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Original Research Article

Studies on the effect of *Celastrus orbiculatus* (Celastraceae) extract on chemosensitivity of liver cancer cells via Wnt/β-catenin pathway

Xiaobo Ding[1], Laijun Song[2], Yunfei Lu[1], Qiting Huang[3], Chengming Jiao[4]*
[1]Jiangyin Hepatobiliary Hospital of Traditional Chinese Medicine, [2]Department of Clinical Laboratory, [3]Department of Hepatobiliary, [4]Department of Administration, Jiangyin Hepatobiliary Hospital of Traditional Chinese Medicine, Jiangyin 214404, Jiangsu Province, China

For correspondence:* ***Email:* *jexa1e@163.com;* ***Tel:*** *+86-051086581366*

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Abstract

Purpose: *To examine the efficacy of Celastrus orbiculatus extract (COE) on the chemosensitivity of liver cancer (LC) cells and its mechanism of action.*
Methods: *Hep G2/ADM cells in the logarithmic growth phase were assigned to a control group (no treatment for cell culture medium only) and a study group (120 μg/ml COE added to the culture medium). After 48 h of incubation, the biological responses were compared. The study group was divided into groups A and B, while control group was divided into groups C and D, with 1 μmol/L XAV939 added in groups A and C. Cell proliferation, cell invasion, cell apoptosis rate, and apoptosis protein in the four groups were evaluated.*
Results: *The study group showed significantly lower values in terms of cell proliferation and cell invasiveness ($p < 0.05$) and a higher apoptotic rate than the control group ($p < 0.05$)). The study group also demonstrated an elevated pro-apoptotic protein Bax level and a declined anti-apoptotic protein Bcl-2 level. In contrast to group B, the proliferation and invasiveness of Hep G2/ADM cells in group A treated with the inhibitor, XAV939, were significantly lower ($p < 0.05$), while the apoptotic rate exhibited a significant increase ($p < 0.05$). There was a rise in the level of pro-apoptotic protein, Bax, and a fall in the anti-apoptotic protein Bcl-2 level in group A. Lower levels of β-catenin, c-Myc, and cyclin D1 protein were observed in the study group compared with the control group ($p < 0.05$). Compared with other groups, the multiplication capacity and invasiveness of cells in group A treated with COE and inhibitor XAV939 significantly declined, while the apoptotic rate increased ($p < 0.05$).*
Conclusion: *COE reverses drug resistance in chemotherapy by inhibiting the expression of Wnt/β-catenin pathway in LC cells. Therefore, COE has potentials for use along with chemotherapeutic agents in the management of liver cancer.*

Keywords: *Celastrus orbiculatus, Liver cancer, Chemosensitivity, Chemotherapy, Apoptosis, Cell invasion*

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INTRODUCTION

Globally, liver cancer (LC) is the most common pervasive malignant tumor [1], which ranks fifth among malignant tumors in the United States. Although the etiology of LC is still elusive, previous research has pointed out several major causes of LC, including chronic alcohol misuse,

hepatitis B or hepatitis C virus infection, and food pollution [2].

Nonetheless, few studies have examined the trends in the occurrence of LC triggered by specific causes. The incidence of LC increased from 471,000 cases in 1990 to 1,007,800 cases in 2016 worldwide, with an increase of 114.0% [3], which is considered a major global public health issue. Notwithstanding the rapid development of detection technology, the disease may have progressed to an advanced stage at time of diagnosis in most cases, resulting in a poor prognosis [4]. Hepatocellular carcinoma (HCC) accounts for over 90 % of LC cases, for which resection is one of the main treatment methods [5]. A prior study has revealed that the postoperative recurrence of tumors has remained one of the pressing clinical issues to be addressed; hence, systemic chemotherapy has captured great attention in clinical practice [6]. However, the sensitivity of chemotherapy has been severely impaired owing to drug resistance and related issues, which hinders the progress of disease treatment.

According to previous research, celastrus orbiculatus is a commonly used herb with various biological activities and multiple functions such as activating blood and dissolving stasis, clearing heat and removing toxicity [7]. Moreover, it has been reported that that *celastrus orbiculatus* has a promising therapeutic efficacy in the treatment of cholecystitis and nephritis [8]. A previous study has stated that *Celastrus orbiculatus* extract (COE) can effectively inhibit various tumors cells. However, the effect of COE is rarely reported in LC. The Wnt/β-catenin pathway is involved in various physiological processes, and it has been reported to be intimately associated with the progression of tumors [9]. Therefore, this research was designed to assess the influence of COE on the chemosensitivity of LC cells and its mechanism.

EXPERIMENTAL

Cell line

Hep G2/ADM cells, a multidrug-resistant cell line of HCC, were purchased from Beina Biotechnology Co. Ltd, a Chinese agent of ATCC.

Cell culture

The Hep G2/ADM cells were cultivated in RPMI 1640 culture medium with10 % fetal bovine serum, followed by cultivation in a humid environment with 5 % CO_2 at 37 °C, prior to trypsin digestion with a cell density reaching 80%. Subsequently, the cells were portioned into culture flasks at a ratio of 1 : 3 for passaging culture.

Determination of the effect of COE on Hep G2/ADM cells

Hep G2/ADM cells were digested by trypsin and counted, with the cell concentration adjusted to 1 × 10^5/mL, followed by the inoculation to a 96-well plate. All cells were distributed to the study group and control group. COE with a concentration of 120 μg/mL was added in the study group, and the culture was continued after treatment. After 48 h, data were collected and analyzed.

MTT assay

Cells were inoculated to a 96-well plate, with the density of 4*103 cells/well, and cultivation was performed at 37 °C for 24, 48, 72, and 96 h, respectively. At each time point, MTT solution (5 μg/mL) (20 μL) was added and cultivated at 37 °C for 4 h, prior to adding 200 μL of dimethyl sulfoxide into each well. Then, the OD value in each group was tested via a V-1200 spectrophotometer at 450 mm wavelength.

Transwell assay

Cells were collected and inoculated in a 24-well plate, with the density regulated to 3 × 104 cells/well, digested by trypsin, and then transferred to the upper chamber. RPMI1640 medium (200 μL) was added in the upper compartment, and RPMI1640 (500 mL) (comprising 10%FBS) was added in the lower compartment. Cultivation of the cells was performed at 37 °C for 48 h. The cells in the upper compartment were wiped off by using wet cotton swabs, rinsed with PBS, and secured with methanol for 30 min. 0.1 % crystal violet was employed for color development for 20 min, and subsequently rinsed with PBS.

Flow cytometry

Trypsin digestion (0.25%) was performed and the cells were rinsed with PBS twice. The binding buffer (100 μL) was added to prepare 1 x 106 cells/mL suspension. AnnexinV-FITC and PI were added for culture at room temperature without light for 5 min. FC500MCL flow cytometry instrument was applied for detection.

Western blot analysis

The study group was divided into group A (containing added 1 μmol/L XAV939) and group

B, and the control group was divided into group C ((containing 1 μmol/L XAV939)) and group D. Proteins were extracted using the RIPA buffer from cultured cells in each group, lysed, and centrifuged at 10000 × g for 20 min to obtain the supernatant. Bicinchoninic acid was employed to detect the protein concentration. The proteins of a same amount were segregated for SDS-PAGE, and transferred to a PVDF membrane. 5% skimmed milk powder was used for blocking at room temperature for 1 h. After electrophoresis, the protein was transferred to PVDF membrane by a membrane transfer instrument. Subsequently, 5 % skim milk was supplemented to block the membrane for 2 h and rinsed. Then, primary antibodies (1: 1000) were added and sealed for overnight cultivation at 4℃, and the membrane was rinsed and added with HRP-labeled goat anti-rabbit secondary antibody (1: 5000).

Statistical analysis

Statistical analysis was performed by using the SPSS 20.0 software, and GraphPad Prism 7 was applied to plot the graphs. The measurement data were represented by (SD ± means) and were analyzed by t-test. The independent-samples t-test was employed for the comparison between groups, expressed in t. One-way ANOVA was used to analyze the comparison among groups. LSD t-test was employed for pairwise comparison. Repeated measures were applied for multi-time point expression. Bonferroni was applied for a post hoc test. P< 0.05 indicated statistical differences.

RESULTS

Biological effects of COE on Hep G2/ADM cells

The study group obtained significantly lower results in terms of multiplication curve and cell invasiveness *(p* < 0.05) and a markedly higher apoptotic rate than the control group *(p* < 0.05). An elevated pro-apoptotic protein Bax level and a reduced anti-apoptotic protein Bcl-2 level was observed in the study group *(p* < 0.05, Figure 1).

Effect of Wnt/β-catenin pathway on HEp G2/ADM cells

The multiplication capacity of Hep G2/ADM cells in group A treated with inhibitor XAV939 was declined *(p* < 0.05). Moreover, the invasiveness capability was also lower than the cells in group B which received no treatment of inhibitor XAV939, while the apoptotic rate in group A was higher than group B *(p* < 0.05). There was an elevation in the pro-apoptotic protein Bax level and a fall in the anti-apoptotic protein Bcl-2 level in group A *(p* < 0.05, Figure 2).

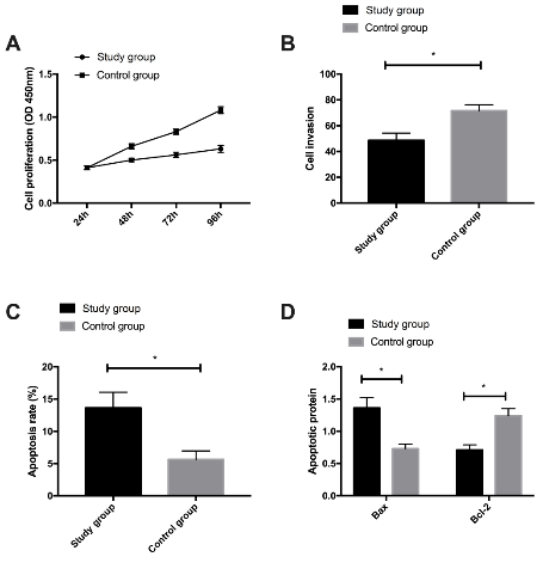

Figure 1: Biological effects of COE on Hep G2/ADM cells. A) Cell proliferation. B) Cell invasion. (C) Apoptosis rate. (D) Apoptosis protein.; *p < 0.05

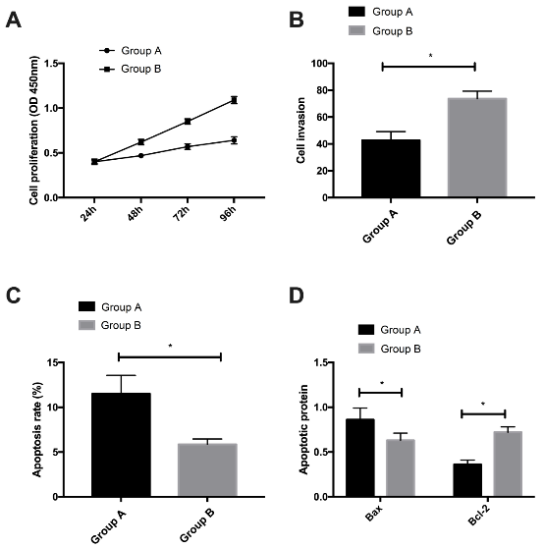

Figure 2: Effect of Wnt/β-catenin pathway on Hep G2/ADM cells. A) Cell proliferation B) Cell invasion. C) Apoptosis. D) Apoptosis protein; *p < 0.05

Expression of β-catenin, c-Myc, and cyclin D1 protein in RG and CG

The study group exhibited lower levels of β-catenin, c-Myc, and cyclin D1 protein expressions than the control group *(p* < 0.05, Figure 3).

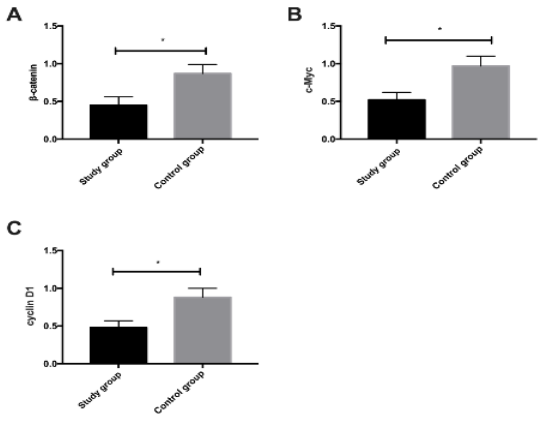

Figure 3: Expression level of β-catenin, c-Myc, and cyclin D1 protein in the study group and control group. (A) Expression of the β-catenin protein in the study group and control group. (B) Expression of c-Myc protein in study group and control group. (C) Expression of cyclin D1 protein in study group and control group. *$P < 0.05$

COE affected the chemosensitivity of Hep G2/ADM cells by regulating Wnt/β-catenin pathway

The multiplication and invasive capacity of cells in group A treated with COE and inhibitor XAV939 were lower than those in other groups, and the apoptosis rates of the cells in group A were increased ($p < 0.05$). Nevertheless, no great disparity in proliferation, invasiveness, and apoptosis between group B (treated with COE alone) and group C (treated with inhibitor XAV939 alone) was found ($p > 0.05$). The cell multiplication and invasiveness of the cells in groups D without COE and inhibitor XAV939 were increased significantly, while the apoptotic rate was diminished markedly ($p < 0.05$) (Figure 4).

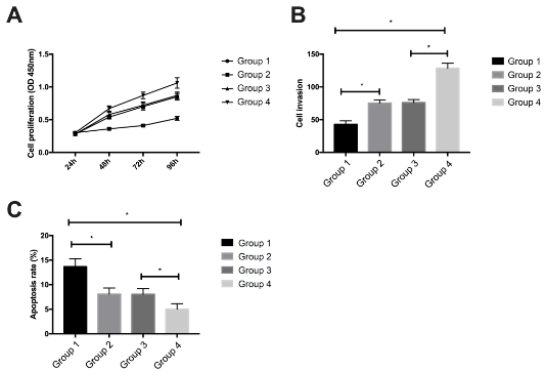

Figure 4: COE affected the chemosensitivity of Hep G2/ADM cells by regulating the Wnt/β-catenin pathway. (A) Cell proliferation. (B) Cell invasion. (C) Apoptosis rate; *$p < 0.05$

DISCUSSION

LC is one of the most pervasive malignancies in the alimentary canal, the incidence of which has witnessed an increasing trend in recent years, with rapid disease progression, serious deterioration, and poor five-year survival [10]. Notwithstanding the implementation of comprehensive treatment methods such as surgical resection and chemotherapy in clinical practice, the prognosis of patients still remains much to be desired. Previous research has revealed that malignant tumors are caused by the uncontrolled mechanism of cell growth, proliferation, and apoptosis [11]. For example, the oncogene is activated while the tumor suppressor gene is inactivated, which leads to the continuous metastasis of cancer cells and the growing severity of the disease. The Chinese herbal medicine, *Celastrus orbiculatus*, is frequently used to dispel wind, promote blood circulation, detoxify and reduce swelling [12]. Herein, the effect and of COE on the chemosensitivity of LC cells and its mechanism were studied.

It was found that the study group had a lower proliferation rate and cell invasiveness than the control group. However, markedly higher apoptosis rate was found in the study group, and study group had an increased pro-apoptotic protein Bax level and a decreased anti-apoptotic protein Bcl-2 level, indicating the anti-cancer role of COE in LC, which is also in line with the findings of a previous study [13], confirming the reliability of our study. It may be attributed to the promising anti-inflammatory and antioxidant effects of COE.

Previous research has revealed that COE can effectively control the activation of NF-kB and mediate the levels of inflammatory factors. Moreover, it can effectively remove oxygen free radicals, inhibit oxidation reactions both inside and outside mitochondria, and increase surface negative charges to avoid damage to the mitochondrial inner membrane [14]. In tumors, COE can significantly reinforce the killing effect on tumor cells through its strong antiviral effect. Although a prior study has pointed out that COE effectively promotes the apoptosis of tumor cells [15], its specific mechanism is still unclear. In was found in this study that the Wnt/β-catenin signal pathway exerts an effect on the influence process of COE. The Wnt/β-catenin pathway is an important path that regulates pluripotency of stem cells and influences cell differentiation during development [16].

It has been confirmed that Wnt/β-catenin is highly activated in LC, which stimulates the growth and development of tumor cells [17]. The findings of this research revealed that the activity of Hep G2/ADM declined and the apoptosis rate increased under the intervention of XAV939, which once again confirmed that the suppression of the Wnt/β-catenin pathway could reverse the drug resistance of LC cells and accelerate the apoptosis of tumor cells.

The results of the present study also revealed that Wnt/β-catenin path-related proteins β-catenin, c-Myc, and cyclin D1 were declined in Hep G2/ADM under the intervention of COE, which indicated that COE could suppress the expression of Wnt/β-catenin pathway in Hep G2/ADM, preliminarily confirming our above speculation. Then, COE was co-cultured with Wnt / β - Catenin path inhibitor. The findings revealed that the activity of Hep G2/ADM cells declined obviously after the co-intervention of COE and XAV939, suggesting that the combination of COE and XAV939 could prominently reinforce the drug resistance of LC cells and the killing effect of chemotherapy drugs on cells. Compared with cells intervened by COE and XAV939, there was no difference in proliferation, invasiveness, and apoptosis, indicating that COE and XAV939 could effectively reverse the drug resistance of LC cells.

The limitations of this study lie in the absence of animal tests and the non-exploration of the optimal dosage for treatment, which may raise some uncertainty regarding the effect of COE on tumors *in vivo*. However, animal tests and optimal dosage exploration will be conducted in future studies.

CONCLUSION

COE reverses drug resistance to chemotherapy by inhibiting the expression of Wnt/β-catenin pathway in LC cells, thus indicating its potentials for further development for use along with chemotherapeutic agents in anti-liver cancer therapy.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaobo Ding conceived and designed the study, and drafted the manuscript. Laijun Song and Yunfei Lu collected, analyzed and interpreted the experimental data. Qiting Huang and Chengming Jiao revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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