

# The anti-inflammatory effects of Yunnan Baiyao are involved in regulation of the phospholipase A2/arachidonic acid metabolites pathways in acute inflammation rat model

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**Abstract.** The traditional Chinese medicine Yunnan Baiyao (YNB) has been reported to possess anti-inflammatory properties, however its mechanism of action remains unclear. It was previously reported that YNB ameliorated depression of arachidonic acid (AA) levels in a rat model of collagen-induced arthritis. In the current study, the capacity of YNB to ameliorate inflammation was compared in carrageenan-induced and AA-induced acute inflammation of the rat paw with celecoxib and mizolastine, respectively (n=24 per group). The capacity of YNB to affect the phospholipase A2 (PLA2)/AA pathway (using reverse transcription-quantitative polymerase chain reaction) and release of inflammatory lipid mediators (by ELISA) were investigated. Celecoxib ameliorated carrageenan-induced paw edema, and mizolastine ameliorated AA-induced rat paw edema. YNB alleviated paw edema and inhibited inflammatory cell infiltration in the two models. YNB inhibited production of 5-LOX AA metabolite leukotriene B4 (LTB4), and suppressed expression of 5-LOX, cytosolic PLA2 (cPLA2), 5-LOX-activating protein, and LTB4 receptor mRNA in the AA-induced inflammation model (P<0.05). YNB Inhibited the production of the COX-2 AA

metabolite prostaglandin E2 (PGE2) and suppressed expression of COX-2, cPLA2, PGE2 mRNA in the carrageenan-induced inflammation mode (P<0.05). Taken together, the data suggest that modulation of COX and LOX pathways in AA metabolism represent a novel anti-inflammatory mechanism of YNB.

## Introduction

Pathological autoimmunity, which can cause conditions such as arthritis, can be treated with glucocorticoids (GCs) and nonsteroidal anti-inflammatory drugs, however their use is often limited by the adverse effects they can cause and novel anti-inflammatory therapies are required (1,2). Traditional herbal medicine has been widely used for treating various inflammatory diseases in Eastern Asia (3,4). Yunnan Baiyao (YNB) is a widely used traditional Chinese herbal remedy, and has since been used to treat various conditions, including bleeding (5), ulcers (6), infection (7), hemangiosarcoma (8) and inflammatory bowel disease (9).

Liu *et al* (10) reported that YNB could reduce ulcer size and accelerate healing in aphthous stomatitis. In a mouse model of colitis, Li *et al* (9) also established that the immunosuppressive activity of YNB involved highly selective cytotoxicity towards B and T lymphocytes, and inhibition of tumor necrosis factor  $\alpha$  and interferon  $\gamma$  expression in the colonic mucosa and plasma. In a previous study, it was identified that YNB could reduce symptoms of rheumatoid arthritis in a mouse model (11). YNB-induced amelioration of foot paw swelling and inflammation was identified to be associated with reduced plasma levels of prostaglandin (PG) E2 (PGE2) and interleukin 1 $\beta$ , and plasma metabolomics analysis indicated that changes in circulating levels of arachidonic acid (AA) were recovered as a result of YNB administration (11).

The phospholipase A2 (PLA2)/AA pathway is a pivotal regulator of inflammatory responses. AA, liberated from the membrane phospholipids by PLA2, acts as a substrate for cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) to produce eicosanoids, including PGs and leukotrienes (LTs); important

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**Abbreviations:** 5-LOX, 5-lipoxygenase; AA, arachidonic acid; cPLA2, cytosolic PLA2; COX-2, cyclooxygenase 2; FLAP, 5-LOX-activating protein; GCs, glucocorticoids; LTB4, leukotriene B4; PGE2, prostaglandin E2; PLA2, phospholipase A2; YNB, Yunnan Baiyao

**Key words:** inflammation, Yunnan Baiyao, paw edema, cyclooxygenase, 5-lipoxygenase, traditional Chinese medicine

inflammatory mediators responsible for characteristic symptoms of inflammation. COX-2, is an inducible enzyme, which is mainly expressed under pathological conditions such as inflammation. Activation of the COX-2 pathway causes PG production. 5-LOX is a major isozyme component of the LOX pathway, which catalyzes biosynthesis of LTs from AA (12). Hence, inhibition of COXs and LOX can modulate AA metabolism and inhibit inflammation. Cytosolic PLA2 (cPLA2) serves an important role in liberation of AA from membrane phospholipids (13,14). Once AA is released, it is metabolized by COXs and 5-LOX pathways (15). The AA pathway generates potent inflammatory mediators: PGs and LTs (16). COX2 catalyzes the two step conversion of AA to PGH2. This transformation begins with formation of the endoperoxide PGG2, which is metabolized into PGH2. LOX catalyzes the incorporation of molecular oxygen into AA to generate 5-hydroperoxyeicosatetraenoic acid that is dehydrated into LTA4. LTA4 is further metabolized to bioactive LTs to LTB4 or LTC4 (17). Production of LTs from endogenous AA requires 5-LOX-activating protein (FLAP), which may serve an essential role in AA transfer to 5-LOX (18). FLAP acts as a scaffold for 5-LOX at the nuclear envelope, where it facilitates access of AA to 5-LOX (19). Gene knockout or pharmacological interference with FLAP fully abolishes the generation of 5-LOX-derived products implying its crucial role in LT biosynthesis (20,21).

As the PLA2/AA pathway is crucial to the pathogenesis of inflammatory diseases, it was hypothesized that the anti-inflammatory effects of YNB may be mediated via regulation of AA metabolism, particularly the COX and 5-LOX pathways. Therefore, the present study focused on the anti-inflammatory mechanisms of YNB, and compared them with those of a selective COX-2 inhibitor, celecoxib, and the histamine receptor antagonist, mizolastine, in two animal models of acute inflammation; carrageenan-induced and AA-induced rat paw edema, respectively (22,23).

## Materials and methods

**Animals.** A total of 192 Specific-pathogen-free male Sprague-Dawley rats, aged between 7 and 8 weeks-old (weight,  $244.01 \pm 10.7$  g), were provided by the Kunming Medical University Animal Center (Kunming, China). The animals were housed under a constant 12 h light/dark cycle in a temperature- and humidity-controlled room, and given free access to solid food and tap water for the duration of the study. Animals were allowed 7 days to adapt to the environment prior to commencement of the experiments. All procedures and animal experiments were approved by the Animal Care and Use Committee of Kunming Medical University (Kunming, China). The rats were sacrificed by cervical dislocation subsequent to blood collection from the abdominal aorta and all efforts were made to minimize suffering.

### Rat paw edema model construction and treatment

**AA-induced edema.** A total of 96 rats were randomly divided into four groups; the control group, model group, mizolastine group and YNB group. Mizolastine and YNB group animals received continuous intragastric administration of 0.3 mg/kg mizolastine (Xian-Janssen Pharmaceutical, Ltd., Beijing, China) or 50 mg/ml YNB (Yunnan Baiyao Group Co.,

Ltd., Kunming, China), respectively, for 7 days, as previously described (11). Model group and control group animals received the same volume of normal saline. A total of 2 h after intragastric administration on day 7, the model group, mizolastine and YNB group animals were subcutaneously injected with 0.1 ml AA (1 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) to the left paw, and injection of 0.1 ml carbonate buffer solution (AA vehicle) to the right paw, as previously described (23). The control group received no injection.

**Carrageenan-induced edema.** A total of 96 rats were randomly divided into four groups; the control group, model group, celecoxib group and YNB group. Celecoxib and YNB group animals received continuous intragastric administration of 10 mg/kg celecoxib (Pfizer, Inc., New York, NY, USA) or 50 mg/ml YNB, respectively, for 7 days, as previously described (24). Model group and control group animals received the same volume of normal saline. A total of 2 h after intragastric administration on day 7, the model group, mizolastine and YNB group animals were subcutaneously injected with 0.15 ml carrageenan ( $150 \mu\text{g paw}^{-1}$ ; Sigma-Aldrich; Merck Millipore) to the left paw and 0.15 ml saline to the right paw as a control. The control group received no injection (22).

**Edema measurements.** The volume (in ml) of the hind paws of control and treated animals were measured with a plethysmometer (model 7150; Ugo Basile, S.R.L., Monvalle, Italy) at 1, 2, 3 and 4 h after stimulus injection. Results are presented as the difference in volume between the test paw and the control paw for each animal at the time shown. Edema ( $\Delta V$ ) was calculated as follows:  $\Delta V = V_t - V_c$ , where  $V_t$  is the edema volume of the left paw;  $V_c$  is the edema volume of the right paw. Edema inhibitory activity was calculated according to the following formula. Percentage inhibition (%) =  $(\Delta V_{\text{model}} - \Delta V_{\text{treated}}) / \Delta V_{\text{model}} \times 100$  (25).

**Histological analysis.** Inflammation paws were collected and fixed in 70% ethanol for 24 h and then preserved in 10% formalin. Subsequently, the paws were dehydrated, blocked in paraffin and then sectioned with a microtome ( $5 \mu\text{m}$ ) (26). The sections were stained with haematoxylin and eosin for the evaluation of histopathological changes.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from edema soft tissue of inflamed and control paws using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RT reactions were performed using the PrimeScript™ 1st Strand cDNA synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China). An equal amount of total RNA ( $2 \mu\text{g}$ ) was used for cDNA synthesis according to the manufacturer's protocol. The reverse transcription reaction products were stored at  $-20^\circ\text{C}$ . Primers for COX-2, PGE2, cPLA2, 5-LOX, LTB4r, FLAP and  $\beta$ -actin are described in Table I. The PCR primers were designed by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). PCR amplification was carried out using the gene-specific PCR primers,  $2 \mu\text{l}$  cDNA,  $12.5 \mu\text{l}$  GoTaq® Green Master Mix 2X

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Pathway	Gene	Sequence (5'-3')		Product length (base pairs)
		Forward primer	Reverse primer	
5-LOX	5-LOX	ATCTGGGTGCGTTCAAGTG	ATGTCCTCGGTGTTGTCCAT	341
	LTB <sub>4</sub> r	TAGCTTTGTCTGTGGAGCA	CAACGTCCTTGTCTTGGGTGT	388
	cPLA2	GCACATAATAGTGGAACACC	ACACAGTGCCATGCTGAACC	530
	FLAP	GGCCATCGTCACCCTCATCA	GATGAGGCAGCGGGGAGA	590
	β-actin	GCCATGTACGTAGCCATCCA	GAACCGCTCATTGCCGATAG	190
COX	COX-2	TACCCGGACTGGATTCTACG	AGTCATCAGCCACAGGAGGA	224
	PGE2	GCATCTCATCTCTCCCAACG	ACTCGCAGCACACCATACAC	228
	cPLA2	GCACATAATAGTGGAACACC	ACACAGTGCCATGCTGAACC	224
	β-actin	GCCATGTACGTAGCCATCCA	GAACCGCTCATTGCCGATAG	323

5-LOX, 5-lipoxygenase; LTB<sub>4</sub>r, leukotriene B<sub>4</sub> receptor; cPLA2, cytosolic phospholipase A2; FLAP, 5-LOX-activating protein; COX, cyclooxygenase; PGE2, prostaglandin E2.

Table II. Effects of YNB on carrageenan-induced rat paw edema.

Group	Paw edema (ΔV) in ml (% inhibition)			
	1 h	2 h	3 h	4 h
Control group	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Model group	0.42±0.03 <sup>a</sup>	0.43±0.02 <sup>a</sup>	0.49±0.02 <sup>a</sup>	0.41±0.02 <sup>a</sup>
Celecoxib group	0.29±0.04 <sup>a,b</sup> (31)	0.22±0.03 <sup>a,b</sup> (50)	0.22±0.03 <sup>a,b</sup> (55)	0.20±0.03 <sup>a,b</sup> (51)
YNB group	0.37±0.02 <sup>a,b</sup> (12)	0.31±0.02 <sup>a,b</sup> (28)	0.29±0.02 <sup>a,b</sup> (41)	0.29±0.02 <sup>a,b</sup> (29)

Paw edema was measured from 1 to 4 h following injection. Values are presented as the mean ± standard error. The suppression rate is shown in parentheses. <sup>a</sup>P<0.05 vs. the control group; <sup>b</sup>P<0.05 vs. the model group. Each group at 1 h, n=24; each group at 2 h, n=24; each group at 3 h, n=12; each group at 4 h, n=12. YNB, Yunnan Baiyao.

Table III. Effects of YNB on arachidonic acid-induced rat paw edema.

Group	Paw edema (ΔV) in ml (% inhibition)			
	1 h	2 h	3 h	4 h
Control group	0.05±0.02	0.01±0.03	0.02±0.04	0.01±0.03
Model group	0.70±0.07 <sup>a</sup>	0.82±0.06 <sup>a</sup>	0.76±0.04 <sup>a</sup>	0.74±0.04 <sup>a</sup>
Mizolastine group	0.55±0.05 <sup>a,b</sup> (21)	0.67±0.04 <sup>a,b</sup> (18)	0.65±0.05 <sup>a,b</sup> (14)	0.63±0.03 <sup>a,b</sup> (15)
YNB group	0.57±0.05 <sup>a,b</sup> (19)	0.71±0.05 <sup>a,b</sup> (13)	0.65±0.06 <sup>a,b</sup> (14)	0.64±0.04 <sup>a,b</sup> (14)

Paw edema was measured from 1 to 4 h after injection. Values are presented as the mean ± standard error. The suppression rate is shown in parentheses. <sup>a</sup>P<0.05 vs. the control group; <sup>b</sup>P<0.05 vs. the model group. Each group at 1 h, n=24; each group at 2 h, n=24; each group at 3 h, n=12; each group at 4 h, n=12. YNB, Yunnan Baiyao.

(Promega Corporation, Madison, WI, USA) and 8.5 μl ddH<sub>2</sub>O. The reaction conditions for cPLA2, 5-LOX, LTB<sub>4</sub>r, FLAP and β-actin were 30 cycles, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 10 min. The reaction conditions for COX-2, PGE2, cPLA2 and β-actin were 30 cycles, denaturation at 95°C for 30 sec, annealing at

59°C for 30 sec and extension at 72°C for 10 min. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. Results are presented as ratios of target mRNA to β-actin mRNA. The band intensity was determined by Quantity One v4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

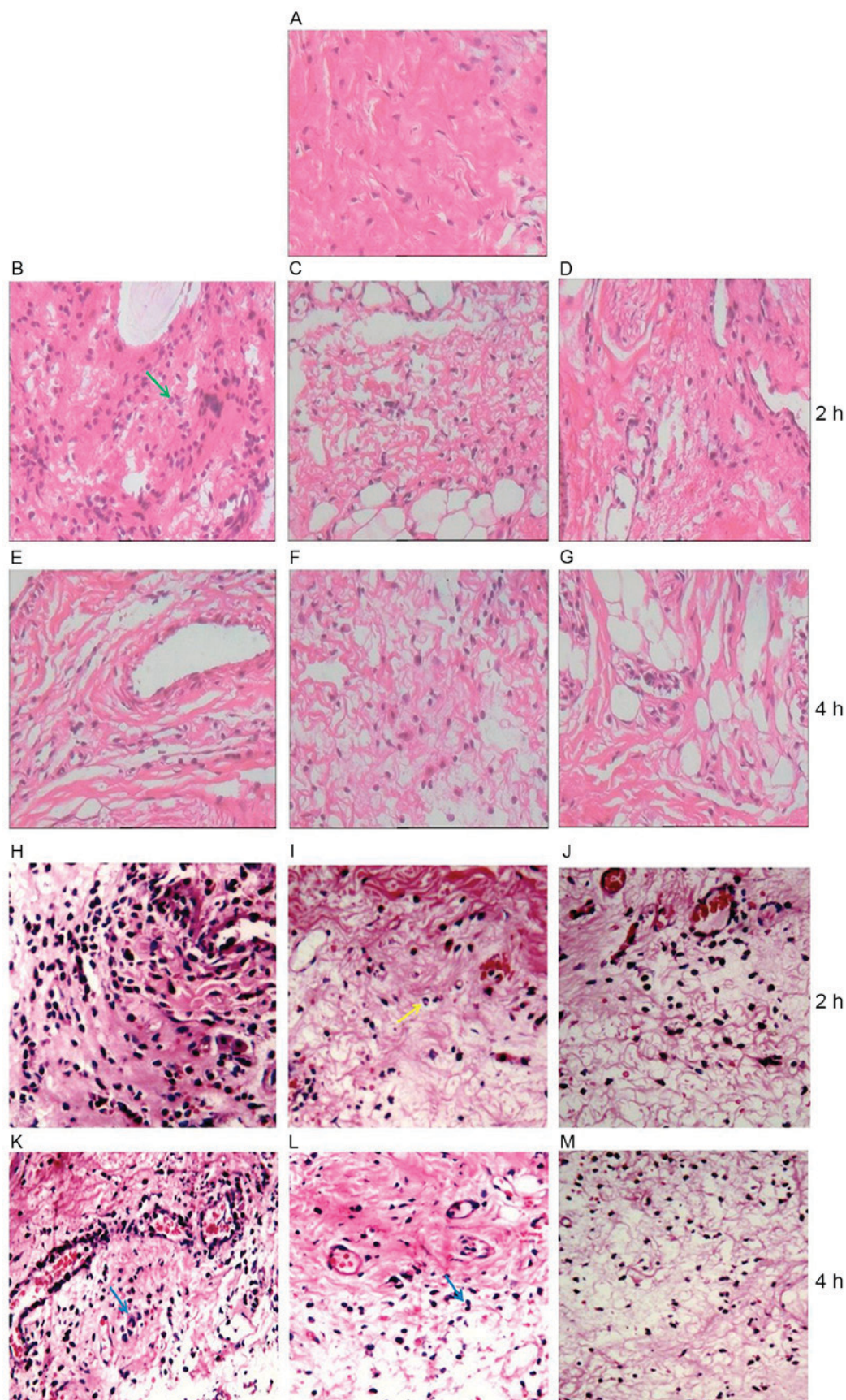


Figure 1. Paw inflammation in control, model and YNB, celecoxib and mizolastine rats. (A) H&E stain of control paw from control group. H&E staining of the inflamed paws was conducted in the carrageenan-induced inflammation model at (B-D) 2 h and (E-G) 4 h. Inflamed paws from the (B and E) model group, (C and F) celecoxib group and (D and G) YNB group are presented. H&E staining was additionally conducted in the arachidonic acid-induced inflammation model for (H-J) 2 h and (K-M) 4 h. The inflamed paws from the (H and K) model group, (I and L) mizolastine group and (J and M) YNB group are presented. Inflammatory cell infiltration was observed in the model groups. Inflammation was alleviated in animals administered with YNB, celecoxib and mizolastine. Green arrows indicate leukomonocytes, blue arrows indicate eosinophils and yellow arrows indicate neutrophils. n=12 in each group; magnification, x400. YNB, Yunnan Baiyao; H&E, hematoxylin and eosin.

**ELISA.** After rat paw volume was measured using a plethysmometer 1, 2, 3 and 4 h after injection. A total of 12 rats were randomly taken from each group at 2 and 4 h, anesthetized with 10% chloral hydrate, and sacrificed after blood was collected from the abdominal aorta. Rat blood was centrifuged for 10 min and the plasma was collected. ELISA kits (Sigma-Aldrich; Merck Millipore) were used to detect plasma levels of LTB<sub>4</sub>, cPLA<sub>2</sub> and 5-LOX in the AA-induced inflammation model and PGE<sub>2</sub>, cPLA<sub>2</sub> and COX-2 in the carrageenan-induced inflammation model, as instructed by the manufacturer.

**Statistical analysis.** The Experimental Data was analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). All data were presented as the mean  $\pm$  standard error and statistical analysis was performed using analysis of variance followed by Dunnett's test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**The acute anti-inflammatory effects of YNB in carrageenan-induced rat paw edema.** The acute anti-inflammatory effects of celecoxib and YNB were investigated in the model of carrageenan-induced edema. In rats administered with carrageenan, significant paw edema was observed from 1 h after injection to 4 h after injection in the sub plantar region of the paw. In animals administered celecoxib paw edema was significantly reduced by 31% at 1 h, and by 50-55% between 2 and 4 h after injection. In animals administered YNB paw edema was also significantly reduced by 12% at 1 h, 28% at 2 h, 41% at 3 h and 29% at 4 h (Table II), indicating that YNB has similar anti-inflammatory effects to celecoxib.

**The acute anti-inflammatory effects of YNB on AA-induced rat paw edema.** AA-induced rat paw edema was also used to evaluate anti-inflammatory activity. In rats administered with AA significant paw edema was observed from 1 h after injection to 4 h after injection in the sub plantar region of paw. In animals administered with mizolastine paw edema was significantly reduced by 21% at 1 h, and by 14-18% between 2 and 4 h after injection ( $P < 0.05$ , Table III). In animals administered with YNB paw edema was also significantly reduced; by 19% at 1 h, 13% at 2 h, 14% at 3 h and 14% at 4 h ( $P < 0.05$ , Table III), indicating that YNB has similar anti-inflammatory effects compared with mizolastine.

**YNB inhibited inflammatory cell infiltration.** In order to evaluate the protective effect of YNB on the histological changes induced in paw tissue during inflammation, the inflamed paw tissue was subjected to H&E staining (Fig. 1). Histopathological changes were observed in the paws of rats administered carrageenan and AA. In the paw tissues of animals in the control group (Fig. 1A) a small number of inflammatory cells were observed in the subcutaneous tissue by light microscopy. In the paw tissues of animals in the model groups (Fig. 1B, E, H and K) numerous inflammatory cells were observed in the subcutaneous tissue. In animals administered with YNB (Fig. 1D, G, J and M), celecoxib (Fig. 1I and L) or mizolastine (Fig. 1C and F) significantly reduced inflammatory cell

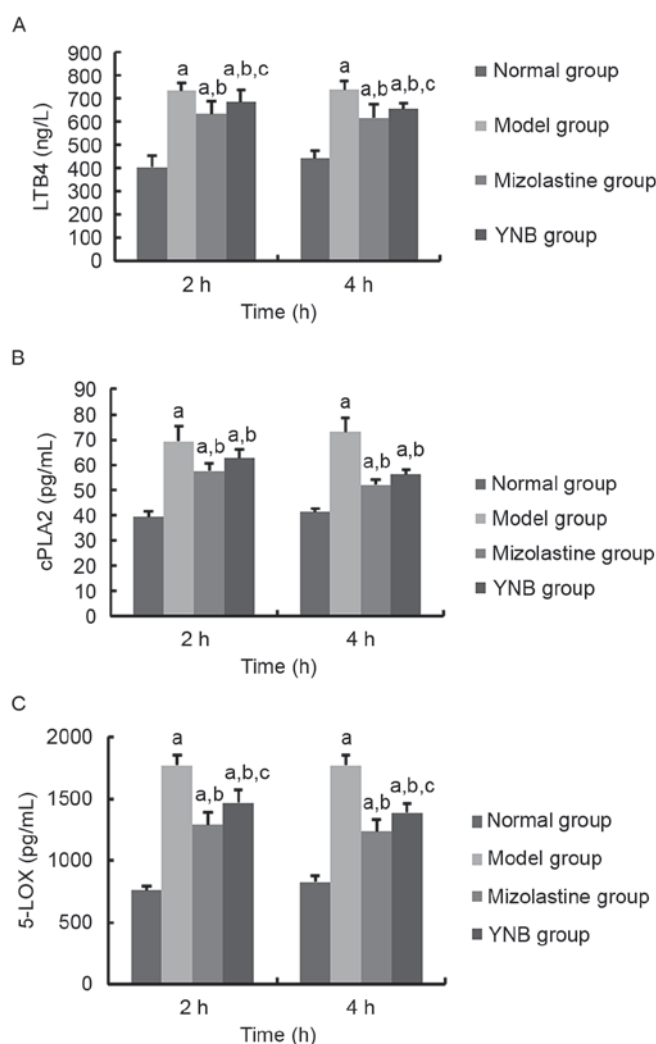


Figure 2. Basic serum biochemical parameters of control, model, YNB and mizolastine groups. Plasma levels of (A) LTB<sub>4</sub>, (B) cPLA<sub>2</sub> and (C) 5-LOX in the arachidonic acid-induced inflammation model were assessed by ELISA 2 and 4 h subsequent to model establishment. Values are presented as the mean  $\pm$  standard error. <sup>a</sup> $P < 0.05$  vs. the control group; <sup>b</sup> $P < 0.05$  vs. the model group; <sup>c</sup> $P < 0.05$  vs. the mizolastine or celecoxib groups. Each group,  $n = 12$ . YNB, Yunnan Baiyao; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; 5-LOX, 5-lipoxygenase.

infiltration was observed, indicating that YNB, celecoxib and mizolastine reduce inflammatory cell infiltration.

**YNB inhibited the plasma levels of 5-LOX AA metabolite pathway key enzymes and inflammatory lipid mediators in the AA-induced inflammation model.** In order to investigate the systemic cytokine response to AA-induced inflammation, plasma levels of LTB<sub>4</sub>, cPLA<sub>2</sub> and 5-LOX were measured at 2 and 4 h by ELISA. Plasma levels of these proteins were all elevated in the AA-induced inflammation model (Fig. 2), both mizolastine and YNB resulted in significantly reduced levels of LTB<sub>4</sub> (Fig. 2A;  $P < 0.001$ ), cPLA<sub>2</sub> (Fig. 2B;  $P < 0.001$ ) and 5-LOX (Fig. 2C;  $P < 0.001$ ), and levels were lower in the mizolastine group compared with the YNB group ( $P < 0.05$ ).

**YNB suppressed expression of 5-LOX, cPLA<sub>2</sub>, FLAP and LTB<sub>4</sub>r mRNA in the AA-induced inflammation model.** The capacity of YNB to suppress mRNA levels of key enzymes

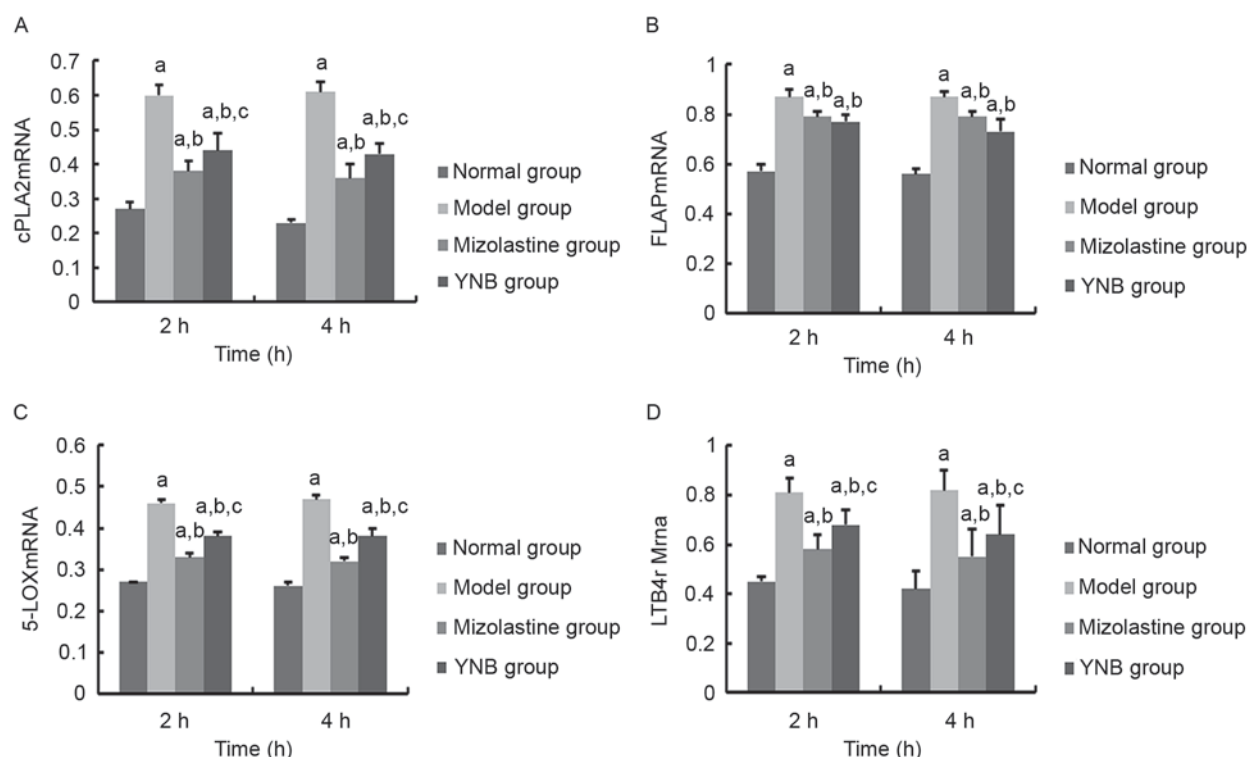


Figure 3. Expression of cPLA2 (A), FLAP (B), 5-LOX (C) and LTB4r (D) in arachidonic acid-induced edema model. mRNA levels were assessed by reverse transcription-quantitative polymerase chain reaction, 2 and 4 h following injection of arachidonic acid. Values are presented as the mean  $\pm$  standard error. <sup>a</sup> $P < 0.05$  vs. the control group; <sup>b</sup> $P < 0.05$  vs. the model group; <sup>c</sup> $P < 0.05$  vs. the mizolastine group.  $n = 12$  per group. cPLA2, cytosolic phospholipase A2; FLAP, 5-LOX-activating protein; 5-LOX, 5-lipoxygenase; LTB4r, leukotriene B4 receptor; YNB, Yunnan Baiyao.

of 5-LOX AA metabolite pathways was investigated. AA significantly increased levels of 5-LOX, cPLA2, FLAP and LTB4r mRNA in the model group (Fig. 3A-C;  $P < 0.001$ ) compared with the control group. Model group and YNB significantly decreased levels of cPLA2 (Fig. 3A;  $P < 0.001$ ), FLAP (Fig. 3B;  $P < 0.01$ ), 5-LOX mRNA (Fig. 3C;  $P < 0.001$ ) and LTB4r (Fig. 3D;  $P < 0.01$ ) compared with the model group. The suppressive effect of mizolastine was stronger than that of YNB on expression of cPLA2 (Fig. 3A;  $P < 0.01$ ), 5-LOX (Fig. 3C;  $P < 0.001$ ) and LTB4r (Fig. 3D;  $P < 0.05$ ).

*YNB inhibited the plasma levels of COX-2 AA metabolite pathway key enzymes and inflammatory lipid mediators in the carrageenan-induced inflammation model.* To investigate the systemic cytokine response to carrageenan induced inflammation, plasma levels of PGE2, cPLA2 and COX-2 were measured at 2 and 4 h by ELISA. Plasma levels of these proteins were all elevated in the carrageenan-induced inflammation model (Fig. 4), both celecoxib and YNB significantly reduced levels of PGE2 (Fig. 4A;  $P < 0.001$ ) in 4 h, cPLA<sub>2</sub> (Fig. 4B;  $P < 0.001$ ) and COX-2 (Fig. 4C;  $P < 0.001$ ) and levels were lower in the celecoxib group compared with the YNB group ( $P < 0.001$ ).

*YNB suppressed expression of COX-2, cPLA2, PGE2 mRNA in the carrageenan-induced inflammation model.* The capacity of YNB to suppress mRNA levels of key enzymes of the COX-2 AA metabolite pathway was investigated. Injection of carrageenan significantly increased plasma levels of COX-2, cPLA2 and PGE2 mRNA compared with the control group

(Fig. 5;  $P < 0.001$ ). Administration of celecoxib significantly reduced levels of COX-2, cPLA2 in the 2 h group, and PGE2 mRNA compared with model group (Fig. 5;  $P < 0.001$ ). Administration of YNB also significantly reduced levels of COX-2 (Fig. 5A;  $P < 0.001$ ), PGE2 (Fig. 5;  $P < 0.001$ ) and cPLA2 (Fig. 5C;  $P < 0.001$ ) in the 2 h group. The suppressive effect of celecoxib was stronger than that of YNB on expression of COX-2 ( $P < 0.05$ ) in the 2 h group, cPLA2 ( $P < 0.001$ ) in 4 h group and PGE2 ( $P < 0.05$ ) (Fig. 5).

## Discussion

Glucocorticoids (GCs) and nonsteroidal anti-inflammatory drugs can effectively reduce inflammation; however adverse effects limit their use. Therefore, efforts have been made to identify novel anti-inflammatory therapies. YNB a herbal medicine, has been used to treat various conditions, including bleeding, ulcers, infections, hemangiosarcoma and inflammatory bowel disease. In a previous study, the effect of YNB was investigated in a mouse model of collagen-induced arthritis, and it was identified that YNB significantly ameliorated the elevated plasma levels of AA (11). It was thus hypothesized that YNB may interfere with AA metabolic pathways. Thus, in the current study, two traditional models of acute inflammation were used, carrageenan-induced paw edema and AA-induced paw edema, to evaluate the effect of YNB on AA metabolic pathways.

Carrageenan-induced paw edema is a well-established model for evaluating the biphasic events of acute inflammation. In the early phase, 0-2 h subsequent to carrageenan

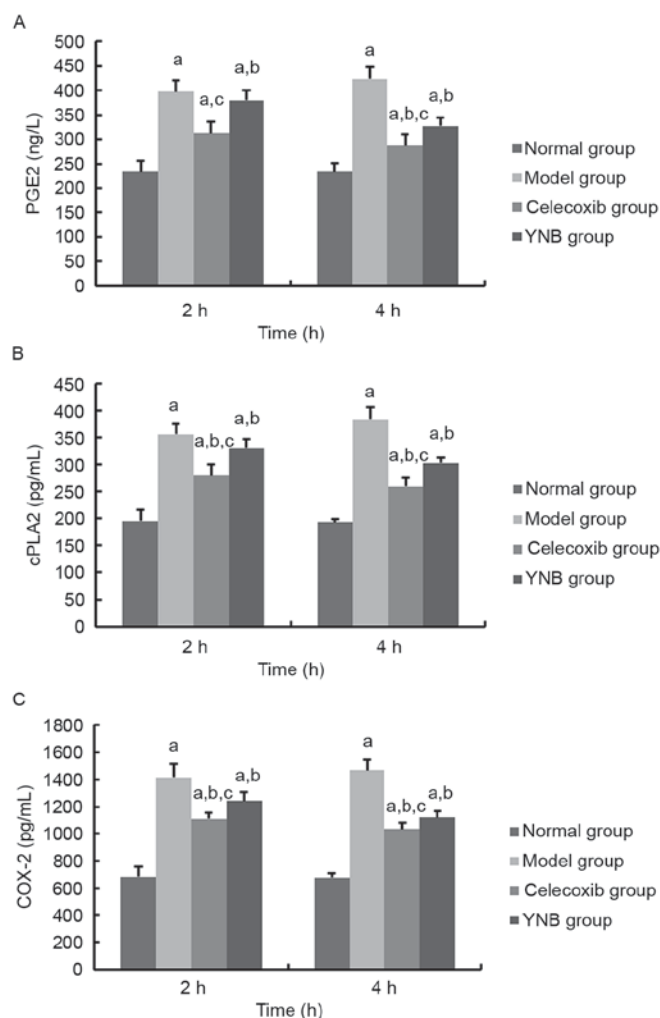


Figure 4. Basic serum biochemical parameters of control, model, YNB and celecoxib groups. Plasma levels of (A) PGE2, (B) cPLA2, (C) COX-2 in the carrageenan-induced inflammation model were assessed by ELISA 2 and 4 h subsequent to model establishment. Values are presented as the mean  $\pm$  standard error. <sup>a</sup> $P < 0.05$  vs. the control group; <sup>b</sup> $P < 0.05$  vs. the model group; <sup>c</sup> $P < 0.05$  vs. the celecoxib group.  $n = 12$  per group. PGE2, prostaglandin E2; cPLA2, cytosolic phospholipase A2; COX-2, cyclooxygenase 2; YNB, Yunnan Baiyao.

injection, histamine release induces vascular permeability. The second phase subsequent to injection of carrageenan is predominantly attributed to enhanced production of PGs. Inflammatory edema has been previously reported to peak 3 h following carrageenan injection (27), which was supported by the results of the current study; paw edema was observed in rats administered carrageenan injection after 1 h, and edema peaked at 3 h and increased local inflammatory cell infiltration. The selective COX-2 inhibitor, celecoxib, ameliorated carrageenan-induced paw edema. AA-induced hind paw edema is another model of acute inflammation, and is ameliorated by the histamine receptor antagonist, mizolastine, however not by COX-inhibitors (28). This model is thus used to investigate the mechanisms of lipoxygenase inhibition. In the present study, paw edema was observed 1 h after AA injection, and peaked after 2 h. Histological analysis indicated increased local inflammatory cell infiltration. AA is predominantly metabolized via the COX-2 and the 5-LOX pathways, leading to the formation of prostanoids and LTs, respectively.

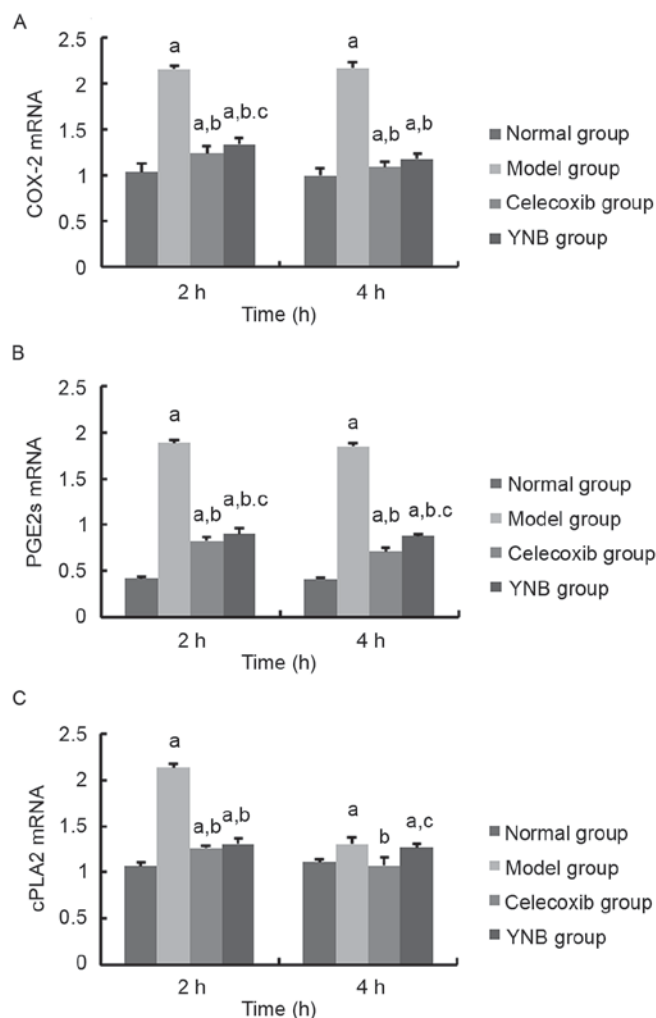


Figure 5. Expression of (A) COX-2, (B) PGE2, (C) cPLA2 in carrageenan-induced edema model. mRNA levels were assessed by reverse transcription-quantitative polymerase chain reaction, 2 and 4 h subsequent to injection of carrageenan. Values are presented as the mean  $\pm$  standard error. <sup>a</sup> $P < 0.05$  vs. the control group; <sup>b</sup> $P < 0.05$  vs. the model group; <sup>c</sup> $P < 0.05$  vs. the celecoxib group.  $n = 12$  per group. COX-2, cyclooxygenase 2; PGE2, prostaglandin E2; cPLA2, cytosolic phospholipase A2; YNB, Yunnan Baiyao.

The carrageen-induced paw edema and AA-induced paw edema activate the COX and 5-LOX pathways, respectively. Thus, these two models were used to investigate whether YNB can alleviate inflammation by inhibiting COX-2 and 5-LOX AA metabolites pathways.

cPLA2 induces AA release from cellular membranes. Blockade of PLA2 thus can reduce inflammation, and PLA2 inhibitors have become a pharmaceutical research focus (29). It was observed that carrageen and AA markedly elevated plasma levels of cPLA2. As predicted, YNB alleviated cPLA2 generation in the two models. Furthermore, YNB significantly reduced levels of cPLA2 mRNA in the paw. These data indicated that YNB could suppress expression of cPLA2 in the transcriptional level and explained why it enabled recovery of AA to normal levels in a previous study (11).

In the present study, it was identified that YNB alleviated paw edema and inhibited inflammatory cell infiltration in the two models. In order to investigate the mechanism of YNB action, the expression of inflammatory mediators was

investigated in the two models. In rats administered with carrageenan, elevated circulating levels of PGE<sub>2</sub>, cPLA<sub>2</sub> and COX-2 were observed, and elevated levels of COX-2, cPLA<sub>2</sub>, PGE<sub>2</sub> mRNA were identified in the paw. YNB inhibited the production of COX-2 AA metabolites, and suppressed expression of COX-2, cPLA<sub>2</sub> and PGE<sub>2</sub> mRNA although less effectively than that of celecoxib. In the present study, it was illustrated that carrageenan markedly activated the COX-2 pathway, while YNB ameliorated carrageenan-induced expression of PGE<sub>2</sub> synthase, and reduced production of PGE<sub>2</sub>, which is consistent with the results of a previous study (11). In contrast, AA activated the 5-LOX pathway. In rats administered with AA we observed elevated circulating levels of LTB<sub>4</sub>, cPLA<sub>2</sub> and 5-LOX, and elevated expression of cPLA<sub>2</sub>, FLAP, 5-LOX and LTB<sub>4</sub>r in the paw. YNB inhibited production of 5-LOX AA metabolites and suppressed the expression of 5-LOX, cPLA<sub>2</sub>, FLAP and LTB<sub>4</sub>r mRNA, although less effectively than that of mizolastine. COX-2 and 5-LOX are dual inhibitors that act on the two major AA metabolic pathways, and have been previously reported to possess superior anti-inflammatory activities (30-32). The data of the current study indicate that YNB can alleviate inflammation by inhibiting COX-2 and 5-LOX pathways. LTs serve a key role in the pathophysiology of inflammatory diseases and host defense reactions. LTB<sub>4</sub>, a potent chemotactic and chemokinetic mediator, can stimulate migration and activation of granulocytes to induce granulocyte adherence to vessel walls, degranulation, release of superoxide and lysosomal enzymes. Furthermore, LTB<sub>4</sub> is associated with increased interleukin production and transcription (33). However, the effects of LTB<sub>4</sub> are all mediated through binding to its receptor (LTB<sub>4</sub>r). The current study observed that YNB significantly ameliorated elevated plasma levels of LTB<sub>4</sub> and LTB<sub>4</sub>r expression in the inflamed paw. LT synthesis requires FLAP, an 18 kDa membrane-bound protein (34). 5-LOX and FLAP are both required for cellular LT synthesis (35). Thus, FLAP expression was analyzed in the inflamed paw. YNB markedly suppressed the expression of FLAP, suggesting that the anti-inflammatory effect of YNB may be partly caused by FLAP inhibition. As LT formation was not an absolute requirement for FLAP, and FLAP can also stimulate the utilization of exogenous AA by 5-LOX, FLAP can bind AA and other cis-unsaturated fatty acids facilitating the presentation of substrate for 5-LOX for optimal synthesis of LTA<sub>4</sub>. Therefore, the inhibitory effect of YNB on LTB<sub>4</sub> may reflect inhibition of FLAP expression. Furthermore, LTB<sub>4</sub> is a potent chemotactic factor for neutrophils, stimulates adhesion of leukocytes to vascular endothelia for extravasation into adjacent tissue, and induces neutrophil degranulation (36). These observations may explain why YNB reduced inflammatory cell infiltration in inflamed paw tissue.

YNB can be prepared in several ways (powder, capsule, aerosol and tincture) for local or oral application. Although the precise herbal formula of YNB is a trade secret, the product is now labeled to identify the major components of each 0.5 g serving (37). The 2011 manufacturer's label listed the following compounds; 200 mg Tienchi ginseng root (*Panax notoginseng*), 85 mg *Ajuga forrestii* Diels plant, 66.5 mg Chinese yam root, 57.5 mg *Dioscorea nipponica* Makino root, 36 mg *Erodium stephanianum* and *Geranium wilfordii* plant, 30 mg *Dioscorea parviflora* ting root and 25 mg *Inula cappa*

plant (5). Which of these components is responsible for the anti-inflammatory activities observed in the present study remains unclear.

In conclusion, YNB alleviated inflammation by inhibiting activation of key AA metabolism enzymes including cPLA<sub>2</sub>, COXs, PGE<sub>2</sub> and 5-LOX, and release of AA pathway-generated inflammatory lipid mediators in two rat models of inflammation. The current study suggests that YNB may modulate cPLA<sub>2</sub>/AA pathways to reduce inflammation. These results provide a theoretical foundation for the clinical application of YNB in inflammatory diseases.

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