Protective effect of hawthorn vitexin on the ethanol-injured DNA of BRL-3A hepatocytes

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Abstract
Vitexin is a natural active ingredient in hawthorn leaves, which has a wide range of anti-tumor effects. This study was conducted to assess the protective effect of vitexin on the ethanol-injured DNA of hepatocytes in vitro and to explore its mechanism. The effect of different concentrations of hawthorn vitexin on ethanol-injured hepatocytes was detected via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method to study the protective effect of hawthorn vitexin on ethanol-injured DNA damage in hepatocytes. Single-cell gel electrophoresis was used to observe the effect of hawthorn vitexin on ethanol-induced DNA damage in hepatocytes, and the Olive tail moment was measured. Cell physiological and biochemical indexes, such as superoxide dismutase activity, malonaldehyde content, and glutathione peroxidase activity, were detected with kits. The mRNA expression of the superoxide dismutase gene was measured via real-time quantitative polymerase chain reaction. It was showed that 0.2, 0.4, and 0.8 mg/mL hawthorn vitexin could significantly repair hepatocyte growth and ethanol-induced DNA damage. This effect was closely related to the improvement in superoxide dismutase, malonaldehyde, and glutathione peroxidase. Hawthorn vitexin could be used to repair ethanol-injured hepatocytes through antioxidation effects, and showed potential for the treatment of liver injury.

Abbreviations: BRL-3A hepatocytes = Big rat liver—3A hepatocytes, CASP = Comet assay software project, DMEM = Dulbecco’s modified eagle’s medium, GSH-Px = Glutathione peroxidase, HPLC = High performance liquid chromatography, LMA = Low melting point agarose, MDA = Malonaldehyde, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NMA = Normal melting point agarose, PBS = Phosphate buffered solution, SOD = Superoxide dismutase, TNF-α = Tumor necrosis factor-α.

Keywords: antioxidation, DNA damage, ethanol-injured hepatocytes, Hawthorn vitexin, physiological and biochemical indexes

1. Introduction
Hawthorn (Crataegus pinnatifida Bunge) belongs to the Rosaceae plant family. In China, it is mainly distributed in Shandong, Anhui, Henan, Jiangsu, Shanxi, Zhejiang, and Jilin. Hawthorn is a traditional Chinese medicine, and its extracts are capable of alleviating liver disease; inhibiting inflammation; regulating blood lipid levels, blood pressure, and hypoxia; increasing coronary flow; and protecting the ischemic myocardium. Subjects found in hawthorn include flavones, flavonoids, cellulose, tannins, flavanes and their polymers, organic acids, steroids, and triterpenes. Among these substances, flavonoids are the main active components of hawthorn. Vitexin, an important class of flavonoids extracted from hawthorn leaves, is the active ingredient of hawthorn and has been previously demonstrated to have a protective effect against liver disease. However, the molecular mechanism of this effect is unclear.

DNA damage includes breakage, mutations, and rearrangements, and causes permanent genetic damage by changing DNA sequences. The result of DNA damage is cell death or canceration, and alcohol is an important inducer of DNA damage. Hepatocytes are vulnerable to alcohol damage. Alcoholic liver disease, which is the most common cause of liver cirrhosis, is caused by long-term heavy drinking. Alcoholic liver disease has been estimated to account for 48% of all deaths from cirrhosis. flavonoids, a common active ingredient of Chinese herbal medicines, have various pharmacological effects, such as antibacterial, anti-inflammatory, and antivirus effects. Flavonoids can protect liver cells by reducing damage and improving activity. However, few studies on the effect of hawthorn vitexin on alcohol-induced DNA damage and its mechanism are available.

The protective effect of Hawthorn vitexin on ethanol-injured BRL-3A hepatocytes was determined via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Single-cell gel
electrophoresis was used to calculate the Olive tail moment and analyze DNA damage. Physiological and biochemical indexes, such as superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, and glutathione peroxidase (GSH-PX) activity, were detected by using kits. Real-time quantitative polymerase chain reaction was applied to detect the mRNA expression of the SOD gene. This study provided a theoretical basis for the application of hawthorn vitexin in medicine and the prevention and treatment of liver diseases.

2. Materials and methods

2.1. Cell and reagents

Rat hepatocyte line BRL-3A (ATCC) was used. DMEM (Dulbecco’s modified eagle’s medium) high glucose medium, fetal bovine serum, Trypsin, and PBS (phosphate buffered solution) were purchased from Gibco (Grand Island, NY). Detection kits of SOD, MDA, and GSH-PX were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cell culture bottle and 96-well plate were obtained from Corning (NY, USA). Thiazolam was purchased from Amresco (Washington, USA).

2.2. Isolation and purification of hawthorn vitexin

Vitexin was extracted from 1 g of hawthorn leaves with 50 mL of ethanol–water (60:40, v/v) for 30 min in an ultrasonic bath. Extraction was repeated twice with 25 mL of ethanol–water. The crude extract of hawthorn vitexin was obtained with HPD-600 maceroreticular resin (Cangzhou Baoen Chemical Co. LTD, China) and ethanol as the eluent.

2.3. Cytotoxicity of hawthorn vitexin to BRL-3A hepatocytes

DMEM culture medium containing 10% fetal bovine serum was used to culture BRL-3A hepatocytes, and the concentration of cells in the logarithmic growth phase was controlled to 1 x 10^5 mL^-1. The cells were inoculated into a cell culture flask at 37°C in a 5% CO₂ incubator (Thermo, Waltham, MA). The cells were digested with trypsin when they grew to 80% confluence. After centrifugation, the cells were suspended in DMEM containing serum, and their concentration was controlled to 5 x 10^4 mL^-1 to prepare a single-cell suspension. The single-cell suspension was inoculated into a 96-well plate. After adherent culture for 24 h, hawthorn vitexin was added into each well at the concentrations of 0.1, 1, 10, and 100 mg/mL^-1. At the same time, the control group without hawthorn vitexin and the zeroing well without cells and hawthorn vitexin were set. Six replicates were set for each group, and the cells were cultured for 24 hours. Subsequently, the absorbance at 490 nm of each well was detected via the MTT method by using a Multiskan GO microplate spectrophotometer (Thermo, Waltham, MA). Then, cell survival rates were calculated as follows: survival rate = Acontrol / Atest x 100%. This study was approved by the ethical committees of Zaozhuang University.

2.4. Effects of ethanol on the survival rate of BRL-3A hepatocytes

The single-cell suspension that was prepared as described in Section “Cytotoxicity of hawthorn vitexin to BRL-3A hepatocytes” was inoculated into a 96-well plate. Then, ethanol was added to each well at the concentrations of 25, 50, 75, and 100 mmol L^-1. The control group and zeroing well were also established as reported in Section “Cytotoxicity of hawthorn vitexin to BRL-3A hepatocytes.” Six replicates were set for each group, and the cells were cultured for 12 hours. Subsequently, the cell survival rate was detected via the MTT method. The ethanol concentration corresponding to 50% survival rate was selected as the test concentration.

2.5. Effects of hawthorn vitexin on the survival rate of ethanol-injured BRL-3A hepatocytes

The prepared single-cell suspension was inoculated into a 96-well plate and cultured for 24 hours. 100 mmol·L^-1 ethanol was added, and cells were cultured for another 12 hours. Then, ethanol was removed by the change of the culture medium. Hawthorn vitexin was added at the concentrations of 0.2, 0.4, and 0.8 mg mL^-1 to each group for 24 hours. Finally, cell survival rates were detected via the MTT method to investigate the effect of hawthorn vitexin on the proliferation of ethanol-injured hepatocytes. The damage model control group (with ethanol) and the normal group (without ethanol and hawthorn vitexin) were set simultaneously.

2.6. Effects of hawthorn vitexin on the SOD activity, MDA content, and GSH-PX activity of ethanol-injured BRL-3A hepatocytes

The method used in this experiment was the same as that reported in Section “Effects of hawthorn vitexin on the survival rate of ethanol-injured BRL-3A hepatocytes.” After the end of culture, the cells were lysed, and SOD activity, MDA content, and GSH-PX activity were detected by using kits.

2.7. Single-cell gel electrophoresis

The method used in this experiment was the same as that presented in Section “Effects of hawthorn vitexin on the survival rate of ethanol-injured BRL-3A hepatocytes.” The cells were collected, and 100 μL of 1% normal melting point agarose (NMA) was spread on a frosted glass slide. The gel was flattened with a cover slide to avoid the formation of air bubbles. The agarose was solidified at 4°C. Then, the cover slide was removed. A total of 10 μL of cell suspension (1 x 10^5 mL^-1) was mixed with 90 μL of 1% low melting point agarose (LMA). The mixture was spread on the NMA and flattened with a cover slide. The gelatinized frosted glass slide was placed in precooled lysate for 4°C for 2 hours in the dark. The slides were removed from the lysate and soaked thrice in precooled deionized water for 5 minutes. Electrophoresis was performed at 25 V for 30 minutes. At the end of electrophoresis, the slides were removed from the...
electrophoresis tank and soaked thrice in precooled neutralization solution for 5 minutes. After staining with 0.5 mg·L\(^{-1}\) propidium iodide, the DNA of a single cell was observed by using a fluorescence microscope (Olympus BX51, Tokyo, Japan). Image analysis was conducted with Comet Assay Software Project (CASP 1.2.3, Wroclaw, Poland).\[21–23\]

2.8. Real-time quantitative polymerase chain reaction

The method used in this experiment was the same as that reported in Section “Effects of hawthorn vitexin on the survival rate of ethanol-injured BRL-3A hepatocytes,” and the cells were collected after treatment. The total RNA of the cells in each treatment group was extracted, and the purity and content of RNA were detected by using NanoDrop One Microvolume UV-Vis spectrophotometers (Thermo, USA). Then, RNA was transcribed into cDNA with a RevertAid First Strand cDNA Synthesis kit (#K1622, Fermentas, EU, Waltham, MA). Real-time quantitative polymerase chain reaction using SYBR Green I (Applied Biosystems, Foster City, CA) was performed for the absolute quantitative detection of the mRNA expression of the SOD gene, and the β-actin gene was used as the internal control. The primers of the SOD gene were AAGGCCTGCATG-GATTCCA and TTGGCCCACCGTGTCT, and the primers of the β-actin gene were GCTCGTCGTCGCA-GCAACGGCCTC and CAAACATGATCTGGGTATTTCT.

2.9. Statistical methods

SPSS statistical software 22.0 was used for paired sample T test. \(P < .05\) was considered to be statistically significant.

3. Results

3.1. Isolation and purification of hawthorn vitexin

Vitexin is a flavonoid, and its chemical structural formula is 8-β-D-glucopyranosyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-Benzopyran-4-one) (Fig. 1A).\[14,15\] Hawthorn vitexin in the crude extract was collected by HPLC in accordance with the elution time of the standard substance, and its purity was determined to exceed 95%. The withdrawal rate of purified vitexin was 0.47\% (Fig. 1B–D).

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**Figure 1.** HPLC chromatograms of hawthorn vitexin. (A) Chemical structural formula of vitexin; (B) standard substance; (C) crude extract of hawthorn vitexin; (D) purified vitexin.
3.2. Hepatotoxicity of hawthorn vitexin

The results showed that 0 to 1 mg·mL⁻¹ hawthorn vitexin had no significant cytotoxicity against the survival of hepatocytes (P > .05). Cell activity decreased significantly when the concentration of hawthorn vitexin reached 10 and 50 mg·mL⁻¹, indicating significant cytotoxicity (P < .01). Therefore, the concentration of hawthorn vitexin in this study was set as 0 to 1 mg·mL⁻¹ (Fig. 2).

3.3. Effects of ethanol on BRL-3A hepatocyte proliferation

The results showed that the cell survival rate of each treatment group was significantly different from that of the control group. The lethal rate of the hepatocytes was 52.7% when the ethanol concentration was 100 mmol·L⁻¹. Therefore, 100 mmol·L⁻¹, which was chosen as the median lethal concentration, was used as the experimental concentration in subsequent experiments in this study (Fig. 3).

3.4. Effect of hawthorn vitexin on the survival rate of ethanol-injured BRL-3A hepatocytes

The results showed that the cell survival rate of the ethanol model group was considerably lower than that of the normal control group, indicating that the model of ethanol-injured hepatocytes was successfully constructed. Compared with the ethanol control treatment, 0.2 mg·mL⁻¹ hawthorn vitexin could significantly improve the survival rate of injured hepatocytes (P < .05), and treatment with 0.4 and 0.8 mg·mL⁻¹ hawthorn vitexin could remarkably improve the survival rate of injured hepatocytes (P < .01) (Fig. 4).

3.5. Effects of hawthorn vitexin on DNA damage in ethanol-injured hepatocytes

Under the fluorescence microscope, DNA was stained orange red by propidium iodide, and the DNA of normal cells showed round bright spots without trailing after electrophoresis (Fig. 5A). The ethanol-injured DNA had a comet-like appearance. Severe DNA injury was reflected by long comet tails (Fig. 5B). Treatment with 0.2, 0.4, and 0.8 mg·mL⁻¹ hawthorn vitexin could improve ethanol-induced DNA damage in hepatocytes and shorten comet tails. Moreover, high hawthorn vitexin concentrations were associated with obvious improvement (Fig. 5C, D, and E). The Olive tail moment of ethanol-injured hepatocytes showed a decreasing trend with the increase in hawthorn vitexin concentration. When the hawthorn vitexin concentration reached 0.8 mg·mL⁻¹, the Olive tail moment of the injured cells decreased to 15.0 ± 3.1 μm. This result further demonstrated the repairing effect of hawthorn vitexin on alcohol-injured hepatocytes, and that this effect was dose-dependent (Fig. 6).

3.6. Effects of hawthorn vitexin on SOD activity, MDA content, GSH-Px activity, and SOD gene expression in ethanol-injured BRL-3A hepatocytes

Comparison with the normal control group revealed that ethanol could significantly reduce SOD and GSH-PX activity and increase MDA content in BRL-3A hepatocytes. Compared with the same
indexes in the ethanol-injured group, the antioxidant indexes of injured cells in the 0.2, 0.4, and 0.8 mg·mL\(^{-1}\) hawthorn vitexin treatment groups had significantly improved. Specifically, SOD and GSH-Px activity increased and MDA content decreased in the hepatocytes in the 0.2 mg·mL\(^{-1}\) group (\(P < .05\)), and SOD and GSH-Px activity remarkably increased and MDA content decreased in the 0.4 and 0.8 mg·mL\(^{-1}\) groups (\(P < .01\)) (Table 1).

Comparison with the normal control group revealed that 100 mmol·L\(^{-1}\) ethanol could significantly decrease the mRNA expression of the SOD gene in cells. Compared with that in the ethanol injured group, the expression of the SOD gene in the hepatocytes in the 0.2, 0.4, and 0.8 mg·mL\(^{-1}\) hawthorn vitexin groups had significantly increased in a dose-dependent manner (Fig. 7).

4. Discussion

DNA damage plays an important role in pathophysiological processes, such as tumorigenesis, aging, and inflammation, as well as in diabetes and neurodegenerative diseases. Oxidative stress, lipid peroxidation, glycosylation, and other cellular...
The model of ethanol-injured hepatocytes was prepared by adding ethanol into the culture medium. The damage mechanism that accounted for the hepatotoxic effect of ethanol was as follows: in hepatocytes, ethanol was metabolized into toxic acetaldehyde, which breaks and damages the DNA in hematopoietic stem cells.[7,8]

In this study, 100 mg·mL⁻¹ ethanol inhibited the survival rate of BRL-3A hepatocytes, injured the DNA of the cells, significantly decreased the activity of SOD and GSH-Px in the cells, and increased the content of MDA. Different concentrations of hawthorn vitexin could alleviate inhibition and damage. Hawthorn vitexin could repair the DNA damage caused by ethanol through an antioxidative effect, which was closely related to the upregulated expression of the SOD gene.

The extraction rate of vitexin, an effective component in hawthorn, can reach 0.87%.[14,15] Experiments on alcoholic liver injury in mice have demonstrated that hawthorn extracts can reduce alcohol-induced liver injury, TNF-α (tumor necrosis factor-α) levels, and cell apoptosis.[25,26] The present study showed that hawthorn vitexin could repair ethanol-injured DNA in vitro. Given the increasing use of hawthorn vitexin in liver injury treatment, the comprehensive study and utilization of hawthorn warrant further development.

5. Conclusions

Hawthorn vitexin could significantly repair hepatocyte growth and ethanol-induced DNA damage via antioxidative effects and showed potential applications in the treatment of liver injury.

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