

Research Article

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Mechanism of oxymatrine-induced human esophageal cancer cell apoptosis by the endoplasmic reticulum stress pathway

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Abstract: Endoplasmic reticulum stress is one of the mechanisms of cell apoptosis. In this study, the mechanism of oxymatrine-induced human esophageal cancer Eca-109 cell apoptosis by the endoplasmic reticulum stress pathway was investigated. Eca-109 cells were cultured *in vitro* with different doses of oxymatrine (0.5, 1, 2 $\mu\text{g}/\text{mL}$) for 48 h. The cell viability and proliferation inhibition rate were examined by MTT assay and cell cycle assay. The apoptosis rate was examined by Annexin V-FITC/propidium iodide assay. The expression of endoplasmic reticulum stress markers, including binding immunoglobulin protein and CCAAT-enhancer-binding protein homologous protein, were determined by real-time quantitative polymerase chain reaction and western blotting, respectively. MTT data showed that oxymatrine significantly inhibited the proliferation of Eca-109 cells. The cell apoptosis rate was quantified by flow cytometry. The expression of binding immunoglobulin protein was markedly downregulated in oxymatrine-treated Eca-109 cells while that of CCAAT-enhancer-binding protein homologous protein was upregulated. Oxymatrine inhibited proliferation and induced apoptosis of human esophageal carcinoma Eca-109 cells. Thus, oxymatrine may be a potential agent for treating human esophageal cancer.

Keywords: Oxymatrine, Human esophageal carcinoma Eca-109 cells, Binding immunoglobulin protein, CCAAT-enhancer-binding protein homologous protein

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1 Introduction

Esophageal cancer is a common cancer of the digestive system, accounting for one quarter of all deaths caused by malignant tumors in China. In particular, esophageal squamous-cell carcinoma has become one of the top ten malignant tumors in China according to the National Health and Family Planning Commission of China [1, 2]. Currently, the prevention and treatment of esophageal cancer using Traditional Chinese Medicine has made progress, but there have been few studies of the molecular mechanisms of oxymatrine's effect on the apoptosis of esophageal cancer Eca-109 cells.

The endoplasmic reticulum (ER) is a necessary organelle involved in the synthesis of nascent proteins, Ca^{2+} storage, glycosylation, and trafficking of newly synthesized membrane and secretory proteins [3]. During hypoxia and nutrient deprivation, Ca^{2+} and protein levels are altered, creating an imbalance in the homeostasis of the endoplasmic reticulum and resulting in ER stress (ERS), which leads to the accumulation of misfolded and unfolded proteins in the ER and activation of the unfolded protein response pathway [4]. Binding immunoglobulin protein (BIP) is thought to be the primary regulator of three unfolded protein response signaling pathways, including pancreatic ER kinase-like ER kinase, activating transcription factor 6, and inositol-requiring enzyme-1. However, if the proteins have aggregated and the ERS cannot be effectively eliminated, apoptosis will occur. When DNA is damaged, CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) may be the most sensitive to ERS [5]. Previously, we found that ER stress contributed to the induction of proinflammatory cytokines in macrophages and may be involved in osteoclastogenesis [6, 7]. In addition, other studies show that some Chinese medicine extracts could induce apoptosis of tumor cells by increasing ERS [8].

Oxymatrine is an alkaloid found in the roots of *Sophora* species. It is known that oxymatrine has anti-

inflammatory, anti-virus, anti-allergic, and liver protection effects, reducing aminotransferase levels and fibrosis [9]. Although the relationship between oxymatrine and cancer cell apoptosis has been well established, little is known regarding oxymatrine's inhibitory effect on esophageal carcinoma Eca-109 cell line with altered expression of the ERS-associated proteins BIP and CHOP. In the present study, we investigated the influence of different concentrations of oxymatrine on the expression of BIP and CHOP in esophageal carcinoma Eca-109 cells cultured *in vitro* to obtain insight into the mechanisms underlying these effects. Our results showed that oxymatrine inhibits the growth of Eca-109 cells and induces cell apoptosis by mediating the expression of the ERS-associated proteins BIP and CHOP. Our findings suggest that these target genes contribute to the apoptosis of Eca-109 cells, providing a theoretical basis for the clinical application of oxymatrine.

2 Material and methods

2.1 Main chemicals and reagents

Oxymatrine injection (Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd., Shanghai, China), batch number 001244, format 2 g/L; fluorouracil (Shanghai Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China), batch number 071001, format 2 g/L; DMEM, trypsin (Gibco, Grand Island, NY, USA); fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China); dimethyl sulfoxide (Shanghai Chemical Reagent Research Institute Co., Ltd., Shanghai, China); and propidium iodide (PI) (Sigma, St. Louis, MO, USA) were used in this study.

2.2 Main instruments

A Thermo Scientific CO₂ incubator (Heraeus, Waltham, MA, USA); FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA); FLUOVIEW FV100 Confocal fluorescence microscope (Olympus, Tokyo, Japan); PCR Cycler (Whatman Biometra, Göttingen, Germany); and Real-time QPCR System (Applied Biosystems, Foster City, CA, USA) were used in this study.

2.3 Cell culture

The Eca-109 cell strain of human esophageal cancer was donated by Professor Kai Juan Wang from School of Public

Health, Zhengzhou University. Cells were cultured in RPMI1640 medium containing a 10% volume fraction of fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C, 5% CO₂, and sufficient humidity. When Eca-109 cells were in the exponential phase of growth, all cells were seeded into 96-well plates (5 × 10³ cells/well) or a culture flask (1 × 10⁵ cells/bottle) for 24 h. Next, Eca-109 cells were treated with 0.5, 1, and 2 µg/mL oxymatrine. After cultivation for 48 h, the cells were collected for further experiments. The negative control group contained the same volume of medium without any drugs. Positive control group cells were treated with 2 mg/mL 5-fluorouracil (5-FU) for 48 h [10].

2.4 MTT assay of cellular proliferative capacity

At the exponential phase of growth, the cells were collected and inoculated into 96-well plates, with each well containing 200 µL (2 × 10³ cells/well). After culture for 24 h, Eca-109 cells were treated with 0.5, 1 and 2 µg/mL oxymatrine. Each group was tested in 6 wells. After treatment with oxymatrine for 48 h, 20 µL 5 mg/mL MTT was added to each well for an additional 4h culture. Living cells can convert MTT to crystal violet formazan, which can be dissolved by dimethyl sulfoxide after the cell supernatant is removed and shocked for 10 min. The absorbance value (A value) was measured at 490 nm in an enzyme-linked immunosorbent assay plate reader. The experiment was repeated three times to obtain the average value of the inhibition rate of proliferation. Proliferation rate (%) = (average A values in experimental group/average A values in control group) × 100%.

2.5 Cell cycle assays

The logarithmic growth cells were collected and inoculated into culture bottle for 24h (1 × 10⁵ cells/L). According to the experimental group, the cells were treated with 0.5, 1 and 2 µg/mL oxymatrine for 48 h, collected and centrifuged 5 min (2000 r/min), fixed by 70% ethanol, saved at 4°C. The cell cycle was determined by Flow Cytometry. The excitation wavelength was 488 nm.

2.6 Apoptosis assays

Apoptosis was detected following the Annexin V/PI Apoptosis Kit instructions. Cells were inoculated into

culture bottles for 24 h (1×10^5 cells/L). Then cells were treated with 0.5, 1 and 2 $\mu\text{g}/\text{mL}$ oxymatrine for 48 h, washed twice with phosphate-buffered saline and digested with 0.25% trypsin for 2–3 min. After centrifugation, the supernatant was removed, the cells were washed twice with cold phosphate-buffered saline, and re-suspended in binding buffer at a concentration of 1×10^6 cells/mL. Cells then were stained with 1 μL fluorescent labeling Annexin V reagent for 20 min at room temperature followed by 5 μL PI for 5 min at 4°C in the dark. Finally, 500 μL binding buffer was added to each tube and the apoptosis rate was measured by flow cytometry within 1 h [11, 12].

2.7 Expression of BIP and CHOP mRNA of Eca-109 cells detected by real-time PCR

Total RNA was extracted from the cells of each group using an RNA Isolation Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Five micrograms total RNA was transcribed into cDNA using a First-stand cDNA Synthesis kit Real-time Quantitative PCR Kit. The polymerase chain reaction (PCR) system was a total of 10 μL and contained the following: 5 mL SYBR Green I Mixture, 0.25 μL upstream primer, 0.25 μL downstream primer, 1 μL template cDNA, 3.3 μL ddH₂O, and 0.2 μL Rox. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing at 58°C for 40 s, and extension at 72°C for 40 s for 45 cycles. The relative expression of the BIP and CHOP genes was standardized according to the β -actin level and quantified using the $2^{-\Delta\Delta\text{Ct}}$ method. $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ (target sample) - ΔCt (control sample), $\Delta\text{Ct} = \text{CT}$ (test gene) - CT (reference gene) [13].

2.8 Expression of BIP and CHOP proteins detected by western blotting

To further investigate BIP and CHOP protein expression in oxymatrine-induced apoptosis, Eca-109 cells were cultured with 0.5, 1, and 2 $\mu\text{g}/\text{mL}$ oxymatrine for 48 h and harvested. Protein was extracted from the cells and protein concentration was measured using the BCA method. The same amount of sample was examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a membrane, and primary and secondary antibodies were incubated, developed, and exposed. The results were displayed and the gray values were scanned. Western blotting was repeated 3 times.

2.9 Data analysis and statistics

All statistical information is described as the mean \pm standard deviation ($\bar{x} \pm S$); SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct statistical analysis. For multi-group statistical information, after testing for homogeneity of variance, one-way analysis of variance was applied, while Student's *t* test was used for inter-group comparison at a significance level of $\alpha = 0.05$.

3 Results

3.1 Effect of oxymatrine on the proliferation capacity of Eca-109 cells

The Eca-109 cell viability was assessed by MTT assay. Cell viability in the treated groups was significantly suppressed and the inhibition rate gradually increased with increasing oxymatrine concentration. Compared to the control group, groups treated with oxymatrine for 48 h showed significantly inhibition effect ($P < 0.05$) (Table 2). The inhibition ratio was gradually increased from 13.2 to 16.5% over the 0.5 to 2 $\mu\text{g}/\text{mL}$ concentration range.

3.2 Effect of oxymatrine on cell cycle

The cell cycle was determined by flow cytometry. The G_0 / G_1 phase cells after treated by different concentration oxymatrine for 48 h was obviously increased, compared with the control group ($P < 0.05$). There was a significant decrease in S stage cells, compared with the control group ($P < 0.05$) (Table 3).

3.3 Oxymatrine treatment induces apoptosis in Eca-109 cells

The cultured Eca-109 cells were treated with 0.5, 1, and 2 $\mu\text{g}/\text{mL}$ oxymatrine for 48 h, and the apoptotic rates were $2.74 \pm 0.29\%$, $9.32 \pm 0.18\%$, and $12.98 \pm 0.54\%$ respectively. The 5-FU group apoptosis rate was $16.22 \pm 0.19\%$. These results demonstrate that oxymatrine treatments increase cell apoptosis. With increasing oxymatrine concentration, the apoptotic rate of Eca-109 cells was increased. The difference was significant compared to the control group ($P < 0.05$) (Figure 1).

Table 1. Oligonucleotides synthesized by Shanghai Generay Biotech Co., Ltd. (China).

	Forward	Reverse	Size (bp)
β -actin	5'-CTACAATGAGCTGGTGTGG-3'	5'-TAGCTCTTCTCAGGGAGGA-3'	108
bip	5'-TCTAGGTGAACGACCCCTAAC-3	5'-GTTCTCTCAATTTCTCCAAC-3	648
chop	5'-CGCCTTCAACGACGAGTTCCTG-3'	5'-GCTGTTCTTATCCACCGACTTC-3'	503

Table 2. Effect of oxymatrine on viability of Eca-109 cells ($\bar{x} \pm s$, $n = 6$).

Category	A Value (490 nm)	Inhibition ratio (%)
Control group	0.91 \pm 0.04	-
Fluorouracil group	0.79 \pm 0.02*	13.2
0.5 μ g/mL Oxymatrine	0.81 \pm 0.03*	11.0
1 μ g/mL Oxymatrine	0.78 \pm 0.02*	14.3
2 μ g/mL Oxymatrine	0.76 \pm 0.03*	16.5

Note: Comparison with the control group: * $P < 0.05$

Table 3. Effect of oxymatrine on cell cycle of Eca-109 cells ($\bar{x} \pm s$, $n = 3$).

Category	G ₀ /G ₁	S	G ₂ /M
Control group	44.25 \pm 1.64	29.21 \pm 0.95	27.54 \pm 1.42
Fluorouracil group	42.56 \pm 2.58	46.32 \pm 2.20*	11.12 \pm 1.52*
0.5 μ g/mL Oxymatrine	55.10 \pm 1.23*	24.15 \pm 2.28*	20.75 \pm 3.03*
1 μ g/mL Oxymatrine	62.45 \pm 1.89*	20.27 \pm 1.54*	17.28 \pm 1.89*
2 μ g/mL Oxymatrine	72.36 \pm 2.16*	15.27 \pm 2.13*	13.54 \pm 2.51*

Note: Comparison with the control group: * $P < 0.05$

3.4 BIP and CHOP mRNA expression

In order to further confirm that oxymatrine induced human esophageal cancer cell apoptosis by the ERS pathway, the expression levels of BIP and CHOP mRNA were detected by real-time quantitative PCR and analyzed by the $2^{-\Delta\Delta Ct}$ method. The results showed that both oxymatrine and 5-FU significantly downregulated the expression of BIP mRNA and upregulated the expression of CHOP mRNA. Additionally, expression in the oxymatrine-treated groups was dose-dependent and showed a significant difference compared to the control group ($F = 129.331$, $P = 0.000$; $F = 14.139$, $P = 0.000$) (Table 4 and Figure 2).

3.5 BIP and CHOP Protein expression

To investigate the apoptotic pathways activated by oxymatrine, we used western blotting to measure the expression of the stress-associated BIP and CHOP proteins (Table 5 and Figure 3). The results indicated that

the protein expression level of CHOP in the oxymatrine treatment groups was significantly higher than in the control group, while BIP was decreased compared to the control group ($P < 0.05$). The expression of BIP and CHOP was dose-dependent. These results suggest that ERS reactions take part in the apoptosis process induced by oxymatrine and are closely related to the changes in BIP and CHOP protein expression.

4 Discussion

The ER is an important organelle in cells for protein synthesis and folding [14]. Triggers such as hypoxia, glucose starvation, oxidation, and glycosylation disorders may cause unfolded or misfolded proteins to accumulate in the ER, leading to ERS. To protect themselves, cells attempt to restore the stability of their internal environment through mechanisms such as initiating unfolded protein reactions, decreasing the rate of protein synthesis, increasing the ER protein processing capacity by upregulating the expression of ER chaperones, and

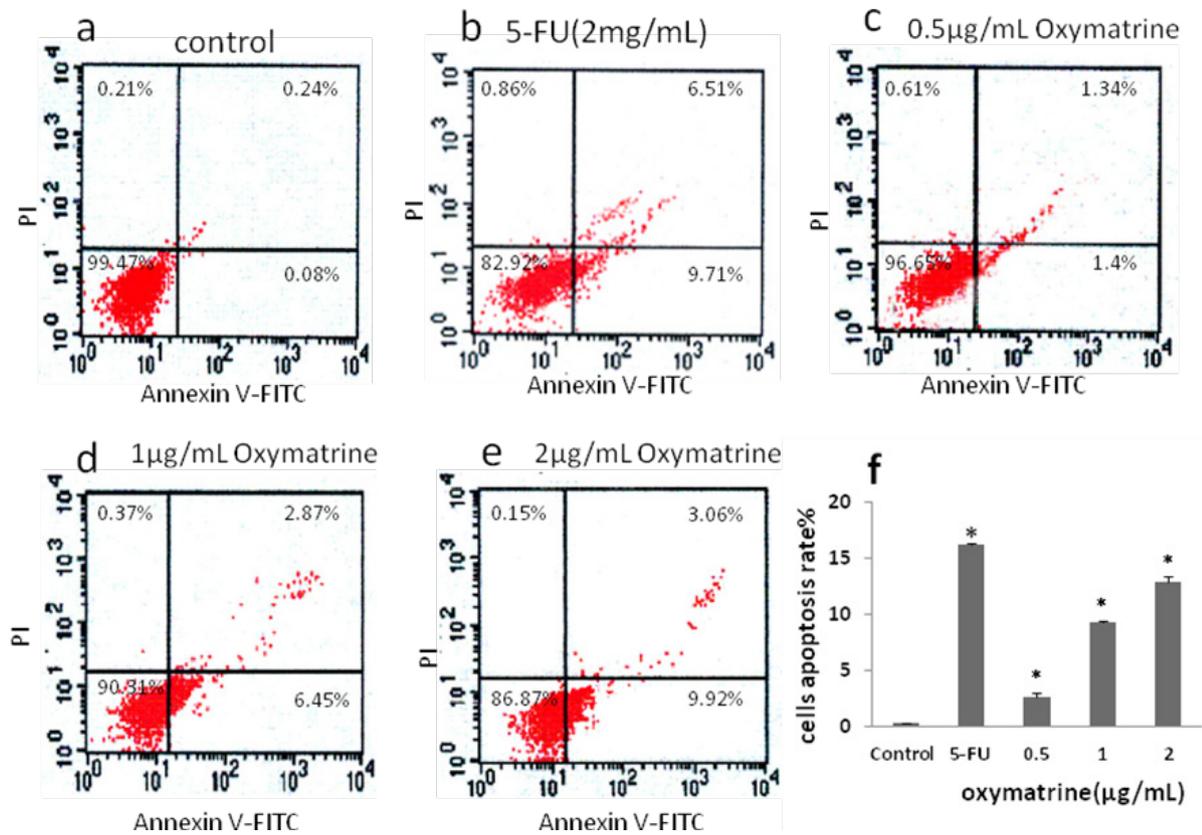


Figure 1. Effects of oxymatrine on apoptotic rate of esophageal cancer Eca-109 cells. After treatment with 0.5, 1, and 2 μg/mL oxymatrine and 2 mg/mL 5-FU for 48 h, the Eca-109 cell apoptosis rate were quantified by Annexin V-FITC and PI double staining. (a) The apoptotic rate was $0.32 \pm 0.08\%$ in controls. (b) The apoptotic rate was $16.22 \pm 0.19\%$ in the 5-FU group. (c) The apoptotic rate was $2.74 \pm 0.29\%$ in the 0.5 μg/mL oxymatrine-treated group. (d) The apoptotic rate was $9.32 \pm 0.18\%$ in the 1 μg/mL oxymatrine-treated group. (e) The apoptotic rate was $12.98 \pm 0.54\%$ in the 2 μg/mL oxymatrine-treated group. (f) The apoptotic rate in all groups is shown in the histogram. Comparison with the control group: * $P < 0.05$.

Table 4. Effects of oxymatrine on expression of Eca-109 cells BIP and CHOP mRNA ($\bar{x} \pm s$, $n = 3$).

Category	BIP mRNA/ β -actin	CHOP mRNA/ β -actin
Control group	11.89 ± 0.22	0.21 ± 0.03
0.5 μg/mL Oxymatrine	$8.73 \pm 0.36^*$	$0.29 \pm 0.04^*$
1 μg/mL Oxymatrine	$4.61 \pm 0.15^*$	$0.32 \pm 0.01^*$
2 μg/mL Oxymatrine	$3.64 \pm 0.48^*$	$0.49 \pm 0.02^*$
Fluorouracil group	$4.28 \pm 0.21^*$	$0.35 \pm 0.02^*$

Note: Comparison with the control group: * $P < 0.05$

Table 5. Effects of oxymatrine on expression of Eca-109 cell BIP and CHOP ($\bar{x} \pm s$, $n = 3$).

Category	BIP/ β -actin	CHOP/ β -actin
Control group	1.03 ± 0.04	0.26 ± 0.06
0.5 μg/mL Oxymatrine	$0.86 \pm 0.03^*$	$0.43 \pm 0.04^*$
1 μg/mL Oxymatrine	$0.57 \pm 0.05^*$	$0.50 \pm 0.02^*$
2 μg/mL Oxymatrine	$0.81 \pm 0.08^*$	$0.86 \pm 0.03^*$
Fluorouracil group	$0.73 \pm 0.04^*$	$0.52 \pm 0.05^*$

Note: Comparison with the control group: * $P < 0.05$

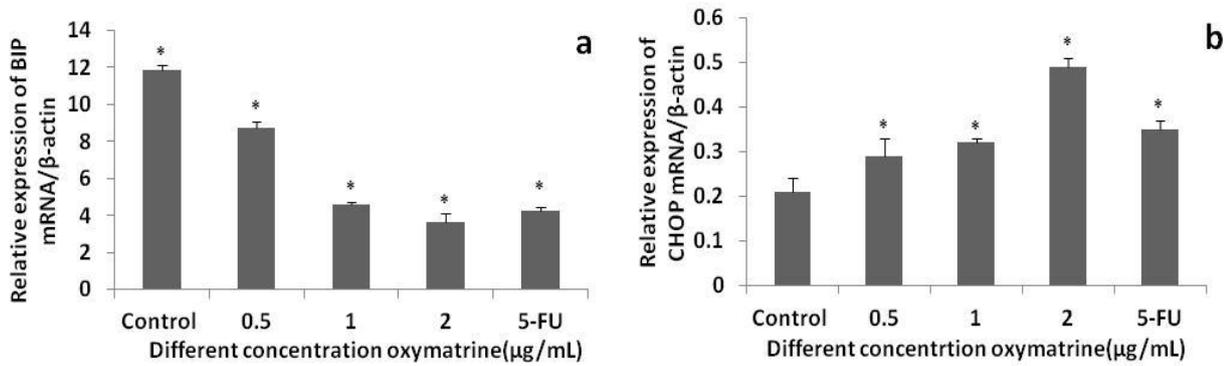


Figure 2. Effects of oxymatrine on the expression of BIP and CHOP in esophageal cancer Eca-109 cells. After treatment with 0.5, 1, and 2 $\mu\text{g}/\text{mL}$ oxymatrine and 2 $\mu\text{g}/\text{mL}$ 5-FU for 48 h, Eca-109 cells were collected and the relative expression levels of BIP, CHOP, and β -actin were analyzed by real-time PCR. (a) Relative expression of BIP mRNA. (b) Relative expression of CHOP mRNA.

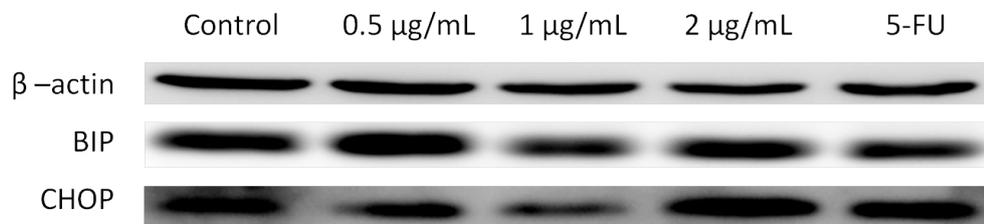


Figure 3. Effects of oxymatrine on the expressions of BIP and CHOP of esophageal cancer Eca-109 cells. After treatment with 0.5, 1, and 2 $\mu\text{g}/\text{mL}$ oxymatrine and 2 $\mu\text{g}/\text{mL}$ 5-FU for 48 h, Eca-109 cells were collected and the relative expression levels of BIP, CHOP, and β -actin were analyzed by western blotting.

reducing the burden on the ER [15]. BIP is also known as glucose regulated protein 78 and is a major glucose-regulated protein. BIP is an ER chaperone that remains in the ER tube. BIP is also considered to be a biomarker of ERS and plays a vital role in protein folding and responding to ER transmembrane signaling. Studies have shown that BIP can be synthesized with unfolded polypeptide chains and play a role in coordinating the correct folding and assembly of proteins, inhibiting protein accumulation, decreasing the production of erroneously folded proteins, and promoting cell survival [16, 17]. Under normal conditions, BIP is bound to RNA pancreatic ER kinase-like ER kinase, inositol-requiring enzyme-1, and activating transcription factor-6, inactivating these three proteins. However, when ERS occurs, BIP releases these proteins, leading to their activation, and downstream target genes are induced, including CHOP [18]. CHOP is a key factor in ERS-induced apoptosis and facilitates apoptosis during this process. To some extent, CHOP reflects the level of apoptosis. When ERS occurs, CHOP can cause apoptosis by inhibiting the transcription of anti-apoptosis factor Bcl-2. CHOP-mediated apoptosis participates in the onset and development of many diseases such as cancer,

diabetes, and Parkinson's disease, and studies have shown that some antineoplastic drugs can upregulate CHOP gene expression and induce cancer cell apoptosis [19]; additionally, over-expression of CHOP plays a key role in drug chemosensitization. Therefore, the involvement of CHOP apoptotic pathways in the onset and development of cancers has gained attention.

Oxymatrine is a mixture of matrine oxide and very small amounts of oxysophocarpine, and is an alkaloid extracted from *Sophora alopecuroides* or the root of *Sophora flavescens* [10]. Current pharmacological studies have shown that oxymatrine can contribute to protection against viruses and inflammation as well as supporting immune function [20]. In addition, oxymatrine has positive effects on the central nervous system and cardiovascular system. Recently, both domestic and international studies found that oxymatrine can significantly inhibit tumor growth, regulate cancer cell periods, and induce cancer cell differentiation [19]. In this study, oxymatrine significantly facilitated the apoptosis of Eca-109 cells and supported the suggestion that oxymatrine can prevent the proliferation of Eca-109 cells. Real-time QPCR revealed higher expression of BIP genes in the control group,

indicating that ERS participates in the proliferation of Eca-109 cells. As tumor tissue grows rapidly, its nutrition supply becomes limited. However, highly-expressed BIP can prevent the apoptosis of relevant cells in the ER so that Eca-109 cells can tolerate hypoxia and acidosis to improve survival.

5 Conclusions

In this study, BIP expression was downregulated in the oxymatrine groups, indicating that the pharmacological effect of oxymatrine in preventing Eca-109 cell proliferation is associated with ERS; in contrast, the expression of CHOP was upregulated, demonstrating that oxymatrine can activate ERS apoptosis signals and induce Eca-109 cell apoptosis. Nonetheless, further studies are required to explore the downstream signal mechanism by which CHOP induces apoptosis. Future studies should evaluate additional apoptotic markers, particularly BCL family genes, and clarify the mechanism of oxymatrine induction of Eca-109 cell apoptosis.

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Conflict of interest: Authors state no conflict of interest.

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