Original Article

*Hericium erinaceus* synergizing with doxorubicin induced SGC7901 cell apoptosis

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Abstract: Our study was to investigate the impact of *Hericium erinaceus* on the antitumor effects of doxorubicin (DOX) in gastric cancer cell line SGC7901 and explore the possible mechanism. Hot water extracts of the fruiting body of *Hericium erinaceus* were used in the study. Hot water extraction was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatography. The purified components (HE) primarily consisted a low-molecular-mass (13 kDa) 1,3-branched-β-1,6-glucan with a triple helix conformation, which we used the concentration of 100 µg/mL in combination with 0-10 µg/mL DOX for the treatment of SGC7901 for 24 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue exclusion. Apoptosis, cell cycle arrest, MMP disruption and ROS production were determined by flow cytometry. We also detected caspase-3 activity by a kit and the expression of caspase-3, pro- and anti-apoptotic proteins, HIF-1α by western blotting (WB). MTT analysis and annexin V-FITC/PI double staining showed that HE alone nearly did not induce SGC7901 cells death under 100 µg/mL while 100 µg/mL HE decreased the IC₅₀ of DOX on SGC7901 cells to 5 µg/mL. Flow cytometry analysis showed that 100 µg/mL HE elevated DOX-induced ROS and the expression of HIF-1α was downregulated by WB detecting. Our results indicate that *Hericium erinaceus* extract (HE) combined with doxorubicin (DOX) maybe a novel strategy for the treatment of human gastric cancer.

Keywords: Combined treatment, ROS, MMP, human gastric cancer, caspase-3, HIF-1α pathway

Introduction

Gastric cancer, a very common disease and the second most frequent cause of cancer death, causes a significant global health care burden [1, 2]. Globally, about one million new cases occur worldwide annually [3]. There is a marked geographic variation in the incidence of gastric cancer [1]. The highest incidence is in men in northeast Asia (Japan, Korea, and China); intermediate incidences occur in Europe and South America; North America, Africa, south Asia, and Oceania (including Australia and New Zealand) are low-incidence regions, with rates of 4-10 cases per 100,000 people [4]. Explanations for these differences in incidence have been sought. High intake of various traditional salt-preserved foods and salt, and low consumption of fresh fruit and vegetables are associated with a raised risk of gastric cancer [5, 6]. The majority of patients newly diagnosed with gastric cancer will present with locally advanced or metastatic disease. Of those fortunate enough to undergo a ‘curative-intent’ resection, approximately 40-60% develop recurrence [7]. Thus, the majority of patients with gastric cancer will have systemic disease at some time during the course of their illness. Surgical resection is the mainstay of curative treatment, but it can be performed in a small subgroup of patients: only 30-50% of patients undergoing surgical exploration can be operated with curative intent, with 5-year survival rates of about 60% and 34% for stage I and stage II disease, respectively [8]. Clinical trials of neoadjuvant and adjuvant therapy have been conducted to improve these results. New cytotoxic agents have been evaluated in large phase III trials in metastatic setting, showing interesting results. In addition, a number of biological agents mod-
Mushrooms have been used as edible and medicinal resources for thousands of years and antitumor substances such as polysaccharides have been identified in many mushroom species [9-12]. *Hericium erinaceus* is an edible mushroom which has been used as a traditional Chinese medicine (TCM) for the treatment of digestive diseases for over 2000 years in China. Present study have shown that for the four *Hericium erinaceus* extracts evaluated, protein, carbohydrate, and mineral (ash) contents were all high, ranging (in % of dry wt) from 35.5 to 38.5, 33.8 to 39.5, and 14.6 to 19.0, respectively [13]. The moisture content was about 8%, and the fiber and fat contents were < 1%. *Hericium erinaceus* polysaccharides have been widely studied and exhibited anticancer, immune stimulation, lowering cholesterol, and stimulating neurite outgrowth activities [14, 15].

Doxorubicin (DOX), an anthracycline antibiotic, is a potent drug in gastric cancer, yielding a response rate of 17% [16, 17]. Its most critical toxicity is irreversible myocardial damage, after exceeding a dose of 550 mg/m² [18]. Cytotoxic combinations in gastric cancer have been derived from single agents and scheduled to maximize the antitumor effect while minimizing the toxicity.

Herein our study was to investigate the impact of *Hericium erinaceus* on the antitumor effects of doxorubicin (DOX) in gastric cancer cell line SGC7901 and explore the possible mechanism.

**Materials and methods**

**Cell culture**

Human gastric cancer cell line SGC7901 was purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Newborn Calf Serum (GIBCO), 4 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin.

**Reagents and antibodies**

Doxorubicin, rhodamine123 (Rh123), dichlorodihydrofluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), catalase (CAT), propidium iodide (PI) and rotenone (ROT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased Promega (Madison, Wisconsin, USA). *Hericium erinaceus* are from Northeast China. Doxorubicin was dissolved in DMSO and kept protected from light. The concentrations of DOX used in the experiments varied between 1-10 µg/mL. In case not specified in the figure legend, values refer to concentration in µg/mL. We purchased HIF-1α antibodies from CST (Cell Signal Technology) companies. Antibodies against β-actin, caspase-3 and the secondary HRP-labeled goat-anti-mouse antibodies were obtained from Byotime Biotechnology.

**Preparation of Hericium erinaceus extracts**

The crude water-soluble polysaccharide, which was obtained from the fruiting body of *Hericium erinaceus* by hot water extraction and ethanol precipitation, was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatography, as previously reported [19]. The purified components (HE) of *Hericium erinaceus* primarily consisted of polysaccharide, which was a low-molecular-mass (13 kDa) 1, 3-branched-b-1, 6-glucan with a triple helix conformation. HE contained a level of endotoxin below the detection limits (0.0015 EU/mL) as assessed by an E-TOXATE kit (Sigma, St. Louis, MO, USA) [20].
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*Cell viability assay (MTT dye assay)*

Cell viability was measured by the MTT method [21]. In brief, cells were collected and seeded in 96-well plates at a density of $5 \times 10^5$ cells/cm². Different seeding densities were optimized at the beginning of the experiments. 20 µL of MTT tetrazolium salt (Sigma) dissolved in Hanks’ balanced solution at a concentration of 5 mg/mL was added to each well with the indicated treatment and incubated in CO₂ incubator for 4 h. Finally, the medium was aspirated from each well, and 150 µL of DMSO (Sigma) was added to dissolve formazan crystals, and the absorbance of each well was obtained using a Dynatech MR5000 plate reader at a wavelength of 490 nm with a reference wavelength of 630 nm. The following formula was used to calculate cell viability: percentage cell viability = (absorbance of the experiment samples/absorbance of the control) × 100%.

*Annexin V-FITC/PI staining*

Annexin V-FITC and PI double staining was employed to determine apoptosis. In brief, cells were treated as indicated and then stained with KeyGen Annexin V-FITC apoptosis detection kit according to the manufacturer’s protocol. Then apoptosis was evaluated using a FACSCalibur flow cytometer (BD Biosciences). Fluorescence was measured with an excitation wavelength of 480 nm through FL-1 filter (530 nm) and FL-2 filter (585 nm).

*Cell cycle analysis*

Cells were treated with HE, DOX, HE + DOX as indicated. After the treatment cells were harvested and washed twice with PBS, then fixed in ice-cold 70% (v/v) ethanol for 24 h at 4°C. Before analysis, cells were washed with PBS, suspended in 1 mL of cold PI solution (50 µg/mL PI, 1% (v/v) TritonX-100, 100 µg/mL RNaseA) and incubated on ice for 30 min in darkness. Cytometric analysis was performed using flow cytometer and Cell Quest software. Fluorescence was measured with an excitation wavelength of 480 nm through FL-1 filter. Apoptotic cells were detected on a PI histogram as a Pro G1 peak.

*Measurement of mitochondrial membrane potential (MMP)*

MMP was measured by flow cytometer using the cationic lipophilic green fluorochrome Rh-123 [22]. Cells were harvested, washed twice with PBS, incubated with 1 µM Rh123 at 37°C for 30 min, and washed twice with PBS. Fluorescence was determined by flow cytometer with an excitation wavelength of 480 nm at FL1 filter.

*Determination of cellular reactive oxygen species (ROS)*

Intracellular ROS was determined by flow cytometer and staining with DCFH-DA [23]. DCFH-DA is deacetylated by intracellular esterase and converted to nonfluorescent dichlorodihydrofluorescein, which is oxidized rapidly to the highly fluorescent compound dichlorofluorescein (DCF) in the presence of ROS. Cellular ROS content was measured by incubating the cells with 10 µM DCFH-DA at 37°C for 30 min. After incubation with the fluorochrome, cells were washed with PBS and analyzed immediately by flow cytometer through FL1 filter with an excitation wavelength of 480 nm.

*Trypan blue exclusion*

The cell death rate was determined by trypan blue exclusion method. Cells in the exponential growth phase were plated at $5 \times 10^4$ cells/well in 24-wells culture plates. After 12 h growth, the medium was replaced by DMEM supplemented with 2% FBS containing various doses of DOX plus 100 µg/mL HE. After incubating for the indicated times, the viable cells and dead cells were counted on optical microscope with hemacytometer. Living cells possess intact cell membranes that exclude trypan blue dyes; however dead cells take up dyes and turn to blue. (Cell death%) = (total number of dead cells per mL of aliquot)/(total number of cells per mL of aliquot) × 100%.

*Caspase-3 activity assay*

Caspase-3 activity was measured using colorimetric assay kits (KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer’s instructions. Briefly, cells as indicated treatment were harvested and lysed, then the lysate was incubated at 37°C for 0.5 h with 200 µM DEVD-pNA (caspase-3 substrate). Samples were read in Synergy™ 2SL (Bio-Tek Instruments). Protein concentration (total protein of the lysate) was determined by Lowry method [24].


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A

B

C

D

E

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Western blot analysis

Cells at $1 \times 10^7$ cells/mL were treated with DMSO, HE alone, DOX alone and the combination of HE and DOX and harvested after the indicated times. After the lysis procedure, the lysates were centrifuged at 12000 g for 10 min at 4°C. The determination of the protein concentration of supernatants using the BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL, USA), equal amounts of protein (50 mg) from each sample were separated by electrophoresis through SDS-PAGE gels (4-12% Tris-HCl, Nu, Invitrogen, Merelbeke, Belgium) and transferred to Hybond-C Super membrane (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

The data were expressed as means ± S.D. Statistical analysis was performed by using Student’s t-test (two-tailed). The criterion for statistical significance was taken as P < 0.05.

Results

Combination treatment of HE and DOX exhibits additive effect in SGC7901

Recent studies indicate that Hericium Erinaceus has potential anticancer effects in different tumor cell types, however these effects have not been investigated in human gastric cancer. To address the therapeutic effects of Hericium Erinaceus in SGC7901, we first investigated the cell death inducing effects of HE with different concentration (10-200 µg/mL).

MTT analysis showed the cell toxicity of HE alone, DOX alone and the combination of HE and DOX. As shown in Figure 1A, HE nearly did not induce SGC7901 cells death below 100 µg/mL. These observations indicate that nearly no apoptotic effect was initiated in SGC7901 cells in response to the HE treatment below 100 µg/mL. And according to Figure 1A, we infer that in the concentration range above 200 µg/mL HE was clearly cytotoxic in SGC7901 cells.

Figure 1B showed that DOX did not induce greatly SGC7901 cells growth below 2 µg/mL, and the IC50 of DOX was about 10 µg/mL. As shown in Figure 1A and 1B, HE or DOX single treatment for 24 h inhibited the growth of SGC7901 cells in a concentration-dependent manner, and the combination treatment (100 µg/mL HE + DOX) produced additive effect on cell growth (Figure 1C and 1D), reducing the IC50 of DOX from 10 µg/mL to 5 µg/mL. In the following study, we use HE + DOX as the symbol of the (100 µg/mL HE + 5 µg/mL DOX) combination. Besides, cell cycle analysis (Figure 1E) showed HE combined with DOX greatly arrest cell cycle at S phase, indicating that the combination of HE and DOX inhibited SGC7901 cell growth by inhibiting the synthesis of DNA.

We also have performed experiments about the effects of the flow part of Hericium Erinaceus extracts from column chromatography on SGC7901 cells. As expected, this part showed little cytotoxicity on SGC 7901 cells and no obvious synergy effect with DOX (data not show).

Combination of HE and DOX increases ROS generation and decreases MMP

ROS are indices of cell redox status. The generation of intracellular ROS and depletion of GSH are always associated with MMP disruption and cell apoptosis [25]. Therefore, we examined the levels of ROS in SGC7901 cells...
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ROS generation that triggered by combination of HE and DOX against SGC7901 originated from mitochondria

We identified and clarified the source of HE-induced ROS burst in SGC7901 cells (Figure 4). Mitochondria are considered to be the main

Antioxidants protected SGC7901 from HE and DOX cytotoxicity

To verify the relationship between ROS generation and cell toxicity, we examined the effects of ROS scavengers, NAC (a well-known antioxidant and GSH precursor) and catalase (CAT, a H$_2$O$_2$-scavenging enzyme), on (HE + DOX)-induced SGC7901 cell death. Before treated with drugs, the cells were treated with 1 mM NAC or 2000 U/mL catalase for 30 min, and then the change of MMP and cell toxicity were determined. As shown in (Figure 3), both NAC and catalase inhibited the loss of MMP and protected cells from HE + DOX cytotoxicity. Further they both significantly reduced (HE + DOX)-induced cell apoptosis as indicated by Hoechst staining and trypan blue exclusion. It is suggested that ROS accumulation caused MMP disruption and mediated cell apoptosis.

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It is estimated that about 2% of the oxygen in a cell leaks from the mitochondrial electron transport chain and becomes ROS under physiological condition \[27\]. We investigated the effect of rotenone (ROT, an inhibitor of mitochondrial electron transport chain complex I) on (HE + DOX)-induced ROS generation. SGC7901 cells were treated with 5 μM rotenone for 30 min, and then treated with or without 100 μg/mL HE +

**Figure 3.** Effects of exogenous application of NAC and catalase (CAT) on the (HE + DOX)-induced ROS generation, GSH depletion, MMP disruption, and cell viability of SGC7901 cells. A. The ROS, GSH and MMP changes were examined by flow-cytometric analysis. B. Cell death rate was determined by trypan blue exclusion. Control, cells treated with solvent as control; HE + DOX, cells treated with HE + DOX for 24 h; CAT + HE + DOX, cells treated with HE + DOX for 24 h after incubated with 2000 U/mL catalase for 30 min; NAC + HE + DOX, cells treated with HE + DOX for 24 h after incubated with 1 mM NAC for 30 min. (The data represent means ± SD of three independent experiments; *P < 0.05, cells treated with HE + DOX after incubated with NAC or catalase were compared with cells treated with HE + DOX alone).

**Figure 4.** Effect of rotenone (ROT) on the ROS induced by the combination of HE and DOX. ROS level was analyzed using flow-cytometric analysis stained with DCFH-DA. Cells co-treated with HE and DOX for 48 h after pretreated with 5 μM rotenone for 30 min. (The data represents the means ± SD of three independent experiments with triplicates. *P < 0.05 compared to HE + DOX alone group).
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DOX for 2 h. Compared with the 2.56-fold increase without any pretreatment, the treatment with rotenone blocked the (HE + DOX)-induced ROS generation significantly (Figure 4). This result suggested that HE triggered the generation of ROS by the mitochondria.

Combination of HE and DOX induces SGC7901 apoptosis in caspase-dependent pathway and in part through inhibition of HIF-1α pathway

Caspases play a critical role in the process of cell apoptosis [28]. Caspase-3 is a major mediator of apoptosis. So we measured the activity of caspase-3 and the expression of Bcl-2 family protein in SGC7901 cells after treatment with HE and DOX. Upon activation, the expression of pro- and anti-apoptotic proteins changes and caspases are cleaved into small, active forms [29]. Therefore, we analyzed changes in the levels of Bcl-2 family protein and cleaved-caspase-3 by Western blot analysis (Figure 5A). Treatment with low doses of HE or DOX induced the up-regulation of Bax and down-regulation of Bcl-2 and Bcl-xl and caspase-3 cleavage. To further determine the activation of caspase-3 by the combination treatment of HE and DOX, the activity of caspase-3 was analyzed using a commercial caspase-3 assay kit. The combination treatment significantly (P < 0.05) increased the activity of caspase-3 (Figure 5B). To confirm whether or not the apoptosis induced by the combination treatment occurs via the caspase-dependent pathway, a general caspase inhibitor z-VAD-fmk was added to the combination treatment. Pretreatment of caspase inhibitor significantly (P < 0.05) reduced cytotoxicity by the combination treatment in a dose-dependent manner (Figure 5C). Thus, these results indicated that low doses of HE and DOX could synergistically enhance caspase activity in HepG2 cells.

It was reported that doxorubicin is dependent on cellular oxygenation for its maximal efficacy [30]. Although the hypoxic acquisition of drug
resistance has been studied with respect to stimulus for some genes, which has been associated with drug resistance [31, 32]. However, the key transcriptional factors that modulate drug-resistance-associated proteins remain to be identified. One possible protein is HIF-1α. HIF-1α is the major transcriptional factor that is significantly activated by hypoxia, and then transactivates many genes involved in tumor development. Recent experimental studies showed that HIF-1α may be the possible factor involved in drug resistance acquired by hypoxia [33]. Our study tested the expression of HIF-1α in SGC7901 cells after treatment with the combination of HE and DOX. Figure 5A shows that HIF-1α decreases significantly, indicating HE synergizes with DOX and HE impairs drug resistance of SGC7901 against DOX in part through HIF-1α pathway.

Discussion

Many food have an ability of curing diseases in accordance with an old Chinese saying that medicine and food are from the same source. Hericium erinaceus is a traditional edible food, which has many activities to be elucidated. DOX is a commonly used anticancer drug, but has side effects like cardiac cytotoxicity, limiting its comprehensive use. Cancer progression is partially affected by the development of cancer cell resistance to apoptosis induced by chemotherapeutic drugs like DOX. Here in our study, we combined HE with low doses of DOX in the treatment of SGC7901 gastric cells, presenting an excellent synergistic effect. Besides, our strategy gives us an outstanding idea of the therapy of gastric cancer using edible food like Hericium erinaceus.

In this study, we showed that HE and low doses of DOX synergize to induce apoptosis in human gastric cancer SGC7901 cells. Caspase-3 was activated in the apoptotic process induced by the combination treatment of HE and DOX. The suppression of caspase activity by a general caspase inhibitor z-VAD-fmk confirmed that the promotion of apoptosis by combination treatment involved a caspase-dependent pathway.

ROS play a pivotal role in apoptosis. The apoptotic effect of HE combined with DOX on SGC7901 cells was associated with an early elevated level of intracellular ROS. Moreover, in order to confirm that the apoptotic effect of HE combined with DOX was mediated by ROS, antioxidants NAC and catalase were used. Both NAC and GSH inhibited the cytotoxicity of HE and DOX associated with suppressed ROS generation and GSH depletion. And they both can ensure cell survival as shown by trypan blue exclusion and significantly reduced cell apoptosis as demonstrated by Hoechst staining. Our results also indicated that HE combined with DOX induced a decrease of MMP in SGC7901 cells. In addition, concerning the time course of ROS burst and MMP depolarization, the ROS burst occurred before intracellular MMP depolarization. This opinion was supported by the results obtained from SGC7901 cells co-incubated with HE and DOX and antioxidant NAC or catalase. Both NAC and catalase blocked the MMP depolarization completely. These results demonstrated that the ROS burst was a prerequisite for MMP collapse and cell death induced by the combination of HE and DOX.

Importantly, many cancer cells have the ability of resistance against chemotherapy drugs. Here we also found that HE can impair DOX resistance of SGC7901 cells by decreasing the expression of HIF-1α, thus enhancing the utility of DOX in therapy of human gastric cancer.

In conclusion, the apoptotic effect of the combination of HE and DOX on SGC7901 cells is mediated by ROS and relied on caspases-cascade. Lower doses of HE or DOX alone had minor cytotoxic effect on SGC7901 cells, while the combination of low doses of both of them has significant apoptotic effect, showing a synergistic effect of Hericium erinaceus.

Disclosure of conflict of interest

None.

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