STUDY OF PHARMACOLOGICAL EFFECTS OF SAPONINS IN GANCAOFUZI DECOCTION BY CLASSIC ANIMAL DISEASE MODELS

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ABSTRACT

Gancaofuzi decoction is the traditional Chinese medicine prescription for the treatment of rheumatic diseases, which consists of R.glycyrrhizae, A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata. The saponins are the main pharmacodynamics compositions in Gancaofuzi decoction for their various. In this study, analgesic, anti-inflammatory and immunomodulatory effects of the saponins components in Gancaofuzi decoction were researched through several classic animal disease models. The results showed that the saponins played a better role on anti-inflammatory and immunomodulatory. This study is of great significance for exploring the pharmacodynamics basis of traditional Chinese medicine. And the study provides a theoretical basis for further study on the compatibility mechanism of traditional Chinese medicine compound.

Keywords: *Gancaofuzi decoction, saponins, analgesic, anti-inflammatory, immunomodulatory.*

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Introduction

Traditional Chinese Formula is an important traditional Chinese medicine's means which is used to treat the diseases in the clinical. The classic Chinese Formula Gancaofuzi decoction plays a role in the treatment of rheumatoid arthritis by restoring antioxidant activity, inhibiting the synthesis of inflammatory cytokines, regulating immune activity and reducing focal bone erosion and bone destruction(1-3). Gancaofuzi decoction is composed of R. glycyrrhizae, A. macrocephalae Rhizoma, Cinnamomum cassia Presl and Radix Aconiti Praeparata(4).

Recently, the study of Gancaofuzi decoction is mainly about its chemical composition and pharmacological effects. The major active ingredients of Gancaofuzi decoction are flavonoids and saponins in R.glycyrrhizae, alkaloids in Radix Aconiti Praeparata. While Cinnamomum cassia Presl and macrocephalae Rhizoma mainly contain essential oil(5-6). Gaos' study demonstrated that Gancaofuzi decoction contained 12 kinds of chemical constituents possessing bioactivities which came from Radix Aconiti Praeparata, A. macrocephalae Rhizoma, R.glycyrrhizae, Cinnamomum cassia Presl(7). In recent years, the saponins being the main composition of Gancaofuzi decoction are concerned for their various pharmacological effects(8-11). The main saponin of Gancaofuzi decoction is Glycyrrhizic Acid for its various farmercutical effects such as its in vivo anti-inflammatory activity(12). Such study could provide a theoretical basis for the further study of compatibility of traditional Chinese medicine from the perspective of intestinal absorption(13-25).

In this study, analgesic, anti-inflammatory and immunomodulatory effects of the saponins components in Gancaofuzi decoction were researched through several classic animal disease models. It is of great significance for exploring the pharmacodynamics basis of traditional Chinese medicine.

Material and methods

Materials

R. glycyrrhizae, A. macrocephalae Rhizoma and Cinnamomum cassia Presl were purchased from Tongrentang Pharmacy of Changchun; Radix Aconiti Praeparata was purchased from Jiangyou Pieces plant of Sichuan; Dulbecco's modified Eagle's medium (DMEM) was purchased from Corning Corporation(USA); Heat-inactivated fetal bovine serum (FBS) was purchased from Biological Industries Israel Beit Haemek ltd (USA); MTT, Trypsin and Penicillin were purchased from Dingguo Corporation (Beijing, China); Streptomycin was purchased from Glenview Corporation (USA); Acetic acid was purchased from Beijing Chemical Plant; Carrageenan was purchased from Nanjing Jiancheng Biological Products Co., Ltd.; complete Freund's adjuvant was purchased from Sigma, USA; mouse macrophage RAW 264.7 was purchased from Shanghai University Library of Chinese Academy of Sciences; Mouse prostaglandin E2 (PGE2), malondialdehyde (MDA) kit was purchased from Nanjing Jiancheng Technology Co., Ltd.; Mouse tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin 2 (IL-2) enzyme-linked immunosorbent kit was purchased from Nanjing Jiancheng Biological Products Co., Ltd.; The rat TNF-α, IL-1β enzyme-linked immunosorbent kit was purchased from Nanjing Jiancheng Biological Products Co., Ltd.

MCO-175 CO2 incubator was purchased from SANYO (Japan); high speed refrigerated centrifuge was purchased from Eppendorf (Germany); enzyme mark instrument was purchased from TECAN (Australia); 96-well culture plate was purchased from Thermo (USA); transwell cell culture plate (1.12cm surface, 0.4μm pore size, 12mm diameter) was purchased from Corning Costar Corporation (USA).

Sample preparation

The Gancaofuzi decoction was extracted twice with boiling water, 40 minutes for the first time and 30 minutes for the second time, and the solutions were filtered through centrifugation. Then, the filtrates were precipitated by 60% alcohol to remove the polysaccharide.

And the filtrates were concentrated to be freeze-dried, then we got the compound. Next, the compond was purified by D101 macroporous resin and ethanol, then we got the saponins whose purity reach 51% through concentrated to be freeze-dried. Followed, these dried extracts were dissolved in DMEM to obtain the working standard solutions to be used in the study of MTT and cell experiment. When we uesd them to animal experiment, the dried extracts were dissolved in water and converted according to the clinical dose of 60 kg adults. And the model group(MX) and the blank group(KB) were supplied by equal amount of water.

Methods

Mouse acetic acid writhing experiment

The acetic acid writhing method is a classic model for screening peripheral analgesics[13-14]. When a mouse with intraperitoneal injection of acetic acid solution has a typical abdominal depression, accompanied by characteristic changes such as torso twist, buttocks elevation and hind limb elongation, it is calculated as a twist. The frequency of the twist of mice between model group and medicine group was counted within 20 min after intraperitoneal injection of acetic acid solution to evaluate the strength of the analgesic effect of the drug. This experiment is simple and reproducible, so it is a common model for evaluating and screening analgesic drugs.

18-22 g male mice were selected for adaptive feeding, and the mice were randomly divided into model group (MX), compound administration group (FF), and saponin group (ZG), with 10 mice in each group. 0.1 mL/10 g body weight was administered by intragastric administration for 8 days. After 1 hour of the last administration, 0.6% acetic acid solution (currently used) was intraperitoneally injected, and the number of writhing of the mice was observed within 20 minutes after the injection. The writhing reaction inhibition rate (%) = (the average writhing number of the model group - the average writhing number of the administration group)/the average writhing number of the model group × 100%.

Mouse hot plate test

The plantar of the mouse touches the hot plate, and the pain response is generated by heat stimulation. The time required for the pain response (latency period) is the pain threshold. The analgesic effect of the drug is reflected by the change of pain threshold (pain threshold discrepancy). This method is simple in operation, convenient and quick to test, the index response is clear[26]. And the incubation period is longer of the pain response, so that the slight difference between the drugs can be compared, so as to compare the strength, the speed and the duration of the analgesic effect of the drug, which is one of the most common methods used at present.

18-22 g female mice were selected for adaptive feeding. Before the experiment, the hot plate instrument was turned on in advance, and the temperature was set at 55 °C±0.2 °C. The qualified mice were first screened. The lick hindfoot time was less than 5 s or more than 60 s, and the hopper were skipped. The screened mice were randomly divided into a blank group (KB), a compound administration group (FF), and a saponin group (ZG), with 10 mice in each group. The mice were administered with a body weight of 0.1 mL/10 g for 7 days, and the pain threshold was measured at 0 min, 30 min, 60 min and 90 min after the last dose. When the mouse still had no reaction during the measurement for 60 s, the mice should be taken out in order to avoid scald, and the pain threshold was calculated as 60 s.

Acute inflammation model caused by carrageenan

The mouse foot swelling model is an acute inflammation model with increased vascular permeability as the main lesion for observing the effect of the test drug on the acute inflammatory process[27, 28]. Carrageenan, a pro-inflammatory agent, can increase the synthesis of prostaglandins (PG) and cause local edema with vasoactive amines and peptides.

After subcutaneous injection of carrageenan into the right hind limb of the mouse, the degree of swelling of the foot was measured by volumetric method, and the swelling rate and the inhibition rate of the drug on the swelling were calculated, which can reflect the anti-inflammatory effect of the test drug. PGE2 and MDA in mouse serum were detected by PGE2 kit and MDA kit to investigate the therapeutic effects of drugs on inflammation and oxidative damage.

18-22 g male mice were selected for adaptive feeding and randomly divided into saline blank group(KB), carrageenan model group(MX), compound administration group (FF), saponin group (ZG). The mice were intragastrically administered for 7 days, and the initial foot volume was measured before the administration of the stomach on the 7th day. The physiological saline solution was injected subcutaneously in the right hind foot pad 30 minutes after the administration or 0.5% carrageen prepared in the previous day and stored at 4 °C.

The paw volume of each mouse was measured at 30 min, 60 min, 90 min, 120 min, 180 min, 240 min after injection of 20 μL of carrageenan, and the effect of the drug-administered groups on the swelling of the mouse foot at different time points were observed: swelling rate (%)) = (paw volume of post-inflammatory - paw volume of pro-inflammatory)/paw volume of pro-inflammatory × 100%. After 240 min, the eyeballs were taken for blood. The blood was centrifuged at 4000 r / min for 15 min at 4° C. The supernatant was taken and frozen stored at -80° C for detected by PGE2 and MDA kits.

Rat adjuvant arthritis model

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic, progressive, symmetrical and aggressive peripheral joint destruction. It is one of the most common disabling diseases. Establishing a simple and reliable animal disease model is an important means to study the pathogenesis of RA and evaluate the role of therapy drug[29, 30]. In this experiment, complete Freund's adjuvant was used to induce adjuvant arthritis (AA) in rats.

After subcutaneous injection of complete Freund's adjuvant in the rat's left hind paw pad, AA was induced. The paw volume was measured before and after administration. The degree of joint swelling was evaluated by arthritis scoring method. ELISA was used to detect TNF and IL-1β content in rat serum. Then the therapeutic effect of each drug on adjuvant arthritis in rats was finally evaluated.

180-200g male rats were selected for adaptive feeding, and the rats were randomly divided into model group (MX), compound administration group (FF), and the saponin group (ZG), with 10 rats in each group injected by complete Freund's adjuvant.. After the model was established for 14 days, continuous intragastric administration sustained for 21 days. After 24 hours of the last administration, the blood, joints and internal organs were decapitated. The blood samples were centrifuged at 4 °C at 4000 r/min for 15 mins, and the supernatant was taken and stored frozen at -80 °C.

Establishment and dosing protocol for immunomodulatory experiment

TNF-α (tumor necrosis factor-α) is mainly secreted by macrophages, which could promote the differentiation of T-cells and participate in the immune regulation of the body. Interleukin (including IL-1, IL-2) secreted by T-lymphocyte and mcrophages, could specific exert immunomodulatory effects, and play a certain role in the inflammatory response[31]. Locally low concentrations of IL-1 mainly plays an immunomodulatory role and can cause the release of inflammatory mediators; a large amount of IL-1 secretion can cause systemic reactions through the

blood circulation, such as the hypothalamus can cause fever, with strong heat effect. IL-2 can participate in the body's immune response, such as active of T cells, promote the production of cytokines; stimulate the proliferation of NK cells, enhance NK killing activity and production of cytokines, induce the production of LAK cells; promote B-cell proliferation and secretion of antibodies.

The mouse macrophage RAW 264.7 was prepared into the cell suspension, seeded in a 96-well plate at a density of 2×10 4 cells/well, cultured in a carbon dioxide incubator for 24 h.

Then the old culture solution was discarded, and the drug solution was added to each drug-administered group for 48 h. The cell supernatant was aspirated, centrifuged at 3000 r/min for 20 min, and the supernatant was carefully collected. The contents of TNF-α, IL-1 and IL-2 secreted by mouse macrophage RAW 264.7 were detected by the ELISA kits. Thus, the immunomodulatory effects of each administration group were examined.

Results and analysis

Inhibition effect on acetic acid-induced writhing in mice

The mouse acetic acid writhing experiment was carried out according to the method in mouse acetic acid writhing experiment.

The effects of each group on the writhing frequency of mice were shown in Table 1. As can be seen from the results, compared with the model group, the number of writhings was reduced after administration and there was a significant difference.

That is to say the compound and the saponins had good analgesic effect. In addition, the compound administration group had the highest inhibition rate on the number of writhing in mice, indicating that the analgesic effect of the compound was better than that of the saponins administration group.

The results indicated that the main analgesic effect ingredients in Gancaofuzi decoction are not the saponins.

Groups	Writhing times in 20mins	Inhibition rate
MX	35.71±9.79	
FF	16.44±3.17*	58.66%
ZG	22.88±10.36*	35.79%

Table 1: Mouse writhing times of each group ($\bar{x}$±SD, n=10).
*Note: *P<0.05 is a significant difference between each group and the blank group.*

Inhibition of hot plate induced pain in mice experiment was carried out according to the method in mouse hot plate test

The effects of the drug groups on the pain threshold and pain threshold difference in mice were shown in Table 2.

It can be seen that there was no significant change in the pain threshold of the blank group between 0 to 90 mins. The pain threshold of the mice in each administration group increased compared with that before administration.

That is to say the compound and the saponins had good analgesic effect. The analgesic effect of the compound administration group was best in at 60 min after administration, while the saponin administration group had the best analgesic effect at 90 min.

Groups	Pain threshold before administration (s)	Pain threshold difference (s)		
		30min	60min	90min
KB	13.58±4.08	0.99±0.70	0.50±0.20	0.68±0.22
FF	13.86±4.49	3.68±1.16*	7.85±2.62*	4.34±1.70
ZG	13.97±6.91	5.00±2.55*	8.78±2.82*	12.40±3.89*

Table 2: Effects on pain threshold in mice ($\bar{x}$±SD, n=10).
*Note: *P<0.05 is a significant difference between each group and the blank group.*

Inhibition of acute inflammation in mice induced by carrageenan

Effects of drugs on swelling rate inhibition of mouse paw

The acute inflammation test of mouse carrageenan was carried out according to the method in acute inflammation model caused by carrageenan. The effects of each group on the swelling rate of the paw of mice were shown in Table 3.

As can be seen from the results, compared with the blank group, the mice in the model group and each of the drug-administered groups experienced different degrees of foot swelling. Compared with the model group, the mice in each drug-administered group had a low rate of swelling, indicating that the compound and the saponin group had an anti-inflammatory effect.

In the compound administration group, the inhibition of mouse paw swelling was good at all times. But during 30 min-120 min, the inhibition of mouse foot swelling by saponin administration group was better than that of compound administration group, indicating saponin component could play a better anti-inflammatory effect.

Groups	30min	60min	90min	120min	180min	240min
MX	14.55±1.32	13.64±0.97	11.36±0.65	9.09±0.74	9.55±0.78	5.91±0.49
FF	4.03±0.78*	5.86±0.65*	7.33±0.44	6.59±0.41	6.59±0.69	4.03±0.36
ZG	2.49±0.65*	3.73±0.11*	5.81±0.77*	6.64±0.98	7.05±0.54	4.98±0.28

Table 3: Effects of drugs on swelling rate inhibition of mouse paw ($\bar{x}$±SD, n=10).
*Note: Note: *P<0.05 is a significant difference between each group and the model group.*

Effects of drugs on the contents of MDA and PGE2 in serum samples of mice

Malondialdehyde (MDA) is the body's production of oxygen free radicals through enzyme and non-enzymatic systems, and oxygen free radicals attack polyunsaturated fatty acids in biofilms, triggering lipid peroxidation, and thus produce lipid peroxides . The final product of lipid peroxidation is mainly malondialdehyde (MDA), which can reflect the degree of lipid peroxidation in the body and indirectly reflect the degree of cell damage. Prostaglandin E2 (PGE2) is the main inflammatory factor in the process of acute inflammation. It causes vasodilatation, fever, pain and other symptoms during inflammation, and its content can reflect the degree of inflammatory damage. As shown in Table 4, the results of detection of MDA and PGE2 in serum samples of mice in each of the drugs were shown. It can be seen from the table that compared with the model group, each administration group could significantly reduce the content of MDA and PGE2 in the serum of mice, thereby alleviated the acute inflammatory reaction and oxidative damage caused by carrageenan, indicating that the compound and saponin components both have anti-inflammatory and antioxidant effects. Compared with the compound administration group, the saponin component did not exert a better effect on reducing the MDA content, but played a better role in reducing the PGE2 content. Perhaps the saponin component was the main anti-inflammatory effect ingredient of the Gancaofuzi decoction.

Groups	MDA (nmol/ml))	PGE2 (ng/L)
KB	3.30±0.02	19.48±0.03
MX	5.09±0.20	52.42±0.15
FF	3.48±0.04*	38.19±0.11*
ZG	3.62±0.06*	25.50±0.04*

Table 4: Effects of drugs on the contents of MDA and PGE2 in serum samples of mice ($\bar{x}$±SD, n=8).
*Note: *P<0.05 is a significant difference between each group and the model group.*

Inhibition on rat adjuvant arthritis model

The degree of joint swelling

The rat adjuvant arthritis experiment was carried out according to the method in rat adjuvant arthritis model. The body weight and the degree of joint swelling of each group were measured pre-modeling and after modeling. And the weight gain and paw swelling rate of the rats on the 35th day of modeling and pre-modeling were calculated. The results were shown in Figures 1 and 2.

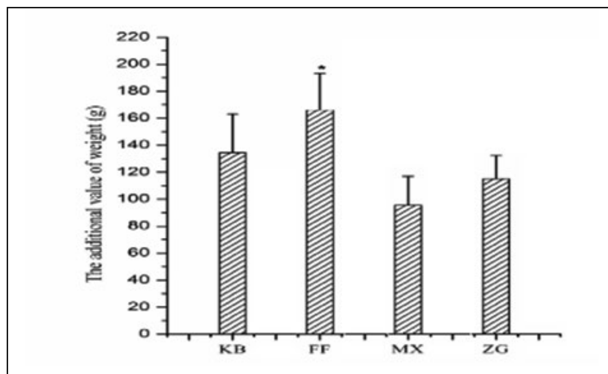

Figure 1: Effect of each drug group on the weight gain of the rats ($\bar{x}$±SD, n=9)
*Note: *P<0.05 is a significant difference between each group and the model group.*

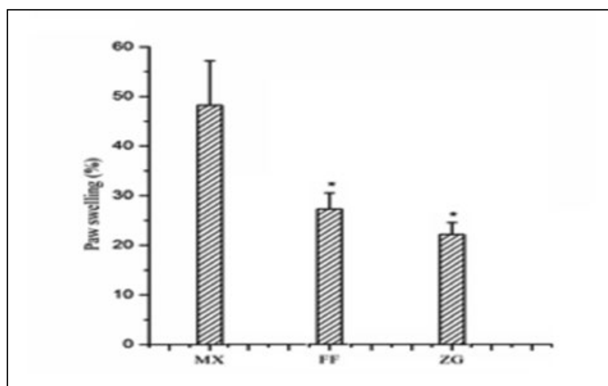

Figure 2: Effect of each drug group on the swelling degree of rat paw ($\bar{x}$±SD, n=9)
*Note: *P<0.05 is a significant difference between each group and the model group.*

In Figure 1, compared with the blank group, the weight gain of the rats in the compound administration group was higher, the sugar content may be beneficial to the weight gain of the rats. And the weight gain of the rats in the saponin administration group was decreased. The post-saponin component did not promote weight gain in rats, and modeling and gavage may affect the rats' feeding and slow their weight gain. In Figure 2, the swelling rate of the paws after the modeling increased significantly, indicating that the modeling was successful. and the swelling rate of the paws in each drug-administered group was

lower than that in the model group, indicating that the compound and saponin components have a significant effect on reducing the swelling rate of the paws. Compared with the compound administration group, the saponin component was more effective in reducing the swelling rate of the paws.

Detection of TNF-α and IL-1β in rats serum by ELISA

The rat adjuvant arthritis experiment was carried out according to the method in rat adjuvant arthritis model. After 24 hours of the last administration, the blood was decapitated, and serum samples were obtained by centrifugation. The serum levels of TNF-α and IL-1β were detected by ELISA. The results were shown in Table 5.

In rheumatoid arthritis, TNF-α can act on osteoclasts, synovial cells and chondrocytes, respectively, resulting in the activation of these cells, producing metalloproteinases, collagenase, basal membrane lytic enzymes and PGE2, further destroying cartilage and causing bone erosion, joint inflammation and cartilage destruction. TNF-α can also cause synovial cells, macrophages, fibroblasts and chondrocytes to produce IL-1, IL-8 and TNF-α itself and aggravate tissue damage.

Therefore, inhibition of the action of TNF-α is very important for controlling the condition of RA and improving the prognosis. IL-1β is the most important pro-inflammatory cytokine in the destruction of articular cartilage. It promotes the proliferation of synovial cells and chondrocytes and stimulates the production of prostaglandins, collagenases and neutral proteases and other inflammatory mediators, which in turn cause cartilage necrosis.

IL-1β also promotes the formation and release of NO, which causes synovial inflammatory response. NO, as an important inflammatory mediator, directly participates in the inflammatory response and joint damage process. Abnormal rise of NO in the body can lead to excessive expansion of blood vessels and increased exudation. It can also promote the release of inflammatory mediators such as PGE2, MMP-2, and IL-1, and aggravate the inflammatory response. The results showed that the serum levels of TNF-α and IL-1β in the rats in each administration group were between the blank group and the model group, indicating that the TNF-α and IL-1β-mediated inflammatory responses were reduced in each administration group.

The anti-inflammatory effect of the saponin component administration group was weaker than that of the compound administration group, indicating that the saponin component was not the main component of the inhibition of TNF-α and IL-1β in the Gancaofuzi Decoction.

Groups	TNF-α	IL-1β
KB	172.42±36.86	11.64±1.67
MX	270.32±52.81	18.53±1.25
FF	208.26±51.95*	14.79±1.06*
ZG	253.93±51.83	15.81±1.71*

Table 5: Effects of drugs on serum TNF-α and IL-1β concentrations in rats ($\bar{x}$±SD, n=9, ng/L).

*Note: *P<0.05 is a significant difference between each group and the model group.*

Effects on immunomodulatory effects

The mouse macrophage immunoregulation experiment was carried out according to the method of establishment and dosing protocol for immunomodulatory experiment, and the contents of TNF-α, IL-1 and IL-2 were detected by ELISA. The results were shown in Table 6.

It can be seen from the results that the compound and saponin components administration groups significantly inhibited the secretion of TNF-α and IL-1 by mouse macrophages compared with the blank group. The drugs could inhibit the cell-mediated immune response by affecting the secretion of macrophage factors. And the drugs could reduce the degree of damage of the immune response to the body throug inhibiting the production of inflammatory mediators such as TNF-α, IL-1 and IL-2.

Thus the inhibition of TNF- α, IL-1 and IL-2 has great significance in the clinical treatment of autoimmune diseases.

Groups	TNF-α	IL-1	IL-2
KB	16.82±0.02	3.98±0.19	2.97±0.03
FF	9.51±0.06*	3.17±0.03	1.83±0.14
ZG	3.38±0.01*	2.38±0.11	0.29±0.02*

Table 6: Effects of different groups of drugs on the secretion of TNF-α, IL-1 and IL-2 in mouse macrophages ($\bar{x}$±SD, n=3, ng/L).

*Note: *P<0.05 is a significant difference between each group and the blank group.*

Conclusions

As the important traditional Chinese medicine prescription for the treatment of rheumatic diseases, Gancaofuzi decoction was mainly studied for its main chemical compositions and pharmacological activity. But the pharmacodynamics basis for differ-

ent pharmacological activity and the compatibility mechanism were not studied deeply and chearly. The saponins are the main pharmacodynamics compositions in Gancaofuzi decoction.

Their pharmacological activities were studied by classic animal disease models to explore their roles in the compound. This study is of great significance for exploring the pharmacodynamics basis of traditional Chinese medicine. And the study provides a theoretical basis for further study on the compatibility mechanism of traditional Chinese medicine compound.

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