Available online at SciVarse ScienceDirect
Chinese Herbal Medicines (CHM)
ISSN 1674-6384
Journal homepage: www.tiprpress.com E-mail: chm@tiprpress.com

Original article

Effects of *Coptidis Rhizoma* on Cell Cycle, DNA Damage, and Apoptosis in L929 Murine Fibroblast Cells

Yan-fei Huang†, Man-man Gu†, Jing Xu, Chun-yang Han, Teng-fei Liu, Cui-yan Liu*

College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China

ARTICLE INFO

Article history

Received: September 28, 2015

Revised: January 21, 2016

Accepted: March 3, 2016

Available online:

July 6, 2016

DOI:

10.1016/S1674-6384(16)60046-1

ABSTRACT

Objective *Coptidis Rhizoma* (CR), a widely used traditional Chinese herbal medicine, is commonly believed to be non-toxic. However, little is known about its cytotoxicity and relevant mechanisms at cellular and genetic levels. The present study was conducted to explore the cytotoxicity of CR and its mechanisms related to cell cycle arrest, DNA damage, cell apoptosis, and mitochondrial membrane potential in L929 murine fibroblast cells. **Methods** The cells were cultured and treated with different concentration of CR aqueous extract for 24 h. Cell viability was determined by CCK-8 method, morphological changes, and mitochondrial membrane potential were observed with an inverted microscope, cell cycle and cell apoptosis were examined by flow cytometry and DNA damages were detected by comet assay. **Results** Our results showed that cell viability was significantly decreased in a dose-dependent manner when concentration was higher than 0.2 mg/mL. A concentration above 1 mg/mL altered the cells morphology. Each DNA damage indicator score increased in the groups with the concentration of above 0.1 mg/mL. Cells at G_2/M phase, cell apoptosis and mitochondrial membrane potential changed in the 2 mg/mL group. **Conclusion** Overall, our study suggests that CR at a high dosage exhibits cytotoxicity on L929 cells, which is likely to be the consequences of cell cycle arrest, DNA damage, cell apoptosis and mitochondrial membrane potential reduction.

Key words

apoptosis; cell cycle; *Coptidis Rhizoma*; cytotoxicity; DNA damage; mitochondrial membrane potential

1. Introduction

Coptidis Rhizoma (CR), the dried rhizome of *Coptis chinensis* Franch., *C. dletoidea* C. Y. Cheng et Hasiao, and *C. teeta* Wall., serves as the medicinal part with bitter taste, cold nature, and tropism to the channels of heart, liver, stomach, and large intestine. It belongs to the antipyretic category because it plays a role in clearing away the damp-heat, purging fire away from the heart, and eliminating toxic materials from the body. Moreover, CR is used as a common traditional herbal drug for treating infectious diseases (Ma, 2013). The main components of CR are isoquinoline alkaloids (Qiu, 2012; Qing et al, 2016), of which berberine is the highest, followed by coptisine, palmatine, epiberberine, and so

* **Correspondence author: Liu CY** E-mail: cyliu@ahau.edu.cn Tel/ Fax: +86-551-6578 6357

† These authors made equal contribution.

Fund: National Natural Science Foundation of China (No. 31172358)

on (Figure 1). Previous pharmacological studies have demonstrated that CR has multiple bioactivities, such as an increase in platelet count, neuro-protective and immune-modulated (Yu et al, 2006), antimicrobial (Choi et al, 2007), anti-inflammatory (Lu et al, 2011), antineoplastic (Tang et al, 2009), anti-Alzheimer, anti-oxidant (Jung et al, 2009), hepatoprotective (Ye et al, 2009), analgesic (Tjong et al, 2011), and antihyperglycemic (Chen et al, 2012) effects. CR has been widely used in clinic for a long time. *Treatise on Exogenous Febrile Disease*, a classical work by Zhong-jing Zhang (a famous doctor in Han dynasty), describes 12 types of prescriptions containing CR. There are at least 50 contemporary Chinese medicines containing CR. According to the *Ministry of Foreign Trade and Medicine Companies' Statistic Data*, the demand of CR in the market reached 2000 tons by 2006. With the discoveries of various efficacies of CR and remarkable curative effects in the treatment of various disorders, the clinical use of CR is quickly expanding.

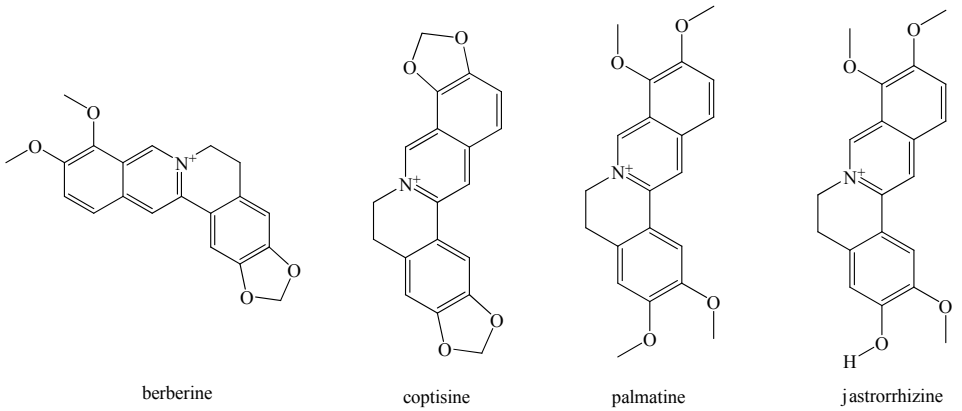

Figure 1 Molecular structures of four primary active constituents from CR

It is widely accepted that traditional Chinese herbal medicines are natural with very few side effect. In addition, CR has been regarded as non-toxic according to its top grade in *Shennong's Herbal Classic*, as well as its essential role in many detoxification prescriptions. But since 1978, there have been some suspected cases reported in Singapore that pregnant women or newborns who took CR or its active principle berberine manifested the defects of glucose-6-phosphat dehydrogenase (G6PD), leading to the neonates suffering hemolytic jaundice or kernicterus. Many studies have been conducted regarding the issue whether CR and berberine are toxic. Yeung et al (1990) and Yang (2000) demonstrated that CR could aggravate the risk of neonatal jaundice in related experiments in 1990 and 2000, respectively. In 2001, Yu et al (2001) implemented a clinical treatment of diabetes with 8−18 g CR grinds powder capsules, adverse reactions emerged, such as nausea, hypoglycemia, and diarrhea. Li et al and Qiu et al found that rats receiving high doses of CR decoction orally showed the dysfunction of gastric mucosal barrier and decrease of prostaglandin E2 (Li et al, 2007; Qiu et al, 2004). Previous reports have also demonstrated that CR and its products induce adverse reactions, including drug eruption, allergies, pancytopenia, dizziness, palpitation, shortness of breath, and joint pain (Feng, 2004; Zhang et al, 2003). These results all strongly suggest that CR has certain toxicity. Therefore, it is reasonable to speculate that CR is not as safe as commonly believed in some cases. However, it remains unclear the toxicity and its relevant mechanisms of CR, especially those at the cellular and genetic levels.

In this study, we used L929 cells line to perform toxicity testing, aiming to explore the cytotoxicity of CR and the related mechanisms. Our study would provide a theoretical basis for its safe applications and allow a rational evaluation of the pharmacological effects.

2. Materials and methods

2.1 Plant materials

Coptidis Rhizoma (CR), national drug standard substance, the dry powder of *Coptis deltoidea* C.Y. Cheng et Hsiao (the batch number is 120913-201310) was purchased from the National Institutes for Food and Drug Control (Beijing, China). CR was extracted as previously described (Ma et al, 2010) with minor modifications based on traditional methods. CR was extracted in boiling distilled water for three times. After filtration, the mixed filtrate was concentrated by a rotary evaporator to 0.1 g/mL at 45 ^{o}C, then the aqueous extract was sterilized by 0.22 μm microporous membrane and saved at 4 ^{o}C. The CR aqueous extract (0.1 g/mL) will be diluted with cell culture medium to the required concentrations for experiments.

2.2 HPLC analysis

Determination of CR contents was performed using HPLC (Agilent 1100 Series: Agilent Technologies Ltd., USA) with a Diamonsil C_{18} column (150 mm × 4.6 mm, particle size 5 μm). In HPLC experiments, the mobile phase was cetonitrile-water solution of 50 mmol/L potassium dihydrogen phosphate (50:50, pH 4.0, containing 15 mmol/L lauryl sodium sulfate), gradient elution was in a mixture of potassium phosphate monobasic (50 mmol/L), and acetonitrile was at column temperature of 30 ºC, with a flow

rate 1.0 mL/min and UV detection (wave length of 345 nm). Epiberberine, coptisine, pamatine, and berberine were used as the standard compounds to identify and quantify the major components of CR.

2.3 Reagents and chemicals

Fetal bovine serum (FBS) was purchased from Life Technologies Co. (Shanghai, China) and high-glucose Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Thermo Fisher Scientific Inc. (Beijing, China). Cell Counting Kit-8 (CCK-8) was acquired from Dojindo Laboratories (Kumamoto, Japan). Cell cycle assay kit and FITC Annexin V Apoptosis Detection Kit were obtained from Becton Dickinson (USA). Mitochondrial membrane potential assay kit with JC−1 was from Beyotime Institute of Biotechnology (Shanghai, China). Low melting agarose (LMA) was supplied by Amresco Corporation (Shanghai, China). DMSO was purchased from Sigma Aldrich (USA). GelGreen was purchased from Biotium, Inc. (USA). All the other materials were of analytical grade.

2.4 Cell culture

L929 murine fibroblast (L929) cell line was purchased from the type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a saturated humidified atmosphere of 5% CO_2, and when 85% confluence was achieved, L929 cells were dissociated using 0.25% trypsin and passaged for experiments.

2.5 Assessment of cell viability

Viability of L929 cells with CR-treatment and control was measured by CCK-8 assay. Briefly, L929 cells were seeded in 96-well tissue culture plate (100 μL/well) at a density of 8×10^4 cells/well with some wells left blank. After the cells formed a 60%–70% confluent monolayer, the culture medium of each well was carefully refreshed by 100 μL CR diluents of various concentration as samples. The conditions in the blank group were the same as the control except for cells. After 24 h, the media were removed and the cells were washed twice with phosphate buffer solution (PBS). Subsequently, a 100 μL CCK-8 working solution was added to each well, the plate was incubated for another 2 h, and the optical density (OD) was read on a microplate reader (Thermo, USA) at a test wavelength of 450 nm against a reference wavelength of 630 nm. The percentages of viability were calculated as follows: cell viability = $(OD_{Sample} - OD_{Blank})/(OD_{Control} - OD_{Blank})$

2.6 Flow cytometry analysis of cell cycle

To investigate the antiproliferative toxicity of CR against L929 cells, the effects of CR on the progression of cell cycle were evaluated. L929 cells in the exponential phase of growth were treated with CR at the indicated concentration (0.25, 1, and 2 mg/mL) for 24 h. Morphological observation of each group was conducted under an inverted 100 × microscope. Subsequently, cell supernatants were collected, and the cells were stained with propidium iodide followed by harvesting. The DNA content of cells was determined with FACSCalibur flow cytometry and ModFit analytic software (Verity Software House, USA).

2.7 Examination of DNA damage by comet assay

In this experiment, we employed comet assay to evaluate the genotoxicity induced by CR on L929 cells with slight modification (Gao et al, 2004; Rivera et al, 2013; Singh et al, 1988). Briefly, cells were induced by the indicated concentration (0.05, 0.1, and 0.2 mg/mL) of CR for 24 h. After trypsinization, cell density was adjusted to 2×10^5 cells/mL. The cell suspension was mixed with 1% LMA at 1:3 and layered on the surface of special slides (40 μL/well) which were made by the covers of 24-well cell culture plates instead of traditional slides. Then, the slides were conducted to gel at 4 ºC for 30 min. Then these slides were immersed in freshly prepared cold lysing solution and refrigerated for 1–1.5 h followed by electrophoresis, despiralization, and neutralization. The dried samples were then stained with GelGreen after dehydration. The whole process was kept cold in dim light to minimize additional repair or damage. Comet images of each slide were taken by a fluorescence microscope (Olympus IX71, Japan), and analyzed with Comet Assay Software Pect (CASP) software (Nandhakumar et al, 2011; Xin et al, 2005).

2.8 Measurement of cell apoptosis

To examine whether cell apoptosis was involved in CR-induced L929 cytotoxicity, two fluorescent dyes Annexin V-FITC/PI were utilized prior to flow cytometric analysis. Cells were washed with PBS and collected by centrifugation after treatment with 2, 1, and 0.25 mg/mL of CR for 24 h. The cell pellets were stained with Annexin V-FITC/PI at room temperature for 15 min. Following incubation, 10 000 cells were analyzed with a flow cytometry and FlowJo 7.6 software was used to analyze cell apoptosis.

2.9 Detection of mitochondrial membrane potential

L929 cells were seeded into the wells of the 6-well plates. After treated by indicated concentration of CR for 24 h, JC−1 work reagent was added to the cells and incubated at 37 °C for 20 min. The mitochondrial fluorescence morphology was observed using fluorescence inversion microscope system (Olympus, Japan).

2.10 Statistical analysis

Statistical analyses were performed by SPSS 19.0 for Windows software and evaluated by One-way analysis of

variance (ANOVA) followed by Duncan's test. Data were presented as $\bar{x} \pm s$, and the significant level was detected at $P < 0.05$.

3. Results

3.1 HPLC analysis

Four main alkaloids (epiberberine, coptisine, pamatine, and berberine) were isolated and analyzed by HPLC (Figure 2), and their mass percents in this extract were 0.16%, 0.59%, 0.3%, and 1.22%, respectively.

3.2 Effects of CR on cell viability

The potential cytotoxicity of CR on L929 by CCK-8 was investigated (Table 1). The viabilities of the cells treated with CR at the concentration lower than 0.2 mg/mL for 24 h were not significantly different from the control. Interestingly, a growth-promoting effect was detected up to 0.1 mg/mL.

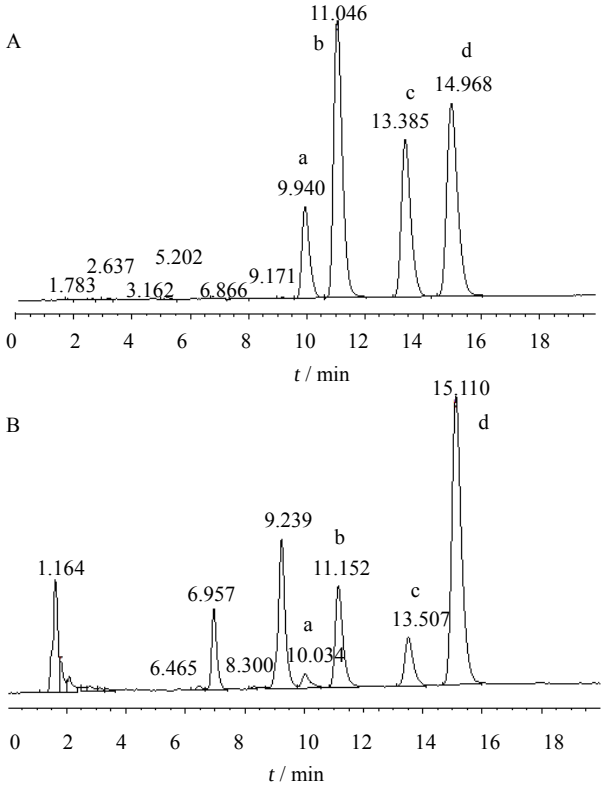

Figure 2 HPLC of reference substances (A) and samples (B) of CR

a: epiberberine b: coptisine c: palmatine d: berberine

Table 1 Effects of CR at different concentration on L929 cell viability ($\bar{x} \pm s$, $n = 4$)

Concentration / (mg·mL^{-1})	OD values	Survival rates / %
0.0000	0.77 ± 0.04	100
0.025	0.80 ± 0.09	103.48 ± 7.50
0.05	0.80 ± 0.09	103.23 ± 9.80
0.1	0.80 ± 0.04	104.41 ± 4.63
0.2	0.70 ± 0.02	88.73 ± 2.67*
1	0.65 ± 0.10*	80.75 ± 17.19**
2	0.49 ± 0.08**	54.75 ± 12.33**
4	0.25 ± 0.03**	14.26 ± 4.73**

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* control. Same as below.

However, the statistically significant decreases were observed when the concentration of CR was higher than 1 mg/mL compared with the control. Notably, when cells were treated with 4 mg/mL CR, the viability of L929 cells was 14.26 ± 4.73, indicating a significant cytotoxicity.

3.3 Effects of CR on cell morphological characteristics

To verify CR-induced cytotoxicity, the changes of cell morphology in L929 cells which was exposed to 0.25–2 mg/mL CR for 24 h were examined. Cells in the control group were of high density and of adherent growth, while cells induced by CR in the groups above 1 mg/mL were shrinking, round and detached. A large number of cells changed their shapes and floated in 2 mg/mL (Figure 3).

3.4 Effects of CR on cell cycle

Figure 4 shows the results of cell cycle analysis. The average proportion of cells in G_0/G_1 phase decreased from (59.15 ± 3.35)% to (46.19 ± 1.78)% at the concentration of 2 mg/mL ($P < 0.01$), while S phase had no significant difference. Importantly, statistically significant increase was demonstrated attributing to the cells proportion in G_2/M phase from (8.74 ± 0.49)% to (18.24 ± 1.69)% at 2 mg/mL group. The results suggested that CR prevented cell cycle progression by arresting cell cycle at G2/M phase, leading to disorders of cell division cycle at the concentration of 2 mg/mL.

3.5 DNA damage

As shown in Figure 5A, only circle fluorescence images could be observed in the control group. With the increase of CR concentration, circular fluorescents become longer, appearing

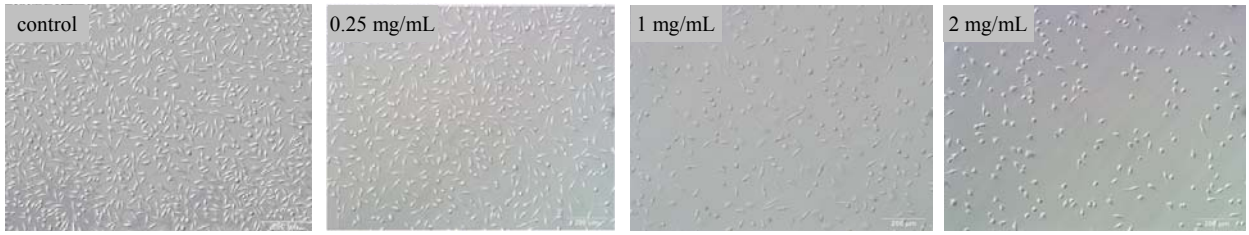

Figure 3 Morphological changes of L929 cells after CR treatment at different concentration for 24 h

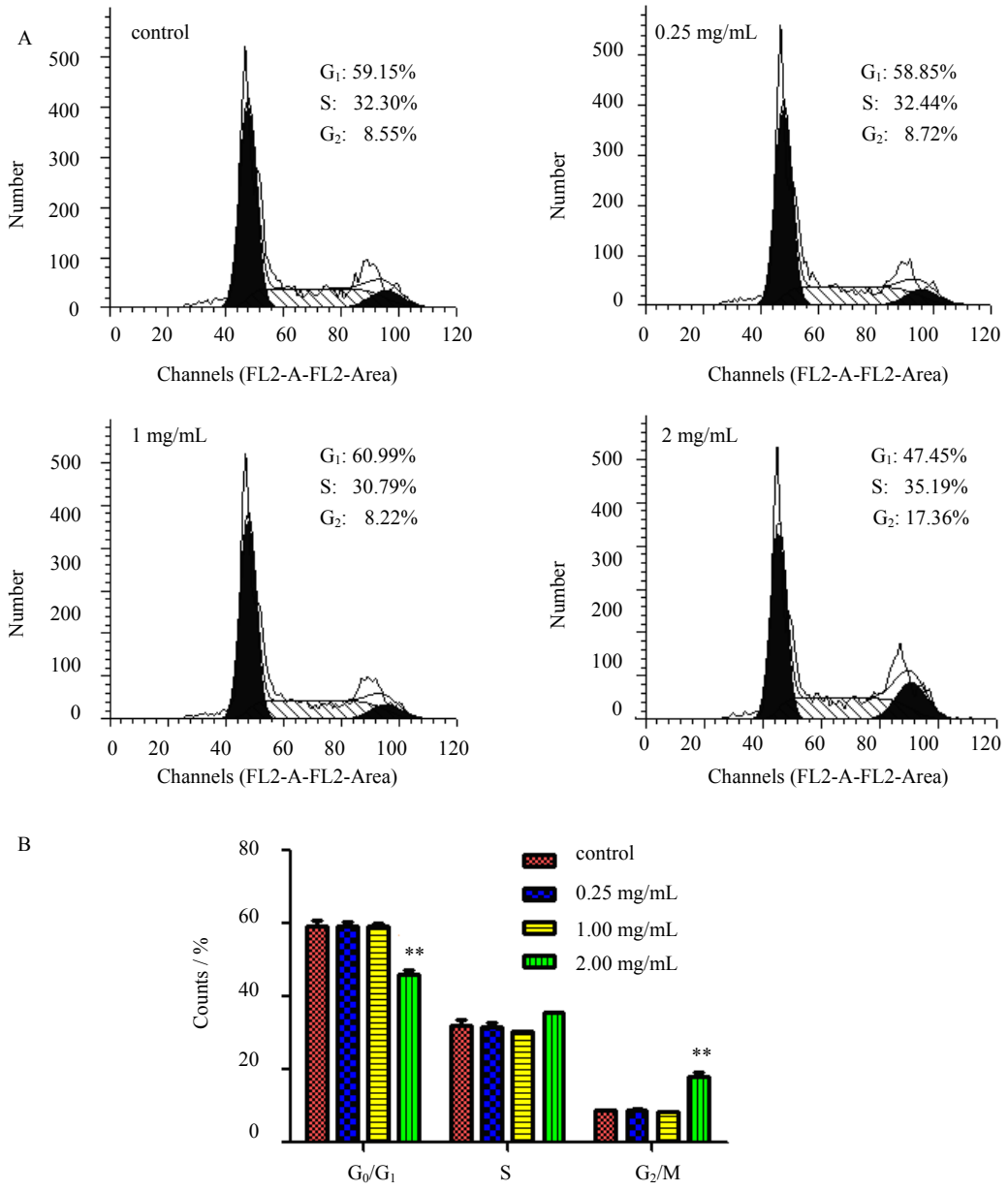

Figure 4 Effects of CR on L929 cell cycle (*n* = 3)

(A) Cells were exposed to different CR concentration for 24 h. Cell cycle after PI staining was detected by flow cytometry. (B) Quantities of cell cycle were analyzed. $^{**}P < 0.01$ *vs* control

comet image apparently. Compared to the control group, no abnormal DNA change was observed at 0.05 mg/mL group; but damage scores were all significantly increased at the concentration of 0.1 and 0.2 mg/mL. The results indicated that the 24 h exposure of L929 cells to CR increased DNA damage in a dose-dependent manner (Figure 5B).

3.6 Effects of CR on cell apoptosis

The results showed that the 2 mg/mL CR-induced almost two-fold increase in the apoptosis rate in contrast to the control and such increase was in a dose-dependent manner (Figure 6). These results indicated that cell apoptosis was involved in CR-induced L929 cell cytotoxicity.

3.7 Effects of CR on mitochondrial membrane potential

In this test, we found that the intracellular mitochondrial membrane potential level in the group of 2 mg/mL was markedly decreased compared with the control (Figure 7). These results indicated that mitochondrial membrane potential was involved in CR-induced cytotoxicity.

4. Discussion

With the growing popularity of traditional Chinese herbal medicines and international applications, the security problem of CR has attracted more and more attention. Some studies have demonstrated the toxic effects of CR, whereas

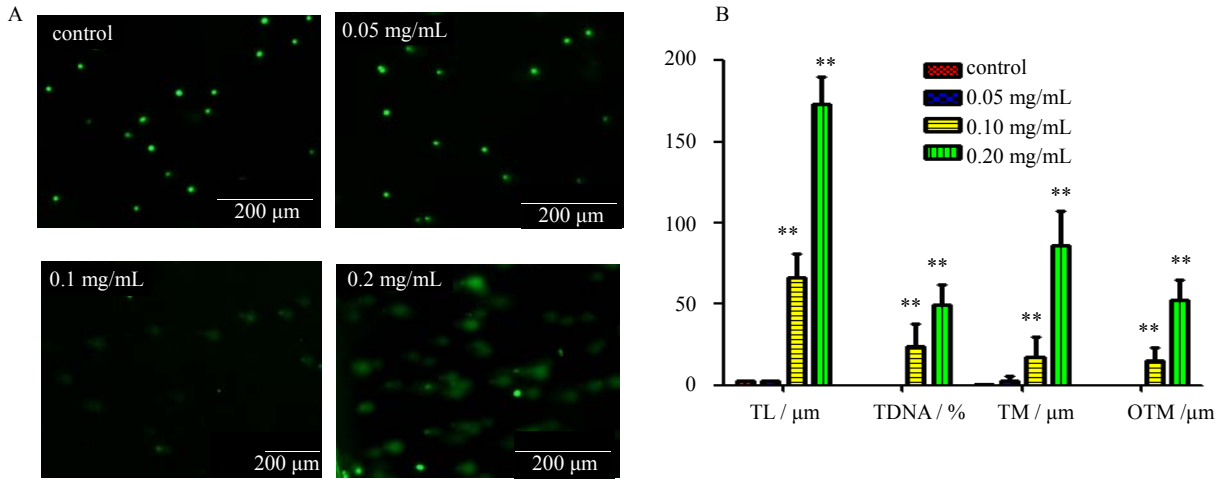

Figure 5 DNA damage of L929 cells stimulated by CR

(A) The indicated concentration of CR was added to cell culture for 24 h. Following dyed with GelGreen, comet images were taken at a magnification of 100 × using a fluorescence microscope. (B) Visual scorings of DNA damage were based on the fluorescence intensity.

TL: tail length TDNA: tail DNA% TM: tail moment OTM: olive tail moment $n = 50$ $^{**}P < 0.01$ *vs* control

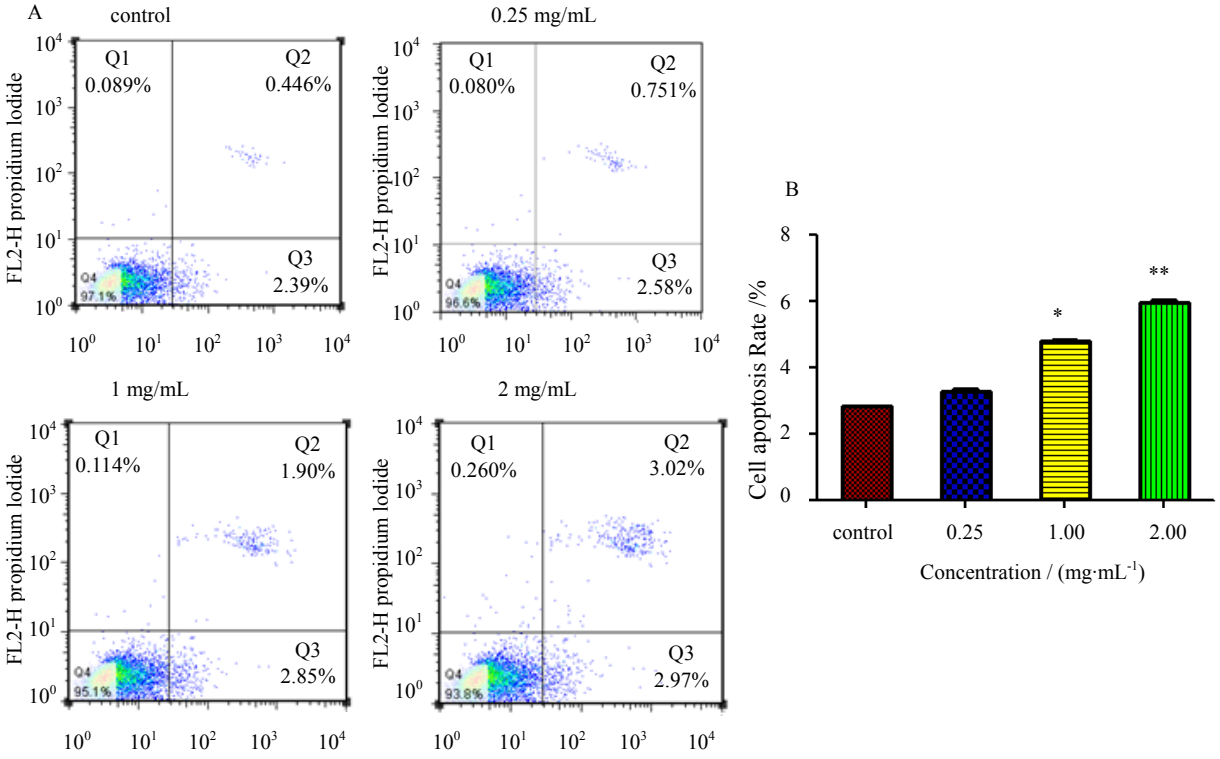

Figure 6 Effects of CR on L929 cell apoptosis rate

(A) The cells were exposed to different CR concentration for 24 h. Cell apoptosis rate after Annexin V-FITC/PI staining was detected by flow cytometry. (B) The quantitative data of apoptosis rate was shown. $n = 3$ $^{**}P < 0.01$ *vs* control.

others have reported the opposite results. For example, Liao et al (1982) pointed out that CR could not cause acute hemolytic jaundice and other adverse effects on healthy neonate or G6PD deficiency of newborns, but it could make a significant reduction in serum total bilirubin. Shen et al (2006) examined the effects of CR on erythrocyte glutathione content of SD rats, and found no significance at low doses. While the results were reduced at a high dose, glutathione dysfunction of maintaining erythrocyte stability was caused. Thus, it is necessary to regulate the CR dosage to ensure its safe application. Yi et al (2013) demonstrated that the currently recommended highest dose of CR is relatively safe because it could not cause any abnormal changes in sub-chronic toxicity tests on rats; Lee et al (2014) showed that the no-observed-adverse-effect level of CR was determined to be 667 mg/kg/d for male and 2000 mg/kg/day for female rats. These results further highlight the necessity to study the safe dosage range of CR.

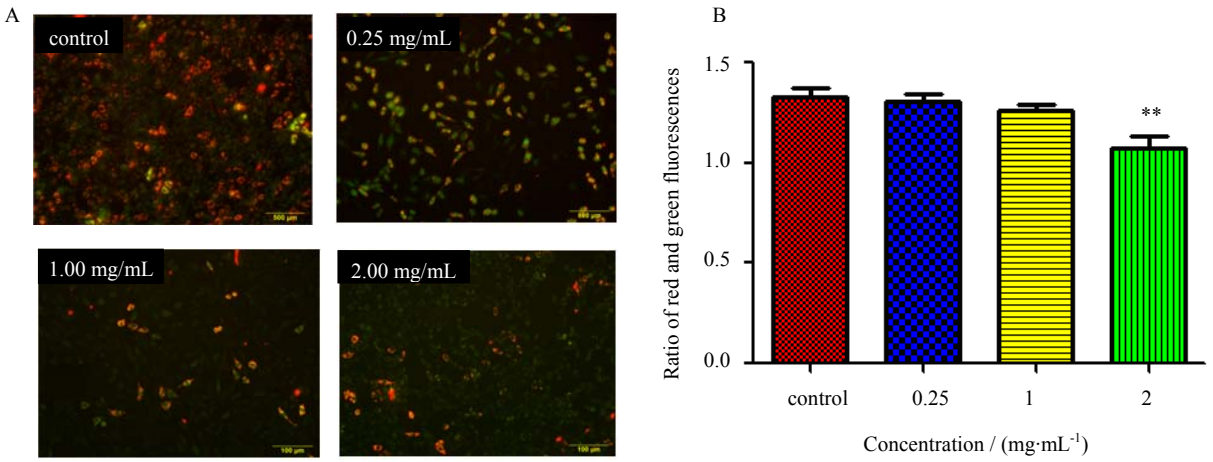

Figure 7 Effects of CR on mitochondrial membrane potential

(A) Cells were incubated with CR in various concentration for 24 h. Mitochondrial membrane potential after JC-1 staining was examined by fluorescence microscope at magnification of 100 ×. (B) Quantitative assessment of mitochondrial membrane potential was determined by GraphPad Prism software. $n = 3$ $^{**}P < 0.01$ *vs* control.

The normal process of cell proliferation was accomplished by an orderly cell division cycle. The conversion of cell cycle relies on the activation and expression of a series of cyclins (e.g., cyclin B, D, and E) and cyclin-dependent kinases (e.g., CDK 2, 4, and 6) (Weinert, 1998). Flow cytometry analysis showed that CR at 2 mg/mL could induce cell cycle change, mainly for the G_2/M phase arrest. While this concentration tested on cell viability has shown remarkably significant inhibition. Previous studies have shown that if the increase of cells in G_2/M phase was not caused by the result of promoted-proliferation, it might be related to DNA damage or other changes. Indeed, we observed G_2/M phase arrest in our test, compatible with this notion. Cell cycle checkpoints were activated to check and repair these damages or changes out of self-protection, eventually leading to cell cycle arrest (Ghosh et al, 2008; Leland et al, 1994). However, this may be the result of berberine. The results suggested that the cytotoxicity of L929 by CR was ascribed to cell cycle arrest partially.

DNA damage could cause distortion or mutant (Yang et al, 2003). We performed the comet assay to check the level of DNA damage, based on the correlation between fluorescence intensity and cell cycle (Kruszewski et al, 2012). Consistent with this report, our results showed that CR induced DNA damage in L929 cells, and four kinds of analysis parameters reached a good consistency. In addition, we observed no significant difference in cell viability at 0.1 mg/mL, but found obvious DNA damage in the comet assay at the same concentration. Therefore, we speculated that the damage of genetic material was prior to biochemical changes in cells. This damage partially accounted for a more potent cytotoxicity of CR on L929 cells.

Apoptosis is the programmed cell death of which mitochondrial pathway is classic (Liu et al, 2013; Song et al, 2014). In the early stage of cell apoptosis, mitochondria structure has changed resulting from the increase of mitochondrial outer membrane permeability and the reduction of mitochondrial transmembrane potential. These can sequentially cause a variety of biochemical changes in the cell. For example, Cyt C will combine with the activation factor 1 of apoptosis enzyme after it is released into the cytoplasm; Under the action of a dATP inducing caspase cascade reaction, the cell structure is destroyed with dysfunction, causing the final apoptosis (Hu and Hu, 2006). Mitochondria can also produce metabolic byproducts-reactive oxygen species (ROS) when generating energy (Cong et al, 2008). All these reasons and results are relevant in the whole process of cytotoxicity.

Many researches at home and abroad suggested the *in vitro* cytotoxicity was demonstrated obvious correlation to the toxicity *in vivo* (Cheong et al, 2008; Peng and Liu, 2007; Ren et al, 2011). The reference index of cytotoxicity LD_{50} was recommended by the National Toxicology Group Program and the Toxicology of the Experimental Evaluation Center (NICEATM). The report said the value of LD_{50} could reveal better the acute toxicity of the compound *in vivo*. The result of a preliminary study of cytotoxicity of CR and berberine on four kinds of cells cultured *in vitro* was demonstrated by our study team. In addition, the *in vitro* evaluation method is adopted at early screening stage of a new chemical substances exploited as a drug, can save time, effort, and money, which has been accepted by many scholars (Zeng et al, 2011; Guo et al, 2013; Liu et al, 2012).

The advantages of this study were as follows. The experiments were performed according to the standard procedures using relatively simple and well established routine methods. More important, berberine cytotoxicity in cell cycle, ROS, DNA damage, and apoptosis was not studied by other. To be pointed out that under the present study in which the cytotoxic strength and toxicity mechanisms of berberine is not clear, the authors chose the international standard (UB/T16886.5-2003) of L929 cells as a test cell for

general toxicity examination. To be sure, L929 is not a very targeted cell. If a deep research due to the results of this study will be carried out, a specific target cell model should be selected based on experimental purposes (such as genetic toxicity, cell cycle progression, etc).

5. Conclusion

In summary, our study demonstrates that a high dosage of CR has exhibited cytotoxicity, which is likely to be the consequences of cell cycle arrest, DNA damage, cell apoptosis, and mitochondrial membrane potential reduction. Further studies are needed to elucidate the complicated mechanisms of CR cytotoxicity, thereby guiding the clinical application.

Acknowledgments

We thank Dr. Yun-hai Zhang and Chao Wei for their kind help.

Conflict of interest statement

All authors declare no conflicts of interest.

References

Chen HY, Ye XL, Cui XL He K, Jin YN, Chen Z, Li XG, 2012. Cytotoxicity and antihyperglycemic effect of minor constituents from *Rhizoma Coptis* in HepG2 cells. *Fitoterapia* 83: 67-73.

Cheong HI, Johnson J, Cormier M, Hosseini K, 2008. In vitro cytotoxicity of eight beta-blockers in human corneal epithelial and retinal pigment epithelial cell lines: Comparison with epidermal keratinocytes and dermal fibroblasts. *Toxicol In Vitro* 22: 1070-1076.

Choi UK, Kim MH, Lee NH, 2007. Optimization of antibacterial activity by gold-thread (*Coptidis Rhizoma* Franch) against Streptococcus mutans using evolutionary operation-factorial design technique. *J Microbiol Biotechnol* 17: 1880-1884.

Cong YM, Li X, Jia HL, Guan WJ, Ma YH, 2008. Relationship of mitochondrial permeability transition with cell apoptosis. *China Anim Husb Vet Med* 35: 35-38.

Feng RJ, 2004. A case of allergic skin reactions and liver dysfunction caused by berberine. *Med J Gentral Equip* 6: 57.

Gao JJ, Feng SM, Jin WF, 2004. Improvement of gel preparation on alkali single cell gel electrophoresis. *Fudan Univ J Med Sci* 31: 311-312.

Ghosh P, Banerjee M, Giri AK, Ray K, 2008. Toxicogenomics of arsenic: Classical ideas and recent advances. *Mutat Res* 659: 293-301.

Guo LX, Yin F, Liu J. H. 2013. Role of pyruvate cycling anaplerosis in glucose-stimulated insulin secretion. *Chin Pharmacol Bull* 6: 748-751.

Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S, Hatzoglou M, Kilberg MS, Sartor MA, Kaufman RJ, 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* 15: 481-490.

Hu S, Hu CP, 2006. Advances in mitochondria and apoptosis. *Int J Respir* 26: 463-470.

Jung HA, Min BS, Yokozawa T, Lee JH, Kim YS, Choi JS, 2009. Anti-alzheimer and antioxidant activities of *Coptidis Rhizoma* alkaloids. *Biol Pharm Bull* 32: 1433-1438.

Kruszewski M, Iwanenko T, Machaj EK, Ołdak T, Wojewódzka M, Kapka-Skrzypczak L, Pojda Z, 2012. Direct use of the comet assay to study cell cycle distribution and its application to study cell cycle-dependent DNA damage formation. *Mutagenesis* 27: 551-558.

Lee YH, Kim D, Lee MJ, Kim MJ, Jang HS, Park SH, Lee JM, Lee HY, Han BS, Woo Chan Son, Seok JH, Lee JK, Jeong J, Kang JS, Kang JK, 2014. Subchronic toxicity study of *Coptidis Rhizoma* in rats. *J Ethnopharmacol* 152: 457-463.

Leland H, Hartwell, Kastan MB,1994. Cell cycle control and cancer. *Am Assoc Adv Sci* 266: 1821-1828.

Li FY, Qiu SH, Yin JK, Sun BQ, Cai Y, 2007. Effects of common-used bitter-cold herbs on gastrointestinal hormone in rats. *J Tradit Chin Med Univ Hunan* 27: 9-11.

Li JC, Meng XL, Fan XJ, Lai XR, Zhang Y, Zeng Y, 2010. Pharmacodyamic material basis of rhizoma coptidis on insulin resistance. *J China Mater Med* 35: 1855-1858.

Liao CL, Le JR, Liu S, 1982. Preliminary observation of Yinchenhao Decoction and *Rhizoma Coptidis* on China neonatal physiological jaundice. *China Med Coll Ann Bull* (Taiwan) 13: 1.

Liu Q, Qin YY, Zhang Y, Wang Q, Zheng XW, Chen C, He Q, Gao H, 2012. Comparison and analysis of *in vitro* cytotoxocity of different Shengmai Injections. *China J Chin Mater Med* 37(5): 643-647.

Liu XT, Wang YR, Zhang M, 201. Mitochondria-mediated apoptosis: A review of recent studies. *J Environ Health* 30: 182-185.

Liu YQ, Cheng MC, Wang LX, Xiao HB, 2010. *Rhizoma Coptidis* and berberine-induced activation of murine microglia N9 cells. *J Ethnopharmacol* 129: 121-126.

Lu J, Wang JS, Kong LY, 2011. Anti-inflammatory effects of Huang-Lian-Jie-Du decoction, its two fractions and four typical compounds. *J Ethnopharmacol* 134: 911-918.

Ma BL, Ma YM, 2013. Pharmacokinetic properties, potential herb-drug interactions and acute toxicity of oral *Rhizoma Coptidis* alkaloids. *Expert Opin Drug Metabol Toxicol* 9: 51-61.

Ma BL, Ma YM, Shi R, Wang TM, Zhang N, Wang CH, Yang Y, 2010. Identification of the toxic constituents in *Rhizoma Coptidis*. *J Ethnopharmacol* 128: 357-364.

Ma XX, Liu JH, Yu BY, 2013. Spectrum-effect relationship of antibacterial activities in *Coptidis Rhizoma*. *Drug Eva Res* 36(3): 171-175.

Nandhakumar S, Parasuraman S, Bhat BV, 2011. Evaluation of DNA damage using single-cell gelelectrophoresis (Comet Assay). *J Pharmacol Pharmacother* 2: 107-111.

Peng SQ, Liu MF, 2007. Alternative methods of toxicology and its application in the evaluation of new drug safety. *J Toxicol* 21(4): 308-309.

Qing DS, Luo WZ, Sun JB, Wang X, Yang Y, Wang DJ, Li LY, Qin Y, 2016. Determination of six alkaloids in crude and processed *Coptidis Rhizoma* with quantitative analysis of multi-components by single marker. *Chin Tradit Herb Drugs* 47(2): 324-329.

Qiu LL, Chen LH, Yan D, Zhong P, Tan MR, Du XX, Xiao XH, 2012. Simultaneous determination of six quaternary ammonium alkaloids in *Coptidis Rhizoma* by UPLC. *Chin Herb Med* 4(2): 164-169.

Qiu SH, Li FY, Yin JK, Wu HJ, Xiao JR, 2004. An experimental study on the effects of nine medicinal herbs with the properties of bitterness and cold on gastrointestinal movement and hepatorenal function of mice. *J Tradit Chin Med Univ Hunan* 24: 1-6.

Ren Y, Dong TT, Zou LB, Li XG, Wang J, 2011. *In vivo* and *in vitro* evaluation method for the toxicity of neural development. *J*

Toxicol 25(1): 61-64.

Rivera N, Rojas M, Zepeda A, Malagón F, Arán VJ, Marrero-Ponce Y, Rivera E, Fortoul TI, 2013. *In vivo* genotoxicity and cytotoxicity assessment of a novel quinoxalinone with trichomonacide activity. *J Applied Toxicol* 33: 1493-1499.

Shen T, Jiang TR, Wu SG, 2006. Change of GSH content in the rat' s red blood cell by compatibility between *Coptidis Rhizoma* and fructus evodiae. *J Sichuan Tradit Chin Med* 24: 16-17.

Singh NP, McCoy MT, Tice RR, Schneider EL, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175: 184-191.

Song BW, Fan QM, He ZY, 2014. Role of mitochondrial calpains in apoptosis. *Chin Pharmacol Bull* 30: 757-760.

Stoddart MJ, 2011. WST-8 analysis of cell viability during osteogenesis of human mesenchymal stem cells. *Methods Mol Biol* 740: 21-25.

Tang J, Feng Y, Tsao S, Wang N, Curtain R, Wang Y, 2009. Berberine and *Coptidis Rhizoma* as novel antineoplastic agents: A review of traditional use and biomedical investigations. *J Ethnopharmacol* 126: 5-17.

Tjong Y, Ip S, Lao L, Fong HHS, Sung JJY, Berman B, Che C, 2011. Analgesic effect of *Coptis chinensis* rhizomes (*Coptidis Rhizoma*) extract on rat model of irritable bowel syndrome. *J Ethnopharmacol* 135: 754-761.

Weinert T, 1998. DNA damage and checkpoint pathways: Molecular anatomy and interactions with repair. *Cell* 94: 555-558.

Xin CH, Li GL, Ji ZY, Gao Y, Yin SN, 2005. Comparison of image analysis program for the comet assay. *J Toxicol* 19: 141-143.

Yang J, Yu Y, Duerksen-Hughes PJ, 2003. Protein kinases and their involvement in the cellular responses to genotoxic stress. *Mutat Res* 543: 31-58.

Yang ZY, 2000. Effects of Chinese traditions customs on neonatal jaundice. *Chin J Pediatrics* 38: 188-189.

Ye XS, Feng YB, Tong Y, Ng KM, Tsao SW, Lau GKK, Sze C, Zhang YB, Tang J, Shen JG, Kobayashi S, 2009. Hepatoprotective effects of *Coptidis Rhizoma* aqueous extract on carbon tetrachloride-induced acute liver hepatotoxicity in rats. *J Ethnopharmacol* 124: 130-136.

Yeung CY, Lee FT, Wong HN, 1990. Effect of a popular Chinese herb on neonatal bilirubin protein binding. *Biol Neonat* 58: 98-103.

Yi J, Ye XL, Wang DZ, He K, Yang Y, Liu XJ, Li XG, 2013. Safety evaluation of main alkaloids from *Rhizoma Coptidis. J Ethnopharmacol* 145: 303-310.

Yu P, Zheng FM, Wu JL, Zhou ZL, Fu ZQ, Zhao L, 2001. Combination therapy of *Rhizoma Coptis* and gliquidone to elderly diabetic in clinic. *Chin J Tradit Med Sci Technol* 8: 186-187.

Yu Y, Wang B, Peng L, Wang J, Zeng C, 2006. Advances in pharmacological studies of *Coptis chinesis*. *J Chongqing Univ* (Natural Science Edition) 29: 107-111.

Zeng LH, Yang XF, Zhao M, 2011. Progress in the study of Substitution method of acute toxicity *in vivo* and *in vitro. Chin J Public Health* 27(10): 1331-1333.

Zhang XY, An LH, Shao Z, 2003. Adverse reactions and therapeutic of *Rhizoma Coptidis* and its products. *Lishizhen Med Mater Med Res* 14: 714.