基础医学研究

70味珍珠丸对CCl_4致急性肝损伤小鼠的保护作用及机制研究

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[摘 要] 目的 70味珍珠丸是珍宝类藏药,由金、银、珍珠、珊瑚、麝香、牛黄、藏红花等70余味动物、植物和矿物药组成,主要用于心血管系统疾病,其保肝作用尚未见报道,本实验研究其对四氯化碳致急性肝损伤小鼠的保护作用及机制研究。方法 ①将小鼠随机分为5组,分别为正常对照组、模型对照组、70味珍珠丸(150、500 mg/kg,po x 7d)和齐墩果酸(25 mg/kg,ipx3 d)组。于末次给药6h后腹腔注射0.1% CCl_4(10 mL/kg),建立急性肝损伤模型。于CCl_4造模18 h后时取材测定小鼠血清ALT和AST活性,HE染色观察肝脏病理组织学变化,RT-PCR观察肝毒性及炎性相关因子表达水平。②随机取小鼠5只,单独给药70味珍珠丸(500 mg/kg,po),连续4 d,RT-PCR检测抗氧化损伤通路基因金属硫蛋白1(MT-1)、核因子相关因子2(Nrf2)、NAD(P)H:醌氧化还原酶1(Nqo1)及血红素氧合酶-1(HO-1)、抗氧化物酶GSH相关基因(Gclc,GST-mu,GST-pi)的表达水平。结果 与正常对照组比较,模型组CCl_4肝损伤小鼠ALT,AST的活性明显升高,与模型组比较,70味珍珠丸高剂量组(500 mg/kg)能显著降低CCl_4所引起的ALT,AST的升高($P<0.05$)。70味珍珠丸高剂量组及齐墩果酸组小鼠肝脏病理损伤程度明显减轻,肝细胞肿胀、变性坏死程度减轻,灶区坏死明显减少,炎性细胞浸润程度有所改善。CCl_4肝损伤模型组肝毒性相关基因Gadd45和Gadd153以及炎性相关因子MIP2,IL-6表达明显升高,70味珍珠丸高剂量组及齐墩果酸组(500 mg/kg)能明显减少Gadd45、Gadd153以及炎性因子MIP2,IL-6的表达($P<0.05$);单独给药组与正常组比较,抗氧化损伤通路基因金属硫蛋白1(MT-1)表达明显升高($P<0.01$),核因子相关因子2(Nrf2)、NAD(P)H:醌氧化还原酶1(Nqo1)及血红素氧合酶-1(HO-1)表达明显升高($P<0.05$),抗氧化物酶GSH相关基因(Gclc,GST-mu,GST-pi)表达明显升高($P<0.05$)。结论 70味珍珠丸对CCl_4致小鼠急性肝损伤具有一定的保护作用,其保护机制与激活MT-1、Nrf2、GSH抗氧化损伤的通路有关。

[关键词] 70味珍珠丸;四氯化碳;氧化损伤;金属硫蛋白;Nrf2 抗氧化通路
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Protective effects of 70Wei Zhen-Zhu-Wan against carbon tetrachloride induced liver injury in mice

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[Abstract] **Objective** 70 Wei Zhen-Zhu-Wan (70W) is a famous Tibetan medicine listed in the 2015 edition of Pharmacopoeia. This study examined its protective effects against carbon tetrachloride (CCl_4)-induced liver injury in mice. **Methods** Mice were given 70W (150,500 mg/kg,po x 7d),or oleanolic acid (25 mg/kg,sc x 3d),or distilled water. Six hours after the last dose,mice were intraperitoneally given 0.1% CCl_4(10 mL/kg for 18 h),and liver injury was examined. **Results** 70W protected against CCl_4induced liver injury,as evidenced by decreased serum enzyme activities and improved histopathology. The high dose of 70W and oleanolic acid re-

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duced the serum ALT and AST levels induced by CCl_4, and ameliorated hepatic inflammation and necrosis. CCl_4 – induced overexpression of DNA – damage inducible genes (Gadd45, Gadd153) and inflammation genes (MIP2, IL – 6) were reduced by 70W and oleanolic acid. 70W alone activated liver detoxification genes such as metallothionein and the Nrf2 antioxidant pathway genes. **Conclusion** 70W had protective effects against acute liver injury induced by CCl_4 in mice, and these effects appear to be mediated via induction of metallothionein and Nrf2 antioxidant genes.

[Key words] 70 Wei Zhen – Zhu – Wan; carbon tetrachloride; oxidative damage; metallothionein; Nrf2 antioxidant pathway

70 Wei Zhen – Zhu – Wan (70W) was developed in the middle of the fifteenth century, and continued to use till today. 70W is listed in the 2015 edition of Pharmacopoeia of the People's Republic of China [1]. 70W is a famous Tibetan medicine, composed of herbo – metallic mixtures prepared by special processing methods of Tibetan medicine [1-3]. The clinical observations showed many therapeutic effects of 70W, including sedation [4], anti – convulsion, improvement of learning and memory [5]. 70W is effectively used in the treatment of cardiovascular diseases [6], such as cerebral ischemia [7], cerebral concussion [8], hypertension [9], heart disease [10] and gastrointestinal diseases. However, its hepatoprotective effect has not been reported; this study aimed to examine its protective effects against carbon tetrachloride – induced liver injury in mice.

1 Material

1.1 Reagents 70W was provided by Tibet Tibetan Medicine Manufacture (Lhasa, China). Product with code number approved by SFDA Z54020062 and detail is listed in Pharmacopoeia of China [1]. 70W was prepared by grinding the pill into powder, adding distilled water to prepare the suspension for oral administration; Carbon tetrachloride (CCl_4) was purchased from Sigma – Aldrich (St. Louis, MO). All other chemicals were commercially available reagents.

1.2 Animal Male Kunming mice SPF – grade were purchased from Animal Experimental Center of Third Military Medical University (Chongqing, China, SCXK – 2012 – 0011). Mice were maintained in the Barrier environment facilities (Certificate No. SYXK 2011 – 004) at Zunyi Medical University, with controlled environment (22 ± 1℃, 50 ± 2% humidity and 12 h: 12 h light: dark cycle) and free access to purified water and standard laboratory chow. All animal care and experimental protocols are complied with the Animal Management Guidelines of the Chinese Ministry of Health and approve by Animal Use and Care Committee of Zunyi Medical University.

2 Methods

2.1 Mice were randomly divided into 5 groups, respectively as normal control, CCl_4 model, CCl_4 + 70W (150, 500 mg/kg, po x 7d) and oleanolic acid (25 mg/kg, ip x 3d as positive control) groups. Twenty – four hr after the last dose of 70W administration, mice were intraperitoneally given 0.1% CCl_4 (10 mL/kg 18 h), and liver injury was examined by serum enzyme activities and by pathology [11-12]. Livers were collected and stored at −80°C for analysis.

2.2 The activity of ALT and AST was determined by kits according to the manufacturer' protocol (Jiancheng, Nanjing, Lot#20160126). Liver samples were fixed in 10% formalin prior to routine processing and paraffin embedding. Liver sections (3 um) were stained with hematoxylin and eosin and evaluated for histopathology.

2.3 Real – time PCR Approximately 50 – 100 mg of tissues was homogenized in 1 ml TRIzol (TakaRa Biotechnology, Dalian, China) and total RNA was extracted according to manufacturer's instructions. The quality and quantity of RNA were determined by the Nano Drop (Thermo Scientific, ND – 2000, USA), with 260/280 ratio (>1.8). Total RNA was reverse transcribed with a High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA,

USA). The primers were designed with Primer3 software and listed (Table 1). The 15 μl PCR reaction mix contained 3 μl of cDNA (10 ng/μl), 7.5 μl of iQTM SYBR Green Supermix (Bio - Rad Laboratories, Hercules, CA), 0.5 μl of primer mix (10 μM each), and 4 μl of ddH2O. After 5 min denature at 95°C, 40 cycles will be performed: annealing and extension at 60°C for 45 seconds and denature at 95°C for 10 seconds. Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. Relative expression of genes was calculated by the $2^{-\triangle\triangle Ct}$ - method and normalized to the house keeping gene β - actin or expressed as % of controls [13].

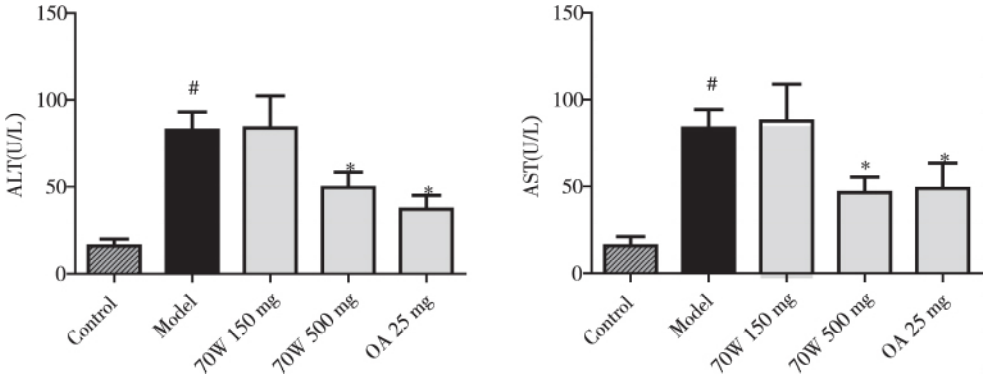
Tab 1 Oligonucleotide sequences of primers for RT - PCR analysis

Gene	Number	Forward	Reverse
β - actin	V01217	TGACCGAGCGTGGCTACAG	GGGCAACATAGCACAGCTTCT
Gadd45	L28177	GCTGCTCAACGTAGACCCCG	CAGCTAGCCGACCCGGGTTG
Gadd153	NM_007837	TCACTACTCTTGACCCTGCG	GACTGGAATCTGGAGAGCGA
MIP2	NM_009140	CCTCAACGGAAGAACCAAAGAG	CTCAGACAGCGAGGCACATC
IL - 6	J03783	GCCCACCAAGAACGATAGTCA	GAAGGCAACTGGATGGAAGTCT
MT - 1	NM_013602	CTCCGTAGCTCCAGCTTCAC	AGGAGCAGCAGCTCTTCTTG
Nrf2	BC026943	CGAGATATACGCAGGAGAGGTAAGA	GCTCGACAATGTTCTCCAGCTT
Nqo1	BC004579	TATCCTTCCGAGTCATCTCTAGCA	TCTGCAGCTTCCAGCTTCTTG
Ho - 1	M33203	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
Gclc	BC019374	TGGCCACTATCTGCCCAATT	GTCTGACACGTAGCCTCGGTAA
GST - mu	NM_010358	CTCCCGACTTTGACAGAAGC	TTGCTCTGGGTGATCTTGTG
GST - pi	D30687	TGGGCATCTGAAGCCTTTTG	GATCTGGTCACCCACGATGAA

2.4 Statistical analysis Data were expressed as mean and standard error. The SPSS 16 software was used for statistical analysis. Data were analyzed using a one - way analysis of variance (ANOVA), followed by Duncan's multiple range test. P value < 0.05 was considered statistically significant.

3 Results

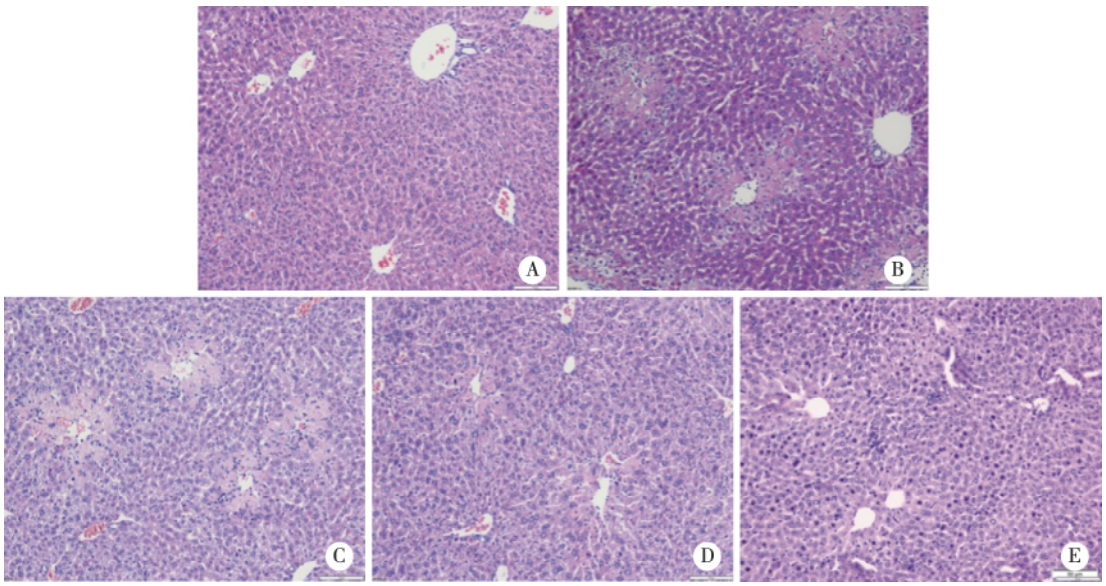
3.1 70W protected against CCl_4 - induced liver injury CCl_4 produced liver injury can be evaluated by increased serum enzyme activities and histopathology. Compared with model group, the high dose of 70W and oleanolic acid reduced the serum ALT and AST activities induced by CCl_4 ($P < 0.05$, Fig 1).



#Compared with control group $P < 0.05$; * Compared with model group $P < 0.05$.

Fig 1 Effect of 70 Wei Zhen - Zhu - Wan (70W) on serum ALT and AST ($\bar{x} \pm s$, n = 10).

3.2 Liver histopathology CCl_4 produced liver injury, as evidenced by hepatocellular necrosis, apoptosis, and inflammation (B), 70W (500 mg/kg) (D) and oleanolic acid groups (E) reduced these pathology lesions (Fig 2).

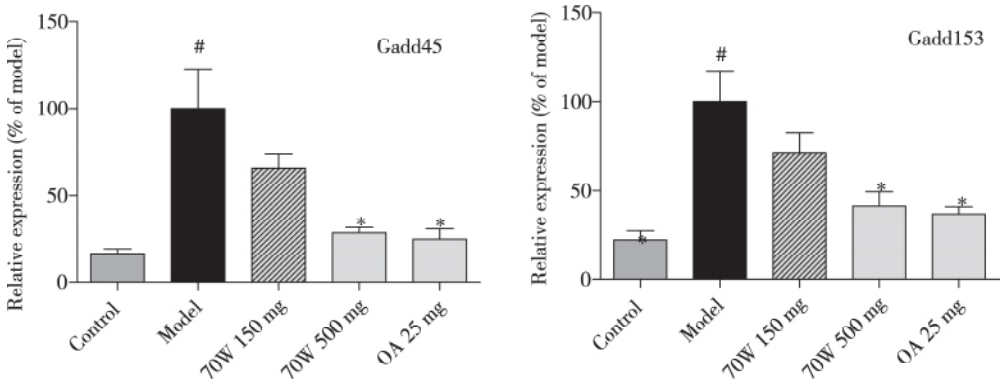


A: Control group; B: CCl_4 model group; C: CCl_4 + 70 W 150 mg/kg; D: CCl_4 + 70 W 500 mg/kg); E: CCl_4 + oleanolic acid 25 mg/kg.

Fig 2　Histopathology (HE, ×200)

3.3　70W reduced CCl_4 - induced toxicity gene expression

3.3.1　DNA damage is a hallmark of CCl_4 - induced liver injury [14-15]. The results of RT - PCR showed that in the model group, the expression of liver toxicity - related genes was increased. Compared with the model group, the expression of Gadd45 and Gadd153 in 70W (500 mg/kg) and oleanolic acid group decreased significantly ($P<0.05$, Fig 3).

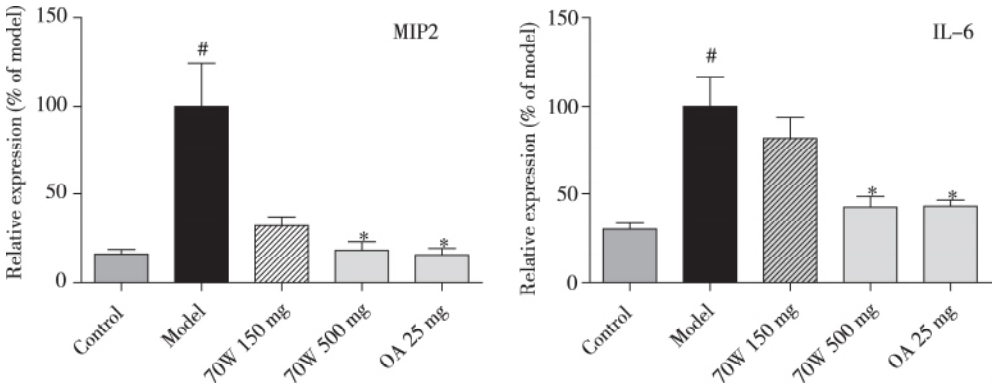


#Compared with model group $P<0.05$, * Compared with Model group $P<0.05$.

Fig 3　Effect of 70 Wei Zhen - Zhu - Wan (70W) on the expression of toxicity - related genes in mice ($\bar{x}\pm s$, $n=10$)

3.3.2　CCl_4 - induced liver injury is characterized by inflammation and pro - inflammatory gene expression　The results of RT - PCR showed that in the model group, the expression of liver inflammatory cytokines increased. Compared with the model group, the expression of MIP2 and IL - 6 in 70W (500 mg·kg^{-1}) and oleanolic acid groups decreased significantly ($P<0.05$, Fig 4).

3.4　70W alone increased hepatic antioxidant components　The hepatic antioxidant genes are mainly metallothionein (MT) and Nrf2 pathway genes [19-20]. Compared with the control group, 70W alone increased MT (3x) and activated Nrf2 (12%), Nqo1 (60%), Ho-1 (60%), Gclc (80%), GST - mu (40%), GST - pi (40%). The expressions of all these genes were significantly increased ($P<0.05$, Tab 2).



Compared with model group $P < 0.05$, * Compared with Model group $P < 0.05$.

Fig 4 The effect of 70 Wei Zhen – Zhu – Wan (70W) on the expression of inflammatory cytokines in mice ($\bar{x} \pm s$, $n = 10$)

Tab 2 The effect of 70 Wei Zhen – Zhu – Wan (70W) on the expression of antioxidative genes

genes	Control	70 W(500 mg/kg)
MT – 1	163 ± 49.4	484 ± 86.9*
Nrf2	86.0 ± 5.38	101 ± 3.92*
Nqo1	21.0 ± 2.90	32.9 ± 2.96*
Ho – 1	83.6 ± 6.14	133 ± 7.23*
Gclc	23.2 ± 3.49	41.9 ± 1.90*
GST – mu	47.7 ± 3.76	68.1 ± 7.31*
GST – pi	68.4 ± 1.23	93.1 ± 7.05*

Relative mRNA (% of β – actin), ($\bar{x} \pm s$, n = 5), * Compared with control group, $P < 0.05$.

4 Discussion

The present study showed that 70W has protective effects against CCl_4 hepatotoxicity, as evidenced by decreased ALT, AST and improved histopathology. Oral administration of 70W alone can significantly increase liver antioxidant capacity. This is the first time to provide a pharmacological evidence for the hepatoprotective effect of 70W.

Oxidative stress plays an important role in CCl_4 hepatotoxicity [16]. The trichloromethyl radical can react with oxygen to form the trichloromethyl peroxy radical $CCl_3OO\cdot$, a highly reactive species that initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, affecting mitochondria, endoplasmic reticulum (ER), and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis and increased permeability of cells leading to cell death [19]. 70W high dose and oleanolic acid significantly reduced liver injury, while 70W low dose group was ineffective, showing that 70W and oleanolic acid can protect against CCl_4 – induced oxidative liver injury, and this effect is dose – dependent.

Gadd45 and Gadd153 are DNA – damage inducible genes and can induce cell cycle arrest and promote DNA repair and cell survival under stress [17]. Elevation of Gadd45 and Gadd153 is a sensitive biomarker of liver injury induced by CCl_4 [18]. The expression of Gadd45 and Gadd153 in the liver of model group was correlated with liver injury. The expression of Gadd45 and Gadd153 in livers of 70W high dose and oleanolic acid group were significantly decreased, indicating 70W and oleanolic acid could decrease CCl_4 – induced DNA damage.

CCl_4 induced liver injury is associated with high expression of inflammatory factors [19]. MIP2 is a chemotactic cytokine secreted mainly by macrophages. In the early stage of inflammation, MIP2 was secreted to cause inflammatory cell infiltration, leading to cell damage in the late stage. It was shown that the apoptosis of the cells might be related to the early release of MIP2 in the early stage of inflammation. IL – 6 is a kind of hormone – like peptide, which is an important immune regulator [20]. IL – 6 can act on target cells by remote secretion, and it is more important in inflammatory response. The present results showed that the expression of IL – 6 and MIP2 was increased by CCl_4. The expression of IL – 6 and MIP2 was decreased in the 70W high dose and oleanolic acid group, indicating that 70W and oleanolic acid could reduce the expression of inflammatory factors to reduce liver injury.

The mechanism of protection against liver injury induced by CCl_4 is related to increased MT – 1 and Nrf2 [19-20]. Induction of MT and Nrf2 is the main mechanism of oleanolic acid hepatoprotection [21].

Pretreatment of animals with 70W could induce body defense mechanisms, a phenomenon called "program the liver" [22], which in turn makes animals resistant to toxicant insult. MT-1 is known as one of the most effective free radical scavenger, has strong antioxidant activity, and plays an important role in the process against various liver damage [20]. Nrf2 is an important transcription factor related to cell self-protection. Nrf2 is normally inhibited by Keapl, in the case of oxidative stress and Keapl is dissociated, and Nrf2 is moved to nuclear to bind antioxidant response element (ARE) to produce antioxidant gene expression, including the phase II detoxification enzymes. The present results showed that MT and Nrf2 was significantly increased by 70W pretreatment, indicating that 70W could "program the liver" to induce the antioxidant capacity of the tissue to eliminate excessive free radicals. The increased expression of Nrf2 further drives the expression of Nqo1, Gclc, GST-mu, GST-pi and HO-1 to produce protective effect.

In summary, 70W has protective effects on acute liver injury produced by CCl_4 in mice in a dose-dependent manner and reduces the expression of DNA damage inducible genes Gadd45, Gadd153 and inflammatory genes MIP2, IL-6. These effects appear to be mediated via induction of metallothionein and Nrf2 antioxidant genes.

[References]

[1] 吴金泉,林兆祥,刘林美,等. 藏药七十味珍珠丸的激光诱导击穿光谱检测 [J]. 中南民族大学学报: 自然科学版,2009,28(2):53-56.

[2] 李玉,伊甫申,李天才,等. 藏药七十味珍珠丸中微量元素的测定 [J]. 广东微量元素科学,2001,8(11):51-52.

[3] 李天才,索有瑞. 藏成药七十味珍珠丸中矿物质元素在体内蓄积性水平研究 [J]. 中国科学院研究生院学报,2003,20(3):348-352.

[4] 海平,周生祥,尚鸿,等. 藏药 70 味珍珠丸抗惊厥作用研究 [J]. 中国民族医药杂志,1998,4(2):45-46.

[5] 朱爱琴,褚以德,Li Q X,等. 藏药七十味珍珠丸对阿尔茨海默病小鼠模型脑组织 Aβ 表达的影响 [J]. 中华精神科杂志,2010,43(2):69-74.

[6] 白振忠,靳国恩,芦殿香,等. 藏药七十味珍珠丸对老年痴呆模型大鼠学习记忆和超氧化物歧化酶、丙二醛的影响 [J]. 青海医学院学报,2011,32(1):29-31.

[7] 郭新庆,薛爱琴,吴穹,等. 70 味珍珠丸对全脑缺血/再灌注大鼠海马 CA1 区神经元的保护作用 [J]. 当代医学,2010,16(25):1-2.

[8] 祁继光. 藏药治疗中风后遗症 54 例疗效观察 [J]. 云南中医中药杂志,2006,27(5):49.

[9] 杜俊蓉,邢茂,林治荣,等. 珍珠七十丸对 SHRsp 脑卒中及高血压防治作用的研究 [J]. 中国中药杂志,2003,28(6):557-559.

[10] 陈惠新,陈秋红. 藏药 70 味珍珠丸对心绞痛疗效微循环等的影响 [J]. 辽宁中医杂志,2000,27(3):109-110.

[11] 胡宗礼,黄晓萍. 虎杖方剂对四氯化碳致大鼠肝损伤的保护作用研究 [J]. 时珍国医国药,2009,20(3):657-658.

[12] 刘晓丽,刘春霞,刘红军,等. 小鼠四氯化碳急性中毒性肝损害模型制备方法探讨 [J]. 新乡医学院学报,2001,18(1):30-31.

[13] Fan G J, Tang J J, Bhadauria M, et al. Resveratrol ameliorates carbon tetrachloride-induced acute liver injury in mice [J]. Environ ToxicolPharmacol, 2009, 28(3): 350-356.

[14] Schaefer K N, Geil W M, Sweredoski M J, et al. Oxidation of p53 through DNA charge transport involves a network of disulfides within the DNA-binding domain [J]. Biochemistry, 2015, 54(3): 932-941.

[15] Lin C R, Yang C H, Huang C E, et al. GADD45A protects against cell death in dorsal root ganglion neurons following peripheral nerve injury [J]. J NeurosciRes, 2011, 89(5): 689-699.

[16] Wu S, Yue Y, Tian H, et al. Carthamus red from Carthamustinctorius L. exerts antioxidant and hepatoprotective effect against CCl_4-induced liver damage in rats via the Nrf2 pathway [J]. J Ethnopharmacol, 2013, 148(2): 570-578.

[17] 冯利娟,段丽娟,张瑞刚,等. JNK 信号通路在大鼠肝再生及肝硬化的细胞增殖和凋亡中作用的比较 [J]. 解剖学报,2014,85(2):345-356.

[18] 杨献光,朱琳,赵卫明,等. GADD45α 对大鼠部分肝切除后肝脏再生的调控作用 [J]. 解剖学报,2016,47(3):323-329.

[19] 陆远富,Connie Wu,刘杰,等. Nrf2 抗氧化损伤通路在 CCl_4 所致小鼠肝毒性中的保护作用 [J]. 遵义医学院学报,2015,38(1):6-14.

[20] Wu Q, Zhang D, Tao N, et al. Induction of metallothionein and the Nrf2-target genes mediates heptoprotective effects of Piper puberllum (Benth.) Maxim [J]. Am J Chin Med, 2014, 42(1): 207-221.

[21] Liu J, Wu Q, Lu Y F, et al. New insights into generalized hepatoprotective effects of oleanolic acid: Key roles of metallothionein and Nrf2 induction. Biochem [J]. Pharmacol, 2008, 76(7): 922-928.

[22] Klaassen C D. Learning to program the liver [J]. Annu Rev Pharmacol Toxicol, 2014, 54(1): 1-8.

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