

# ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF ETHANOL EXTRACT OF WU-ZI-YUAN-CHUNG-WAN IN MICE

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The aim of this study was intended to investigate the analgesic and anti-inflammatory activities of ethanol extract of Wu-Zi-Yuan-Chung-Wan (WZYCW). We examined the analgesic activity of WZYCW (0.25, 0.5 and 1.0 g/kg, p.o.) by acetic acid-induced writhing response. The anti-inflammatory activity of WZYCW (0.25, 0.5 and 1.0 g/kg, p.o.) was assessed using the  $\lambda$ -carrageenan induced paw edema model. The results showed that the WZYCW (0.5 and 1.0 g/kg, p.o.) decreased the acetic acid-induced writhing response. Moreover, WZYCW also significantly decreased the paw edema induced by  $\lambda$ -carrageenan and the level of MDA in the edema paw. WZYCW increased the activities of SOD, GSH-Px and GSH-Rd. In conclusion, these results suggest that WZYCW possessed analgesic and anti-inflammatory activities. The anti-inflammatory mechanisms of WZYCW may be related to decreasing the level of MDA in the edema paw via increasing the activities of SOD, GSH-Px and GSH-Rd in the liver. WZYCW may be used as a pharmacological prescription in the prevention or treatment inflammatory disorders.

**Key words:** Wu-Zi-Yuan-Chung-Wan, anti-inflammation, analgesia, carrageenan, acetic acid

## Introduction

According to the statistical analysis of Department of Health, Executive of Yuan, cancer was ranked first at the top ten diseases which caused

death during 1982 to 2006. Cancer is top one disease which affects the health of the population in Taiwan. The primary phase of cancer was resulted from inflammatory response. The chronic inflammation is the major incident cause of cancer<sup>1</sup>. Therefore,

developing novel anti-inflammatory drugs is very important nowadays.

Pro-inflammatory (e.g. TNF- $\alpha$ ) stimuli activate cellular responses that increase production of many cytokines, including prostaglandins (PGs) and NO, during the response to inflammation<sup>2</sup>. Inflammation, which is typically characterized by redness, swelling, pain, and heat, is one of the most important host defense mechanisms against invading pathogens. Some researches indicate that the increases in the immunity which possesses powerful defensive system decrease the incidence of cancer<sup>3</sup>. If the inflammatory response is continuing, it increases the incidence of cancer<sup>4</sup>. The inflammatory cytokines (NO, PGs) could increase the incidence of cancer<sup>5</sup>. The reason is NO combines with superoxide anion ( $\cdot O_2^-$ ) to produce peroxynitrite (ONOO<sup>-</sup>). The peroxynitrite results in DNA modification which produces gene toxicity and initiation of carcinogen<sup>6,7</sup>. It is important to decrease the proliferation of free radicals. The superoxide dismutase (SOD), glutathione reductase (GSH-Rd) or glutathione peroxidase (GSH-Px) play an important role in decreasing proliferation of free radicals ( $H_2O_2$  and  $\cdot O_2^-$ ), then decreasing cellular ROS and injury, and the propagation of inflammatory response<sup>8,9</sup>.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are important therapeutic agents used for treating pain and inflammation. Though their main functions suppress the COX activeness, the NSAIDs used clinically are often of limited application due to their common side effects, such as gastrointestinal (GI) hemorrhage<sup>10</sup>. Therefore, the aim of the present study was intended to search for anti-inflammatory agent that has low side effect from traditional Chinese medicine.

Wu-Zi-Yuan-Chung-Wan (abbrev. WZYCW),

firstly described in Shung-Jie-Lou which is an ancient Chinese book, was composed of *Plantago asiatica* L., *Lycium chinense* L., *Cuscuta chinensis* Lam., *Rubus idaeus* Hu., and *Schizandra chinensis* Baill., *Lycium chinense* and *Cuscuta chinensis* possess germination and kidney function-enhancing effects. *Rubus idaeus* and *Schizandra chinensis* possess benefiting jinq for promoting production of blood. *Plantago asiatica* possesses diuretic effect<sup>11</sup>. WZYCW possesses supplementing kidney-energy for enriching sperms (or supplementing essence), coursing kidney qi, becoming pregnant for propagation and uses to treat sterility<sup>12</sup>. In modern medicine researches, WZYCW possesses protective function of germination in testis, regulatory function of hypothalamus-pituitary-sex gland axis, anti-aging, hypoglycemic, anti-oxidative and immunological enhancing effects<sup>13,14</sup> and is the first choice formula for treating sterility. WZYCW possesses anti-aging effect via improving symptoms of aging caused by kidney function decay, regulating the sex hormone level, decreasing LPO level in the blood, and elevating the activity of SOD<sup>15</sup>. The diverse medical properties and the presence of anti-oxidation in the WZYCW prompted us to investigate its analgesic and anti-inflammatory activities.

Anti-inflammatory and analgesic activities of the herbal extract are usually evaluated using  $\lambda$ -carrageenan, acetic acid-induced writhing response. The animals were induced writhing response after 1% acetic acid injected intraperitoneally. The acetic acid-induced writhing response is a standard model for screening for analgesic compounds<sup>16</sup>.

The  $\lambda$ -carrageenan, histamine, 5-HT, dextran, bradykinin and prostaglandin are the inflammatory mediators. The cellular and molecular mechanism of the  $\lambda$ -carrageenan-induced inflammation is well

characterized, because this model of inflammation is a standard model for screening for anti-inflammatory compounds<sup>17</sup>.  $\lambda$ -carrageenan, origin from carra-heen town in Ireland, is extracted from Irish sea moss (*Chondrus crispus*). The initial phase of edema (0-1 h), contributes to the release of histamine, bradykinin and 5-hydroxytryptamine (5-HT), produces cytokine (TNF- $\alpha$ ) production which stimulates productions of IL-1 $\beta$ , IL-6, and IL-8. Then COX proliferation induces inflammatory pain. The histamine, bradykinin, and 5-HT also stimulate cNOS protein activation which promotes NO release. The iNOS will be induced and promote NO production at the late phase of edema (1-6 h)<sup>18</sup>.

This study investigated the anti-inflammatory and analgesic activities of WZYCW. The analgesic activity of WZYCW was evaluated using acetic acid-induced writhing response. The anti-inflammatory activity of WZYCW was determined using the carrageenan-induced paw edema model. This study was also detected the level of malondialdehyde (MDA) in the edema paw and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) in the liver at the 3rd hr after  $\lambda$ -carrageenan injection to investigate the relationship between the anti-inflammatory mechanism of the WZYCW and antioxidant enzymes activities. Indomethacin was used as a positive control.

## Materials and Methods

### Plant material and crude extract preparation

According to the 4<sup>th</sup> edition Chinese pharmacopeia record, the Wu-Zi-Yuan-Chung-

Wan was composed of *Plantago asiatica* L., *Lycium chinense* L., *Cuscuta chinensis* Lam., *Rubus idaeus* Hu., and *Schizandra chinensis* Baill at the ratio of 1: 4: 4: 2: 0.5. After mixing the five herbal medicines, the 1.8 kg mixture was extracted with 10 L 75% ethanol for four times. Ethanol was removed by distillation under reduced pressure and the remaining solution was lyophilized to yield the crude ethanol extract of WZYCW which was then stored in -20 °C refrigerator. The yield ratio of WZYCW was 22.2%. For the pharmacological tests, the extract was dissolved in saline solution prior to its use.

All of the herbal medicines were purchased from Shin-Long Drug store in Taichung city and identified by Dr. Kuo, Chao-Lin, leader of the School of Chinese Medicine Resources.

### Experimental animals

Male ICR mice were obtained from the National Laboratory Animal Center. Male ICR mice (18-22 g) were used in the experiment. They were housed in standard cages at a constant temperature of  $22 \pm 1$  °C, relative humidity  $55 \pm 5\%$  with 12 h light-dark cycle (08:00 to 20:00) for 1 week at least before the experiment. Animals used in this study were housed and cared in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### Experimental groups

Sixty mice were random divided into six groups: control, positive control, three doses (0.25, 0.5, and 1.0 g/kg) of WZYCW groups. The number of mice was ten per group.

## Experimental methods

### Acetic acid-induced writhing response

The writhing test in mice was carried out by using the method of the previous study<sup>16</sup>. The writhes were induced by intraperitoneal injection of 1.0% acetic acid (v/v, 0.1 ml/10 g body weight). There are three different doses (0.25, 0.5, and 1.0 g/kg) of WZYCW administered orally to each groups of mice, 60 min before chemical stimulus. Indomethacin as a positive control was administered 30 min prior to acetic acid injection. The number of muscular contractions was counted over a period of 5 min after acetic acid injection. The data represented the total numbers of writhes observed during 10 min.

### $\lambda$ -Carrageenan-induced mice paw edema

The anti-inflammatory activity of WZYCW was determined by the  $\lambda$ -carrageenan-induced edema test in the hind paws of mice. Male ICR mice (six per each group), were fasted for 24 h before the experiment with free access to water. Fifty microliter of 1%  $\lambda$ -carrageenan suspension (Sigma Co., USA) in saline was injected into the plantar side of right hind paws of the mice<sup>18</sup>. Paw volume was measured immediately at 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> h after the administration of the  $\lambda$ -carrageenan, using a plethysmometer. The degree of swelling was evaluated by the delta volume (a-b), where a and b are the volume of the right hind paw after and before the  $\lambda$ -carrageenan treatment, respectively. Indomethacin (10 mg/kg) was administered intraperitoneally 30 min before  $\lambda$ -carrageenan injection. WZYCW (0.25, 0.5, and 1.0 g/kg) was orally administered 60 min before  $\lambda$ -carrageenan injection. The control was given an equal volume of saline.

In the secondary experiment, the whole right hind paw tissue and liver tissue were taken at the 3<sup>rd</sup> h. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in four times their volume of cold normal saline and homogenized at 4°C. Then the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained and stored at -20°C refrigerator for the MDA assay.

The whole liver tissue was rinsed in ice-cold normal saline, and immediately placed in one time their volume of cold normal saline and homogenized at 4°C. Then the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained and stored at -20°C refrigerator for the antioxidant enzymes (SOD, GSH-Px, and GSH-Rd) activity assays.

### *Superoxide dismutase (SOD) Assay*

SOD enzyme activity was determined according to the method of Woolliams *et al.*<sup>19</sup> at room temperature. One hundred microliter of tissue extract was added to 880  $\mu$ l (0.05 M, pH 10.2, 0.1 mM EDTA) carbonate buffer. Twenty microliter of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture at 480 nm for 4 min on a Hitachi U 2000 Spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50% which is equal to 1 unit.

### *Glutathione reductase Assay*<sup>20, 21</sup>

The 100 microliters liver homogenate was added into test tube contained 100 microliters iced dist. water and stored at 2~8°C. The mixture was centrifuged at 1878 g for 5 min. The 100 microliters supernatant was mixed with 1.9 ml normal saline. The 50  $\mu$ l mixture, 50  $\mu$ l glutathione reductase buffer (250 mmol/L pH 7.3 Kupfer

phosphate and EDTA 0.5 mmol/L), GSSG (2.2 mmol/L) and NADPH (0.17 mmol/L) were mixed and measured the absorbance at 340 nm. The glutathione reductase activity in the liver was expressed as U/g protein.

#### ***Glutathione Peroxidase Assay***

Glutathione peroxidase (GSH-Px) enzyme activity was determined according to the method of Kraus and Ganther<sup>22</sup> at 37°C. A reaction mixture was composed of 500 µl phosphate buffer, 100 microliters 0.01M GSH (reduced form), 100 microliters 1.5 mM NADPH and 100 microliters GSH-Rd (0.24 units). One hundred microliter of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Then 50 microliters of 12 mM *t*-butyl hydroperoxide was added to 450 microliters tissue reaction mixture and measured at 340 nm for 180 s. The molar extinction coefficient of  $6.22 \times 10^{-3}$  was used to determine the enzyme activity. One unit of activity is equal to the mM of NADPH oxidized/min per mg protein.

#### ***Total Protein Assay***<sup>23</sup>

0.5 g liver tissue and 0.5 ml 0.9% normal saline were added into test tube and homogenized. 50 microliters homogenate and 350 microliters normal saline were mixed. The mixture was added 0.1 ml Biuret reagent (sodium hydroxide 100 mmol/L, Na-K-tartrate 16 mmol/L, potassium iodide 15 mmol/L, cupric sulphate 6 mmol/L) and detected the absorbance per 25 s for 15 min at 550 nm. Albumin (6.0 g/dl) was used as protein standard. Sodium hydroxide (100 mmol/L) and Na-K-tartrate (16 mmol/L) was used as Blank reagent. Total protein level in liver was expressed as mg/g tissue.

#### ***MDA assay in edema paw***<sup>24</sup>

0.2 ml homogenate of the edema paw, 0.8 ml

phosphate buffer saline, 0.0025 ml BHT and 0.5 ml TCA (30%) were mixed and centrifugated at 1878 g for 15 min after 2 hr later. The 1.0 ml supernatant, 0.075 ml EDTA (0.1 M) and 0.025 ml TBA (1% in 0.05 N NaOH solution) were mixed and heated on the water bath for 15 min and detected the absorbance of the reaction mixture at 532 nm and 600 nm. The MDA level of the edema paw could be calculated according to the formula  $[(A532-A600)/1.56/100000]$  and expressed as nmol MDA/mg protein.

#### **Statistical analysis**

All the data were expressed as mean  $\pm$  S.E.M. Statistical analysis was carried out using one-way ANOVA followed by Scheffe's multiple range test. The criterion for statistical significance was  $P < 0.05$ .

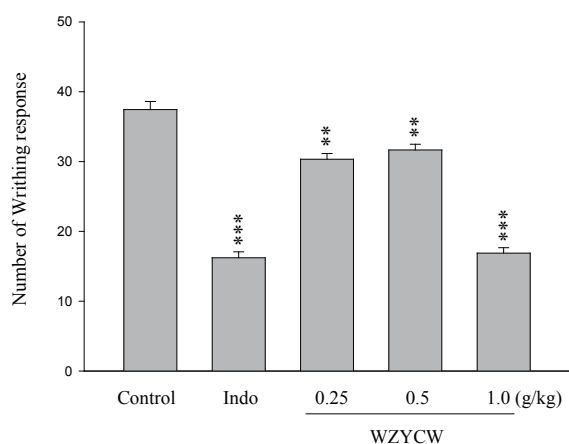
## **Results**

### **Effect of WZYCW on Acetic acid-induced writhing response**

Fig 1 shows the acetic acid-induced writhing responses in mice, indicative of the analgesic activity of WZYCW. Both 0.25-1.0 g/kg WZYCW and indomethacin significantly reduced writhing responses induced by acetic acid when compared with the control group.

### **Effect of WZYCW on carrageenan-induced paw edema**

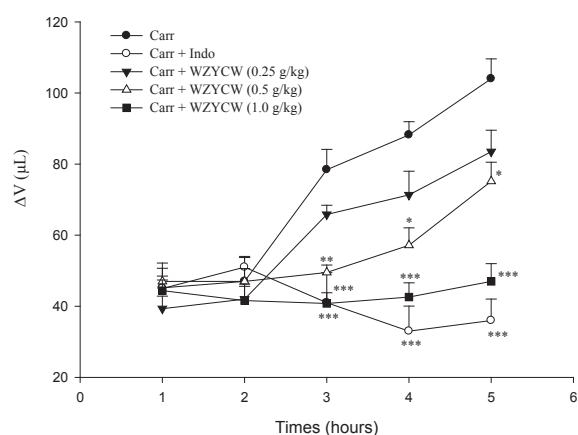
Carrageenan-induced paw edema was significantly reduced by administering WZYCW and indomethacin at 3, 4, and 5 h after the carrageenan injection (Fig 2). Therefore, we detected the activity of the anti-oxidative enzymes in the liver at 3<sup>rd</sup> hr after the carrageenan injection.



**Fig. 1.** Analgesic effect of the WZYCW and indomethacin (Indo) on acetic acid-induced writhing response in mice. Each value represents as mean  $\pm$  SEM (n = 8). \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  as compared with the control group. (One-way ANOVA followed by Scheffe's multiple range test).

### Effects of WZYCW on the activities of antioxidant enzymes

At the 3<sup>rd</sup> h following the intrapaw injection of  $\lambda$ -carrageenan, liver tissues were also analysed for the biochemical parameters such as SOD, GSH-Px and GSH-Rd activities. As showed in Table 1, SOD, GSH-Px and GSH-Rd activities in liver tissue were decreased significantly after  $\lambda$ -carrageenan administration for 3 hr. SOD, GSH-Px and GSH-



**Fig. 2.** Effects of the WZYCW and indomethacin on hind paw edema induced by  $\lambda$ -carrageenan in mice. Each value represents as mean  $\pm$  SEM (n = 8). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  as compared with the  $\lambda$ -carrageenan (Carr) group. (One-way ANOVA followed by Scheffe's multiple range test).

Rd activities were increased significantly after treated with WZYCW (0.5-1.0 g/kg) and 10 mg/kg indomethacin ( $P < 0.05-0.001$ ).

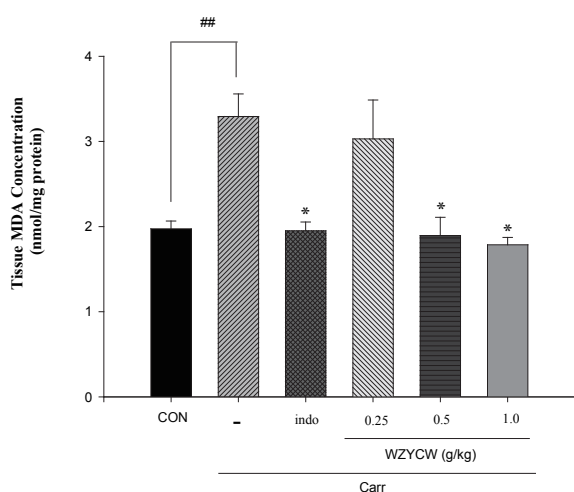
### Effects of WZYCW on MDA level

MDA level in the edema paw induced by  $\lambda$ -carrageenan was increased significantly. However, MDA level was decreased significantly by treatment with 0.5 g and 1.0 g/kg WZYCW, as well as 10 mg/

**Table 1.** The effects of WZYCW on the  $\lambda$ -carrageenan (Carr)-induced liver SOD, GSH-Rd and GSH-Px activities in mice.

Treatments	Dose (g/kg, p.o.)	SOD (U/mg protein)	GSH-Rd (U/g protein)	GSH-Px (U/g protein)
Control		32.98 $\pm$ 0.86	173.80 $\pm$ 4.92	39.80 $\pm$ 2.32
Carr		24.39 $\pm$ 1.39 <sup>##</sup>	80.02 $\pm$ 4.51 <sup>###</sup>	17.65 $\pm$ 1.04 <sup>###</sup>
Indo		42.27 $\pm$ 3.57 <sup>***</sup>	163.24 $\pm$ 11.59 <sup>***</sup>	36.82 $\pm$ 2.10 <sup>***</sup>
Carr + WZYCW	0.25	27.99 $\pm$ 0.35	94.60 $\pm$ 4.46	21.38 $\pm$ 1.08
	0.5	34.49 $\pm$ 1.49 <sup>*</sup>	92.69 $\pm$ 4.58	26.28 $\pm$ 1.03 <sup>**</sup>
	1.0	37.07 $\pm$ 1.71 <sup>**</sup>	119.62 $\pm$ 5.5 <sup>**</sup>	27.25 $\pm$ 1.09 <sup>***</sup>

Each value represents as mean  $\pm$  SEM (n = 10). <sup>##</sup> $P < 0.01$ , <sup>###</sup> $P < 0.001$  as compared with the Control group. <sup>\*\*\*</sup> $P < 0.001$  as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test).



**Fig. 3. Effects of the WZYCW and indomethacin (Indo) on the MDA concentration of edema paw in mice. Each value represents as mean  $\pm$  SEM (n = 8). ### $P$  < 0.01 as compared with the CON group. \* $P$  < 0.05 as compared with the Carr group. (One-way ANOVA followed by Scheffe's multiple range test).**

kg indomethacin (Fig. 3). This result suggested that the anti-inflammatory effect of WZYCW was related to the decrease in the MDA level in the edema paw of mice.

## Discussion

The acetic acid-induced writhing response belongs to chemical stimulating pain, was described by Siegmund *et al.*<sup>25</sup> The mice were induced writhing response after acetic acid injected intraperitoneally for 5 min<sup>26, 27</sup>. Both of NSAIDs and SAIDs could decrease the acetic acid-induced writhing response. This model was used to screen the analgesics. In the acetic acid-induced writhing response, the visceral pain model, the analgesic mechanism of abdominal writhing induced by acetic acid involves the release of arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis. Test results indicate

that the number of acetic acid-induced abdominal constrictions was reduced significantly by WZYCW (0.5-1.0 g/kg) and indomethacin (10 mg/kg) (Fig 1). The mechanism of the analgesic effect of WZYCW may be blockage of arachidonic-acid metabolite synthesis.

Among the several models of acute inflammation,  $\lambda$ -carrageenan-induced inflammation has been well established as a valid model to study free radical generation in liver tissue after inflammatory states<sup>18</sup>.  $\lambda$ -carrageenan, extracted from Irish sea moss (*Chondrus crispus*) is used to induce inflammatory response in animals and possesses high production<sup>28</sup>. The cellular and molecular mechanisms of the  $\lambda$ -carrageenan-induced inflammation are well characterized. In this study, both WZYCW and indomethacin significantly decreased carrageenan-induced paw edema. WZYCW had an anti-inflammatory effect on carrageenan-induced mice paw edema.

The development of edema in the hind paw following injection of carrageenan has been characterized as a biphasic event in which various mediators operate in sequence to generate this inflammatory response<sup>29</sup>. The initial phase of edema (0-1 h) is not inhibited by NSAIDs such as indomethacin or aspirin<sup>30</sup>. Conversely, the second phase, in which swelling accelerates (1-6 h), is correlated with elevated production of NO and PGs<sup>31</sup>. The paw edema induced by  $\lambda$ -carrageenan was raised to maximum at the third hour<sup>32</sup>. Free radical and prostaglandin will be released when administrating with  $\lambda$ -carrageenan for 1 to 6 hours<sup>31</sup>. The  $\lambda$ -carrageenan-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such

as hydrogen peroxide, superoxide and hydroxyl radicals, as well as the release of other neutrophil-derived mediators<sup>33</sup>. A drug could decrease the ROS level during inflammation possesses anti-inflammatory effect<sup>34</sup>. Janero demonstrates that MDA production is due to free radicals attack plasma membrane<sup>35</sup>. Liver tissue contains many anti-oxidative enzymes that could prevent from free radicals damage. Therefore, we detected the activity of the anti-oxidative enzymes in the liver at 3<sup>rd</sup> hr after the carrageenan injection. In this study, there is significantly increased in SOD, GSH-Rd and GSH-Px activities with WZYCW treatment. Furthermore, there is significantly decreased in MDA activity with WZYCW treatment. We assume the suppression of MDA production is probably due to increasing SOD, GSH-Rd and GSH-Px activities. The antioxidant activity of WZYCW explains at least in part the mechanism of its anti-inflammatory activity. The anti-inflammatory effect of WZYCW was not described in the ancient books and was the new finding in this study. WZYCW possesses anti-ROS and immune enhancing functions<sup>14</sup>. Whether the mechanism of anti-inflammation of WZYCW was related to the increase in the level of glucocorticosteroid hormone or not, it need to be further study in the future.

These results indicated that WZYCW possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of WZYCW might be related to the decrease in the level of MDA in the edema paw *via* increasing the activities of SOD, GSH-Px and GSH-Rd in the liver. WZYCW can be developed as a pharmacological agent for the prevention of inflammatory disorders.

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## 五子衍宗丸對小鼠之鎮痛及抗發炎作用

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本研究先以醋酸扭體試驗評估五子衍宗丸之鎮痛作用，並以 $\lambda$ -角叉菜膠誘導小鼠足蹠腫脹試驗探討其抗發炎作用，並分析肝臟中之SOD、GSH-Px、GSH-Rd活性及發炎足蹠組織中MDA的含量之變化以探討其抗發炎作用之機轉。

實驗結果顯示，在鎮痛實驗中五子衍宗丸(0.25, 0.5, 1.0 g/kg)可減少由醋酸所引起疼痛之扭體次數。於抗發炎試驗中，五子衍宗丸(0.5, 1.0 g/kg)可抑制 $\lambda$ -carrageenan誘導的急性足蹠腫脹。肝組織抗氧化酵素活性測定中，五子衍宗丸對肝臟中之SOD、GSH-Px、GSH-Rd活性均具增強作用並會減少小鼠發炎足蹠組織中MDA的含量。

綜合以上結果顯示，五子衍宗丸具有鎮痛及抗發炎作用。其鎮痛及抗發炎之作用機轉可能與提升肝臟中抗氧化酵素之SOD、GSH-Px及GSH-Rd活性、減少發炎足蹠組織中MDA的含量有關。

**關鍵字：**五子衍宗丸、抗發炎、鎮痛、角叉菜膠、醋酸

