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ORIGINAL ARTICLE

COMBINATION TREATMENT OF *LITHOSPERMUM ERYTHRORHIZON* POLYSACCHARIDE AND AUTOPHAGY INHIBITORS ON HEPATOMA CELLS INHIBITION AND THE UNDERLYING MECHANISM

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Abstract

Our paper evaluated the effects of *Lithospermum erythrorhizon* polysaccharide (LEP) combined with an autophagy inhibitor on hepatoma cells BEL-7404. Human hepatoma BEL-7404 cells cultured *in vitro* were treated with LEP alone or with the autophagy inhibitor 3-methyladenine (3-MA) (LEP+3-MA group) or chloroquine (CQ) (LEP+CQ group), respectively. MTT assay was used to detect cell proliferation, flow cytometry was used to detect the apoptosis rate, and Western blot was used to detect the changes of autophagy proteins LC3-I, LC3-II, p62 protein and anti-apoptotic protein BCl-2 (B-cell lymphoma-2). Our results showed that LEP could induce autophagy, promote apoptosis and inhibit the proliferation of hepatoma cells. The autophagy of LEP on hepatoma cells experiences the formation process of autophagosome in the early stage of cell autophagy, and autophagosome degradation occurs in the late stage of autophagy. The addition of CQ can inhibit the degradation of autophagosomes and further promote cell autophagy.

Rezumat

A fost evaluat potențialul efect al polizaharidului provenit de la *Lithospermum erythrorhizon* (LEP), combinat cu un inhibitor de autofagie, asupra celulelor de hepatom BCL-7404. Celulele au fost tratate doar cu LEP sau împreună cu inhibitorul de autofagie 3-metiladenină (3-MA) (grup LEP+3-MA) sau, respectiv, clorochină (CQ) (grup LEP+CQ). Testul MTT a fost folosit pentru a evidenția proliferarea celulară, citometria în flux a fost utilizată pentru a detecta rata apoptozei și Western blot pentru a evalua modificările proteinelor de autofagie LC3-I, LC3-II, proteinei p62 și proteinei anti-apoptotice BCl-2. Rezultatele noastre au arătat că LEP ar putea induce autofagia, favorizează apoptoza și inhibă proliferarea celulelor de hepatom.

Keywords: *Lithospermum erythrorhizon* polysaccharide, autophagy, liver cancer, apoptosis, proliferation

Introduction

The *Lithospermum erythrorhizon* (*Boraginaceae*) is a perennial herb whose rhizome is rich in a purple substance. During the spring and autumn seasons, the rhizome is dug up and obtained after removing silt and drying. It contains lipid-soluble anthraquinone compounds (shikonin and its derivatives) and water-soluble *Lithospermum erythrorhizon* polysaccharide (LEP), which represents only 2% of the total components [1, 2]. Previous studies have shown that the LEP possesses several physiological and pharmacological properties, like anti-inflammatory, antipyretic, analgesic and anti-tumour effects [3]. It also decreases blood temperature, activates blood circulation, detoxifies and treats the rash. The anti-tumour activity of *Lithospermum erythrorhizon* is mainly due to its active component, LEP [4]. Autophagy is a lysosome dependent intra-cellular degradation process which is ubiquitous in eukaryotic cells.

Autophagy removes damaged or senescent organelles and biomacromolecules accumulated within cells. Autophagy regulates the self-homeostasis process, which affects multiple physiological functions of the cells. Studies showed that promoting autophagy can inhibit the proliferation of tumour cells [5]. 3-MA (3-methyladenine) and chloroquine (CQ) which are autophagy inhibitors, act *via* different mechanisms. 3-MA acts as an autophagy inhibitor by blocking the assembly process of autophagosomes in the early stage of autophagy by inhibiting type III phosphatidylinositol-3 kinase (PI3K) [6]. CQ, which is similar to ammonium chloride, can neutralise the acidic pH inside lysosomes to inhibit the enzyme's proteolytic activity [7]. Currently, the beneficial therapeutic effects of the phyto-complexes is well-known [8] and LEP seems to be no exception.

The mechanisms of action of LEP on the proliferation and autophagy in the human hepatoma cells are not clear. Therefore, the effects of LEP on autophagy, cell proliferation and apoptosis in BEL-7404 cells were investigated. The effects of autophagy inhibitors on the mechanism of LEP in hepatoma cells were also explored.

Materials and Methods

Cells line

Human hepatoma cell line (BEL-7404) was purchased from the Wuhan Procell Life Technology Co., Ltd., China and preserved in the hospital laboratory. The cells were cultured in RPMI 1640 (Roswell Park Memorial Institute 1640) medium (Gibco™, Thermo-Fisher Scientific, USA) containing 10% foetal bovine serum (Gibco™, ThermoFisher Scientific, USA) in a cell incubator saturated with 5% CO_2 at 37°C.

Preparation of LEP

About 100 g of *Lithospermum erythrorhizon* (Ningshan Guosheng Biotechnology Co., Ltd., China) powder was weighed. The crude powder was degreased three times with 600 mL petroleum ether (Jinan Huijinchuan Chemical Co., Ltd., China) at 30 - 60°C and dried. Then, 1000 mL of distilled water was added to the dried powder for extraction and kept for 5 h. It was filtered hot, and the extraction procedure was repeated three times. The water extracts were combined and concentrated to a quarter of the original volume. Absolute ethanol equivalent to about three times the volume of the above concentrate was added. It was filtered after keeping it stand overnight. The precipitate was washed with absolute ethanol, acetone and ether and dried *in vacuum* to obtain LEP.

The proliferation inhibition rate was detected by MTT (2,5-diphenyl-2H-tetrazolium bromide) assay

The BEL-7404 cells in the logarithmic growth phase were taken and inoculated into 96 well plates at a cell density of 5.0×10^3 cells/well. The experimental groups (LEP + 3-MA group, LEP + CQ group and LEP group) and control group were set up. After cell adhesion, the cells in LEP + 3-MA and LEP + CQ groups were pre-treated with 3-mA (Ningshan Guosheng Biotechnology Co., Ltd., China) (5 mmol/L) and CQ (Ningshan Guosheng Biotechnology Co., Ltd., China) (5 μg/mL), respectively, for 1 h. LEP (2.5 μg/mL) was added to the cells of the experimental groups. The control group cells received no treatment. Five compound holes were set in each group. After co-culturing for 12 h, 24 h and 48 h, respectively, 10 μL MTT solution was added to each well, followed by incubation in the dark for 4h. The culture medium of each well was sucked out as gently as possible, and 150 μL DMSO (dimethyl sulfoxide) was added to each well. The optical density (OD) of each pore was measured by an enzyme-labelled instrument at 490 nm, and the inhibition rate of cell proliferation was calculated [9]. The MTT cell proliferation assay kit was purchased from Invitrogen, ThermoFisher Scientific Inc., USA.

Cell apoptosis was detected by flow cytometry

The BEL-7404 cells from each treatment group were digested with 0.25% trypsin, collected into centrifuge tubes (1.0×10^6 cells), and centrifuged at 1,200 r/min for 10 min. The digested cell suspension was washed twice with precooled PBS (phosphate-buffered saline) solution and resuspended in 400 μL of binding buffer. Following the Annexin V FITC/PI apoptosis detection kit (Shanghai BestBio Biotechnology Co., Ltd., China) protocol, 10 μL of FITC labelled Annexin V (fluorescein isothiocyanate labelled Annexin V) was gently mixed with the suspended cells and kept in the dark. Later, it was placed in a refrigerator at 4°C for 15 min, and 5 μL of the PI (propidium iodide) solution was added. Flow cytometry was performed immediately after placing the cells in the refrigerator at 4°C for 5 min. FlowJo software (BD Biosciences, USA) was used to analyse data.

Observation of autophagosome formation by fluorescence staining

The cells in the logarithmic growth phase were prepared into a single cell suspension at a density of 5×10^6 L^{-1}. Later, the suspension was added to a culture dish with a cleaning cover slide placed in advance, and a cell climbing slide was made on the cover glass. After the cells adhered to the wall, the drug treatment was performed. The experimental groups (LEP + 3-MA group, LEP + CQ and LEP groups) and blank control group were set up. The cells were pre-treated with 3-MA (5 mmol/L) and CQ (5 μg/mL) for 1 h in the LEP + 3-MA and LEP + CQ groups, respectively. To each well in the experimental group, 2.5 μg/mL LEP was added, whereas only a culture medium was added to the blank control group. The cell climbing slide was removed, washed with sterile PBS solution, dried in the air, and stained with 0.05 mmol/L MDC (monodansylcadaverine) solution. After incubating the slide for 40 min at 37°C in the dark, it was washed twice with PBS solution and observed under a laser confocal microscope (Olympus Europa SE & Co. KG, Germany).

Western blot

The BEL-7404 cells in the logarithmic growth phase were inoculated into the 6-well plates at a density of 1.5×10^5 cells/well. The experimental groups were divided into three groups: the LEP + 3-MA group (3-MA pre-treatment for 1 h), the LEP+CQ group (CQ pre-treatment for 1 h) and LEP single drug group in doses presented before. The cells suspended with only a culture medium were set as the control group. After 24 h, the cells were collected for Western blot analysis to determine LC3-I, LC3-II, p62 and Bcl-2 (B-cell lymphoma-2) levels. The proteins underwent gel electrophoresis and blotted on the PVDF (polyvinylidene difluoride) membranes. Then the membranes were incubated with the antibodies against LC3-I (microtubule-

associated protein light chain 3I) (1:2500), LC3-II (microtubule-associated protein light chain 3II) (1:1000), p62 (1:40000) and Bcl-2 (B-cell lymphoma-2) (1:5000)-(Proteintech Group, Inc (Wuhan, China) at room temperature for 1 h. Then the membrane was washed 3 times for 10 minutes each with TBST (Tris-buffered saline with 0.1% Tween® 20 detergent) and incubated with the second antibody – IgG-HRP-conjugated anti-rabbit (1:1000) (LC3-I and LC3-II) and IgG-HRP-conjugated anti-mouse IgG (1:10000) (p62 and Bcl-2) for 1.5 h at room temperature and washed 3 times for 10 minutes each with TBST [9]. Finally, the membrane was incubated with Chemiluminescent-HRP substrate according to the manufacturer's instructions. The integral optical densities of LC3-I, LC3-II, p62 and Bcl-2 protein bands were measured by ImageJ software.

Statistical analysis

SPSS19.0 statistical software (IBM, USA) was used for the data analysis. The data were expressed in the form of media ± standard deviation. One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) was used to compare the two groups. A value of $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Inhibition of the proliferation of BEL-7404 cells by the autophagy inhibitors

The BEL-7404 cells in the logarithmic phase were used for the experiment. Autophagy inhibitors, 3-MA and CQ, were co-cultured with LEP for 12 h, 24 h and 48 h, respectively, and the control group without any drug was set up. MTT results showed that inhibition of the proliferation of BEL-7404 cells increased gradually over time (Figure 1). After 24 h, inhibition of the proliferation of the LEP group was significantly increased compared with the control group ($p < 0.05$), indicating that LEP could inhibit the proliferation of BEL-7404 cells. Moreover, LEP associated with autophagy inhibitors, 3-MA and CQ, determined a more statistically significant effect than the control group ($p < 0.01$).

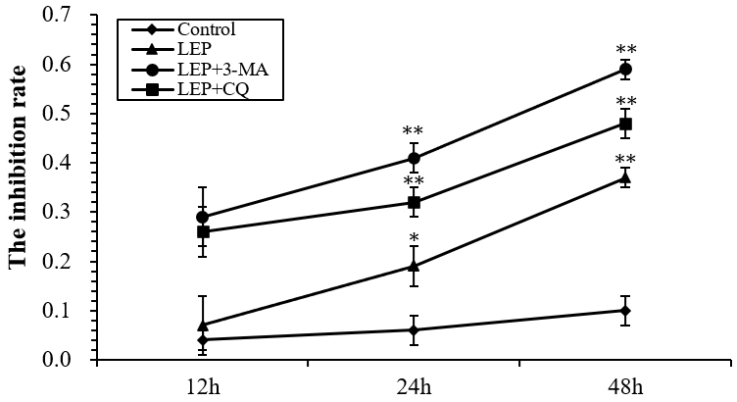

Figure 1.

The cell proliferation inhibition rate detected by the MTT assay compared with the control group, * $p < 0.05$, ** $p < 0.01$

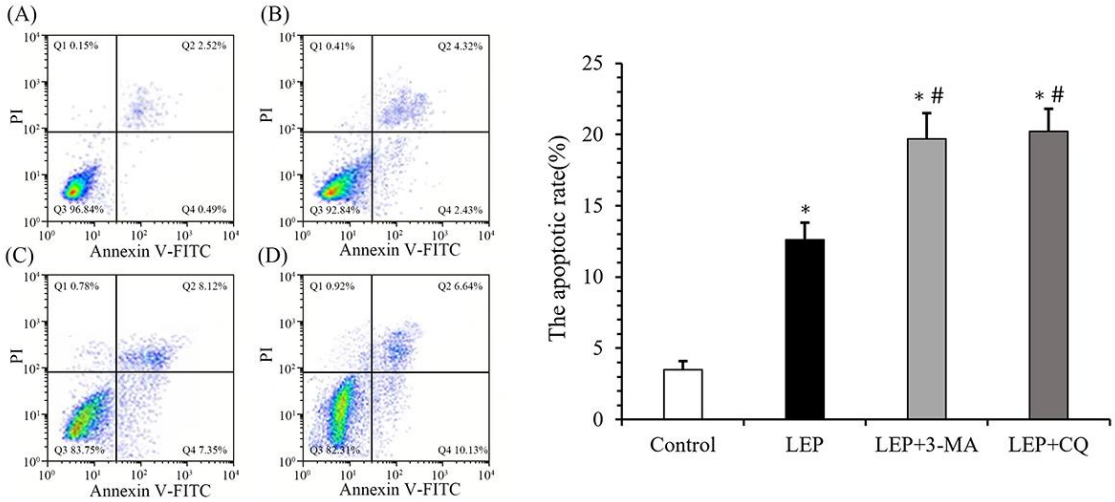

Figure 2.

Results of flow cytometry A. Control group; B. LEP group; C. LEP +3-MA group; D. LEP + CQ group; * $p < 0.05$ *vs*. Control group; # $p < 0.05$ *vs*. LEP group

Apoptosis of BEL-7404 cells by Flow cytometry

The results of flow cytometry showed that the apoptosis rate of BEL-7404 cells in the LEP group was significantly higher compared with the control group ($p < 0.05$). The apoptosis rates of the BEL-7404 cells treated with 3-MA or CQ combined with LEP for 24 h were significantly higher than those of the LEP group ($p < 0.05$) (Figure 2).

Detection of autophagy positive BEL-7404 cells

The weakly basic fluorescent dye selectively concentrated on the acidic autophagosome inside the cells. As shown in Figure 3, the number of autophagy positive cells in the LEP group was significantly higher compared to the control group ($p < 0.05$). However, there was no significant difference in the number of autophagy positive cells between the control and LEP+3-MA groups ($p > 0.05$). Notably, the number of autophagy positive cells in the LEP+CQ group was significantly higher compared with the LEP group ($p < 0.05$).

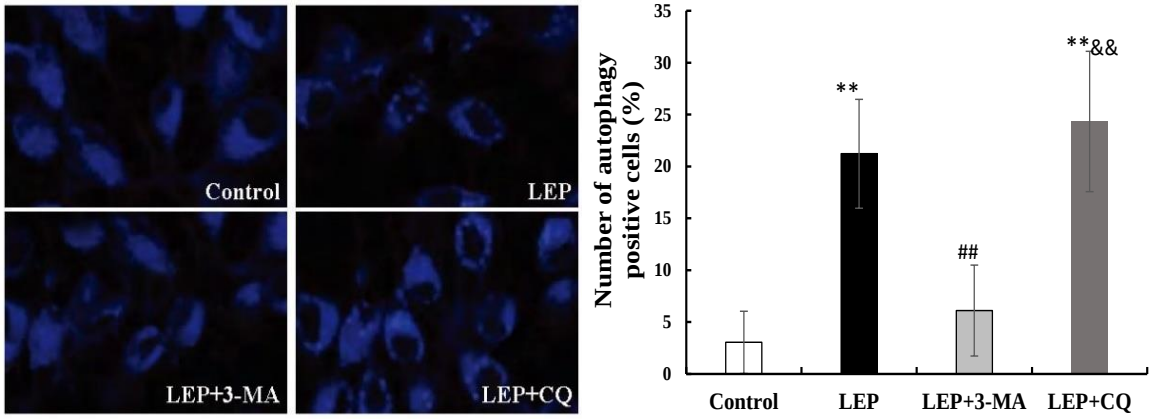

Figure 3.

Effects of LEP and autophagy inhibitors on the autophagosome formation in BEL-7404 cells (× 200).
** $p < 0.01$ *vs*. Control group; $^{\#\#}$ $p < 0.01$ *vs*. LEP group; $^{\&}$ $p < 0.01$ *vs*. LEP + 3-MA group

Results of Western blot analysis

The expression level of LC3-II and ratio of LC3-II to LC3-I in the LEP group were significantly increased compared with the control group ($p < 0.05$). After pre-treatment with CQ, the expression level of LC3-II was significantly increased compared to the LEP group ($p < 0.05$), but the ratio of LC3-II to LC3-I was not significantly different ($p > 0.05$). However, after adding 3-MA, the expression level of LC3-II and the ratio of LC3-II to LC3-I were significantly decreased compared with the LEP group ($p < 0.05$), but the expression level of LC3-I was significantly increased ($p < 0.05$).

The expression level of p62 protein in the LEP group was significantly lower than that in the control group ($p < 0.05$), but there was no significant difference between the LEP and LEP + CQ groups ($p > 0.05$). There was also no significant difference between the LEP + 3-MA and control groups, but it was significantly higher compared to the LEP group ($p < 0.05$). In addition, the expression level of Bcl-2 protein in the LEP group was significantly lower than that in the control group ($p < 0.05$). Compared to the LEP group, LEP + 3-MA and LEP + CQ groups showed a significantly decreased level of Bcl-2 protein ($p < 0.05$). However, there was no significant difference between LEP + 3-MA and LEP + CQ groups ($p > 0.05$) (Figure 4).

During the autophagosome formation process, the conversion of LC3-I to LC3-II occurs in cells. The cytoplasmic free form of LC3-I combines with PE of autophagosome membrane to form LC3-II [10]. As the process of autophagy progresses, the autophagosome will degrade, and the content of the LC3-II will also decrease. The autophagy induced early increase and late decrease in the level of LC3-II is known as LC3 turnover [11]. In LC3 turnover, the decrease of LC3-II in the late stage of autophagy may be confused with the result of not inducing autophagy [12]. To solve this problem, lysosome inhibitors were introduced, such as CQ, to inhibit the hydrolysis process in the late stage of autophagy and prevent the reduction of LC3 levels [13, 14].

The results of our study showed that the increase in expression levels of LC3-II after pre-treatment with CQ was significantly higher than that of the LEP alone [15, 16]. The CQ inhibited the lysosome activity and prevented subsequent autophagosome degradation. Thus, it was proved that LEP-induced autophagosome degradation occurs in the late stage of autophagy [17].

The autophagy inhibitor 3-MA works in the early stage of autophagy [18, 19]. In the presence of 3-MA, LEP cannot induce the increase and aggregation of LC3-II in HCC cells. Hence autophagy cannot be initiated [20, 21]. This further confirmed that LEP induces the

formation of autophagosomes in the early stage of autophagy in hepatoma cells.

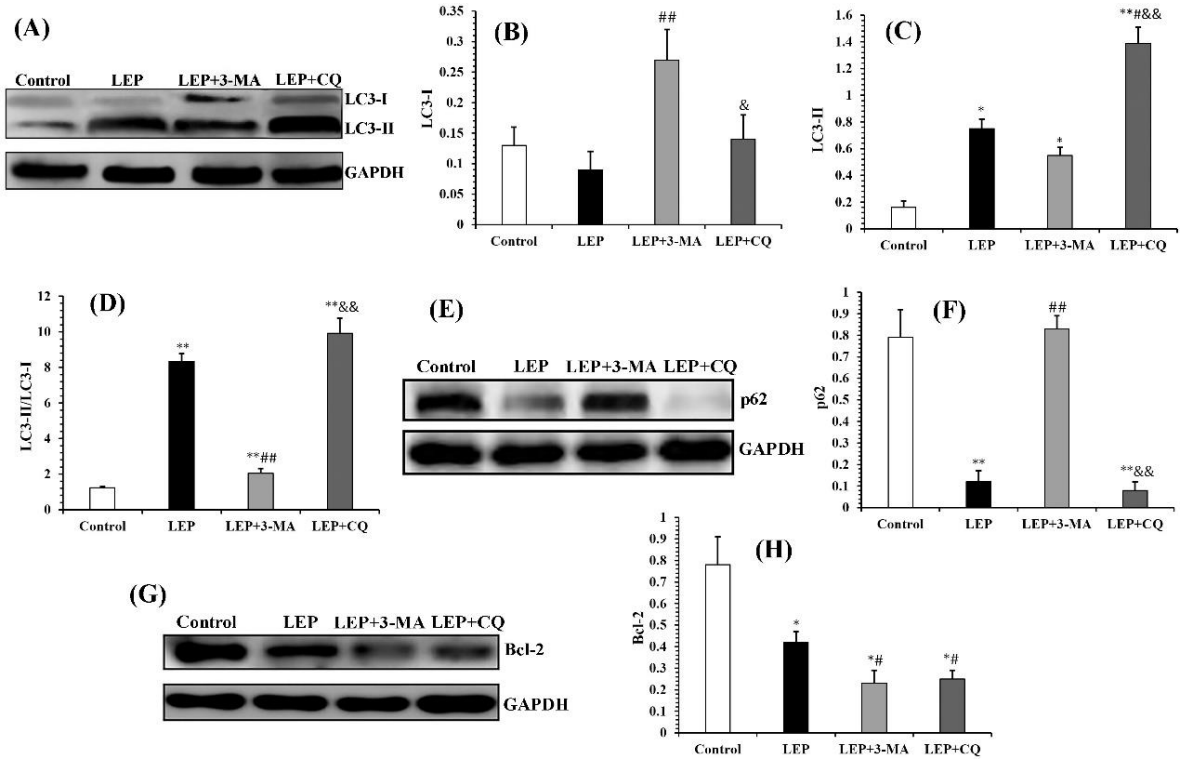

Figure 4.

Results of Western blot analysis. A. LC3-I and LC3-II proteins; B. LC3-I relative grey value statistical chart; C. LC3-II relative grey value statistical chart; D. LC3-II/ LC3-I ratio; E: Western blot analysis results of p62 protein; F. p62 relative grey value statistical chart; G. Western blot analysis results of Bcl-2 protein; H. Bcl-2 relative grey value statistical chart. * $p < 0.05$, ** $p < 0.01$ *vs*. Control group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs*. LEP group; $^{\&}$ $p < 0.05$, $^{\&\&}$ $p < 0.01$ *vs*. LEP + 3-MA group

In addition, the results of this study showed that LEP could reduce the expression level of p62 in hepatoma cells, which indicates that autophagic lysosome-mediated degradation of p62 protein occurs in hepatoma cells [22]. There is no significant difference in the expression level of p62 protein between LEP + 3-MA and control groups, indicating that LEP induced autophagy is inhibited [22]. The expression level of Bcl-2 protein in the autophagy inhibitor group is significantly decreased, while the cell proliferation rate and apoptosis rate are significantly increased after adding autophagy inhibitors. The results are consistent with the previous studies, indicating that autophagy inhibitors can inhibit the proliferation and promote apoptosis of the tumour cells [23, 24].

Conclusions

LEP can induce autophagy, promote apoptosis and inhibit the proliferation of hepatoma cells. The autophagy of LEP on hepatoma cells experiences the formation process of autophagosome in the early stage of cell autophagy, and autophagosome degradation occurs in the late stage of autophagy. Further studies should confirm the efficacy of LEP in cancer treatment.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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