Antinociceptive effect of glucosides of *Chaenomeles speciosa*

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Abstract: AIM To observe the antinociceptive effects of glucosides of *Chaenomeles speciosa* (GCS) and to study their relative mechanism. **METHODS** The effects of GCS on normal and inflammatory animals were observed in mouse acetic acid writhing test, mouse formalin test and arthritic flexion test of adjuvant arthritis (AA) rats; the concentration of prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) secreted by the synovial cells of AA rats were measured by radioimmunoassay. **RESULTS** Different doses of GCS (60, 120, 240 $mg \cdot kg^{-1}$ for mice and 30, 60, 120 $mg \cdot kg^{-1}$ for rats, ig) inhibited mice's writhing response and second phase of formalin response. It also suppressed the increased arthritic flexion scores in AA rats. On 28 d after inflammation induction, GCS (60, 120 $mg \cdot kg^{-1}$) decreased the concentration of PGE_2 and TNF-α of synovial cells in the AA rats. **CONCLUSION** GCS have antinociceptive effects, which related to its inhibitory effects on peripheral inflammatory mediators.

Key words: analgesia test; *Chaenomeles speciosa*; glucoside; dinoprostone; tumor necrosis factor

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Fructus Chaenomeles (also called *Chaenomeles* fruit) is the ripe fruit of *Chaenomeles speciosa* (Sweet) Nakai, which grows mainly in Xuancheng, Anhui Province, China. According to Traditional Chinese Medicine (TCM) theory, Fructus Chaenomeles could subdue liver-qi and lessen contracture, regulate stomach function and dispel damp[1]. In TCM clinical practice, it has been widely used in the treatment of rheumatoid arthritis, prosopalgia and hepatitis. Modern pharmacological studies have also demonstrated its analgesic, anti-tumor, antibacterial and immuno-regulatory effects[2,3].

From the fruit of *Chaenomeles speciosa* (GCS), glucosides were extracted with ethanol and purified for the first time in our lab. The identified components include stearic acid, β-sitosterol, palmitic acid, ursolic acid-3-*O*-behenate, ursolic acid, 3-acetyl ursolic acid, 3-acetyl pomolic acid, daucosterol and betulinic acid. In previous study, we found that GCS has anti-inflammatory and immuno-regulatory properties. It can obviously ameliorate the inflammation in adjuvant arthritis (AA) and collagen-induced arthritis (CIA) rats[4,5]. In order to investigate the antinociceptive profile of GCS, several pain models, acute as well as chronic, were applied in the study.

1 MATERIALS AND METHODS

1.1 Plant material, preparation of extracts, fractions and phytochemical analysis

Botanical material was collected in Xuancheng, south of Anhui Province. The optimum preparation process of GCS was found out by using orthogonal design. GCS was determined by colormetric method. The optimum ethanol-extracting factors were 8-fold as refluxing and extracting in 80% ethanol for 1 h, and repeating for 3 times. The extract rate of the crude product from the herb is about 10.37%. The water solution of the ethanol-extracts was absorbed for 1 h with a column of macroporous resin (1.5 cm × 16 cm) and the resin was washed with 100 mL of distilled

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water, then washed with 100 mL of 20% ethanol. After that, 5.09% of the purified product was obtained. The content of GCS in this purified solid is about 61.33%.

1.2 Animals

Sprague-Dawley (SD) rats (male, 160 – 200 g, Grade Ⅱ, Certificate №006) and Kunming mice (male and female, 18 – 22 g) were obtained from the Animal Department of Anhui Medical University, China. All rats and mice were acclimatized under standard laboratory conditions for at least 3 days before testing. During the experimental period, tap water and commercially available food were given freely. The lighting duration in the breeding room was 12 h (7:00 to 19:00). Experiments were performed in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for pain experiments in conscious animals[6].

1.3 Acetic acid writhing test

Acetic acid writhing test was carried out by intraperitoneal injection of acetic acid (0.6%), resulting in a contraction of abdominal muscle together with a stretching of hind limbs. Mice were pretreated intragastrically once a day with vehicle (0.5% sodium carboxymethylcellulose, CMC-Na), GCS (60, 120 and 240 $mg \cdot kg^{-1}$) or ibuprofen (50 $mg \cdot kg^{-1}$) for four consecutive days. All of the drugs were administrated at 0.02 $mL \cdot g^{-1}$. On the last day of drug administration, acetic acid was injected intraperitoneally 1 h after drug administration. Then, mice were placed in separated boxes and the number of abdominal constrictions was cumulatively counted over a period of 15 min. All the experiments were carried out at 20 – 22℃.

1.4 Formalin test

Mice were divided into five groups, which were respectively administered ig with vehicle (0.5% CMC-Na) and GCS(60, 120 and 240 $mg \cdot kg^{-1}$) once a day for four consecutive days or hydrochloric pethidine(20 $mg \cdot kg^{-1}$) only once on the last day. All of the drugs were administrated at 0.02 $mL \cdot g^{-1}$. One hour before the last administration, Formalin (2% formaldehyde diluted in saline) was injected subcutaneously into the plantar of the right hind paw of mice. Animals were observed from 0 – 60 min after injection. The time spent for licking the injected paw was timed with a chronometer and was considered as an indication of pain. The first or early phase occured 0 – 5 min after the Formalin injection. The second or late phase occured 15 – 60 min after injection.

1.5 Adjuvant arthritis induction and evaluation

Adjuvant arthritis was induced on d 0 by intradermal injection of complete Freund's adjuvant (CFA), containing 10 mg heat-inactive BCG in 1 mL paraffin oil, into the rats' left hind paws, 0.1 mL for each rat.

The rats with AA were divided randomly into five groups which were AA model group, GCS (30, 60, and 120 $mg \cdot kg^{-1}$) groups and ibuprofen(25 $mg \cdot kg^{-1}$) group. GCS or ibuprofen were given intragastrically, once a day from d 17 to d 24 after AA induction, while an equal amount of vehicle was given to the control normal and AA model groups. All of the drugs were administrated at 10 $\mu L \cdot g^{-1}$.

The ankle flexion procedure was applied to evaluate the hyperalgesic state. It involved holding the rat comfortably and gently extending the right hind paw metatarsally. The test was repeated 5 times at 5 s intervals; a score of 1 or 0 was given according to whether the animal vocalized (1) or not (0). For each rat, the total score was calculated by the accumulated times of the animal vocalizing, thus it was ranged from 0 to 5[7,8].

1.6 Synoviocyte culture

Rats were sacrificed on d 28 after AA induction. Synoviocytes from rat knees were excised and dispersed with sequential digestion of 0.4% (*W/V*) collagenase type Ⅱ and 0.25% (*W/V*) trypsin. Synoviocytes were resuspended in RPMI-1640 medium at a concentration of 1×10^9 L^{-1}. The cells suspension 500 μL and lipoplysaccharide (LPS, mediator) 500 μL with the final concentration of 10 $mg \cdot L^{-1}$ were added to 24-well plate. After incubation at 37℃ in 5% CO_2 atmosphere for 48 h, the supernatant containing TNF-α and

PGE_2 was collected and then stored at −20℃[9].

1.7 Prostaglandins E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) radioimmunoassay (RIA)

TNF-α and PGE_2 in supernatant produced by cultured synoviocytes were measured according to the procedures offered in TNF-α and PGE_2 ^{125}I RIA kits (Spring Bioscience Co., USA).

1.8 Statistical analysis

$\bar{x} \pm s$ were calculated for quantitative data. Statistical analysis of the quantitative data for multiple comparisons was performed by one-way analysis of variance (ANOVA) followed by Duncan's test. The frequency data such as the hyperalgesic scores were also expressed as $\bar{x} \pm s$, but statistical analysis of them was compared using Kruskal-Wallis rank sum test. A level of $P < 0.05$ was accepted as statistical significant.

2 RESULTS

2.1 Effect of GCS in writhing test

After ig administration of GCS (60, 120 and 240 mg·kg^{-1}) for 4 d, mice's writhing responses were decreased significantly (Fig 1). And this effect of GCS was dose-independent. The GCS at 240 mg·kg^{-1} has the similar inhibitory effects as ibuprofen (50 mg·kg^{-1}). Irritation or repression was not observed in mice after drug administration.

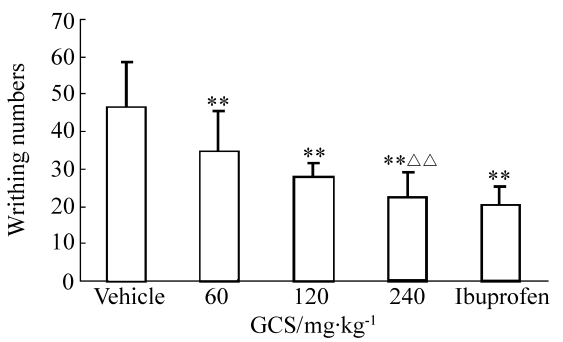

Fig 1. Effect of glucosides of *Chaenomeles speciosa* (GCS) on mouse writhing test. Mice were pretreated intragastrically with vehicle (0.5% CMC-Na), GCS or ibuprofen (50 mg·kg^{-1}) once a day for four consecutive days. Mice's abdominal constrictions were cumulatively counted over a period of 15 min. $\bar{x} \pm s$, $n = 10$. $^{**}P < 0.01$, compared with vehicle group; $^{\triangle\triangle}P < 0.01$, compared with GCS 120 mg·kg^{-1}.

2.2 Effect of GCS in Formalin test

The results of Formalin test has been summarized in Fig 2. After four-day ig administration of GCS (120 and 240 mg·kg^{-1}), the second-phase responses in Formalin test were reduced compared with vehicle group, but GCS failed to exert any antinociceptive effect in the first phase. Irritation or repression was not observed in mice after GCS administration. Different from GCS, hydrochloric pethidine could inhibit both the first and the second phase of formalin test, and it reduced mice's activity frequency.

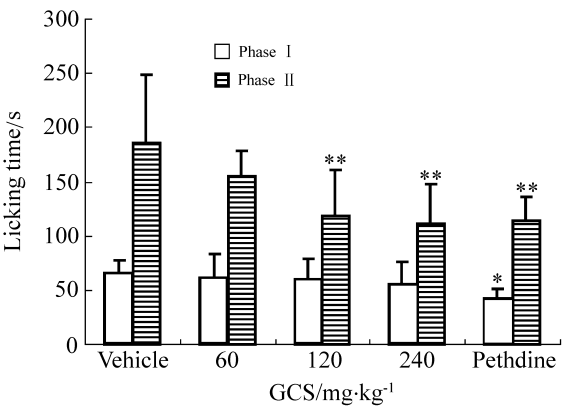

Fig 2. Effect of GCS in mouse Formalin test. See Fig 1 for vehicle and GCS treatments. Hydrochloric pethidine (20 mg·kg^{-1}) was given only once. The time spent for licking the injected paw was timed. 0 – 5 min after Formalin injection first or early phase. 15 – 60 min after injection was the second or late phase. $\bar{x} \pm s$, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with vehicle group.

2.3 Effect of GCS on ankle flexion in AA rats

In AA rats, the scores in arthritic flexion test were remarkably higher than that in normal rats beginning on 14 d after AA induction. The scores increased consecutively and peaked on the d 24. However, from 17 d to 24 d after AA induction, the scores of GCS (30, 60 and 120 mg·kg^{-1}) groups were remarkably lower than that of AA model groups (Fig 3).

2.4 Effect of GCS on PGE_2 and TNF-α level

In AA rats, on d 28 after AA induction, the concentrations of PGE_2 and TNF-α in synovial cells were both decreased. GCS (60, 120 mg·

kg^{-1}) induced the levels of PGE_2 and TNF-α in GCS groups. Ibuprofen (25 mg·kg^{-1}) showed the stronger inhibitory effects on PGE_2 and TNF-α than GCS(Fig 4 and Fig 5).

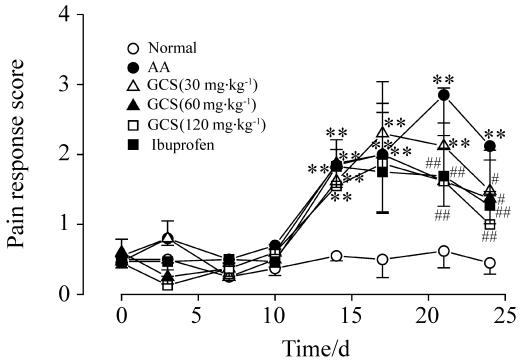

Fig 3. Therapeutical effects of GCS on pain response in adjuvant arthritis rats. The rats were treated with drugs from d 17 to d 24 after acetic acid(AA) induction. The test was repeated 5 times with 5 s intervals; score 1 or 0 was recorded for vocalizing or not. The pain response score was the accumulated vocalizing times of the animal. $\bar{x} \pm s$, $n = 8-10$. $^{**}P < 0.01$, compared with normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with AA model group by Kruskal-Wallis rank sum test.

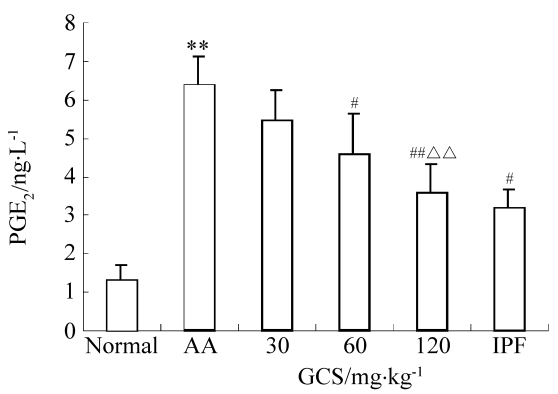

Fig 4. Effect of GCS on prostaglandin E_2 (PGE_2) concentration in culture supernatant of synoviocytes from adjuvant arthritis rats. IPF: Ibuprofen 25 mg·kg^{-1}. Synoviocytes were harvested from AA rat knee joints on d 28 after AA induction and cultured *in vitro*. PGE_2 produced by cultured synoviocytes were measured with PGE_2 ^{125}I RIA kits. $\bar{x} \pm s$, $n = 4-6$. $^{**}P < 0.01$, compared with normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with AA group; $^{\triangle\triangle}P < 0.01$, compared with GCS 60 mg·kg^{-1} group.

3 DISCUSSION

Fructus Chaenomeles has been traditionally used in TCM to relieve pain and to treat inflammatory disease such as rheumatoid arthritis. In the present study, the analgesic effects of GCS and its possible mechanism were investigated.

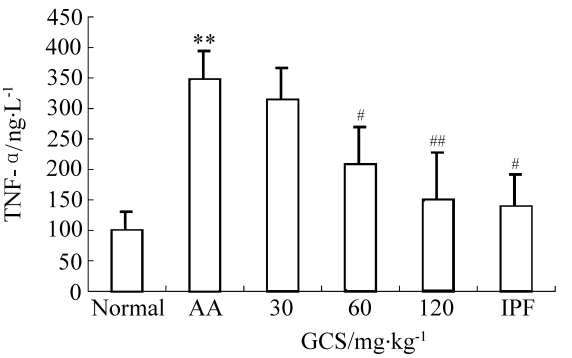

Fig 5. Effect of GCS on tumor necrosis factor-α (TNF-α) concentration in culture supernatant of synoviocytes from adjuvant arthritis rats. See Fig 4 for experimental scheme. TNF-α concentration measured with TNF-α ^{125}I RIA kits. $\bar{x} \pm s$, $n = 4-6$. $^{**}P < 0.01$, compared with normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with AA group.

Writhing test is a classical pain model which has high sensitivity but low selectivity. In this model, chemical stimulants are known to sensitize primary afferents and to recruit silent nociceptors[10,11]. Our study showed that GCS(60, 120, 240 mg·kg^{-1}) decreased the mice's writhing times after acetic acid was injected intraperitoneally. High dose of GCS(240 mg·kg^{-1}) showed the similar antinociceptive potency with a non-steroid anti-inflammatory drug(NSAID), ibuprofen.

Formalin test is different from most traditional tests in that it is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissue[12]. Its early phase (0 – 5 min) was mediated by the central stimulation on nociceptors and release of substance P or bradykinin, and the late phase (15 – 60 min) was mediated by the peripheral effect of some chemical transmitters(histamine, serotonin, prostaglandins, kinins, *etc*.)[13,14]. In our study, after ig administration of GCS, the second-phase responses of mice but not the first-phase were reduced. No obvious activity change was found. Therefore, the result we obtained indicates that the antinociceptive effect of GCS might be mediated by its peripheral action.

Hyperalgesia in AA model, whose characteristic is chronic, is similar to pain in RA patients. Arthritic ankle flexion test has been used to monitor hyperalgesia and drug effects on pain in AA rats in a simple way since 1970s[15]. But this method has its own weakness that it can not be carried out with a consistent pull strength, which may result in uncertainty of the experimental data. Anyway, the results of our study show that GCS has the reducing effect on increased ankle flexion scores in AA rats without any effect on rats' regular activity. It suggested that GCS suppress the chronic inflammatory pains like arthritic pain.

It is well known that products derived from arachidonic acid *via* both the cyclooxygenase and lipoxygenase pathways play a role in inflammatory pain. For example, prostaglandins (PGs), particularly PGE_2, contribute to the development of hyperalgesia[16]. Besides, the released TNF-α, rather than the prostaglandins, mediates the hyperalgesia by stimulating vagal afferents which induce the inflammatory pain[17]. Since the result of Formalin test gave us the clues that GCS's analgesic effect is related to its peripheral action, it is rational to attribute its antinociceptive effect to these imflammatory mediators.

In this study, we focus on the synoviocytes of ankle joint in AA rats. We found that increased levels of PGE_2 and TNF-α secreted by synoviocytes in AA rats were both decreased by GCS *in vivo*. This result confirmed our suggestion that the antinociceptive effect of GCS is related to its peripheral inhibitory action. However, the question whether GCS affects these mediators directly or indrectly is to be clarified and studies in this respect are now under way.

In conclusion, GCS possesses antinociceptive effects. In the arthritic pain model, this effect of GCS might relate to its inhibition on peripheral inflammatory cytokines.

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木瓜苷的镇痛作用

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摘要：目的　观察木瓜苷的镇痛作用并研究其相关机制。**方法**　通过小鼠乙酸扭体反应，甲醛实验及佐剂性关节炎大鼠屈伸关节实验等疼痛模型，观察木瓜苷的镇痛作用并检测佐剂性关节炎大鼠滑膜细胞分泌的前列腺素 E_2（PGE_2）及肿瘤坏死因子-α（TNF-α）的含量。**结果**　不同剂量的木瓜苷（小鼠 60，120 和 240 mg·kg^{-1}，ig，大鼠 30，60 和 120 mg·kg^{-1}，ig）可以抑制小鼠的乙酸扭体反应和甲醛第二相反应。木瓜苷（60，120 mg·kg^{-1}）可使佐剂性关节炎大鼠致炎 d 28 关节滑膜细胞升高的 PGE_2 和 TNF-α 水平显著降低。**结论**　木瓜苷具有镇痛作用，其可能机制与其抑制外周炎症介质有关。

关键词： 镇痛试验；木瓜苷；地诺前列酮；肿瘤坏死因子

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会　议　信　息

第五届金属硫蛋白（metallothionein，MT）国际会议将于 2005 年 10 月 8～12 日在北京召开。

1. 会议名称　The Fifth International Conference on Metallothionein
简称　MT-2005
主题　Metal and Metallothionein in Biology and Medicine

2. 日　期　2005 年 10 月 8～12 日

3. 地　点　中国·北京·友谊宾馆

4. 主办单位　北京大学、中国生物化学与分子生物学会、中国毒理学会和加拿大毒理学会

5. 会议名誉主席　J.H.R.Kagi 教授（University of Zurich，Switzerland）
C.D.Klaassen 教授（University of Kansas，USA）
会 议 主 席　Binggen Ru 教授（中国北京大学）
M.G.Cherian 教授（University of Western Ontario，Canada）

6. 会议规模　共约 300 多人，国外约 200 人

7. 大会内容　大会设有 Plenary lectures，Symposium 和 Poster 3 种形式进行学术交流。

Plenary lectures：

（1）Dr.JHR Kagi（瑞士）：Metallothionein and Glutathione，Yin and Yang in Cellular Zinc Assimilation

（2）Dr.C.D.Klaassen（美国）：Metallothionein and Metal Toxicity

（3）Dr.Kazuo T.Suzuki（日本）：Biological Roles of Metallothionein Based on Mercaptide Binding

Symposium：

（1）MT and Homeostasis of Zinc，Copper and Other Metal Ions and Related Diseases

（2）MT in Brain and Related Diseases

（3）Metals，MT and Tumors

（4）Metals，MT and Cardiovascular Diseases and Diabetes

（5）Metals，MT and Liver，Kidney Diseases and Oxidative Stress

（6）MT-like Molecules in Other Organisms，Environmental Applications of MT

（7）Basic Research：Biochemistry，Molecular Biology，Structure and Function，Spectroscopic Studies，and Other Theoretical Studies on MT

Chinese Symposium（专为广大中国科学家设置的一个特殊报告会），主题：“Progress of MT Research in China”

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