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Original Research Article

Brucea javanica oil emulsion induces autophagy in glioma cells via lncRNA-p21/miR-17-5p/PTEN pathway

Ying Xu, Zeng Wang, Hongyan Zhang, Qiong Wang*
Department of Pharmacy, The Cancer Hospital of the University of Chinese Academy of Sciences (Zhe jiang Cancer Hospital), Institute of Basic Medicine and Cancer (IBMC), Chinese Academy of Sciences, Hangzhou, Zhejiang 310022, China

For correspondence:* ***Email:* *wangqiong_0819@163.com;* ***Tel:*** *+86-0571-88122425*

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Abstract

Purpose: *To investigate the possible effects of Brucea javanica oil emulsion in the progression of glioma, and elucidate the possible regulatory mechanism.*
Methods: *MTT assays were performed to assess the effects of BJOE on the viability of glioma cells, and flow cytometry (FCM) was conducted to evaluate its effect of the oil on cell apoptosis. Immunoblot assays were carried out to investigate its effects on glioma cell autophagy and LincRNA-p21/miR-17-5p/PTEN axis of glioma cells.*
Results: *Administration of BJOE (1, 2 and 4 mg/mL) increased the protein levels of LC3 II/ LC3 I and Beclin-1, but decreased the protein levels of p62 ($p < 0.05$). These changes were reversed by knockdown PTEN. Moreover, the expression of lncRNA-p21 and PTEN mRNA were significantly elevated, while the expression of miR-17-5p significantly decreased in BJOE-treated U251MG cells ($p < 0.05$).*
Conclusion: *BJOE may serve as a novel therapeutic drug for the treatment of glioma; The antitumor effect of BJOE is based on its ability to regulate lncRNA-p21/miR-17-5p/PTEN pathway.*

Keywords: *Glioma, Brucea javanica oil emulsion, Autophagy, Phosphatase and tensin homolog (PTEN)*

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INTRODUCTION

Glioma (GBM) is the most common primary intracranial tumor, accounting for about 80% of primary intracranial malignant tumors. In the past few decades, the treatment of glioma has become more and more diverse [1]. At present, the most common treatment for glioma is surgery. However, glioma is a highly invasive tumor that cannot be completely cured by surgical treatments, and it has a poor prognosis. Temozolomide (TMZ), the first-line chemotherapy drug for glioma, is used within 30 days after surgery as the standard care for gliomas patients [2]. Unfortunately, these treatments have little effect on improving patient survival. Therefore, it is still necessary to explore alternative therapies to provide new clinical guidance for the treatment of glioma.

Autophagy, also known as autophagic cell death, is an evolutionarily conserved intracellular self-

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digestion process which aids the maintenance of cellular homeostasis through a lysosome-dependent process [3]. Autophagy plays an important role in suppressing tumors. Previous studies have shown that many anti-tumor drugs activate autophagy [4]. Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene which is frequently mutated in GBM. Phosphatase and tensin homolog (PTEN) encodes a dual-specific phosphatase that negatively regulates the PI3K/AKT/mTOR pathway, which is a key regulator of autophagy [5]. microRNA-17 prolongs the survival time of glioma cells by inhibiting PTEN and regulating HIF-1α, as well as inducing angiogenesis and promoting stem cell-like aggregation [6]. Long non-coding RNA-p21 inhibits the proliferation and invasion of glioma cells. In addition, lncRNA-p21 also acts as a competitive endogenous RNA (ceRNA) to adsorb miR-17-5p [7,8].

Brucea javanica oil emulsion (BJOE), extracted from the dried mature fruits of *Brucea javanica*, possesses various pharmacological attributes such as anti-fungal, anti-oxidant, anti-inflammatory and anti-cancer biological activities that can inhibit cell proliferation and induce apoptosis in various malignant tumors [9,10]. In addition, BJOE inhibits the proliferation and invasion of glioma cells [11]. Brucein D, another bioactive constituent of *Brucea javanica*, exerted anti-tumor activity in gastric cancer through the regulation of Linc01667/miR-138-5p/CyclinE1 pathway [12].

The purpose of this study was two-fold: (1) to determine the effect of BJOE on glioma cells; and (2) to investigate whether it enhances glioma cells autophagy through of targeting lncRNA-p21/miR-17-5p/PTEN pathway.

EXPERIMENTAL

Cell viability assays

To assess cell viability, U251MG cells were trypsinized, counted and replated in 96-well plates (5000 cells per well). After treatment with BJOE at different concentrations, viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) kit as per manufacturer's instructions.

Flow cytometry

U251MG cells were seeded into 6-well plates and treated with three doses of BJOE (1, 2 L and 4 mg/mL). Then, the cells were trypsinized and washed with PBS 3 times, the cells were incubated with propidium iodide (PI) and FITC-conjugated Annexin V for 15 min in the dark, and evaluated by flow cytometry.

Western blot assay

This method is based on a previous study [13]. Cell lysates were prepared in RIPA buffer (Beyotime, Hangzhou, China) with protease and phosphatase inhibitors, and the protein concentration was evaluated with a BCA Protein assay kit (Beyotime, Hangzhou, China). Proteins were separated using SDS-PAGE and transferred to NC membranes. Blots were blocked with 5 % Bovine serum albumin (BSA) in (TBS+Tween) TBST buffer at room temperature for 1 h, and subsequently incubated with primary antibodies against P62 (Abcam, Cambridge, UK), LC3 (Abcam, Cambridge, UK), Beclin-1 (Abcam, Cambridge, UK), PTEN (Santa Cruz, California, USA) and GAPDH (Santa Cruz, California, USA) overnight at 4 °C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime, Jiangsu, China) for 1 h and measured by Luminata Creseendo Western HRP substrate through Molecular Imager ChemiDoc XRS System (Bio-Rad, Philadelphia, PA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted by TRIzol reagent (Life Technologies, Rockville, MD). The RNA was reverse-transcribed into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA). Real-time polymerase chain reaction (RT-PCR) was used to aassess the gene expression of lncRNA-p21, miR-17-5p and PTEN. The qPCR was performed by using the SYBR Green Master Mix (Applied Biosystems).

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis which was performed using unpaired two-tailed Student's t test. Data are presented as mean ± standard error of the mean (SEM). Differences were considered significant when $p < 0.05$.

RESULTS

Effect of *Brucea javanica* oil emulsion on U251MG cells

In order to determine whether BJOE could suppress the growth of glioma cells, the viability of U251MG cells was measured. As shown in Figure 1 A, the administration of BJOE (2 and 4

mg/mL) significantly reduced the viability of U251MG cells. In agreement with cell viability, the apoptosis ratio of U251MG cells was increased by the administration of BJOE in a dose-dependent manner (Figure 1 B and C, $p < 0.05$). Since the most effective dose of BJOE in U251MG cells was 4mg/mL, the dose of 4mg/mL was used for the subsequent experiments.

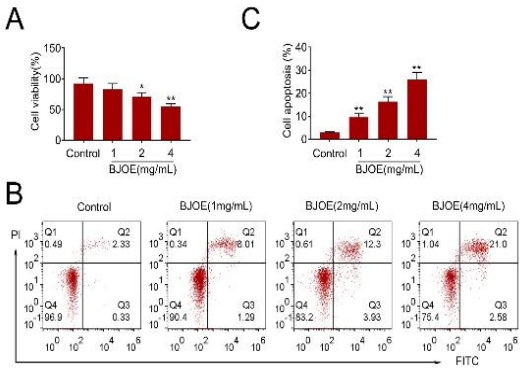

Figure 1: *Brucea javanica* oil emulsion promoted the apoptosis in U251MG cells. A. Dose-dependent effect of BJOE on cell viability. B. Representative images of flow cytometry for apoptosis assay. C. Percent apoptosis. Data are expressed as means ± SEM (n = 5; $^{**}p < 0.01$, $^{*}p < 0.05$ vs control

Effects of *Brucea javanica* oil emulsion on autophagy-related proteins in U251MG cells

As displayed in Figure 2, the protein levels of p62 was significantly down-regulated. However, the protein levels of LC3 II / LC3 I and Beclin-1 were markedly increased in BJOE-treated U251MG cells.

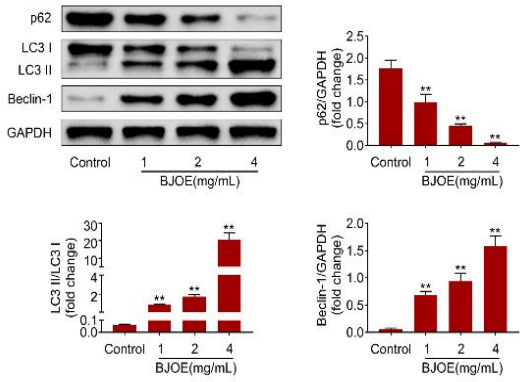

Figure 2: *Brucea javanica* oil emulsion increased the autophagy-related protein in U251MG cells. A. Representative images of Western blot results. B - D. Optical density for the protein blot of p62, LC3 and Beclin-1 against GAPDH. Data are expressed as mean ± SEM (n = 5); $^{**}p < 0.01$ vs control

Effect of *Brucea javanica* oil emulsion on lncRNA-p21/miR-17-5p/PTEN in U251MG cells

To further investigate the mechanism by which BOJE promoting the autophagy of U251MG cells, the protein levels of PTEN (a tumor suppressor gene), and the mRNA expression of lncRNA-p21, miR-17-5p and mRNA level of PTEN was detected. As displayed in Figure 3A, the expressions of lncRNA-p21 and PTEN were significantly elevated in BJOE-treated U251MG cells, while the expression of miR-17-5p was decreased after administration of BJOE. In agreement with the mRNA level of PTEN, the protein level of PTEN was markedly up-regulated in BJOE-treated U251MG cells.

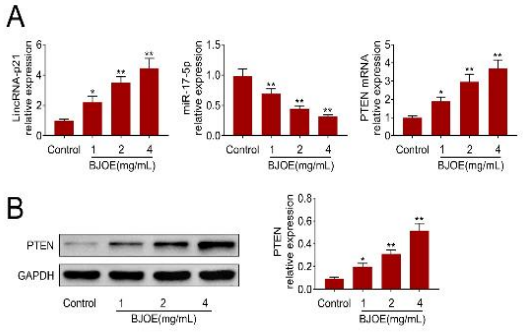

Figure 3: *Brucea javanica* oil emulsion regulated lncRNA-p21/miR-17-5p/PTEN pathway in U251MG cells. A. Dose-dependent effects of BJOE on the expression of lncRNA-p21, miR-17-5p and mRNA level of PTEN. B. Representative images of Western blot results (left panel), Optical density for the protein blot of PTEN against GAPDH (right panel). Data were expressed as mean ± SEM (n = 5); $^{**}p < 0.01$, $^{*}p < 0.05$ vs control

Knockdown of PTEN abolished the apoptosis-induced and autophagy-induced effect of *Brucea javanica* oil emulsion

Expectedly, the administration of BJOE at the dose of 4mg/mL decreased the viability of U251MG cells and increased the Annexin-V positive cells, which were reversed in the presence of PTEN siRNA (Figure 4 A and B). At the same time, the decrease in p62 protein level and the increase in LC3 II / LC3 I and Beclin-1 protein levels in BJOE- treated U251MG cells were reversed in the presence of PTEN siRNA (Figure 4C).

DISCUSSION

In this study, the effect of BJOE on glioma and the underlying mechanisms were explored using U251MG cells which are glioma cell.

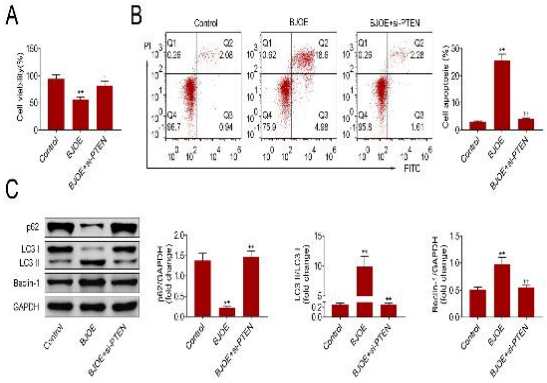

Figure 4: Knockdown of PTEN protect BJOE-treated U251MG cells from autophagy. A. Cell viability. B. Representative images of flow cytometry assay (left panel), percentage of apoptosis (right panel). C. Representative images of Western blot results (left panel), Optical density for the protein blot of p62, LC3 and Beclin-1 against GAPDH (right panel). Data are expressed as mean ± SEM (n = 5); $^{**}p < 0.01$ vs control; $^{++}p < 0.01$ vs BJOE

The results showed that apoptosis level was significantly increased upon the administration of BJOE, accompanied by the up-regulation of LC3 II/LC3 I, PTEN, Beclin-1, lncRNA-p21 expression, and the increase number of Annexin-V positive cells; as well as the down-regulation of the protein levels of p62 and the expression of miR-17-5p. Knockdown of PTEN restored the protein levels of p62, LC3 II/LC3 I and Beclin-1, as well as cell viability, accompanied by alleviation of cell apoptosis in BJOE-treated U251MG cells. To the best of our knowledge, this is the first study that sought to demonstrate that BJOE is a novel anti-glioma drug which exacerbated glioma cells autophagy via a mechanism involving targeting lncRNA-p21/miR-17-5p/PTEN.

It is well-known that activating cell death in tumor plays a critical role in antitumor drugs. Autophagy is believed to be a major type of cell death in glioma [14]. Autophagy generally protects cells and organisms from stressors such as nutritional deficiencies, which also plays an important role in cancer. Autophagy inhibited tumor growth, and the loss of autophagy-related genes can lead to tumorigenesis [15].

It has been revealed that Beclin-1, a tumor suppressor gene, is one of the critical regulators of apoptosis and autophagy [16]. LC3 I (16kDa) was transformed into LC3 II (14 kDa) at the beginning of autophagy [17]. P62 protein, encoded by the SQSTM1 gene, is a receptor for selective autophagy[18] It acts as a "bridge" between ubiquitinated protein and autophagosomes in cells. When stress (such as cytotoxic conditions) is detected, misfolded proteins produced in cells are ubiquitinated, linked to p62 protein, and then transported to the autophagosome where it is degraded after fusion with the lysosome [19]. The accumulation of misfolded proteins disrupts protein homeostasis, which is the major characteristic of senescent cells [20]. The p62 protein maintains protein homeostasis by promoting selective autophagy and degrading the misfolded proteins into amino acids which can be recycled.

Brucea javanica oil emulsion was extracted with petroleum ether from the dried ripe fruit of *Brucea javanica*. The main components are oleic acid and linoleic acid [21]. In the present study, the protein levels of Beclin-1 and LC3 II/ LC3 I were significantly increased, accompanied by p62 protein level which markedly decreased in BJOE-treated U251MG cells, suggesting the occurrence of autophagy. A number of studies have already reported that BJOE can be used in the treatment of lung cancer, brain metastasis as well as digestive tract cancer. It has been shown that BJOE improves the effect of radiotherapy on esophageal cancer cells by targeting cyclin D1-CDK4/6 or TLR4/ NF-κB pathway in rats with Crohn's disease [9,22]. In this study, BJOE could serve as a promising drug for the treatment of glima.

PTEN plays an important role in the formation of tumors. It is abnormally expressed in a variety of tumor tissues. In addition, there are frequent deletions or mutations of PTEN in tumor tissues [23,24]. LncRNA is a type of non-coding RNA which has multiple roles in the occurrence and development of tumors. On the one hand, lncRNA can be used as a competitive endogenous RNA (ceRNA) to adsorb miRNA, and to participate in the regulation of the expression of target genes. On the other hand, lncRNA may cooperate with transcription factors so as to activate the expression of related genes or bind proteins, and in order to prevent protein degradation and promote protein-related functions [25]. In the present study, BJOE was able to activate PTEN. In the BJOE-treated U251MG cells, miR-17-5p which is a PTEN inhibitor, was decreased. Meanwhile, lncRNA-p21, a sponge RNA of miR-17-5p, showed an upsurge after the administration of BJOE. In the present study, the results showed that BJOE suppressed glioma by targeting lncRNA-p21/miR-17-5p/PTEN. Furthermore, knockdown of PTEN restored apoptosis ratio and autophagy level, which recorded an increase due to the administration of BJOE in U251MG cells, confirming the link between BJOE and PTEN.

CONCLUSION

The findings of the present study indicate that BJOE exerts significant suppressive effect on glioma. Administration of BJOE leads to cell apoptosis and autophagy in glioma, by promoting lncRNA-p21 and PTEN expressions, as well as suppression of miR-17-5p. Furthermore, PTEN is the potential target of BJOE via the knockdown of PTEN. Thus, BJOE is a potential novel therapeutic agent for the management of glioma; its antitumor effect regulates lncRNA-p21/miR-17-5p/PTEN pathway.

DECLARATIONS

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Competing interests

No conflict of interest to disclose.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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. Ying Xu designed the study and supervised the data collection; Zeng Wang and Hongyan Zhang analyzed and interpreted the data; Qiong Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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