Suppression of human hepatocellular cancer cell proliferation by *Brucea javanica* oil-loaded liposomes via induction of apoptosis

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Abstract

Introduction: Hepatocellular carcinoma (HCC) is a type of malignancy with high incidence and poor prognosis. *Brucea javanica* is extracted from Simaroubaceae plants. It is found to have low toxicity but high anti-cancer efficiency. The aim of this study is to determine the effects of *Brucea javanica* oil-loaded liposomes (BJOL) on human hepatocellular cancer cell line HepG2. The related molecular mechanisms were determined.
Material and methods: Morphologic changes of HepG2 cells were observed by transmission electron microscope after treatment with BJOL *in vitro*. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after cell treatment with different doses of BJOL. Flow cytometry was performed. Nude mice were divided into 4 groups randomly and treated with different doses of BJOL. The apoptosis hepatocellular carcinoma was detected by TUNEL.
Results: Proliferation of HepG2 was inhibited significantly by BJOL in a dose-dependent manner (2.5 mg/l or 5 mg/l). Compared with the animal models treated with the negative control, the animal models in the BJOL group had higher weight and lower metastasis rates ($p < 0.01$). The rate of apoptosis in hepatocellular carcinoma tissue of the BJOL groups was increased when compared with the control group ($p < 0.05$).
Conclusions: *Brucea javanica* oil-loaded liposomes inhibits proliferation of HepG2. The effect appears to be dose-dependent, possibly by inducing apoptosis of cancer cells.

Key words: *Brucea javanica* oil-loaded liposomes, hepatocellular carcinoma, apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is a malignancy with high incidence, early metastasis, poor prognosis, and lack of effective treatment [1–4]. Chemotherapy and radiotherapy treatment efficacies are unsatisfactory, and therefore biological treatments are receiving more and more attention.

Brucea javanica is extracted from Simaroubaceae plants. It is found to have low toxicity but high anti-cancer efficiency [5–10]. It is reported that the *Brucea javanica* extract has potent toxic effects with an IC_{50} value of 0.44 ±0.02 µg/ml on HCC cells, but the cell cytotoxic activity

is not detectable in peripheral blood mononuclear cells that are treated with *Brucea javanica* extract with a concentration less than 30 µg/ml, which is a high dosage [8]. Treatment of non-small-cell lung cancer cells, hepatocellular carcinoma cells, breast cancer cells, and oesophageal squamous cell carcinoma cells with *Brucea javanica* extracts indicate antiproliferative effects and apoptosis-inducing activities [9]. Selective cytotoxicities are also found when cervical cancers and other cancers are treated with the *Brucea javanica* extracts [10]. In addition to the aqueous extracts, it is shown that emulsion extracted from *Brucea javanica* can effectively prevent tumor proliferation and infiltration [11, 12]. *Brucea javanica* oil emulsion (BJOE) is a non-specific anti-cancer reagent, having an inhibitory effect on tumor cells in G0, G1, S, G2, and M phases [13, 14]. It significantly inhibits DNA, RNA and protein synthesis of tumor cells, and interferes with the formation of peptide tendon [15]. It is reported that BJOE inhibits cell growth and DNA synthesis of lung cancer cells *in vitro* [16]. Li *et al.* found that BJOE prevents progress from G0/G1 to S phase of human renal granulosa cell carcinoma cell line GRE-1 and inhibits DNA synthesis [17]. *Brucea javanica* oil emulsion also prevented cell cycle transition of BIU-87 cells from G0/G1 into S phase progress and inhibited DNA synthesis [18]. However, BJOE and the aqueous extracts have to be used at a relatively high dose, which often results in high non-specific toxicity to normal cells [8–10, 12–17]. Therefore, novel therapeutic methods need to be studied.

Liposome technology is an important pharmaceutical technology [19]. It is found that liposome-vectored chemotherapy drugs can significantly alleviate the side effects and increase efficacy. Yachi *et al.* found that treatment of mice with metastatic liver cancers using liposome-vectored doxorubicin significantly increases the survival time of mice [20]. In this study, liposome technology was studied to determine the effects of *Brucea javanica* oil-loaded liposomes (BJOL) on hepatocellular carcinomas. Tumor suppression of BJOL was determined in comparison to BJOE *in vivo* and *in vitro*.

Material and methods

Reagents and cell lines

HepG2 cells were provided by the Laboratory of the Department of Hepatobiliary Surgery, School of Medicine, Xi'an Jiaotong University. The MTT and TUNEL assay kits were purchased from Promega Corporation (Madison, USA). Annexin V/PI kit was purchased from Sigma Company (St. Louis, USA). *Brucea javanica* oil emulsion injection was purchased from Zhejiang Sanjiu Bangerkang Co., Ltd (Hangzhou, China). DMEM, trypsin and other cell culture reagents were purchased from Gibco Company (Grand Island, USA).

Preparation of BJOE and BJOL

Film-ultrasound dispersion was used to prepare BJOE [11, 12]. *Brucea javanica*, lecithin, and cholesterol were mixed with a ratio of 2 : 3 : 1 [6, 14]. Then an appropriate amount of phosphate buffer with a pH of 7.4 was added. The mixture was vibrated ultrasonically for 5 min to obtain a BJOL suspension [14]. This suspension was examined under a light microscope and a transmission electron microscope to determine the quality of BJOL [14].

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cells were collected, and then 150 µl of DMSO was added after the supernatant was removed. After oscillation for 10 min, the A490 value was determined by an enzyme-linked immunoassay analyzer. The HepG2 cell inhibition rate of each drug concentration = $(1 - A$ value of dose solution group/A value of negative control group) × 100%.

Flow cytometry

The cells were suspended in 200 µl of binding buffer, and 10 µl of Annexin V-FITC and 5 µl of PI were mixed. After gently mixed and keeping in the dark at room temperature for 30 min, 300 µl of binding buffer was added and the solution was tested.

Transmission electron microscopy

Cells were washed twice with PBS, and fixed with 25 ml/l glutaraldehyde for 30 min. Then, cells were fixed with 10 g/l osmic acid for 1 h. Cells were placed in acetone for gradient dehydration and for displacement in embedding medium (1 : 1) for 30 min. After 2 h in embedding reagents, cells were observed under a transmission electron microscope.

HepG2 tumor mice model

The HepG2 cells (0.2 ml) in the logarithmic growth phase were subcutaneously injected into the back of 4-week-old male nude mice. The animals were raised in flow chamber for 28 days until the diameters of subcutaneous nodules reached 1 cm. Tumor tissue was removed under sterile conditions and preserved in DMEM containing 20 ml/l FCS at 37°C, and was divided into tumor tissue pieces (2 mm × 2 mm × 2 mm).

The 4- to 6-week-old male nude mice were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Tumor tissue pieces were inoculated into the left lateral lobe. Twenty-four tumor-bearing mice were intraperitoneally

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injected (2 ml/day) and were randomly divided into four groups: the negative control group (saline), the small-dose BJOL group (BJOL, 2.5 mg/l), the high-dose BJOL group (BJOL, 5 mg/l), and the positive control group (BJOE, 25 mg/l). Mice were kept under the same conditions in the lower stream chamber for 40 days, and were all sacrificed. The weight of each animal model was recorded and specimens of the liver were taken. All animal experiments were conducted according to the ethical guidelines of Xi'an Jiaotong University, China, which fulfill the legal requirements in Poland and the European Communities, and the National Institute of Health Guide.

HE staining and TUNEL apoptosis detection

Conventional liver tissue was embedded in paraffin and sectioned successively. Some of the liver tissues were HE-stained and were observed under an optical microscope [12]. Some slices were detected by TUNEL [12, 15]. Terminal deoxynucleotidyl transferase reaction solution (100 μl) was added onto each slice. A slice was covered with a plastic cover, and incubated at 37°C for 60 min. 3,3'-Diaminobenzidine solution (100 μl) was added to each piece until there was a light brown background. For each sample, 200 cells were counted under the microscope. Brown particles in cells were considered apoptotic cells. The positive rate of apoptotic cells was calculated.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). SPSS 13.0 software was used for statistical analyses. Value of $p < 0.05$ was considered statistically significant.

Results

BJOL has more significantly inhibitory effects on HepG2 cells than BJOE

To determine the effects of BJOL on HepG2, BJOL was prepared and examined under a light microscope. The liposomes were of the same size of about 20–40 μm, which is consistent with the size of a single chamber liposome. HepG2 cells in the logarithmic phase were treated with the negative control (saline), BJOL (2.5 mg/l or 5 mg/l), and BJOE (25 mg/l), respectively. The MTT results (Table I) showed that the HepG2 cell inhibition rates gradually increased ($p < 0.05$) when the concentrations of BJOL were increased from 2.5 mg/l to 5 mg/l and the treatment times were increased. The results in Table I indicated that the inhibitory effects of BJOL on HepG2 were more significant than the effects of BJOE.

HepG2 cells in the logarithmic phase were treated with the negative control (saline), BJOL (2.5 mg/l or 5 mg/l), and BJOE (25 mg/l) for 1 h. The results of flow cytometry indicated that there were 0.13 ±0.21% ($p < 0.05$) apoptotic HepG2 cells in the negative control group. As the concentration of BJOL increased from 2.5 mg/l to 5 mg/l, the HepG2 cell apoptosis significantly increased, with a significant difference between groups ($p < 0.05$, Figure 1). These results indicated that BJOL (5 mg/l) induces apoptosis of HepG2 cells more effectively than BJOE (25 mg/l).

Morphological changes of HepG2 cells induced by BJOL

HepG2 cells in the logarithmic phase were treated with the negative control (saline), BJOL (2.5 mg/l or 5 mg/l), and BJOE (25 mg/l) for 6 h. By transmission electron microscopy, irregular cell morphology and integral cell membrane structure were visible in the negative control group (Figure 2). It was found that when cells were treated with BJOL (5 mg/l) for 6 h, the activity of HepG2 tumor cells was affected and apoptosis was induced without detectable cytotoxicity (Figure 2).

Survival and weight changes of tumor-bearing mice

Twenty-four tumor-bearing mice were intraperitoneally injected (2 ml/day) and were ran-

Table I. Inhibition rates of HepG2 cells after treatment with BJOL

Groups	Absorbance value (*A*)			Inhibition rates (%)		
	1 h	3 h	6 h	1 h	3 h	6 h
Negative control (saline)	0.442 ±0.0032	0.462 ±0.0021	0.481 ±0.0065	0.003 ±0.02	0.003 ±0.01	0.004 ±0.16
BJOL (2.5 mg/l)	0.112 ±0.0024[#]	0.101 ±0.0041[#]	0.060 ±0.0038[*]	33.31 ±1.13[#]	62.37 ±3.24[#]	85.17 ±2.22[*]
BJOL (5 mg/l)	0.082 ±0.0065[*]	0.065 ±0.0037	0.053 ±0.0053	45.23 ±0.72[*]	70.65 ±2.14	90.62 ±3.27
BJOE (25 mg/l)	0.126 ±0.0031	0.125 ±0.0042	0.070 ±0.0045	20.33 ±1.36	33.47 ±2.18	70.43 ±3.32

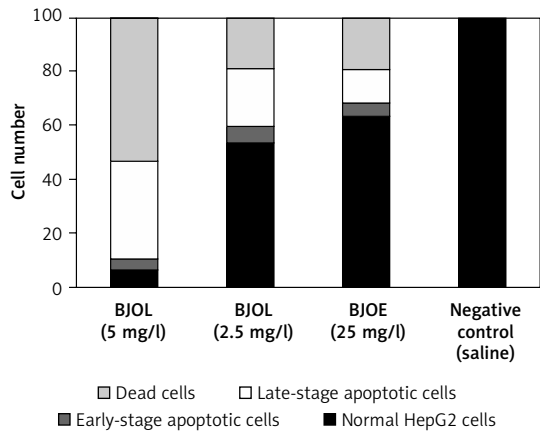*[*]P < 0.05, compared with BJOL (2.5 mg/l) group; [#]p < 0.05, compared with BJOE group.*

Figure 1. HepG2 cells were treated with BJOL. BJOL was prepared and examined under a light microscope. The liposomes were examined under a transmission electron microscope (15 000×). The cells were treated for 1 h as indicated in the figure. Numbers of HepG2 cells were counted

Table II. Comparison of mice receiving different treatments of BJOL

Groups	Weight when sacrificed [g]	Intrahepatic metastasis rates (%)
Negative control (saline)	20.15 ±1.96	100
BJOL (2.5 mg/l)	22.99 ±1.23	16.7*
BJOL (5 mg/l)	24.93 ±1.32*	1.2*
BJOE (25 mg/l)	22.68 ±1.89	25.0*

*$P < 0.05$, compared with negative control group.

domly divided into 4 groups (6 mice/group): the negative control group (saline), the small-dose BJOL group (BJOL, 2.5 mg/l), the high-dose BJOL group (BJOL, 5 mg/l), and the positive control group (BJOE, 25 mg/l). Mice were kept under the same conditions in the lower stream chamber for 40 days, and were all sacrificed after the

treatments were completed. The weight of each animal model was recorded and specimens of liver were examined. The results in Table II indicated that, when compared with the other 3 groups, BJOL increased the weight of mice and decreased the intrahepatic metastasis rate significantly ($p < 0.05$), suggesting the therapeutic effects of BJOL *in vivo*.

By direct observation and light microscopy, two types of liver pathology were found (Figure 3) in these mice. For type 1 (Figure 3 A, left panel), liver volume was decreased and the texture was hard in the BJOL (2.5 mg/l) group. Visible nodules appeared in the original tumor, showing satellite lesions and signs of partial intestinal obstruc-

Negative control (saline)

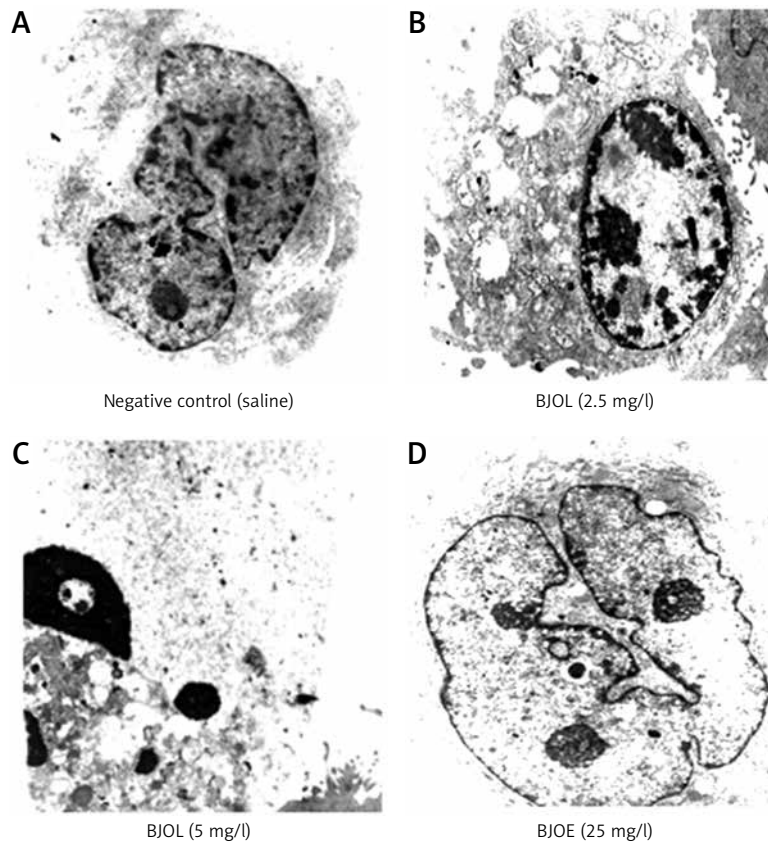BJOL (2.5 mg/l)

BJOL (5 mg/l)

BJOE (25 mg/l)

Figure 2. Morphology under transmission electron microscope (5000×) of HepG2 cells treated with BJOL or the controls. **A** – The tumor cell was normal, **B** – tumor cell in stage 1 apoptosis, **C** – tumor cell in stage 2 apoptosis, **D** – vacuolated tumor cells
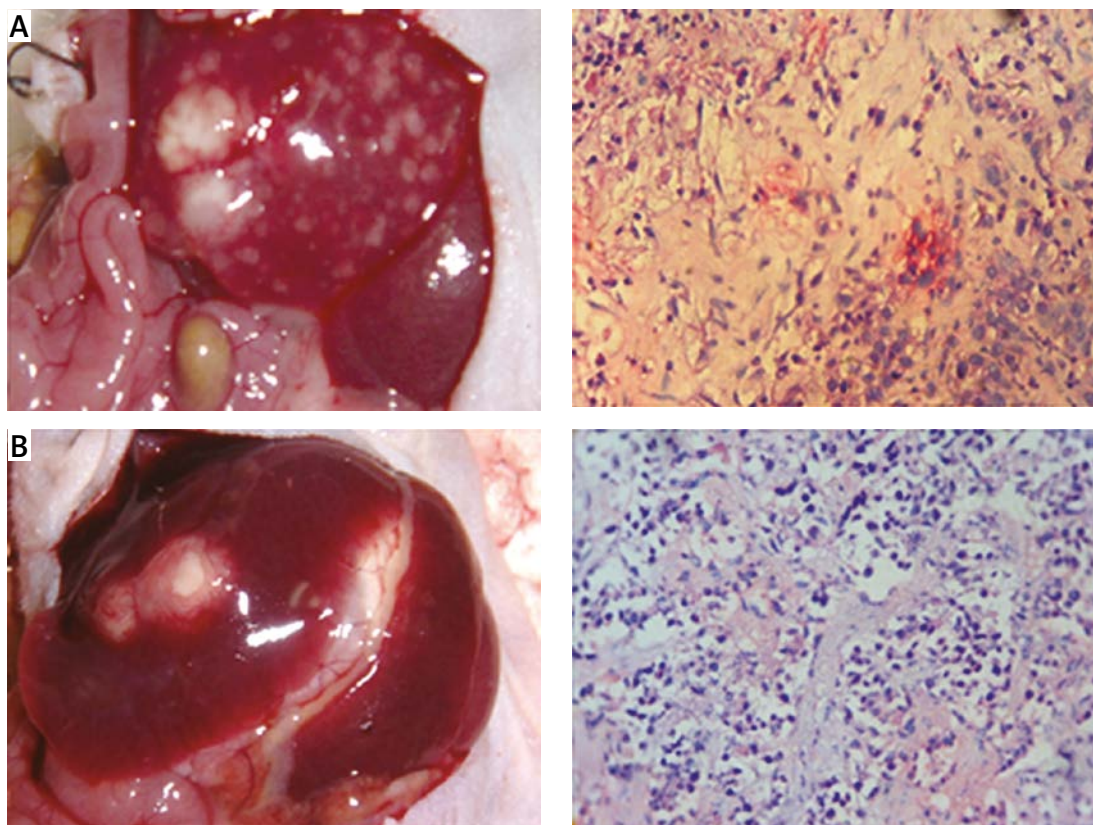
Figure 3. Morphology of liver tissues of mice treated with BJOL. **A** – Type 1 pathology changes of tissues (left panel, direct observation; right panel, under microscope). **B** – Type 2 pathology changes of tissues (left panel, direct observation; right panel, under microscope)

tion. Under microscopy (Figure 3 A, right panel), tumor cells were arranged in disorder and nuclei were lightly stained in an irregular shape. Nuclear heterochromatin atypia was abundant and mitosis was common. The liver tissue was nearly invisible.

For type 2 (Figure 3 B, left panel), the liver volume was changed insignificantly to a bright red color, soft texture and smooth surface in the BJOL (5 mg/l) group. The tumor was in the left lobe of the original tumor inoculation site with a clear boundary of normal liver tissue, without liver metastases. Under microscopy (Figure 3 B, right panel), the tu-

mor nest was visible with partial apoptosis of the tumor cells. Lobular remnants were visible at the tumor edge. Apparently, the type 1 tissues were more malignant than the type 2 tissues.

All mice in the negative control group (saline) had type 1 liver tissues. Most of the mice (93.8%) in the BJOL (5 mg/l) group had type 2 liver tissues, while the mice in the BJOL (2.5 mg/l) group and in the BJOE group all had type 1 liver tissues ($p < 0.05$).

Liver tissue apoptosis induced by BJOL

TUNEL staining was performed to detect liver tissue apoptosis in the mice treated with BJOL. The brown staining indicated apoptotic cells. Statistical analysis showed that in the mice treated with BJOL (5 mg/l), cell apoptosis ratios were significantly higher than in mice treated with BJOL (2.5 mg/l) or BJOE (25 mg/l) ($p < 0.05$) (Table III).

Discussion

Chemotherapy is the primary treatment of liver cancers, but the long-term efficacies are not good enough. Therefore, novel chemotherapy needs to be studied. An important anticancer mechanism of chemotherapy is induction of apoptosis. *Bru-*

Table III. TUNEL apoptosis analysis of tumor-bearing mice treated with BJOL

Groups	TUNEL positive cell number	Ratio (%)
Negative control (saline)	4.4 ±1.9**	1.45**
Low dose BJOL (2.5 mg/l)	22.3 ±2.2	4.61
High dose BJOL (5 mg/l)	34.2 ±3.3*	7.82*
BJOE (25 mg/l)	23.6 ±2.8	3.87

*$P < 0.05$, compared with low dose BJOL (2.5 mg/l) group and BJOE group; **$p < 0.01$, compared with the other 3 groups.

cea javanica oil emulsion can induce apoptosis of the bladder cancer cell line BIU-87 and human promyelocytic leukemia cell line HL-60 [21, 22]. It was also found that BJOE can induce apoptosis of human pancreatic adenocarcinoma cells [23]. However, BJOE has been used with a high concentration and thus the toxic effects of BJOE on the normal cells are relatively high. In this study, we studied the effects of BJOL on liver cancer.

We used transmission electron microscopy to observe the typical morphological changes of apoptosis, which showed that BJOL induced apoptosis significantly when compared with the negative control and BJOE that served as the positive control. The flow cytometry results indicated that with the increase of BJOL concentrations, the HepG2 cell apoptosis rate was increased, suggesting that the action of BJOL may be related to the inhibition of DNA synthesis and blockage of G0/G1 development to S phase.

In this study, all mice were alive at 40 days after intraperitoneal injection of BJOL. Furthermore, the treatment did not result in laparotomy intraperitoneal adhesions, or kidney and pulmonary congestion. Therefore, the side effects of BJOL were relatively limited. Our experimental results have shown the efficacy of BJOL in the treatment of liver tumors. Since *Brucea javanica* extracts have antiproliferative and apoptosis-inducing activities in multiple cell lines including the non-small-cell lung cancer cells, hepatocellular carcinoma cells, breast cancer cells, oesophageal squamous cell carcinoma cells, cervical cancers, and other cancers [9, 10], the efficacy of BJOL in the treatment of these tumor types should be determined using methods similar to the animal model reported in this study.

It is reported that *Brucea javanica* extracts reduce the protein expression levels of c-Myc, cyclin D1, survivins [8], but increase caspase 3 activity [9] and p53 protein level [10]. The effects of BJOL on the pro-apoptotic signaling pathway, which is related to pancreatic cancers, should be studied in the future [24]. In addition, the molecular biomarkers or DNA polymorphisms for carcinomas [25, 26] might be useful for study of the effects of BJOL on cancers.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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