

## Original Research

### Anti-inflammatory, anti-nociceptive and sedative-hypnotic activities of lucidone D extracted from *Ganoderma lucidum*

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Received December 10, 2018; Accepted April 15, 2019; Published April 30, 2019

Doi: <http://dx.doi.org/10.14715/cmb/2019.65.4.6>

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**Abstract:** Inflammation and insomnia are two types of symptoms very likely occur in life, seriously perplexing people's work and life. How to alleviate these symptoms is an urgent medical problem. Lucidone D (LUC) is a terpene from the ethanol extract of *Ganoderma lucidum* fruiting body. Triterpenoids are also the main pharmacological components of *Ganoderma lucidum*. In recent years, people pay more and more attention to its anti-inflammatory effect. In this study, LPS induced RAW264.7 macrophage inflammatory response model was used to evaluate the anti-inflammatory activity of LUC. The results showed that LUC could significantly inhibit the production of inflammatory mediators NO, which may play a role by down-regulating the expression level of iNOS and COX-2 proteins. Meanwhile, the production of TNF- $\alpha$  and IL-6 was significantly inhibited. These results indicate that LUC has obvious anti-inflammatory activity. Writhing and sedation tests in ICR male mice showed that LUC showed significant analgesic and sedative effects. In conclusion, these results suggest the anti-inflammatory, analgesic and sedative effects of LUC in vitro and in vivo.

**Key words:** Ganoderma lucidum; Lucidone D, Anti-inflammatory; Antinociceptive; Sedative-hypnotic activities.

## Introduction

Inflammation is a complex defense response to harmful stimuli, and it is also a complicated pathological process (1). The clinical features of inflammation are local redness, swelling, heat and pain and the whole body dysfunction. There are a large number of inflammatory cells infiltrating in the inflammatory site, accompanied by tissue proliferation and tissue necrosis (2, 3). At present, the commonly used anti-inflammatory drugs are non-steroidal anti-inflammatory drugs and steroidal anti-inflammatory drugs. Although these two kinds of anti-inflammatory drugs have certain clinical anti-inflammatory effects, long-term use can also produce a series of adverse reactions, such as damage of gastric mucosa, liver, kidney and so on (4-6). Natural drugs, with the characteristics of wide source, low price and small side effects, have been gradually studied. Some natural drugs have been recognized for their anti-inflammatory and analgesic effects (7). Therefore, the search for anti-inflammatory and analgesic active components from traditional Chinese medicine has become the current research hotspot.

Sleep plays an important role in maintaining human health. It is an important physiological function of integrating information, synthesizing new proteins and restoring tissue function (8). Insomnia is a common sleep disorder, long-term insomnia is accompanied by attention loss, mental disorders, hypomnesia and many other mental and physical diseases (9). The incidence of insomnia in the population is 10% to 35%, which is one of the most common diseases in clinical practice, which

seriously affects people's daily life (10). At present, the main treatments for insomnia, depression and other central nervous diseases are barbiturates, benzodiazepines and non-benzodiazepines (11, 12). However, western medicine generally has the disadvantages that long term use easy to produce dependence, large toxic and side effects, etc., which makes it have some limitations in clinical application (13). The curative effect of effective components of many traditional Chinese medicines is definite in the treatment of mental diseases with small side effects. Therefore, a new way has been opened up for the treatment of mental diseases from traditional Chinese medicine and natural medicine.

*Ganoderma lucidum* (Leyss. ex Fr.) Karst is a fungus of the genus *Ganoderma* belonging to the Hymenozymetes, aphylophorales, *Ganoderma lucidum*, which has a long medicinal history in our country, having the functions of tonifying qi and soothing the nerves, relieving cough and asthma. The dried fruiting body of *Ganoderma lucidum* (Leyss. ex Fr.) Karst and *G. sinense* was identified as the medicinal product of *Ganoderma lucidum* by Chinese pharmacopoeia (14). Since ganoderic acid A and ganoderic acid B of *Ganoderma lucidum* were reported in the 1980s, the chemical constituents and biological activities of *Ganoderma lucidum* have been extensively studied at home and abroad. Triterpenoid is the main secondary metabolite of *Ganoderma lucidum* and is also an important bioactive substance of *Ganoderma lucidum* (15). *Ganoderma lucidum* has shown a wide range of biological activities, such as anti-tumor, liver protection, anti-hypertension, inhibition of hyperglycemia and anti-HIV-1 protease, inhibition of

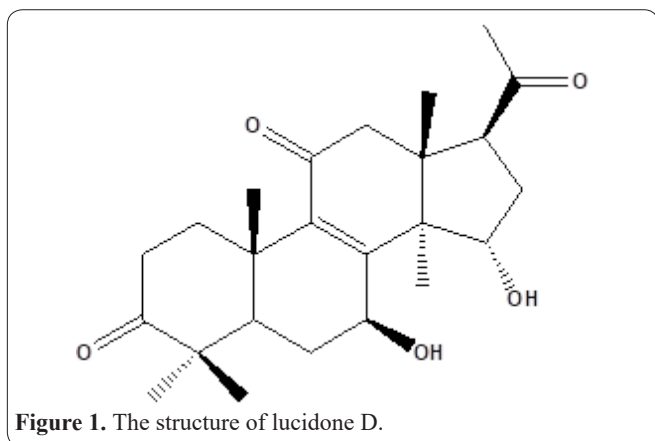


Figure 1. The structure of lucidone D.

platelet aggregation, inhibition of NO release, antioxidant activities (16, 17). Therefore, the study of its related pharmacodynamic substances has attracted much attention, among which triterpene is an important class (18). Lucidone D (LUC, Fig.1) is a new terpene isolated from the ethanol extract of *Ganoderma lucidum* fruiting body. In this study, RAW264.7 macrophages were used to evaluate the anti-inflammatory activity of LUC and ICR male mice were used to detect the analgesic and sedative effects of LUC.

## Materials and Methods

### Materials and reagents

Lucidone D (Shanghai Yihe Biotechnology Co., Ltd.) was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical) as a stock solution. Sodium carboxymethyl cellulose (Sinopharm Chemical Reagent Co., Ltd.); Aspirin enteric-coated tablets (Bayer Schering Pharma); Acetic acid (Shanghai Shenbo Chemical Industry Co., Ltd.); Normal saline (Kunming Nanjiang Pharmaceutical Co., Ltd.); 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT); Pentobarbital sodium (Sigma company); Antibodies to COX-2, TNF- $\alpha$ , IL-6 and  $\beta$ -actin (Santa Cruz, CA, USA); ELISA kit (GAD Technology Co., Ltd., USA) and other reagents are domestic analytical pure grade.

### Cell culture

Mouse mononuclear macrophage cell line RAW264.7 (ATCC; Manassas, VA, USA) was cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin streptomycin. It was placed in a incubator with 5% CO<sub>2</sub> at 37 °C and logarithmic growth of cells were taken for follow-up experiment.

### Effect of LUC on proliferation of RAW264.7 cells by MTT assay

Cytotoxicity was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) assay (19). The RAW264.7 cells were seeded onto 96-well culture plates at a density of  $1 \times 10^4$  cells/well. After incubation with various concentrations of LUC in serum-free RPMI medium for 24 h, 20  $\mu$ L of MTT solution (0.5 mg/mL) was added to each culture well and the cells were further incubated at 37 °C for 4 h. Then, the MTT solution was removed, 200  $\mu$ L of DMSO was added and the mixtures were shaken, and the crystals were fully dissolved for about 10 min. The absorbance

of the solubilized formazan was read at 540 nm using a microplate reader (Infinite F200 Pro, Tecan, Switzerland). The absorbance was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

### Effect of LUC on NO release from RAW264.7 cells by Griess reagent assay

RAW264.7 cells were inoculated on 12-well plate with  $2 \times 10^5$  cells per well. After 24 hours of adhesion, RAW264.7 cells were divided into three groups: blank control group, LPS treatment group and drug+LPS treatment group. Before adding LPS (1  $\mu$ g/mL), the cells were treated with LUC in serum-free medium for 2 hours. The supernatant of cell culture was collected after 24 hours of culture and centrifuged for 20 min at 3000 r/min. The supernatant 100  $\mu$ L was operated according to the flow chart of NO detection kit. The OD value at the wavelength of 550nm was measured by ultraviolet spectrophotometer, and the content of NO secreted by RAW264.7 cells in each group was calculated. The experiment was repeated three times.

### Effect of LUC on the secretion of TNF $\alpha$ and IL-6 in RAW264.7 cells by ELISA assay

RAW264.7 cells were inoculated on 12-well plate and treated as the above experiment. After 24 hours, the supernatant of the cell culture was obtained. According to the steps of ELISA kit, the absorptivity of TNF  $\alpha$  and IL-6 were measured at the wavelength of 450nm and 630nm by the microplate reader, and the secretory volume of TNF  $\alpha$  and IL-6 were calculated. The experiment was repeated three times.

### Analysis of iNOS and COX-2 protein expression levels by Western blot

RAW264.7 cells were cultured in a 6-well plate for 24 h, then treated with LPS (1  $\mu$ g/ml) and different concentrations of LUC for 18 h. According to the method in previous study (20), RIPA cell lysis solution (20mM Tris-HCl (Ph7.5), 150mM NaCl, 1mM Na<sub>2</sub>EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 $\mu$ g/mL leupeptin) was used to lyse cells. The total protein was extracted and the content of total protein was determined by Coomassie brilliant blue method. 50 $\mu$ g of total protein samples were collected from each group and separated on 10% polyacrylamide gel electrophoresis. The separated protein was transferred to the nitrocellulose membrane and incubated in the blocking solution at room temperature for 2 h. 1: 1000 TBS diluted rabbit anti-mouse COX-2 and iNOS monoclonal antibodies were added and incubated overnight in a 4 °C shaker. The membrane was rinsed consecutively by TBST (pH7.6) for 3 times with each time for 5 min. The second antibody of rabbit IgG labeled with horseradish peroxidase was diluted with 1: 5000 TBS and incubated at room temperature for 2 h. The membrane was rinsed consecutively by TBST (pH7.6) for 3 times with each time for 5 min. The color development was performed in enhanced chemiluminescent liquid (ECL), and the film exposure was carried out in the darkroom. The film was scanned and processed by Image J software. The experiment was repeated three times.

## Experimental animal

The healthy ICR male mice was obtained from the Shanghai Jiao Tong University experimental animal research center and the average body weight of (20 ± 2) g. The mice were fed in separate cages and placed in a quiet, suitable temperature and humidity, well-ventilated environment, drinking water freely and feeding reasonably. The experiment began after 1 week of adaptation to the environment.

## Writhing test of mice induced by acetic acid

According to the method in previous research (21), male ICR mice were randomly divided into 5 groups with 10 mice in each group, namely model control group, aspirin group (40 mg/kg), low, medium and high dose of LUC group (the doses were 20 mg/kg, 40 mg/kg and 80 mg/kg, respectively). The model control group was given 0.5% sodium carboxymethylcellulose solution with the volume of 20 mL/kg, the other groups were given the corresponding drugs by the volume of 20 mL/kg, once a day for 7 days. One hour after the last administration, 6% glacial acetic acid solution (prepared by pure water) was injected intraperitoneally with the volume of 10 mL/kg. The number of writhing reactions (abdominal fovea, extension of hind limbs, hip elevation) was recorded in mice within 15 min after injection, and the writhing inhibition rate was calculated by the following formula:

Writhing inhibition rate (%) = (1 - writhing times in administration group / writhing times in model control group) × 100%.

## Effects of LUC on sleep time and sleep latency induced by threshold dose pentobarbital sodium in mice

Male ICR mice were randomly divided into 5 groups with 10 mice in each group: blank group (intra-gastric administration of distilled water), diazepam group (intra-gastric administration of diazepam), low, medium and high dose of LUC group (the doses were 20 mg/kg, 40 mg/kg and 80 mg/kg, respectively). The mice in 5 groups were given intra-gastric administration once a day for 7 days. 30 min after the last administration, the mice in each group received intraperitoneal injection of pentobarbital sodium at a threshold dose of 49.5 mg/kg. The number of mice with righting reflex disappeared for more than 1 min within 30 min was observed. The onset time of sleeping and waking time of each mouse were recorded at the same time, namely the sleep latency period and sleeping time of mice.

## Statistical method

In this study, GraphpadPrism7.0 statistical software was used for statistical analysis of the research data. The measurement data was expressed as mean ± standard deviation ( $\bar{x} \pm SD$ ). Single factor analysis of variance (ANOVA) was used for multi-group comparison, and Student-Newman-Keuls test (SNK) was performed for post-comparison. For the subhypnotic dose of pentobarbital test, the chi-square test was used to compare the number of sleeping mice.  $P < 0.05$  represents significant difference in statistics.

## Results

## Effect of LUC on proliferation of RAW264.7 cells by MTT assay

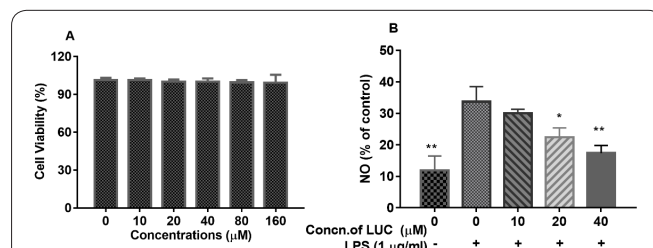
MTT assay was used to detect the cytotoxicity of LUC after 48 hours of treatment with different doses. The results showed that LUC had no significant effect on the proliferation of RAW264.7 macrophages at the concentrations of 10, 20, 40, 80 and 160  $\mu\text{M}$  (Fig. 2A). That is, the drug has no cytotoxicity within this concentration range.

## Detection of NO release by LUC on RAW264.7 cells by Griess reagent assay

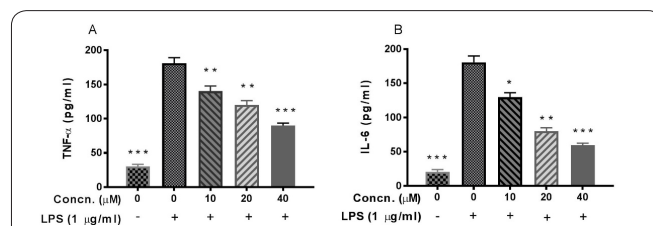
Griess reagent assay was used to detect the level of NO released by LUC in RAW264.7 cells. Compared with the control group, the level of NO secreted by RAW264.7 cells was significantly increased after LPS treatment ( $P < 0.05$ ). Pretreatment with LUC reduced the secretion of NO compared with that of LPS (Fig. 2B) and showed obvious concentration dependence.

## Detection of effect of LUC on the secretion of TNF $\alpha$ and IL-6 in RAW264.7 cells by ELIAS assay

In order to investigate whether LUC inhibits TNF- $\alpha$  and IL-6, the production of TNF- $\alpha$  and IL-6 in RAW264.7 cell culture supernatant induced by LPS was quantitatively determined by ELIAS assay. As shown in figure 3, compared with the control group, cells in the LPS group (1  $\mu\text{g}/\text{mL}$ ) released a large number of inflammatory factors, and the production of TNF- $\alpha$  and IL-6 increased significantly. However, pretreatment of



**Figure 2.** Effects of LUC on cell viability and NO production in RAW264.7 macrophages. (A) RAW264.7 cells ( $1 \times 10^5$  cells/ml) were treated with 10, 20, 40, 80 and 160  $\mu\text{M}$  OLE for 48 h, and cell viability was determined by the MTT assay; (B) RAW264.7 cells were pre-incubated with 10, 20 and 40  $\mu\text{M}$  LUC for 2 h, and then treated with 1  $\mu\text{g}/\text{ml}$  LPS for an additional 24 h. Data represent the mean  $\pm$  SD of at least three independent experiments. The statistical significance (\*,  $P < 0.05$ , \*\*,  $P < 0.01$ ,) of results were determined through Student's t test vs. control.



**Figure 3.** Effects of LUC on LPS-induced proinflammatory cytokine production in RAW264.7 cells. RAW264.7 cells were treated with LPS (1  $\mu\text{g}/\text{ml}$ ) in the absence or presence of LUC at the indicated concentration for 24 h. (A) TNF- $\alpha$  and (B) IL-6 in the cultured supernatant were measured by ELISA. Data represent the mean  $\pm$  SD of at least three independent experiments. The statistical significance (\*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ) of results were determined through Student's t test vs. control.

RAW264.7 cells with different concentrations of LUC significantly inhibited the production of TNF- $\alpha$  and IL-6 in a dose-dependent manner ( $P < 0.05$ ).

#### Analysis of iNOS and COX-2 protein expression levels by Western blot

The effect of LUC on the expression of iNOS and COX-2 proteins in RAW264.7 macrophages was detected by western blot assay. As shown in figure 4, 1  $\mu\text{g}/\text{mL}$  LPS stimulation could significantly up-regulate the expression of iNOS and COX-2 protein in RAW264.7 macrophages, while the effect of LPS on iNOS and COX-2 decreased with the increase of LUC concentration, and the inhibitory effect was concentration-dependent ( $P < 0.05$ ).

#### Writhing test of mice induced by acetic acid

LUC was intragastric administered continuously at doses of 20mg/kg, 40mg/kg and 80mg/kg for 7 days. Compared with the model control group, 20mg/kg, 40mg/kg and 80mg/kg LUC all decreased the times of writhing reaction induced by acetic acid in mice ( $P < 0.05$ ), indicating that LUC had obvious analgesic effect (Fig. 5).

#### Effects of LUC on sleeping time and sleep latency induced by pentobarbital sodium in mice

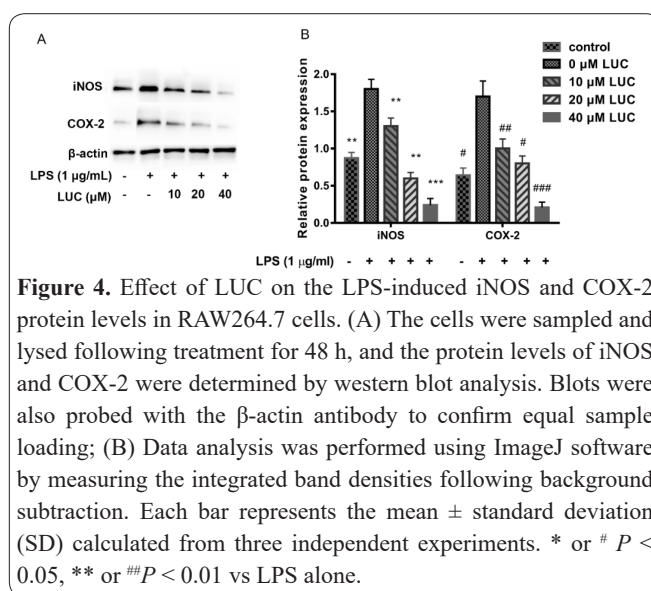
The sleeping time and sleep latency of each group were compared. Compared with the blank group, the sleeping time of diazepam group was prolonged ( $P < 0.05$ ), and the sleep latency was shortened ( $P < 0.01$ ). Meanwhile, compared with the blank group, the mice in 40mg/kg and 80mg/kg LUC groups had longer sleeping time and shorter sleep latency ( $P < 0.01$ ), but there was no significant change in sleeping time and sleep latency in 20mg/kg LUC group (Fig. 6).

#### Discussion

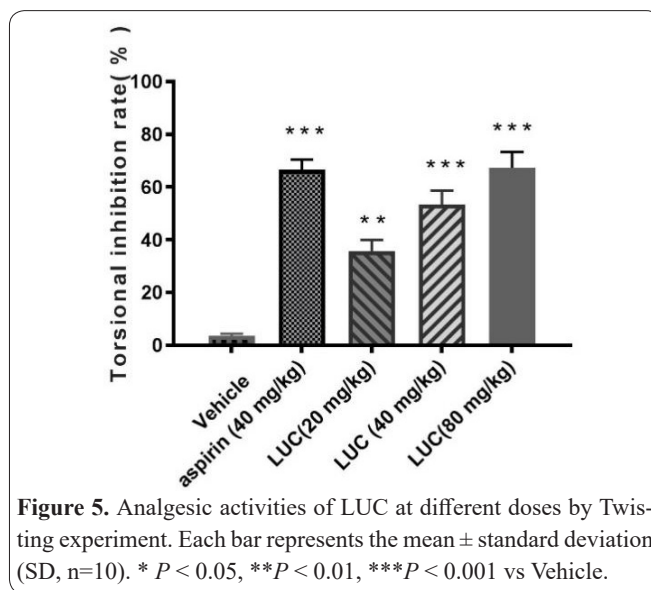
Inflammation is a physiological defense response of active tissues to harmful stimuli such as pathogens, damaged cells, or irritants (22). After stimulating the body, inflammatory factor activates the macrophages to synthesize and release many kinds of inflammatory mediators, such as prostaglandin ( $\text{PGE}_2$ ), nitric oxide (NO), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) and so on, increases vascular permeability and promotes chemotaxis of inflammatory cells, thus inducing inflammation (23). However, chronic inflammation can lead to many diseases and even organ failure (24). In this study, mouse macrophage RAW264.7 cell lines were used to investigate the potential therapeutic effect of LUC in regulating cytokine and NO production, thereby inhibiting inflammation.

First, MTT assay was used to detect the cytotoxicity of mouse cells treated with different doses of LUC for 48 hours. The results showed that LUC had no effect on the proliferation of RAW264.7 macrophages at the highest concentration, indicating that the drug had no cytotoxicity within this concentration range.

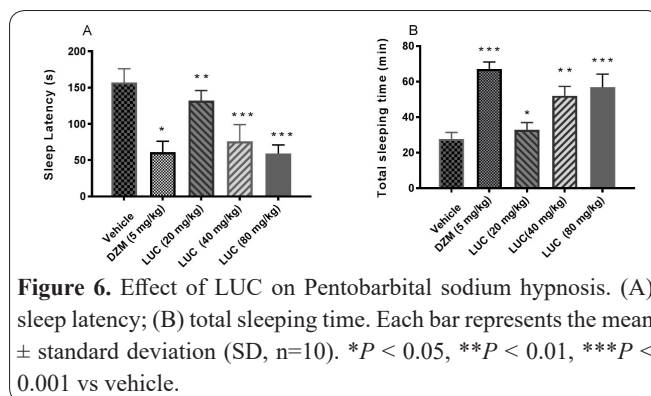
Bacterial lipopolysaccharide (LPS) is the most common inflammatory factor, which stimulates the synthesis and release of many inflammatory mediators in macrophages (23). NO is one of them, which is synthesized



**Figure 4.** Effect of LUC on the LPS-induced iNOS and COX-2 protein levels in RAW264.7 cells. (A) The cells were sampled and lysed following treatment for 48 h, and the protein levels of iNOS and COX-2 were determined by western blot analysis. Blots were also probed with the  $\beta$ -actin antibody to confirm equal sample loading; (B) Data analysis was performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents the mean  $\pm$  standard deviation (SD) calculated from three independent experiments. \* or #  $P < 0.05$ , \*\* or ##  $P < 0.01$  vs LPS alone.



**Figure 5.** Analgesic activities of LUC at different doses by Twisting experiment. Each bar represents the mean  $\pm$  standard deviation (SD,  $n=10$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  vs Vehicle.



**Figure 6.** Effect of LUC on Pentobarbital sodium hypnosis. (A) sleep latency; (B) total sleeping time. Each bar represents the mean  $\pm$  standard deviation (SD,  $n=10$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  vs vehicle.

by arginine through three types of nitric oxide synthase (NOSs): inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) (25). A large number of studies have shown that iNOS is the main enzyme that promotes the production of NO in inflammatory response(26-27). Prostaglandin E2 ( $\text{PGE}_2$ ) is one kind of prostaglandin, a metabolite of arachidonic acid cyclooxygenase (COX). There are three subtypes of cyclooxygenase (COX): COX-1, COX-2 and COX-3, among which COX-2 is also called inducible enzyme. Because COX-2 is not expressed under normal conditions, and it is significantly up-regulated when cells are stimulated by LPS or other factors (28). Inflammatory cytokines are cytokines in-

volved in inflammatory reactions. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) is the most important inflammatory mediator in the process of inflammation, because it activates neutrophils and lymphocytes and increases the permeability of vascular endothelial cells, then promote the synthesis and release of other cytokines (29). Interleukin (IL-6) is involved in the body immune response and promotes the occurrence of inflammation. After IPS activates macrophages, proinflammatory mediators and cytokines are produced, including prostaglandin (PEG<sub>2</sub>), nitric oxide (NO), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6). Thus, down-regulation of these mediators and cytokines produced by macrophages is critical to inhibiting inflammation and provides a mechanism for the development of drugs that may have been used to treat a variety of inflammatory diseases.

In this study, the effect of LUC on the release of NO in RAW264.7 cells were detected by Griess reagent assay, and the effect of LUC on the release of TNF  $\alpha$  and IL-6 inflammatory factors in RAW264.7 cells was detected by ELISA assay. It has been demonstrated that LUC can decrease the secretion of NO in a concentration dependent manner. With the increase of LUC concentration, iNOS and COX-2 were also down-regulated, and showed a concentration correlation. This also suggests that LUC is a potential drug for the treatment of inflammation.

The pain writhing model of mice induced by acetic acid is a common method for the screening of analgesic drugs at home and abroad. The main purpose is to investigate the peripheral analgesic effects of the tested drugs and to study the pharmacology of central analgesic drugs (30). The chemical stimulation produced by acetic acid can cause persistent abdominal pain in mice, accompanied by acute inflammatory reaction, which stimulates the visceral and parietal peritoneum and causes painful writhing reaction in mice. The results showed that LUC could significantly reduce the number of painful writhing in mice and had a certain analgesic effect.

The classical experiment to study the hypnotic function of drugs is the synergistic sleep test of barbiturates. Pentobarbital sodium prolongs sleep time by interfering with the polysynaptic transmission of the brainstem system and enhancing the inhibitory effect on the central nervous system (31). The results indicated that 40 and 80 mg/kg LUC successfully affected the sleep latency and total sleeping time of mice in subthreshold and threshold dose of pentobarbital sodium synergistic experiments. The significant reduction in sleep latency and total sleeping time induced by pentobarbital sodium proved the sedative effect of LUC. Therefore, it can be concluded that LUC has sedative activity in addition to its analgesic and anti-inflammatory effects, suggesting that it may be effective in controlling pain and related diseases.

In conclusion, through a series of experiments, it is proved that LUC has anti-inflammatory, analgesic and sedative effects, but the deeper action mechanism still needs to be further studied.

### Acknowledgements

We thank the Pei lab members for advice and support.

### Conflict of Interest

There are no conflicts of interest in this study.

### Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yan Wang; Xia Feng, Yan Wang collected and analysed the data; Xia Feng wrote the text and all authors have read and approved the text prior to publication.

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