in normal mice and mice without adrenals, leukocyte migration experiments, and carrageenin-induced peritonitis experiments, taking JZXS as the positive control.
Results: AIG and JZXS prevented xylene-induced ear edema in normal mice but showed no effects when adrenals were removed. Additionally, AIG and JZXS inhibited leukocyte migration, reduced prostaglandin E2 (PGE2) level in inflammatory exudates and nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-6 and 8 (IL-6 and IL-8) levels in serum.
Conclusions: AIG and JZXS showed significant anti-inflammatory activities depending on pituitary-adrenal axis, thereby inhibiting leukocyte migration and reducing cytokines and mediators.

1. Introduction

Jinxuan Zhike Xunxi San (JZXS) as a prescription of traditional Chinese medicine is one of the main products of Mayinglong Pharmaceutical Group Co., Ltd. It has been used in clinical field for several years for the treatment of the anal swelling and pain caused by hemorrhoid surgery or inflammatory external hemorrhoids, prompting that it shows the activities of detumescence, relieving pain, dispelling wind, and removing dampness. But the preparation process of JZXS is very rough and the dosage must be very high to ensure effective. For more precise and stabile preparation and smaller dose, it is necessary to carry out enriched experiments on its active ingredients. In our previous studies (Zhang et al., 2010), the paw edema experiments, xylene-induced ear edema experiments, and the hot plate-induced pain experiments were adopted to screen the active section of JZXS, indicating an active ingredients group (AIG) containing saponins, flavonoids, and minerals was the main constituent with anti-inflammatory and anti-nociceptive effects.

Thus, to explore AIG as a natural agent for the treatment of anal swelling and pain, further anti-inflammatory activities as well as the potential mechanism of AIG have been performed in the present study. This study had the following objectives: (a) to evaluate the anti-inflammatory effects of AIG by xylene-induced ear edema experiments compared with JZXS; and (b) to infer the possible mechanisms by evaluating anti-inflammatory effect of AIG on mice without adrenals and the effects of AIG on leukocyte migration, prostaglandin E_2 (PGE_2) level in inflammatory exudates, nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-6 and 8 (IL-6 and IL-8) levels in serum.

2. Materials and methods

2.1. Materials

JZXS (no. 090311) was purchased from Mayinglong Pharmaceutical Group Co., Ltd (Hubei, China); (λ)-carrageenan, ELISA assay kits for IL-6, IL-8, and TNF-α were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Xylene (no. 091204) was purchased from Tianjin Yuanli Chemical Co., Ltd. (Hebei, China).

2.2. Preparation of test samples

Preparation of JZXS solution: powdered JZXS dissolved in water (containing 0.5% Tween 80) and diluted to a concentration of 0.59

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and 0.38 g powdered material per ml as the high concentration for mouse and rats, respectively; the high concentration diluted to 0.30 and 0.19 g powdered material per ml as the low concentration for mouse and rats, respectively.

Preparation of AIG solution: Lonicerae japonicae Flos (dry flower buds or blooming flowers from *Lonicera japonica Thunb*. of caprifoliaceae, identified by professor Hezhen Wu in Hubei University of Chinese Medicine), Schizonepetae Herba (dry ground part from *Schizonepeta tenuifolia Briq.* of Lamium plants, identified by professor Wu in Hubei University of Chinese Medicine), and Portulacae Herba (Dry ground part from *Portulaca oleracea L.* of Portulaca secco plant, identified by professor Wu in Hubei University of Chinese Medicine) were mixed (3:4:3) according to the proportion of prescription, and CO_2 supercritical fluid extracted to obtain a extract (named Extract 1, the yield was 2.36 g/100 g of powdered material); the residue after supercritical fluid extraction was percolated by 60% EtOH and the extract was condensed and purified on a D101 macroporous resin column eluted with H_2O followed by 70% EtOH, the fraction eluted by 70% EtOH was condensed and dried to obtain Powder 2 (the yield was 5.35 g/100 g of powdered material); powered *Natrii sulfas exsiccatus* and *Calcined Alumen* were mixed according to the proportion in the prescription to obtain Powder 3; Extract 1, Powder 2 and Powder 3 were mixed according to the proportion of the prescription (2.36:5.35:90) as AIG and dissolved in water (containing 0.5% Tween 80) and diluted to a concentration of 0.59 and 0.38 g powdered material per ml as the high concentration for mouse and rats, respectively; the high concentration diluted to 0.30 and 0.19 g powdered material per ml as the low concentration for mouse and rats, respectively.

2.3. Preliminary chemical assay of AIG

AIG is a mixture containing three parts, Extract 1, Powder 2 and Powder 3. The content of pulegone in Extract 1 was determined by GC method as previously described (Tang et al., 2008). In Powder 2, the luteolin was detected by a HPLC method (Wang and Yang, 2007) and total flavonoids were analyzed by a spectrophotometric method at 505 nm using the luteolin as a control, total saponins were determined also by a spectrophotometric method at 405 nm using the hederagenin as a control and the chlorogenic acid was detected by a HPLC method (Tang, 2009) and total organic acid were analyzed by a potential titration method (Zeng et al., 2010). Sodium sulfate and aluminium potassium sulfate in Powder 3 were analyzed by the standard methods (National Pharmacopoeia Committee of China, 2010).

2.4. Animals

Male Kunming mice weighing 18–22 g and Wistar rats weighing 120–150 g were procured from The Center for Disease Prevention and Control in Hubei province, China (reg. no. SCXK (Hubei) 2008-0005). They were housed at 22 ± 2 °C under a 12-h light/12-h dark cycle and with access to food and water ad libitum. The animals were acclimatized and habituated to the laboratory for at least a week before testing and were used only once throughout the experiments. The study has been carried out along the "Principles of Laboratory Animal Care" (Xu et al., 2003).

2.5. Xylene-induced ear edema in normal mice

The xylene-induced ear edema test was performed as previously described (Luo et al., 2008). Administration by washing the ear with 1 ml of vehicle (water), high and low concentrations of AIG or JZXS (10 each) twice daily before inducing ear edema were conducted for 6 consecutive days. Thirty minutes after the last administration of tested drugs, a total of 0.05 ml of xylene was applied to the inner and outer surface of the right ear of each mouse. The left ear remained without treatment. Fifteen minutes later, the mice were sacrificed by cervical dislocation and the plugs were removed with a cork borer (9 mm in diameter) from both the right and left ear. The difference in weight between the two plugs divided by the weight of the left plug was taken as a measure of edematous response (%).

2.6. Xylene-induced ear edema in mice without adrenals

When the mice were acclimatized and habituated to the laboratory, bilateral adrenal enucleation was performed. After that, for anti-infection, the mice were feed with 5% glucose saline solution and were given intramuscular injection with penicillin in the next 3 consecutive days. On the 4th day, the mice were divided into 5 groups (10 each) randomly and administrated by washing the ear with drug samples, induced ear edema, sacrificed, removed the plugs and measured edematous response according to the above methods (see Section 2.5).

2.7. Leukocyte migration assay in mice

The test was carried out using the technique of (Kou et al., 2005). 5 groups (12 each) of mice underwent rectal administration of 0.2 ml drug solution (water, high and low concentration of AIG or JZXS) twice daily. After 6 days of administration, the air pouch was formed in the intrascapular region of mice by initial subcutaneous injection of 5 ml sterile air. The next day, 1 h after the last administration of tested drugs, animals received an injection of 5 ml 1.5% carboxymethylcellulose sodium (CMC-Na) solution in the air pouch, and then 0.1 ml of pouch fluid were obtained after 1, 3, 6 h of injection, respectively. The number of leukocytes was counted under a light microscope.

2.8. Effects on PGE_2 level in inflammatory exudates and NO, TNF-α, IL-6 and IL-8 levels in serum

6 groups (10 each) of rats were rectally administrated 1 ml drug solution (water, high and low concentration of AIG or JZXS) twice daily. After 6 days of administration, the peritonitis was formed by intraperitoneal injection of 1% carrageenin saline solution in the dose of 2 ml/kg, while the rats in normal control were injected with saline solution in the same vehicle. 4 h after the peritonitis formed, all animals were rectally administrated for the last time. And another 3 h later the rats were sacrificed; the blood was collected and centrifuged to obtain the serum; 2 ml physiological saline was used to wash the inflammatory exudates and centrifuged to obtain supernatants.

Then isomerization reaction done for 20 min at 50 °C, absorbency value of the mixture, which represented the content of PGE2, was determined by 751 ultraviolet spectrophotometer at 450 nm after diluted to 4 ml with methanol (Wu, 1991) content. The serum was assayed for TNF-α, IL-6 and IL-8 by respective ELISA kits according to the kit instructions. Levels of the NO in serum were determined using a colorimetric assay based on the Griess reaction (Sherman et al., 1993).

2.9. Statistical analysis

All data are presented as means ± S.E.M. One-way ANOVA was first used to assess the differences among multiple groups, followed by the Dunnett post hoc test. The data was assessed by the statistical package from the Social Science Software (SPSS) program. The levels of significance are indicated as difference letters.

3. Results

3.1. Preliminary chemical assay of AIG

AIG obtained from JZXS is a mixture made from Extract 1, Powder 2 and Powder 3, respectively. Sodium sulfate and aluminium potassium sulfate in Powder 3 were about 49.94% and 40.72%, respectively, analyzed by the standard methods (National Pharmacopoeia Committee of China, 2010).

3.2. Xylene-induced ear edema in normal mice and mice without adrenals

Table 1 shows the effects of AIG and JZXS on Xylene-induced ear edema in normal mice and mice without adrenals. AIG as well as JZXS significantly prevented xylene-induced ear edema in a dose dependent manner after administration. The maximal inhibitions were both about 55.1% vs. vehicle-treated mice exhibited by AIG and JZXS at the concentration of 0.59 g powdered material per ml, and AIG showed the same effect as JZXS in both high and low concentrations. When bilateral adrenals of the mice were removed, AIG and JZXS showed no effects on xylene-induced ear edema ($P > 0.05$).

3.3. Leukocyte migration assay in mice

The results of leukocyte migration experiments are presented in Table 2. It can be found that CMC-Na elicited significant leukocyte migration in the air pouch of the mice. Both AIG and JZXS at the concentration of 0.59 g powdered material per ml markedly decreased leukocyte counts with inhibition of about 56.1% after 1 h of CMC-Na injected. But after that, the leukocyte counts increased fast, and there was no difference between drugs treated groups and control group till 6 h after injection of CMC-Na ($P > 0.05$).

3.4. Effects on PGE_2 level in inflammatory exudates and NO, TNF-α, IL-6 and IL-8 levels in serum

After 6 days of administration, in carrageenin-induced peritonitis experiments, both high and low concentrations of JZXS and the high concentration of AIG significantly reduced PGE_2 levels ($P < 0.05$) in inflammatory exudates compared to the control group (Fig. 1A). Additionally, both AIG and JZXS at both concentrations showed significant inhibiting effects on production of NO ($P < 0.05$) (Fig. 1B). Also, it can be clearly found that AIG and JZXS reduced the TNF-α, IL-6 and IL-8 levels in serum, in different degrees (Fig. 2A–C), which are mast cell-derived pro-inflammatory cytokines playing critical biological roles in inflammation.

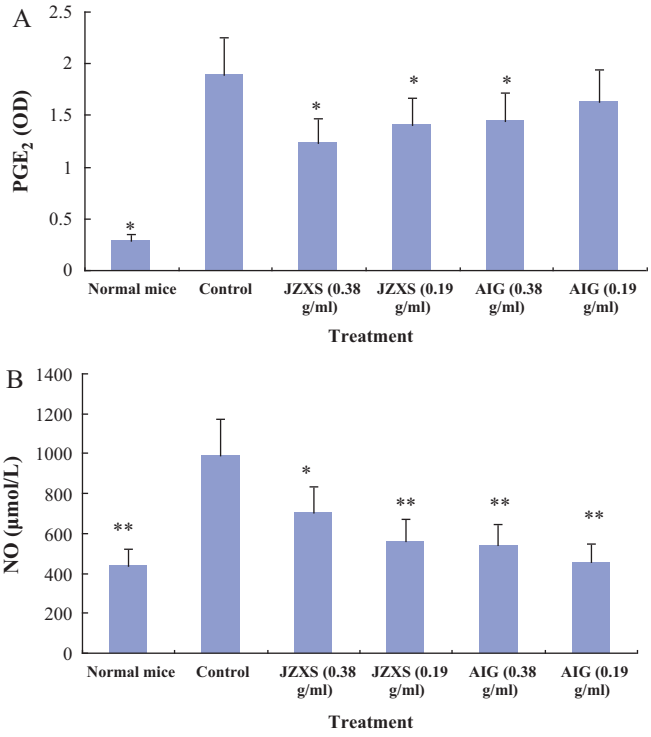

Fig. 1. PGE_2 (A) and NO (B) levels of each group in the carrageenin-induced peritonitis test. ($n = 8$–12, mean ± S.E.M.). $^*P < 0.05$ and $^{**}P < 0.01$ when compared with control (ANOVA followed by Dunnett's test).

Table 1
Effect of AIG and JZXS on xylene-induced ear edema ($n = 8$–12, mean ± S.E.M.).

Treatment	Edematous response (%)	
	Normal mice	Mice without adrenals
Control	64.41 ± 26.18	77.94 ± 36.66
JZXS (0.59 g/ml)	28.92 ± 11.91**	93.47 ± 47.88
JZXS (0.30 g/ml)	38.97 ± 11.92*	99.73 ± 30.29
AIG (0.59 g/ml)	28.91 ± 18.21**	88.10 ± 33.26
AIG (0.30 g/ml)	38.05 ± 12.42*	100.40 ± 28.23

* $P < 0.05$.
** $P < 0.01$ when compared with control (ANOVA followed by Dunnett's test).

Table 2
Effect of AIG and JZXS on leukocyte migration ($n = 8$–12, mean ± S.E.M.).

Treatment	Leukocyte count (10^4 cells/ml)		
	1 h	3 h	6 h
Control	3.42 ± 1.71	3.98 ± 1.88	5.73 ± 1.62
JZXS (0.59 g/ml)	1.58 ± 0.52**	2.56 ± 0.85*	7.09 ± 1.47
JZXS (0.30 g/ml)	1.69 ± 0.91*	3.54 ± 2.51	5.84 ± 3.82
AIG (0.59 g/ml)	1.43 ± 0.45**	2.14 ± 1.36*	6.16 ± 3.63
AIG (0.30 g/ml)	2.11 ± 1.88	3.44 ± 1.69	6.25 ± 3.99

* $P < 0.05$.
** $P < 0.01$ when compared with control (ANOVA followed by Dunnett's test).

4. Discussion

In our previous studies (Zhang et al., 2010), the paw edema experiments, xylene-induced ear edema experiments, and the hot plate-induced pain experiments were adopted to screen the active section of JZXS to obtain an active ingredients group AIG. To further confirm whether the anti-inflammatory effect of AIG is the same as or even higher than that of JZXS, whether the dose of AIG is lower, and what are the anti-inflammatory mechanisms of JZXS and AIG, in the present study, the anti-inflammatory activities as well as the mechanisms of AIG were evaluated, taking JZXS as the positive control.

Anti-inflammatory effects of natural medicine are mainly dependent on the system of hypothalamic-pituitary-adrenal axis, thereby interfering with the metabolism of arachidonic acid, inhibition of the synthesis, release or action of inflammatory mediators, inhibition of the formation of NO, etc. In clinical method, JZXS shows quick and short-term effect on hemorrhoid swelling. So, it is mainly used for the treatment of acute inflammation. In this study, to investigate the mechanisms of action of AIG and JZXS on acute inflammation, (1) xylene-induced ear edema experiments in normal mice and mice without adrenals were performed to confirm whether or not the anti-inflammatory effects are dependent on pituitary-adrenal axis; (2) leukocyte migration experiments were used to analyze whether AIG and JZXS showed inhibition of leukocyte migration; (3) Carrageenin-induced peritonitis experiments were carried out to analyze the effects of AIG and JZXS on cytokines

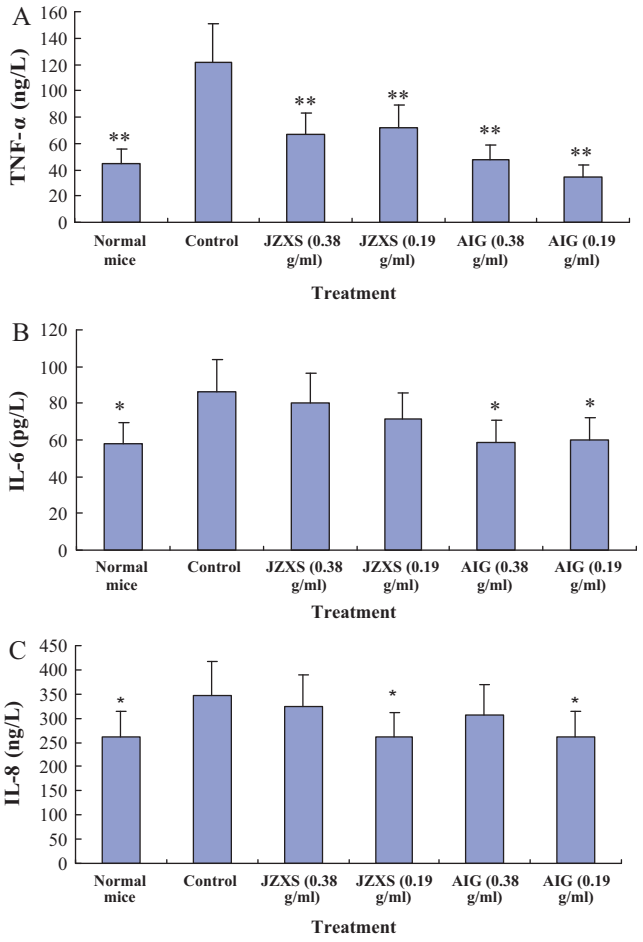

Fig. 2. TNF-α (A), IL-6 (B) and IL-8 (C) levels of each group in the carrageenin-induced peritonitis test. (n = 8–12, mean ± S.E.M.). *P < 0.05 and **P < 0.01 when compared with control (ANOVA followed by Dunnett's test).

(including TNF-α, IL-6 and IL-8) and mediators (including PGE_2 and NO).

Before animal experiments, to confirm the main active components of AIG, preliminary chemical assay was performed by spectrophotometric, GC, HPLC and potential titration methods. As a result, AIG mainly contained the pulegone (Liu et al., 2006), total flavonoids (including the luteolin), total saponins, total organic acid (including the chlorogenic acid), sodium sulfate and aluminium potassium sulfate.

From the ear edema test in normal mice, AIG as well as JZXS significantly prevented xylene-induced ear edema in a dose dependent manner after administration. The maximal inhibitions were both about 55.1% exhibited by AIG and JZXS at the concentration of 0.59 g powdered material per ml. However, when bilateral adrenals of the mice were removed, AIG and JZXS showed no effects on ear edema. Thus, it can be inferred that the anti-inflammatory effects of AIG and JZXS mainly depended on the system of pituitary-adrenal axis.

The results of leukocyte migration experiments showed that both AIG and JZXS at the concentration of 0.59 g powdered material per ml markedly decreased leukocyte counts after 1 h of CMC-Na injected, indicating AIG and JZXS can reduce leukocyte chemotaxis in inflammatory tissues thereby diminishing inflammation. But after that, the leukocyte counts increased fast. Thus, the characteristics, which were quick and short-term, of anti-inflammatory effects of AIG and JZXS were in accordance with that of JZXS in clinical application.

In inflammation process, a series of cytokines and inflammatory mediators contribute to evoking and regression of inflammation. As a mediator, a certain concentration of PGE_2 can increase inflammatory response synergized with bradykinin and leukotriene. In this study, both high and low concentrations of JZXS and the high concentration of AIG significantly reduced PGE_2 levels in inflammatory exudates, indicating that AIG and JZXS might exhibit their anti-inflammatory effects by means of the inhibition of cyclooxygenase (COX-1 and/or COX-2) expression, which ultimately led to the inhibition of the synthesis, release or action of PGE_2 involved in inflammation. Literatures (Wang et al., 2007; Yang et al., 2009) have reported that the luteolin and chlorogenic acid showed the inhibiting activities on PGE_2. Thus, the inhibition of PGE_2 activities by AIG and JZXS might be due to the action of total flavonoids (including luteolin) and total organic acid (including chlorogenic acid).

NO as another mediator and regulator of inflammatory responses is produced in high amounts by inducible nitric oxide synthase (iNOS) in activated inflammatory cells (Korhonen et al., 2005). COX-2 also can be affected directly at its enzymatic effect by nitric oxide and iNOS (Tsatsanis et al., 2006). From the results both AIG and JZXS at both concentrations showed significant effects on the mediator. It might be another mechanism of anti-inflammatory action. The literature (Kim et al., 1999) has reported that luteolin showed the inhibiting activities on iNOS and NO. Thus, the inhibition of NO activities by AIG and JZXS might be due to the action of total flavonoids (including luteolin).

Mast cell-derived pro-inflammatory cytokines, particularly TNF-α, IL-6 and IL-8, have critical biological roles to play in inflammation. These cytokines are released as pre-stored cytokines but can also be newly synthesized upon mast cell activation (Hide et al., 1997). The reduced level of pro-inflammatory cytokines released from mast cells is a key indicator of reduced inflammatory symptoms. In this study, we demonstrated that AIG and JZXS reduced the TNF-α, IL-6 and IL-8 levels in serum, in different degrees, which might be another mechanism of anti-inflammatory action. Literatures (Yang et al., 2009; Xagorari et al., 2001) have reported that luteolin and chlorogenic acid showed the inhibiting activities on TNF-α and IL-6. Thus, the inhibiting TNF-α and IL-6 activities by AIG and JZXS might be due to the action of total flavonoids (including luteolin) and total organic acid (including chlorogenic acid).

In conclusion, AIG obtained from JZXS with the yield 10.36 g/100 g of powdered material showed almost the same anti-inflammatory activities as JZXS, and the anti-inflammatory effects were dependent on the system of pituitary-adrenal axis, thereby inhibition of leukocyte migration, reducing cytokines (TNF-α, IL-6 and IL-8) and mediators (PGE_2 and NO), which contribute to evoking and regression of inflammation. Because the molecular mechanisms underlying inflammation are very complicated, further pharmacological evaluations are required to confirm more comprehensive anti-inflammatory mechanisms of AIG and JZXS.

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