

# Effects of Gastrodin on Analgesia and Inhibition of Ferroptosis

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## Research

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# Abstract

**Background:** Gastrodin possesses low toxicity and a broad range of pharmacological activities and exhibits beneficial effects in neurological diseases. This study investigated the effects of gastrodin (GAS) on analgesic, anti-inflammatory, anxiolytic and inhibition of ferroptosis.

**Materials and Methods:** The chronic inflammatory pain model of C57BL/6J mice was established by hindpaw injection of complete Freund's adjuvant (CFA). After GAS treatment, Thermal hyperalgesia test, Mechanical allodynia test, Elevated plus-maze (EPMT) and Open-field test (OFT) were performed to assess the behavioral changes of pain and anxiety. mRNAs of FTHI, GPX4, HO-1 and PTGS2 were measured by RT-qPCR.

**Results:** In CFA-injected C57BL/6 mice, we found that the mechanical and thermal pain threshold was increased with treatment of GAS. In EPMT, the number of entries in open arms and retention times of open arms were increased by GAS. In the OFT, the time spent in the central area was also increased. Furthermore, GAS enhanced mRNA expressions of FTHI, GPX4 and HO-1, as well as decreased the expression of PTGS2 in a dose-dependent manner.

**Conclusion:** GAS is effective in the treatment of mice chronic inflammatory pain and anxiety-like behaviors. It maybe exhibit potential neuroprotective effects through inhibition of ferroptosis.

## Background

Ferroptosis is a unique iron-dependent form of regulated cell death(1). The accumulation of lipid peroxidation products and lethal reactive oxygen species (ROS) is the main characteristic of ferroptosis(2). Ferroptosis, as a way to promote cell death, may be implicated in the occurrence and development of many diseases. Studies have shown the importance of ferroptosis in many diseases of the central nervous system, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Traumatic brain injury (TBI)(3).

Gastrodiae Rhizoma (Tianma), a notable Chinese herb, is dry tubers of *Gastrodia elata* Blume which belongs to Orchidaceae. *Gastrodiae Rhizoma* is considered a top-grade medicine described to treat the hypertension of liver-yang hyperactivity in the tradition Chinese medicine. Studies have shown that the application of *gastrodia elata* has biological activities of anticonvulsion, antioxidation, neuroprotection, anti-denguevirus, anti-cardio-cerebral-vascular diseases, anti-inflammation(4). The major active component and material basis of *Gastrodia elata* is Gastrodin (GAS). GAS, a chemical compound that known as 4-hydroxybenzyl alcohol-4-O- $\beta$ -D-glucopyranoside, is isolated from the rhizome of *Gastrodia elata*. Furthermore, the molecular formula of GAS is  $C_{13}H_{18}O_7$ , and its chemical structural formula is shown in Fig. 1. GAS has numerous pharmacological activities including analgesic(5), antidepressant(6), anxiolytic(7), anti-inflammatory(8), antiobesity (9) and memory and retrieval improvements(10, 11). Among them, analgesic, antioxidant, anti-inflammatory and neuroprotective effects are the main research hotspots in recent years. Recent findings suggest that GAS exerts a protective effect on primary neural

progenitor cells (NPCs) by resisting amyloid $\beta$  (A $\beta$ ) (1–42)-induced neurotoxicity(12). In the meantime, GAS increased the expression of HO-1, Nrf2 and GPX4 protein in Rat Glioma Cell Line C6, which protected Rat Glioma Cell Line C6 from ferroptosis induced by H<sub>2</sub>O<sub>2</sub>(13). In recent years, several neuroprotective mechanisms of GAS have been found. However, the study regarding to the effects of Gas on ferroptosis is rare. GAS was also reported to display powerful anti-inflammation properties. Based on the above research progress and analysis, it is speculated that GAS might be a potential therapeutic for the inhibition of ferroptosis. This study was designed to explore the effects of GAS on analgesic, anti-inflammatory and anxiolytic. We also examined whether GAS can exhibit neuroprotective effect through inhibition of ferroptosis.

## Materials And Methods

### Materials

GAS was purchased from Nanjing Baide Biotechnology Co., Ltd. (>99% purity, Nanjing, China). Complete Freund's adjuvant (CFA) and Von Frey filaments were purchased from Sigma (St. Louis, MO). Elevated Plus Maze Video Tracking System was purchased from Shanghai Xinruan Information Technology Co., Ltd. (Shanghai, China). YLS-6A Intelligent hot plate was purchased from Jinan Yiyuan Technology Development Co., Ltd. (Shandong, China). ABI7500 Real-Time PCR Detection Systems was purchased from Bio-Rad (Hercules, California). AxyPrep™ Multisource Total RNA was purchased from AXYGEN (Silicon Valley, California). SYBR Green qPCR Mix(2X) was purchased from Beyotime Biotechnology (Shanghai, China). D7260 Prime Script TMRT Reagent Kit was purchased from TaKaRa (Liaoning, China); RR037A primer was purchased from Sangon Biotech (Shanghai, China).

### Animals and grouping

Male C57BL/6J mice (aged 8 weeks, weighing 21-25g) were purchased from Chengdu Dashuo Laboratory Animal. Animals were housed in groups of six mice with a temperature (20 ± 2°C), humidity (55 ± 15%) and lighting (12 h light/dark cycle, lights on at 7:00 AM). All animals must adapt to conditions for at least 7 days after they arrived. Food and water were freely available.

The rats were randomly divided into four groups of six individuals each as follows: Blank group (0.9% physiological saline (SAL)-treated group, n = 6), Model group (The CFA-induced plus SAL-treated group, n = 6), the CFA-induced plus 100mg/kg GAS-treated group (CFA + 100 group, n = 6), the CFA-induced plus 200mg/kg GAS-treated group (CFA + 200 group, n = 6).

### Experimental designs and GAS treatment

10ul CFA (50%) was injected intraplantar subcutaneously into the left hindpaws of mice to established chronic peripheral inflammatory pain. In the control group, the same volume of SAL was injected into the hindpaws of mice. GAS was dissolved in saline before use. The mice were intraperitoneally injected with GAS (100, 200 mg/kg) after CFA insult GAS or saline was used repeatedly in mice once a day for 2 weeks.

## Mechanical allodynia

Mechanical allodynia was assessed with a set of von Frey filaments on day 1,4,7, and 14. Mice were placed on a wire mesh covered with organic glass and acclimated to the environment at least 30 minutes prior to test. Start with 0.4 mN(#2.44) filament and stimulate the center of left hindpaw until filament bending for 3s, and the mice have reactions like licking foot or foot lifting.

## Thermal hyperalgesia

After 14 days of administration, the temperature of the hot plate was set to 55°C. The left hindpaw of mice was placed on the hot plate, and time was recorded when the mice had reactions like foot lifting.

## Elevated plus-maze test

Mice were placed in the central zone of the maze facing the closed arm, and the time was recorded for 5min. Outcome measures: the number of entries in open arms, retention times of open arms, the number of entries in closed arms, retention times of closed arms. The number of entries in open arms and retention times of open arms were negatively correlated with anxiety in mice.

## Open field test

Mice were placed in the center of the box, and the time of mice entering the central area was videotaped. The observation time is 5min.

## Real-time Quantitative PCR (qRT-PCR)

The total RNA was extracted from the ACC and the spinalcord of the rat lumbosacral enlargement(L4-5) using TRIZOL reagent. D7260 Prime Script™ RT Reagent Kit performed reverse transcription for the synthesis of cDNA. Reverse transcription was the performed via Real-Time PCR System in a 20uL reaction mixture and while following the manufacturer's instructions. SYBR Green qPCR Mix(2X) was used for QRT-PCR. The primers utilized here are shown in (Table 1).

Table 1  
Specific gene primes sequences

Gene name	Forward prime (5' to 3')	Reverse prime (5' to 3')
HO-1	AGACACCGCTCCTCCAGT	TCAGGTATCTCCCTCCATT
FTH1	GCAGGATATAAAGAAACCAGA	TCTCAATGAAGTCACATAAGT
GPX4	GTCTGGCAGGCACCATGT	GTGACGATGCACACGAAACC
PTGS2	TGGAGGCCAAGTGGGTTTTA	GAGTGGGAGGCACTTGCATT
GAPDH	GCAGAATTCCTGGCCAAGGTCATCCATGAC	GCAGGTACCGGGGCCATCCACAGTCTTCTG

# Statistical Analysis

All results are presented as mean  $\pm$  standard deviation (S.D.) and were analyzed using SPSS (Version 13.0, Chicago, USA). A p value  $< 0.05$  was considered to be statistically significant.

## Results

### Effects of GAS on CFA-induced mechanical and thermal hypersensitivity

After CFA was injected into mice, mechanical thresholds were determined on day 1, 4, 7, and 14. As shown in Fig.2, on the first day after CFA injection, the mechanical pain threshold of the model group was significantly lower than blank group, and the left hindpaw of mice was obviously swollen, indicating that the chronic inflammatory pain model was successfully established. The paw withdrawal threshold of CFA-injected mice significantly decreased after CFA injection for 1-4 days. Meanwhile, the administration of GAS (100 and 200 mg/kg) increased the paw withdrawal threshold in CFA-injected mice. GAS also attenuated thermal hyperalgesia in CFA-injected mice (Fig.3). Moreover, GAS dose-dependently increased the mechanical and thermal pain threshold in mice.

### Effects of GAS on CFA-induced anxiety-like behavior

Anxiety-like behaviors of animal are determined by EPMT and OFT. In EPMT, after CFA injection, the number of entries in open arms and retention times of open arms significantly decreased. Moreover, compared with the model group, the number of entries in open arms and retention times of open arms in the GAS-treated group were increased (Fig.4A, B). In the OFT, compared with the blank group, the time spent in the central area decreased in the model group. while the GAS (100 and 200 mg/kg) reversed the reduction caused by CFA(Fig.5). The results show that GAS attenuated CFA-induced anxiety-like behavior.

### mRNA expression changes GAS on chronic inflammatory pain model

The mRNA expressions of FTH1, GPX4, HO-1 and PTGS2 in the ACC and L4-5 of experimental mice on day 14 after CFA injection were detected through Real-time Quantitative PCR. The relative expression levels of each group of genes were shown in Fig 6(A, B).

In the ACC and the spinalcord of the rat lumbosacral enlargement, both FTH1 and GPX4 were significantly decreased on the model group as compared with blank group. Meanwhile, we found that CFA elevated the expressions of PTGS2 and HO-1. Finally, compared with the model group, FTH1, GPX4 and HO-1 in GAS groups were significantly increased while PTGS2 decreased in a dose-dependent pattern. Take together, GAS improved FTH1, GPX4, HO-1 and PTGS2 mRNA expressions, and decreased the production of ROS.

## Discussion

Acute inflammatory pain induced by injection of CFA. In this process, rats were allergic to mechanical allodynia and thermal hyperalgesia, and the pain-induced anxiogenic effect lasted for more than 14 days(14). Clinically, it has been reported that chronic pain leads to mental problems such as anxiety and depression, which seriously reduces the quality of life of patients and hinders their normal life(15). GAS is a phenolic glucoside with significant analgesic and anti-inflammatory effects. In the CFA-induced chronic inflammatory pain model, we found that mechanical and thermal pain threshold was increased with treatment of GAS in a dose dependent pattern. In addition, the number of entries in open arms and retention times of open arms were increased by GAS. These studies further confirmed that GAS has powerful analgesic, anti-inflammatory and anti-anxiety effects in the chronic inflammatory pain model of mice. GAS exerted analgesic and anti-inflammatory effects by decreasing the activation of astrocyte and microglia and the induction of TNF- $\alpha$  and IL-6 in the ACC(16). In a mouse model of chemotherapeutic agent-induced neuropathic pain, 5-HT 1A receptor can mediate the powerful antinociceptive of GAS(5).

Inflammatory disease (ID) is a series of diseases characterized by inflammatory response, and ferroptosis is closely related to inflammatory response(17). There are some inflammatory factors related to the metabolism of peroxides and arachidonic acid in ferroptosis tissues(18). Studies have shown that both ferroptosis and inflammatory diseases have the depletion of Gx4 and GSH, the increase of lipid peroxidation products, and the interruption of iron metabolism(19). At present, although a variety of molecular mechanisms and signaling pathways can lead to ferroptosis, Iron metabolism and lipid peroxidation signaling are the main way to regulate ferroptosis(20). During iron metabolism, Excessive iron leads to ferroptosis by producing ROS. Ferritin heavy chain 1(FTH1), as an iron storage protein complex, is involved in the uptake of excessive iron(2). we found that GAS increased the expression of FTH1, and thus balanced intracellular iron levels. The heme oxygenase-1 (HO-1), a major intracellular source of iron(21), plays an important role in ferroptosis and inflammation. It was reported that p38 MAPK phosphorylation could mediate the protective effect of GAS on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress(22). GAS could ameliorate MPP<sup>+</sup>-induced oxidative stress by regulating the expression of HO-1 in human dopaminergic cells(23). We also demonstrated that GAS increases HO-1 expression, which accelerates the decomposition of heme and inhibits inflammation. In addition, the expressions of glutathione peroxidase4 (GPX4) and prostaglandin-endoperoxide synthase2 (PTGS2) are also important for the induction of ferroptosis.

In our experiments, GAS significantly upregulates the expression of FTH1 and GPX4, decreases PTGS2 expression, suggests that GAS against ferroptosis by reducing lipid peroxidation. CFA-induced chronic inflammatory pain is accompanied by the ferroptosis of neuronal cells, and GAS has an inhibitory effect on ferroptosis, which is one of the possible mechanisms to protect neuronal cells. In order to make the argument more sufficient, in the future, it is necessary to further study the mechanism of GAS inhabiting ferroptosis at cellular levels.

## Conclusion

In conclusion, our research shows that GAS has obvious analgesic and anti-anxiety effects on CFA-induced chronic inflammatory pain in mice. At the same time, GAS may exhibit potential neuroprotective effects through inhibition of ferroptosis. The data from the present study provide a theoretical basis for GAS in the treatment of neurological diseases, and promote in-depth research and application of GAS.

## **Abbreviations**

GAS, Gastrodin; CFA, Complete Freund's adjuvant; EPMT, Elevated plus-maze test; OFT, Open-field test; GPX4, Glutathione peroxidase; FTH1, Ferritin heavy chain 1; HO-1, The heme oxygenase-1; PTGS2, Prostaglandin-endoperoxide synthase2; ROS, Reactive oxygen species; ACC, anterior cingulate cortex; Nrf2, nuclear factor erythroid 2-related factor 2.

## **Declarations**

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### **Authors' contributions**

XW and XL conceived and designed this study. ZXH and JYW collected and analyzed data. ZXH and JYW drafted the manuscript. XW revised the paper. All authors read and approved the final manuscript.

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval and consent to participate**

All experimental procedures were approved by the Animal Ethics Committee of Southwest Jiaotong University and were conducted in accordance with the university's animal experiment guidelines.

### **Consent for publication**

Not applicable.

## Competing interests

There are no conflicts of interest.

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## Figures

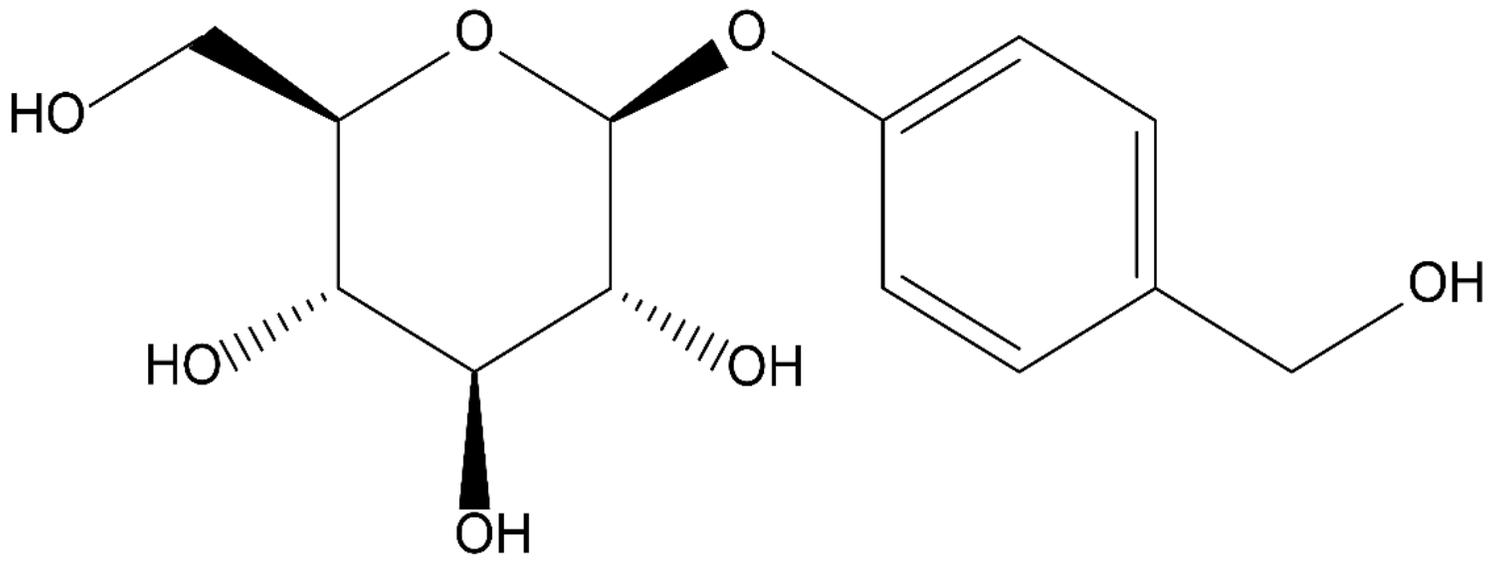


Figure 1

The chemical structural formula of GAS.

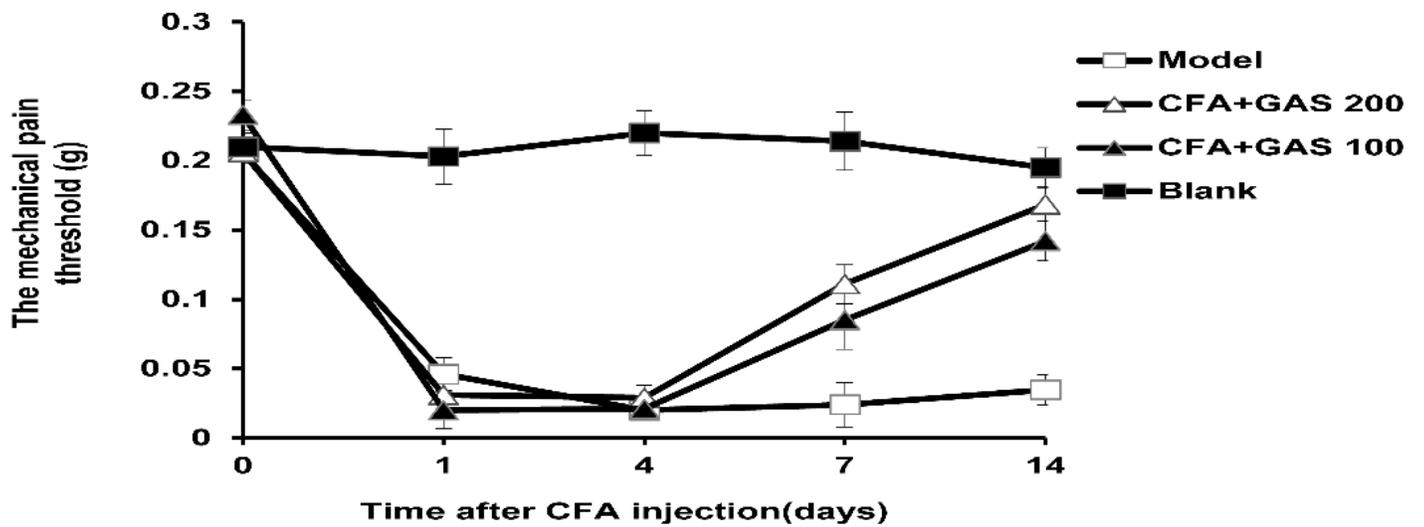


Figure 2

Changes of the paw withdrawal threshold after CFA injection

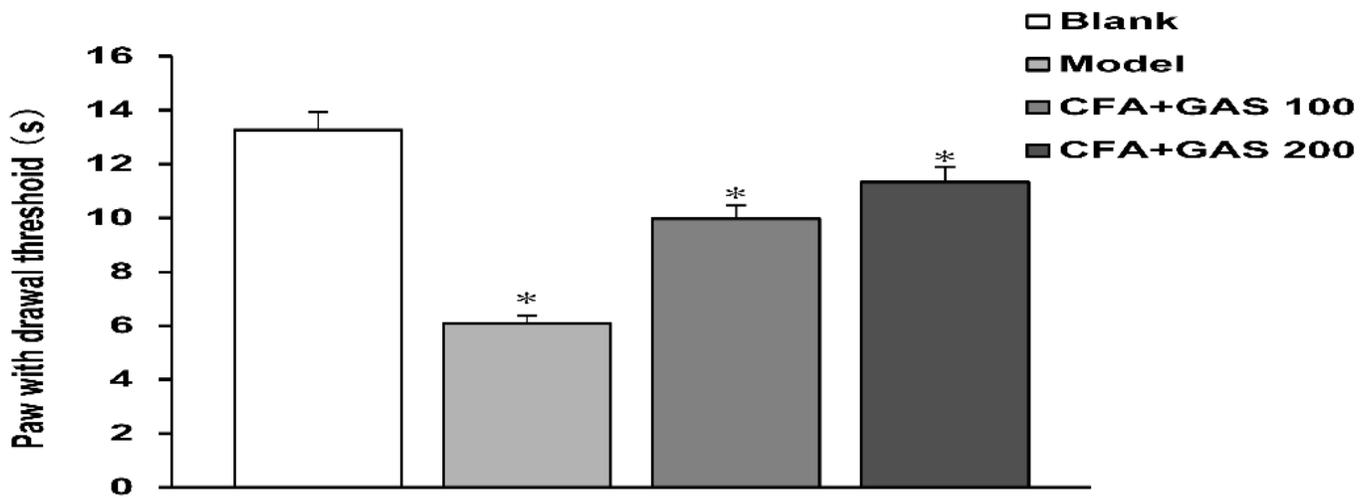
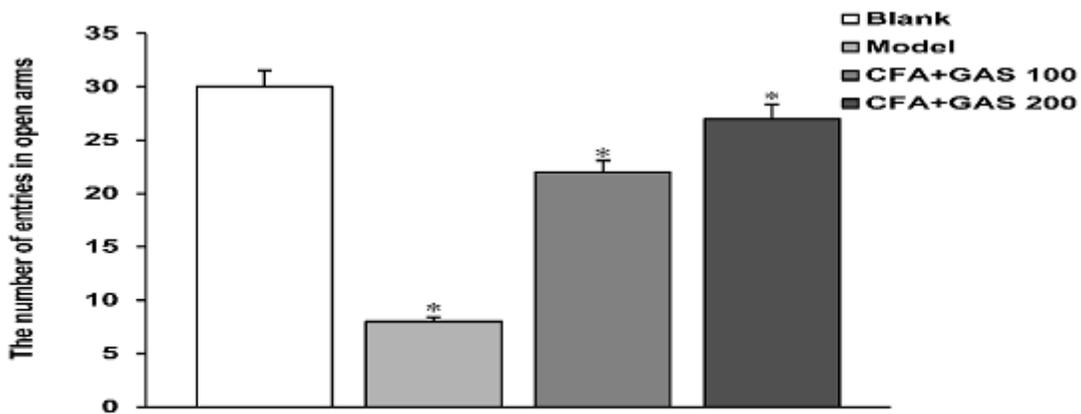
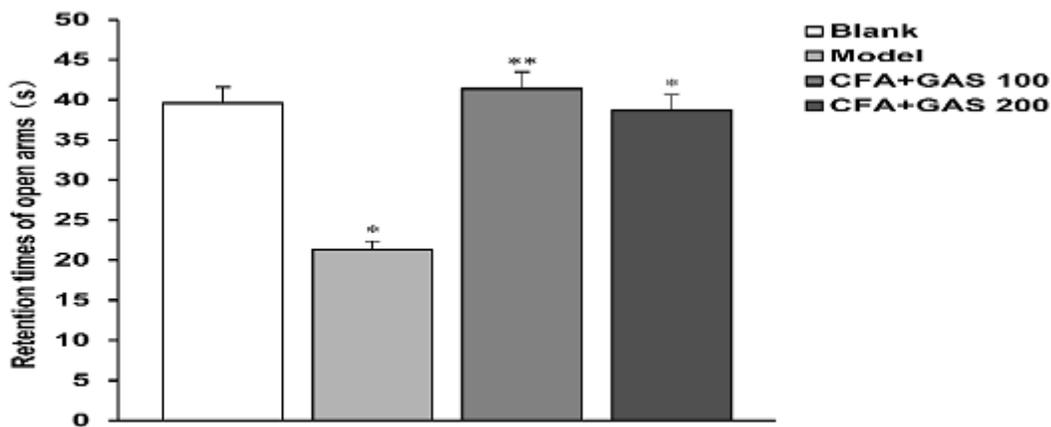


Figure 3

The paw withdrawal latency of mice(s) (\*P<0.05, \*\*P<0.01 compared with blank group)



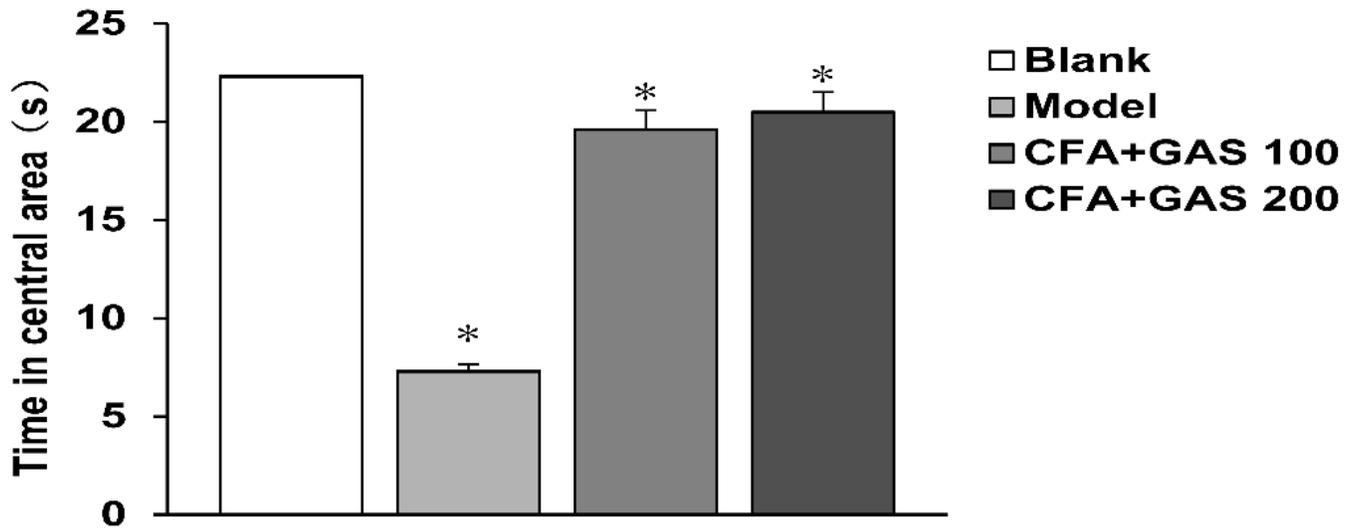
A



B

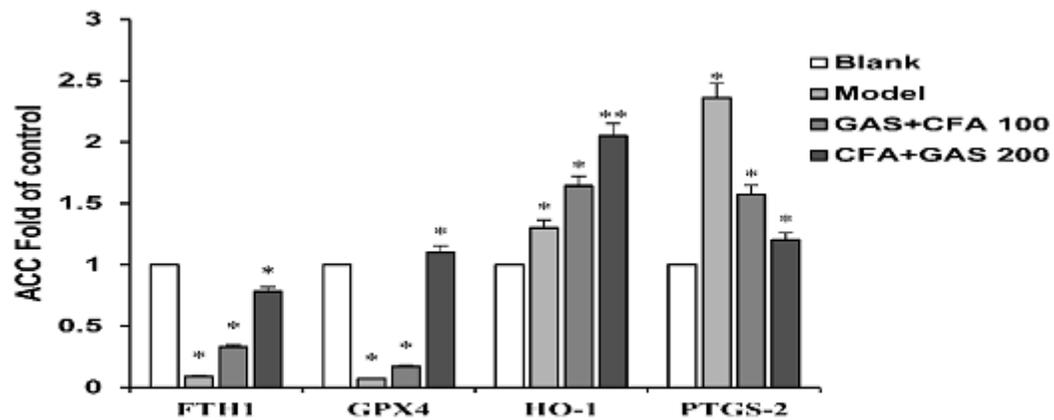
**Figure 4**

Effect of GAS on results of Morris water maze test A the number of entries in open arms B retention times of open arms (\*P<0.05, \*\*P<0.01 compared with blank group)

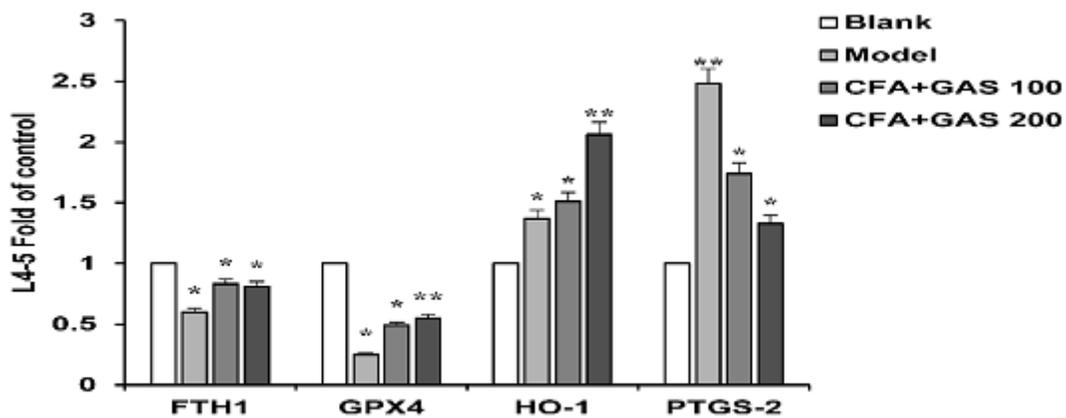


**Figure 5**

Effect of GAS on results of OFT (\*P<0.05 compared with blank group)



A



B

Figure 6

$2^{-\Delta\Delta Ct}$  value of genes in ACC and L4-5 (\* $P < 0.05$ , \*\* $P < 0.01$  compared with blank group)