Matrine Suppresses the ER-positive MCF Cells by Regulating Energy Metabolism and Endoplasmic Reticulum Stress Signaling Pathway

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Matrine (C15H24N2O), an alkaloid that is one of the main active components from Sophora flavescens. Matrine has been demonstrated to have therapeutic effects on various solid tumors, including breast cancer, but the mechanism still needs further study. Endoplasmic reticulum (ER)-positive Michigan Cancer Foundation cells were cultured, and matrine was added in various amounts to measure the dose-dependent and time-dependent cytotoxicity. Hoechst 33258 staining was used to observed nuclear morphological changes. Apoptosis was measured by AnnexinV/PI double staining assay kit. Intracellular adenosine triphosphate and glycometabolism were detected by assay kit. The protein levels GRP78, p-eIF2α, CHOP, cytochrome c, and HexokinaseII were analyzed. Mechanistic investigations revealed that matrine treatment causes ER dilation and up-regulated the expression of ER stress markers GRP78, eIF2a, and CHOP, increases the levels of apoptotic in Michigan Cancer Foundation cells, subsequently, blocking the ER stress-mediated apoptosis pathway, significantly decreased matrine-induced apoptotic but still has significant difference between control group. In addition, matrine not only promoted the occurrence of ER stress but also inhibited the expression of hexokinase II, down-regulated energy metabolism. In summary, the present study suggests that the induction of ER stress-mediated apoptosis by matrine and down-regulated energy metabolism may account for its cytotoxic effects in human breast cancer cells. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: matrine; ER-positive MCF cells; endoplasmic reticulum stress; energy metabolism; hexokinase II.

INTRODUCTION

There is a long history of the use of traditional Chinese medicines (TCMs) to treat solid tumors in China. In the past several decades, many TCMs have been approved by the Chinese State Food and Drug Administration and used in the clinical treatment of various types of solid tumor. Kushen (Sophora Flavescentis) is a widely used TCM for a series of diseases including viral hepatitis, cardiac arrhythmia, and cancer. The compound Kushen injection also has been used for clinical oncology diseases. An update systematic review and meta-analysis indicated that compound Kushen injection has obviously therapeutic effect in hepatocellular carcinoma. (Ma et al., 2016) But because of the different methods of preparation and different parts of plant used for herbal extracts, therefore, systematic reviews are not devoid of limitations, particularly in the field of herbal medicine. (Izzo et al., 2016). So looking for the active product is an important approach to the development for new drugs. Matrine (C15H24N2O), an alkaloid that is one of the main active components from Sophora flavescens. More recent studies have indicated that matrine exhibits potent anti-tumor activity in various cancer cell lines in vitro, including hepatoma, breast carcinoma, gastric carcinoma, multiple myeloma, and osteosarcoma.

Breast cancer was the second leading cause of cancer related death among women worldwide. Although concerted efforts have been made in breast cancer therapy, it is still estimated that over 1,000,000 women are newly diagnosed with breast cancer every year worldwide and that more than 400,000 cases will die from breast cancer (Li et al., 2015; Shao et al., 2013; Li et al., 2010). Matrine was reported to effectively inhibit the proliferation of breast cancer cells, including endoplasmic reticulum (ER)-positive Michigan Cancer Foundation (MCF) cells, HER2-positive BT-474 cells, and highly metastatic MDA-MB-231 cells. The death of cancer cells was observed in the matrine treatment group. Studies indicated that matrine triggers intrinsic apoptotic cascades, involved in the regulation of NF-κB and Akt. However, the mechanism of matrine-induced apoptosis in breast cancer remains to be further explored (Yang and Sk, 2015; Xie et al., 2014b; Wang et al., 2013).

Endoplasmic reticulum and mitochondrial are important organelles to maintain normal cell function (Nami et al., 2016; Liao et al., 2016). The unfolded protein response (UPR) is a cellular signaling pathway in the ER that is activated in response to overloading of unfolded or misfolded peptide chains. The ER UPR is a survival mechanism utilized by tumors to buffer proteotoxic stress (Fujimoto et al., 2016; Kaira et al., 2016). Cancer cells rely on the ER UPR to cope with...
numerous genetic and proteotoxic insults, including hypoxia, oxidative stress, genetic instability, and the overexpression of numerous wild type or mutant oncoproteins (Lee et al., 2016; Piton et al., 2016; Suyama et al., 2011). But overloading the UPR can result in catastrophic ER stress, activated apoptosis pathway, leading to cell death. In addition, continuous ER stress caused the occurrence of mitochondrial dysfunction, resulting in abnormal mitochondrial energy metabolism, cytochrome c release. In addition, hexokinase (HK) also is another key enzyme for mitochondrial function (Shi et al., 2011; Xiang et al., 2011). This enzyme transfers a phosphate group from adenosine triphosphate (ATP) to glucose, forming glucose-6-phosphate. Glycolysis of cancer cell metabolism is associated with the binding of HK II to the outer mitochondrial membrane protein voltage-dependent anion channel (Kwee et al., 2012), and this association appears important for mitochondrial homeostasis (Neary and Pastorino, 2013). And studies show Akt phosphorylation involved in its regulation. In this report, ER stress-induced cell death and Akt/HKII pathway in breast cancer cells which both influence by matrine were examined (Majewski et al., 2004; Zhuo et al., 2015).

### MATERIALS AND METHODS

**Reagents.** The ER-positive MCF cell line was provided by the cell bank of the Chinese Academy of Sciences (Shanghai, China). Matrine, 4-phenylbutyric acid (4-PA, ER stress inhibitor), and ATP detection kits were obtained from Sigma Chemical Co (St. Louis, MO, USA). Antibodies for GRP78, Phospho-eIF2a, CHOP, HK II, Cytochrome c, and GAPDH were purchased from Abcam (Abcam, UK).

**Cell culture and experimental groups.** Endoplasmic reticulum-positive MCF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FBS) and 1% penicillin/streptomycin under a humid 5% CO2 atmosphere at 37 °C, and trypsinized every 3 days.

The ER-positive MCF was randomly divided into different groups as follows:

1. **The control group,** in which the ER-positive MCF were incubated in normal solution throughout the experimental period;
2. **matrine group,** in which the cells were treated with the addition of matrine in various amounts (0, 20, 40, 80, 160, 320, 640, or 1280 μg/mL) for various times (24, 48, or 72 h);
3. **MP:** matrine (2 mM) and 4-PA (30 nM);
4. **4-PA (30 nM).**

**Cell culture cell viability and morphological changes.** 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was employed to measure the dose-dependent and time-dependent cytotoxicity of mistletoe alkaloid fractions. ER-positive MCF cells were cultured in 96-well microplates at a density of 5 × 10^4 cells/mL in 100 μL DMEM containing 10% FBS, and matrine or phosphate-buffered saline (PBS) (as control) was added to the culture media in various amounts (0, 20, 40, 80, 160, 320, 640, or 1280 μg/mL) for various times (24, 48, or 72 h). At the end of treatment, MTT reagent (5 mg/mL, 20 μL) was added to each well, and cells were incubated for 3 h. Finally, medium was removed, and 150 μL DMSO was added into each well and mixed by gentle shaking for 15 min, and optical density value of each well was determined using a plate reader at 490 nm (Infinite M200 Pro, Switzerland). Relative cell viability determined by MTT assay on treatment with matrine (2 mM) and MP (matrine 2 mM + 4-phenylbutyric acid), 4-PA (ER stress inhibitor) was also detected.

**Hoechst 33258 staining.** Hoechst 33258 nucleic acid staining was used to observed nuclear morphological changes. Briefly, cells were seeded in 24-well microplates and treated with matrine (0.5, 1, and 2 mM) and MP (matrine 2 mM + 4-phenylbutyric acid), 4-PA (ER stress inhibitor) for 24 h. Following treatment, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at 37 °C, then stained in dark with Hoechst 33258 for 20 min at 37 °C, washed with PBS for three times, and photographed using florescence microscope (Nikon Eclipse Ni; Nikon Corporation, Japan).

**Annexin V/PI double-staining assay.** Apoptosis was measured by flow cytometer using AnnexinV/PI double staining assay kit. Cells were seeded in 48-well microplates and treated with matrine (2 mM) and MP (matrine 2 mM + 4-phenylbutyric acid), 4-PA (ER stress inhibitor) for 24 h. According to the manufacturer’s instructions, cells were collected, washed with cold PBS twice, and re-suspended in 195 μL binding buffer. Annexin V-FITC 5 μL and propidium iodide 10 μL were added to each tube and then incubated for 15 min at 37 °C in the dark. The amount of apoptotic cells were determined by a flow cytometer (Becton Dickinson, CA, USA) at 525 and 575 nm.

**Western blotting.** Michigan Cancer Foundation cells were harvested after treatment and lysed with lysis buffer on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. Total protein concentration was determined using Bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, China). The protein samples were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were blocked with TBST buffer (Beyotime Institute of Biotechnology, China) containing 5% free fat milk for 1 h and then incubated with specific primary antibodies overnight at 4 °C Phospho-eIF2a, CHOP, GRP78 (1:500; Abcam), Cytochrome c, HK II (1:1000; Abcam), and GAPDH (1:2000; Abcam). After incubation, three times washing with TBST buffer, each membrane was incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies(1:3000) for 1 h at room temperature, then each membrane washed three times with TBST buffer, and the signal was detected using an enhanced chemilmunescence detection kit from Millipore (Billerica, MA, USA). The immunoreactive bands were analyzed by Copyright © 2017 John Wiley & Sons, Ltd. Phytother. Res. 31: 671–679 (2017)
Adenosine triphosphate assay and cytosolic translocation of cytochrome C. Adenosine triphosphate assay kit was used to detect the change of intracellular ATP according to the manufacturer’s protocol. Supernatant and cells were collected from six groups and measured by spectrophotometry. Standard curve was drawn, control wells were set, and the experiments were repeated three times.

Cytochrome C levels were also measured. Cells were washed with PBS and extracted by lysis buffer, were collected by centrifugation (1000 g, 5 min, 4 °C), and the supernatant was discarded. The cells pellet was resuspended with Digitonin cell permeabilization buffer, vortexed and incubated on ice for 5 min. Cells were then centrifuged (1000 g, 5 min, 4 °C). The supernatants were saved, as they contained the cytosolic fraction of cytochrome C. There maining pellet was then resuspended with Radio-Immunoprecipitation Assay (RIPA), cell lysis buffer 2, vortexed and incubated on ice for 5 min. The lysate was vortexed and centrifuged (1000 g, 10 min, 4 °C). The supernatants were saved, as they contained the mitochondrial fraction of cytochrome C.

Caspase activity assay. The activities of caspase-3 were measured with caspase-3 activity kits (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China), respectively, according to the manufacturer’s instructions. Cells were harvested and lysed, total protein was extracted, and the protein concentration was determined. A mixture of 10 μL of protein extracts, 80 μL of reaction buffer, and 10 μL of caspase-3 substrate was then incubated in 96-well plates at 37 °C for 4 h. The optical density was subsequently measured at 405 nm using a microplate reader. The relative caspase activity was expressed as the percentage of enzyme activity relative to the control. The experiments were repeated in triplicate.

Hexokinase II overexpression. Cells were incubated in DMEM containing 2% FBS and adenovirus containing a full-length human HK II construct (50 moi for 6 h). The media was replaced with 10% FBS in DMEM, and the cells allowed to incubate overnight before the experiments were performed. HK II overexpression was confirmed by immunoblot analysis.

Glucose consumption, lactate production, and adenosine triphosphate production in hexokinase II overexpression cell. Glucose consumption, lactate production, and ATP production were determined by an assay Kit (Sigma Chemical Co.). Lactic acid was quantified on the basis of its lactate oxidase-catalyzed conversion to pyruvate and hydrogen peroxide (H₂O₂), followed by peroxidase-catalyzed conversion of the chromogen precursor ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)) to its chromogen in the presence of H₂O₂. The chromogen was quantified spectrophotometrically at 548 nm, and its appearance was strictly proportional to the lactic acid concentration. This assay is linear between 0.020 and 13.32 mM/L. Manual dilution was performed before quantification in the case of the highest concentrations. Lactate production was calculated as the lactate concentration measured in the cell supernatants minus the lactate concentration measured in the cell medium.

The glucose concentration is directly proportional to the generated NADH concentration that is spectrophotometrically measured at 340 nm. This assay is linear between 0.28 and 44.40 mM/L. Samples containing higher concentrations were diluted manually before quantification. Glucose uptake was calculated as the glucose concentration of the medium minus the glucose concentration measured in the cell supernatant. The level of ATP in cell lines was determined using the ATP assay Kit according to the manufacturer’s protocol.

Statistical analysis. The results are expressed as the mean ± standard deviation. All experiments were repeated at least three times. Statistical analysis was performed with Student’s t-tests or Mann–Whitney test. Differences at the level of P ≤ 0.05 were regarded as statistically significant.

RESULTS

Matrine induces cell growth inhibition in human breast cancer cells; remarkably, matrine still shows poisonous to the cells when combined used with 4-phenylbutyric acid

We assessed the effect of matrine on cell viability or growth of the human breast cancer cell lines, using

![Figure 1.](image-url)
various concentrations at different times. The IC₅₀ values represented the mean ± SD of three different experiments. The matrine group showed the significant specificity cytotoxicity to ER-positive MCF cells at an IC₅₀ value of 528.6 ± 34.6 μg/mL; remarkably, matrine still show poisonous to the cells when combined used with 4-phenylbutyric acid, but the cell viability increased obviously (Fig. 1).

Matrine induced the endoplasmic reticulum-positive Michigan Cancer Foundation cells apoptosis, and decreased under combined with 4-phenylbutyric acid, but apoptosis still exist

Hoechst 33258 staining and Annexin V-FITC assay were used in evaluation for apoptosis. It was observed that matrine showed significant apoptosis against ER-positive MCF cells as shown in Fig 2. The numbers of apoptotic cells increased in a dosage-dependent manner (Fig. 2II–IV). On the contrary, the percentages of apoptotic cells were reduced in matrine pretreatment with 4-phenylbutyric acid group at 24 h after incubation (Figs. 2V). These findings indicated that the matrine obviously promoting the ER-positive MCF cells apoptosis, and the effect was decreased significantly under combined with 4-PA. But it is worth noting that, although the apoptosis significantly decreased, there were still significant differences compared with the control group.

> Figure 2. Hoechst 33258 staining of cell apoptotic (I–VI) in control (I), matrine (0.125, 0.5, 2 mM, II–IV), and MP (matrine 2 mM + 4-phenylbutyric acid, V). [Colour figure can be viewed at wileyonlinelibrary.com]

Endoplasmic reticulum stress is mainly involved in the apoptosis in human breast cancer cells induced by matrine

We used western blot analyses to investigate whether matrine-induced ER stress in human breast cancer cells. Phospho-eIF2α was elevated in matrine-treated MCF-7 cells after 24 h compared with control cells (Fig. 4). Furthermore, GRP78 and CHOP-activated were significantly induced compared with control cells; these results indicate that matrine induced ER stress in MCF-7 cells. But given the ER stress inhibitor, the ER stress effect is decreased obviously in MP group.
Matrine inhibits mitochondrial function, and promoted the release of cytochrome C and enhanced caspase-3 activity, combined with 4-PA, the effect still exists.

Adenosine triphosphate was detected to investigate the energy metabolism in ER-positive MCF-7 cells. The results revealed that ATP concentration of MCF-7 cells was 314.2 ± 38.7 mM/grot after treated with matrine (Fig. 5), and the ATP value was also significantly lower than control group in MP group (MP: 489.8 ± 56.1; control 653.1 ± 47.8 mM/grot) ($P < 0.05$). Taken together, the content of ATP, representing energy metabolism activity, was down-regulated in MCF-7 cells after treating with matrine. ER stress could lead to mitochondrial energy metabolism dysfunction, but the role of matrine which reduced mitochondrial energy metabolism has not disappeared, when combined with ER stress inhibitors.

Levels of cytochrome c in the cytosol and mitochondria were also measured. Mitochondrial dysfunction leads to cytochrome c release; released of cytochrome c from mitochondria into the cytosol initiates apoptosis. The level of cytosolic cytochrome c was markedly increased; meanwhile, the mitochondria cytochrome c was falling in MCF-7 cells treated with matrine. This effect weakened when combined with 4-PA but still exist significant differences compared with control group. The activity of caspase 3 was also detected; the result indicated that the activity of caspase 3 was significantly enhanced in MCF-7 cells treated with matrine. This effect weakened when combined with 4-PA, but still also exist significant differences compared with control group (Fig. 5).

Matrine inhibits mitochondrial function, and promoted the release of cytochrome C and enhanced caspase-3 activity, combined with 4-PA, the effect still exits.
consumption, lactate production, and ATP content, were examined in MCF-7 cell over-expressing HK II. As shown in Fig. 6, the overexpression of HK II increased glucose consumption and lactate production in MCF-7 cell, while simultaneous pretreatment of matrine caused no significant change in glucose consumption and lactate production, indicating that HK II overexpression leads to an increase in cancerous glycolytic metabolism, which could later be blocked by matrine. We also examined whether ER stress expression affected the glycolytic metabolism of MCF-7 cells. Glucose consumption, lactate production, and ATP content were examined in MCF-7 cell. As shown in figure 6, pretreatment with 4-PA in cells, the glucose consumption and lactate production have no significant difference to control group; meanwhile, 4-PA also have no significant influence on matrine pretreatment cells. That indicated that the impact of matrine on glucose metabolism is not related to ER stress.

**DISCUSSION**

Cancer is a chronic disease with high mortality due to its high metastatic ability and resistance to chemo-therapy and radio-therapy. Natural product anticancer agents activate multiple pathways that inhibit tumor growth survival, invasion, and metastasis (Safe and Kasiappan, 2016; Arumuggam et al., 2015). The ER stress response
has also been a recent focus from a cancer therapeutic perspective, given its emerging role as a protective mechanism against chemotherapy induced cytotoxicity and as a potential initiating step in the development of chemoresistance (Amantini et al., 2016; Giglio et al., 2015; Jalota et al., 2016). Overload ER stress is considered to be an important regulator of several cellular pathological processes, including cancer cell death pathways in response to anticancer drugs. In addition, excessive ER stress can also affect the cell mitochondrial function, to further promote the occurrence of apoptosis (Finley, 2016; Vannuvel et al., 2016; Burton et al., 2016).

Breast cancer is the most common cancer in women worldwide (Golubnitschaja et al., 2016; Chen et al., 2016). Meanwhile, some breast cancers develop multidrug resistance to chemotherapies rapidly, and acquired multidrug resistance severely blocks the effective therapies for breast cancer. Matrine has been demonstrated to have therapeutic effects on various solid tumors, including breast cancer (Li et al., 2015; Xie et al., 2014a; Li et al., 2012). Matrine injection has been used in the treatment of tamoxifen resistance breast cancer. Matrine also promotes the efficacy and safety of platinum-based doublet chemotherapy for advanced non-small cell lung cancer (Rong et al., 2015), but the mechanism still needs further study.

In this study, cell viability was measured using the MTT assay firstly. The growth of ER-positive MCF-7 cells was significantly inhibited in a dose and time-dependent manner when treated with matrine. Then, we focus on the effects of matrine on apoptosis whether via ER stress in current studies. ER stress is considered as a vital regulator of various cellular pathological processes, including cancer cell death pathways in response to anticancer drugs. Mounting data indicate that ER stress plays an important role in the regulation of apoptosis. The CAAT/enhancer binding protein homologous protein (CHOP) has been reported to be a crucial ER stress responsive factor that executes apoptosis, which can be induced and unregulated by all three arms of the UPR signaling pathways (Park et al., 2016; Lei et al., 2016). In this study, we demonstrated that (i) matrine induced the up-regulation of GRP78, p-eIF2α; (ii) matrine also increased the expression of CHOP, which is an important apoptotic inducer; (iii) blocking ER stress by ER stress inhibitor 4-PBA not only effectively decreased matrine-induced GRP78 and CHOP expression but also significantly decreased matrine-induced apoptosis.

Breast cancer cells’ mitochondrial function also is evaluated. Continuous ER stress reduced mitochondrial dysfunction and then leads to apoptosis. HK II is also the critical part in mitochondrial function regulation (Martin et al., 2016). HK II association with the mitochondrial outer membrane is involved in mitochondria-mediated apoptosis and increased resistance of cancer cells to chemotherapeutic drugs (Seccia et al., 2016). In this study, we demonstrated that (i) matrine inhibited the mitochondrial ATP production and promoted the mitochondrial cytochrome C release; (ii) blocking ER stress by ER stress inhibitor 4-PBA, the ATP, and cytochrome C regulated by matrine was weaken but there was still a significantly difference compared with control group; (iii) the establishment of high expression plasmid transfected HK II cell, matrine could significantly inhibited HK II expression.
(iv) matrine could significantly inhibited glucose, lactate, and ATP production in adenosine transfection HK II cells.

In conclusion, matrine promoted the apoptosis of breast cancer ER-positive MCF-7 cells through up-regulation of ER stress and down-regulation of HK II expression. These findings suggest that matrine has potential to be developed as a new natural anti-gastric cancer agent and used in the treatment of drug resistant breast cancer cells.

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Conflict of Interest

There have no conflicts of interest to this work.

REFERENCES


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