Department of General Surgery[1]; Department of Thoracic Surgery[2], First Affiliated Hospital of Liaoning Medical University, Jinzhou, Liaoning, China

Oxymatrine mediates Bax and Bcl-2 expression in human breast cancer MCF-7 cells

Benrui Lin[1], Dinuo Li[1], Lan Zhang[2]

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Lan Zhang. No. 2 People's Avenue Section 5, Jinzhou, Liaodong, China, Postal Code: 121000. lanzhang_ln@sina.com

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The aim of this study was to explore the effects of oxymatrine in treating breast cancer patients using biomolecular methodology. Human breast cancer MCF-7 cells were treated with oxymatrine at concentrations of 0 (control), 25, 50 and 100 μg/mL. Apoptosis assay by Annexin/PI staining was performed to examine the effects of oxymatrine on apoptotic rates of MCF-7 cells at time points of 24 h, 48 h, and 72 h after treatment. Real-time PCR was performed for the mRNA abundance of Bax and Bcl-2 after the cells were treated with oxymatrine at concentration of 0, 25, 50, and 100 μg/mL at the time points of 24, 48, and 72 h. Western blotting was performed when the cells were treated with oxymatrine at various concentrations for 72 h. High concentration of oxymatrine at 100 μg/mL enhanced apoptosis by 6.4-fold at 72 h compared with control (33.16% vs. 4.47%; $t = 9.82$, $p < 0.001$). Oxymatrine at 100 μg/mL up regulated Bax mRNA abundance by 169 % at 72 h ($t = 18.32$, $p = 0.001$), and reduced Bcl-2 mRNA abundance by 24 % at 72 h ($t = 6.30$, $p = 0.001$) compared with control. Oxymatrine enhanced the expression of Bax protein while reduced the expression of Bcl-2 protein. Oxymatrine treatment showed pro-apoptotic effects in breast cancer MCF-7 cells, and these effects correlated with the up regulation of Bax transcription and protein expression and the down regulation of Bcl-2 transcription and protein expression in a time- and dose-dependent manner. Conclusion: Oxymatrine had effects in promoting apoptosis in human breast cancer MCF-7 cells by mediating the mRNA and protein expression levels of Bax and Bcl-2.

1. Introduction

Breast cancer is one of the most common malignant tumors in females and it has the highest morbidity and fatality than any other tumors affecting females in the world (Matsen and Neumayer 2013). Approximately 30% of breast cancer patients suffer from relapse and metastasis, even when they have been treated at an early stage of the disease (Hutchinson 2010). As a heterogeneous disease, breast cancer can be divided into five categories according to molecular markers and gene expression profiles (Hutchinson 2010). The heterogeneous expressions of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) are characteristic of the classification they determine chemotherapy choices and patient outcome (Van't Veer et al. 2002). The five categories of breast cancer include (1) Luminal A, (2) Luminal B, (3) Basal, (4) Claudin-low, and (5) HER2 (Holliday and Speirs 2011). Approximately 80 % of breast patients at primary diagnosis present with estrogen receptor positive (ER^+) breast cancers (Martin and Dowsett 2013). The MCF-7 cell line has been established as the *in vitro* model for breast cancers characteristic as ER^+ (Holliday and Speirs 2011).

Conventional treatments can control the progression of metastasis in breast cancer patients after initial pathological diagnosis; however, most of the patients experience exacerbation towards mortality over time (Hutchinson 2010). Cancer can be as a succession of genetic changes, during which normal cells are transformed into malignant cells, while evasion of cell death is crucial changes in the disordered cell cycle (Wong 2011). Currently, three pathways of apoptosis have been identified (Elmore 2007). The two well-studied pathways are extrinsic pathway (or death receptor) and intrinsic (or mitochondrial) pathway. A less well-known apoptosis initiation pathway is arguably the intrinsic endoplasmic reticulum pathway (Elmore 2007; Wong 2011). BCL-2 proteins are crucial for regulating cell differentiation and apoptosis through the intrinsic pathway (Youle and Strasser 2008). A multitude of studies have focused on the role of BCL-2 proteins for identifying therapeutic targets for breast cancer patients who are estrogen-receptor positive (Martin and Dowsett 2013; Redondo 2013). Based on their function and conformation, the proteins of BCL-2 family (BCL-2 refers to the super family proteins) are categorized into three groups: (1) anti-apoptotic proteins containing the four BCL-2 Homology (BH) domains, such as Bcl-2, Bcl-XL, Mcl-1, Bcl-W; (2) pro-apoptotic proteins containing BH1, 2, and 3 domains, such as Bax and Bak; and (3) pro-apoptotic proteins containing only the BH3 domain (Youle and Strasser 2008). Bcl-2 protein plays crucial roles in the carcinogenesis of breast cancer by blocking apoptosis and cooperating with the transcription expression of MYC in cell differentiation (McDonnell and Korsmeyer 1991). In addition, Bcl-2 protein enhances both carcinogenesis and metastatic potential in MCF-7 breast cancer lines (Del Bufalo et al. 1997).

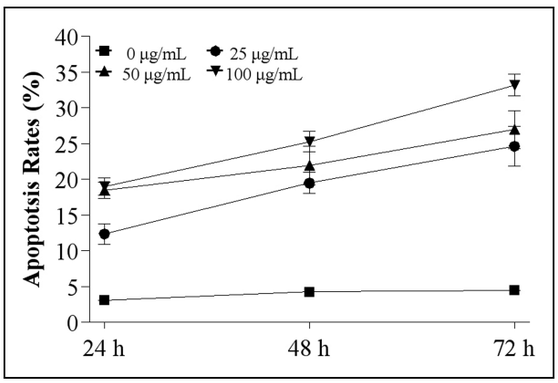

Fig. 1: Effects of oxymatrine treatment on apoptosis rates at 24, 48, and 72 h.
*significant difference vs. control.

Oxymatrine, which is a major bioactive extract from Kushen (Radix Sophorae flavescentis), has shown pharmacological effects of anti-inflammation, anti-allergy, inducing of cell cycle arrest, and accelerating apoptosis (Wang et al. 2015). Oxymatrine diminishes the side population and inhibits the expression of β-catenin in MCF-7 breast cancer cells. Oxymatrine has inhibitory effects on the proliferation and vascular endothelial growth factor A (VEGF-A) expression in human lung denocarcinoma (SPC-A-1) cell (Liang et al. 2012). Oxymatrine inhibits the proliferation of prostate cancer cells (Wu et al. 2015). These studies showed that oxymatrine plays roles in the pathogenesis and progression of various cancers; however, the exact mechanisms of anti-cancer effects remain undefined. In this study, the effects and mechanisms of oxymatrine on breast cancer cells were examined using the MCF-7 cell line. Flow cytometry was performed to evaluate the relative abundance of apoptosis in MCF-7 cells treated by oxymatrine. Immunocytochemistry, Western blotting, and quantitative real-time PCR were performed to explore the mechanism of oxymatrine exerting its effects.

2. Investigations and results

2.1. Oxymatrine enforced apoptosis in MCF-7 cells dose- and time-dependently

MCF-7 cells were treated or not with oxymatrine at three concentrations of 25, 50, or 100 μg/mL for 24, 48, or 72 h before the apoptotic rates were assayed by flow cytometry. Multivariate analyses showed that oxymatrine at the three concentrations induced increased apoptosis at 24, 48, and 72 h, respectively, as compared with vehicle control (Fig. 1). Notably, high concentration of oxymatrine at 100 μg/mL enhanced apoptosis by 6.4-fold at 72 h compared with control (33.16% vs. 4.47%; $t = 9.82$, $p < 0.001$). Further, high concentrations of oxymatrine treatment induced larger apoptotic rates compared with lower concentrations.

2.2. Oxymatrine dose-dependently mediated the mRNA abundance of Bax and Bcl-2

The MCF-7 cells were treated with oxymatrine at three concentrations of 25, 50, or 100 μg/mL for 24, 48, or 72 h, and the mRNA abundances of Bax and Bcl-2 were then assayed by real-time PCR, with the statistics normalized to the respective vehicle controls (Fig. 2A). Oxymatrine treatment at 100 μg/mL significantly up regulated the Bax mRNA abundance by 169 % at 72 h ($t = 18.32$, $p = 0.001$) compared with control (Fig. 2B). In contrast, oxymatrine treatment at 100 μg/mL significantly reduced the Bcl-2 mRNA abundance by 24 % at 72 h ($t = 6.30$, $p = 0.001$) compared with control (Fig. 2C). In addition, lower concentration of oxymatrine showed slight effects compared with the high concentrations, suggesting that the concentrations of oxymatrine at 25 μg/mL appeared lower than was capable of producing statistically significant effects given the present sample size (Fig. 2C) .

2.3. Oxymatrine mediated the expressions of Bax and Bcl-2 proteins

The MCF-7 cells were treated or not with oxymatrine at three concentrations of 25, 50, or 100 μg/mL for 72 h, and then the expression levels of Bax and Bcl-2 proteins were examined by Western blotting (Fig. 3A). Oxymatrine treatment at 100 μg/mL up regulated the expression of Bax protein (Fig. 3B) by 5.0-fold compared with control ($t = 28.54$, $p < 0.001$), while oxymatrine treatment at 100 μg/mL significantly reduced the Bcl-2 expression (Fig. 3C) by 82% ($t = 14.4$, $p < 0.001$). Furthermore, dosage of oxymatrine treatment correlated significantly to the expression levels of Bax ($r = 0.87$; $p < 0.001$) and Bcl-2 proteins ($r = -0.89$; $p < 0.001$).

3. Discussion

In this study, breast cancer MCF-7 cells were utilized to explore the potential anti-cancer effects of oxymatrine. Apoptosis assay showed enhanced apoptosis in MCF-7 cells after oxymatrine treatment (Fig. 1). Further, real-time PCR revealed significant increases in the Bax mRNA abundance as well as marked decreases in the Bcl-2 mRNA abundance, when MCF-7 cells were treated with high concentration oxymatrine (Fig. 2). In the end, Western blotting revealed enhanced expression of Bax protein and attenuated the expression of Bcl-2 protein (Fig. 3).
For both apoptosis induced by extrinsic or intrinsic pathways, caspase 3 is referred to the executioner caspase, activated by cytochrome c to form a complex known as apoptosome, leading to the destined cell death (Kroemer et al. 2007). Notably, human breast cancer MCF-7 cells do not express caspase 3 that was thought be a critical component in the apoptosis cascade (Liang et al. 2001). Therefore, researches on breast cancer using MCF-7 cell line focused on the expression of Bax and Bcl-2 to evaluate the effects of drugs being tested (Liang et al. 2001). The absence of caspase 3 in the MCF-7 cell line is not an abnormality in carcinogenesis, since cancerous cells evade apoptosis via three major mechanisms: disruption of the balance of pro-apoptotic and anti-apoptotic proteins; (2) reduced caspase function; and (3) impaired death receptor signaling from the extrinsic pathway. Therefore, a multitude of studies focused on Bax and Bcl-2 as well as the Bax/Bcl-2 ratio when evaluating therapeutic effects of potential drugs for breast cancer (Liang et al. 2001; Wong 2011; Leung and Wang 1999). Surprisingly, the well-studied gene p53 is not expressed at baseline, nor up regulated with apoptosis reduction in the MCF-7 cell line (Wong 2011). Therefore, the validity and representativeness of MCF-7 cell line in breast cancer research needs to be further evaluated. Since tamoxifen has long been indicated for breast cancer patients due to its proven effects (Fisher et al. 1998; Osborne et al. 2003), the combined effects of tamoxifen and oxymatrine on the phenomenology of apoptosis in MCF-7 cell line will be explored in our future studies.
In conclusion, oxymatrine treatment showed pro-apoptotic effects in breast cancer MCF-7 cells, and these effects correlated with the up regulation of Bax transcription and protein expression as well as the down regulation of Bcl-2 transcription and protein expression in a time- and dose-dependent manner.

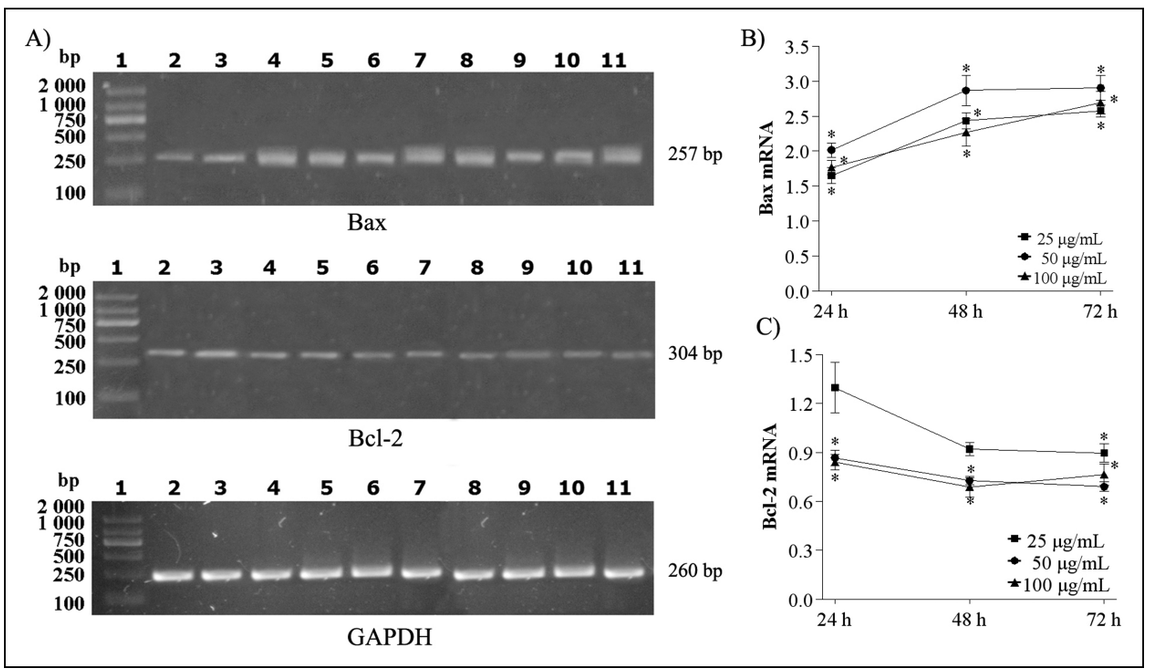

Fig. 2: Effects of oxymatrine on Bax and Bcl-2 mRNA abundance in MCF-7 cell line measured by real-time PCR. A): Lane 1 was marker; lane 2 was control; lane 3–5 were oxymatrine at 25 μg/mL; lane 6–8 were oxymatrine at 50 μg/mL; lane 9–11 were oxymatrine 100 μg/mL; lanes 3, 6, 9 represented 24 h; lanes 4, 7, 10 represented 48 h; lanes 5, 8, 11 represented 72 h. B, C): Bax and Bcl-2 mRNA abundance normalized to vehicle control; *significant difference vs. control.

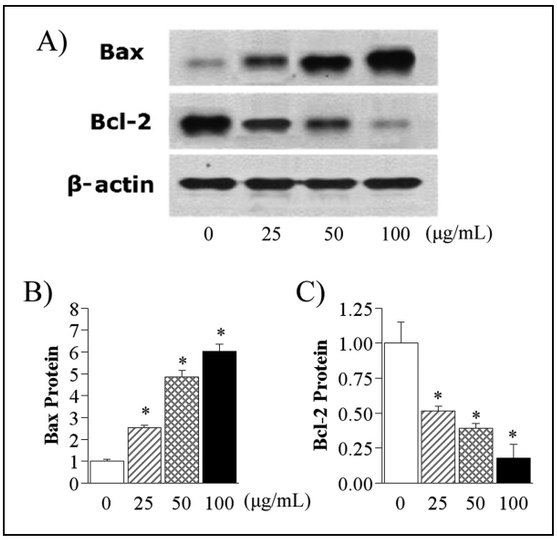

Fig. 3: Effects of oxymatrine treatment at 72 h on the expressions of Bax and Bcl-2 proteins in MCF-7 cell line. A): Western blotting of Bax and Bcl-2 proteins; B, C): Relative densitometry of Bax and Bcl-2 expression normalized to vehicle control; *significant difference vs. control.

Oxymatrine has potential therapeutic effects in treating breast cancer patients with the biomolecular targets as Bax and Bcl-2 proteins.

4. Experimental

4.1. Cell culture and treatment

The materials and reagents used in the cell culture included (1) the human breast cancer MCF-7 cells (American Type Culture Collection, New York, U.S.); and (2) oxymatrine (≥ 98%; Product Batch No.: 201414230; Huaming Pharmaceutical Co., Shandong, China). MCF-7 cells were cultured in RPMI 1640 media (*Invitrogen*, Germany) containing 10% fetal bovine serum and placed in an incubator containing 5% CO_2 at 37 °C. The incubation medium was changed every 1 to 2 days. The cells were passaged every 3 to 4 days. After five passages, the logarithmic-phase MCF-7 cells were harvested and subjected to oxymatrine treatment at three concentrations of 25, 50, and 100 μg/mL at cell density of 2×10^5 cells/mL, respectively, for further experiments in this study. Cells treated with vehicle acted as control.

4.2. Apoptosis assay by Annexin/PI flow cytometer

The logarithmic-phaseMCF-7 cells incubated in RPMI 1640 culture medium (1% double-antibody, 10% fetal bovine serum) was adjusted to cell concentration at 3×10^5/mL. The cell suspension was added to the 12-well cell culture microplate (2 mL/well). The cells were then placed in an incubator containing 5% CO_2 at 37 °C for 24 h. PBS buffer solution served as the blank control with three duplicate wells per place. The cells were then incubated with 5% CO_2 at 37 °C for 24 h, 48 h, or 72 h, respectively. The cells were then centrifuged before discarding the supernatant. The BD FACSAria II flow cytometer (Becton Dickinson, New Jersey, USA) and Annexin V/PI double labeling method were used to detect the apoptosis and compute the apoptotic rate (Ivanović-Matić et al. 2014). This experiment was done in triplicate for samples in each group. The Cell Quest software system was used to analyze the results.

4.3. RNA extraction and quantitative real-time polymerase chain reaction

The RNA extraction, cDNA synthesis, and real-time PCR were performed according to previously published method with slight modifications (AbuHammad and Zihlif 2013). All operations were performed on ice in an RNase-free environment. The total RNA of the MCF-7 cells were extracted with TRIzol using the Universal MicroRNA Kit (*Invitrogen*, CA, U.S.) 48 h after the MCF-7 cells had been treated or not with oxymatrine at three concentrations of 25, 50, and 100 μg/mL. cDNA was reversely transcribed with the TaqMan MicroRNA Reverse Transcription Kit (ABI, CA, U.S.). The concentration of the total RNA was determined by measuring the absorbance at 260 (A_{260}) and 280 (A_{280}) nm in a spectrophotometer (NanoDrop ND-1000). The constituents of the total 20 μL reaction mixture for cDNA synthesis included 15 μL of 100 mM dNTPs, 1.00 μL of MultiScribe reverse transcriptase, 1.50 μL of 10 × reverse transcription buffer, 0.19 μL of RNase inhibitor, 4.16 μL of nuclease-free water, 3 μL of hsa-miR-4458 or U65 × RT primer, and 5 μL of RNA sample (10 ng Total RNA). The reaction mixture was incubated 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. Synthesized cDNA was stored at -20 °C for later use.
The real-time PCR assays were run on an ABI 7500 real time PCR system (Applied Biosystems, Life Technologies, Germany). The PCR reaction was

Table 1: Senses and optimized annealing temperatures in real-time PCR experiment

Gene	Primer sequence	Size (bp)	Anneal (°C)
Bcl-2	F: CTGGGATGCCTTTGTGGAAC R: GGCAGGCATGTTGACTTCAC	304	56
Bax	F: GGATGCGTCCACCAAGAA R: GCACTCCCGCCACAAAGA	257	56
GAPDH	F: TGACGTGGACATCCGCAAAG R: CTGGAAGGTGGACAGCGAGG	260	56

repeated in duplicate for each sample. The constituents of the total 20 μL mixture subjected to PCR reaction included: 1.33 μL of cDNA, 1 μL of hsa-miR-4458 or U6 20 × real time primer, 10 μL of TaqMan 2 × universal PCR master MIX, and 7.67 μL of nuclease-free water. After optimization for reaction volume and annealing temperatures, the parameters set for the real-time PCT program were as follows: 10 min of initial denaturation at 95 °C, 15 s of denaturation at 95 °C, 60 s of annealing at 60 °C, 60 s of extension at 60 °C, with 40 cycles repeated (Table 1). Aragose electrophoreses were performed using 2.5 μL ethidium promide. The electrophoresis was then run at 104 V for 45 min, and the DNA bands are visualized using a UV-transilluminator (Bio-Rad UV Transilluminator 2000). This experiment was replicated in triplicates for samples in each group. Statistical analyses on ΔCT were performed using the non-parametric procedure Wilcoxson Rank Sum Test.

4.4. Western blotting of Bax and Bcl-2 proteins

The Western blotting of Bax or Bcl-2 proteins was performed by a previously published method with slight modifications (Siddiqa et al. 2008). Briefly, the MCF-7 cells that were treated or not with oxymatrine at the three concentrations for 72 h were collected. The experimental cells were transferred to a centrifuge (12,000 × g) for protein extraction and quantification in accordance with the manufacturer's instructions. An average of 30 μg of protein was subjected to 10% SDS-PAGE. The gel containing proteins of interest after electrophoresis was sliced off and marked. Then, the protein was transferred to PVDF membrane by electrophoresis, and blocked in 5% non-fat milk for 2 h at room temperature. The blocked PVDF membrane was treated by murine monoclonal anti-Bax (dilution: 1:250; Sigma-Aldrich) or anti-Bcl-2 antibodies (dilution: 1:250; Sigma-Aldrich) diluted in TBST and placed on a mechanical shaker at 4 °C overnight. The PVDF membrane was then treated by goat anti-mouse secondary antibody (dilution: 1:500; Sigma-Aldrich) marked by horse radish peroxidase (HRP) for 2 h at room temperature. The membrane was then subjected to DAB development and ECL luminescence. This experiment was replicated in triplicates for samples in each group. The BioDoc-IT 220 Gel-Imaging system (UVP, NY, U.S.) was used for visualization, and the Quantity One software was used for analyzing densitometry.

4.5. Statistical analyses

Statistical analyses were performed using SAS 9.2 on Windows 7. Univariate analyses were performed using the Mixed Linear Model (PROC MIXED of SAS) with treatment and time as factors as well as the potential interaction effects of time × treatment. Post hoc comparisons were performed using the LSMEANS statement in the PROC MIXED of SAS with Sidak adjustment. Assumptions of normality and homoscedasticity were checked by residuals as well as predictions generated from the PROC MIXED procedure. Correlations of oxymatrine treatment with dependant variables of interest were analyzed using PROC REG of SAS. Data were expressed by Mean ± SD. $P < 0.05$ was considered statistically significant.

Conflicts of interest: This study was not sponsored by any governmental or non-governmental organization. The authors of this research declare no conflicting interests existed.

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