Original Article

Comparison of the effects of rutaecarpine on molecular subtypes of breast cancer

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ABSTRACT

Objective: Natural compounds have gained considerable attention in recent years due to disadvantages and properties of current chemotherapy drugs in cancer therapy. In addition, the impact of these compounds is specific for each type and/or subtypes of cancer due to different treatment response. Rutaecarpine, an alkaloid obtained from Evodia Rutaecarpa Chinese herb, has anticancer activity by inhibiting topoisomerase and/or cyclo-oxygenase-2 levels. However, the effectiveness of rutaecarpine has not been well known in breast cancer in terms of subtype. Therefore, we investigated the potential therapeutic effects of rutaecarpine on two different subtypes of breast cancer cells.

Materials and Methods: The cytotoxic and apoptotic effects of rutaecarpine on MCF-7 and MDA-MB-231 cells were analyzed by WST-1, Annexin V, cell cycle, and acridine orange staining.

Results: WST-1 results indicated that rutaecarpine significantly inhibited the growth of both cancer cells for 48 h ($P < 0.05$). In addition, rutaecarpine treatment caused apoptotic cell death through chromatin condensation and nuclear blebbing and G0/G1 arrest in both breast cancer cells. However, the efficacy of rutaecarpine was more profound in MCF-7 cells than MDA-MB-231 cells.

Conclusions: Consequently, rutaecarpine has a potential therapeutic effect on breast cancer. However, the effectiveness of rutaecarpine is dependent on the subtype of breast cancer.

KEY WORDS: Apoptosis, breast cancer, rutaecarpine, subtype

INTRODUCTION

Breast cancer is the most common type of cancer diagnosed by women in the world. According to GLOBOCAN data, more than 2 million new cases are detected worldwide in 2018 and its incidence is expected to increase in further.[1,2] In developing countries, breast cancer is the first cancer among women when examined on cancer-related mortality and is the second most lethal form of cancer in developed countries.[3] In addition, breast cancer subtype, tumor size, stage, etc., often affect treatment response and survival rate due to heterogeneous properties.[4] It is therefore important to evaluate the effects of existing chemotherapy or new therapeutics drugs and/or compounds on each subtype of breast cancer to ensure successful clinical implications.

Rutaecarpine (from Evodia Rutaecarpa) is an alkaloid that has been used for thousands of years to treat various diseases such as gastrointestinal diseases, amenorrhea, and postpartum bleeding, obtained from a traditional Chinese herb. Rutaecarpine is known to have many biological effects including anti-inflammatory, antiobesity, and antitumor activity.[5-8] In the literature, rutaecarpine inhibits topoisomerase I and topoisomerase II and demonstrates a cytotoxic effect by intercalation due to the inability of supercoils to be opened during DNA replication.[9] Topoisomerase inhibitors are antineoplastic agents commonly used alone or in combination with other drugs that inhibit a group of key enzymes in clinical practice. These agents interfere with the function of the cell during DNA replication and transcription, create a cytotoxic effect, and play a crucial role in the treatment of cancer and other neoplastic diseases.[9,10] Another feature of rutaecarpine is thought to mediate the antineoplastic effect is that it has an inhibitory effect on cyclo-oxygenase-2 (COX-2). In studies, priority is given to the effect of inflammatory

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diseases with COX-2 inhibition by rutaecarpine.[11] However, COX-2 expression increases in different malignant tumors including breast cancer. In addition, COX-2 expression affects the invasive properties of cancer cells, causing the cells to become more invasive and leading to increase in the level of matrix metalloproteinase-2. As a result of higher COX-2 expression, inflammation due to increased prostaglandin levels and neoplastic proliferation is accelerated by facilitating the release of some growth factors in the tumor microenvironment.[12-14] In addition, COX-2 expression has been shown to increase in 63%–85% of premalignant cases of breast cancer. Similarly, the concentration of COX-2 is found to increase by nearly 40% of human breast tumors.[15] A number of studies have investigated the cytotoxic effect of rutaecarpine on various cell lines such as renal and lung cancer cells.[16] However, there is limited literature On the potential effects of ruteacarpine on breast cancer treatment. Therefore, we have compared the potential therapeutic effects of rutaecarpine on two different subtypes of breast cancer cell line due to different prognosis and treatment response.

MATERIALS AND METHODS

Cell culture conditions

Human estrogen receptor positive (ER+) breast cancer cell line MCF-7 (ATCC® HTB-22™) and human triple-negative breast cancer cell line MDA-MB-231 (ATCC® HTB. 26™) were purchased from the American Type Culture Collection (ATCC). Both cells were incubated in Dulbecco's Modified Eagle Media (DMEM, Gibco) with 10% fetal bovin serum (FBS, Gibco) and 1% pen/strep (Gibco) at 37°C in 5% CO_2 incubator (Thermo Fisher Scientific).

Cell viability analysis

Rutaecarpine was provided from Sigma-Aldrich. WST-1 reagent was used to determine the antiproliferative effect of rutaecarpine on MCF-7 and MDA-MB-231 cells. The cells were seeded in 96 well plates (2×10^4 cells per well) in 100 µl cell culture media and cultured overnight at 37°C in 5% CO_2 incubator. Cells were incubated with different concentrations (0–160 µM) of rutaecarpine. After 24 and 48 h treatment with rutaecarpine, 10 µL WST-1 reagent (Biovision) was added to the each well, and absorbance was measured at 450 nm using a microplate reader (Allsheng, China) ($n = 3$).

Annexin V and dead cell assay

The cells were seeded in 6 well plates (1×10^5 cells per well) and incubated overnight. After treatment of rutaecarpine (80 µM and 160 µM) for 48 h, the cells were digested by trypsin, and the suspension of cells was centrifuged for 5 min at 1500 rpm. The cell pellet was washed twice with PBS, then Muse™ Annexin V and Dead Cell Kit (Merck Millipore) were performed and analyzed by Muse™ Cell Analyzer (Merck Millipore, Germany). All experiments were performed three times, independently.

Cell cycle assay

The effect of rutaecarpine on cell cycle distribution was determined by Muse™ Cell Cycle Kit. Breast cancer cell lines were seeded in 6 well plates (5×10^5 cells per well) and treated with rutaecarpine (80 µM and 160 µM) for 48 h. After digestion by trypsin and centrifuge, the pellet was washed twice with PBS. Then, control group and rutaecarpine-treated cells were fixed in ice-cold 70% ethanol at −20°C for at least 3 h. Before the analysis, 200 µl of Muse cell cycle reagent added to the tubes and incubated at room temperature conditions for 30 min. After treatment of rutaecarpine, cell cycle distribution was analyzed by Muse™ Cell Analyzer (Merck Millipore).

Acridine orange staining

The apoptotic morphology of cells were monitored with acridine orange (AO) staining. The cells were incubated with 80 and 160 µM rutaecarpine for 48 h and then fixed in 4% paraformaldehyde solution for 30 min. Each well was washed with PBS three times and then 1 mL AO (100 mg/ml) was added. After staining in the dark for 30 min, EVOS FL Cell Imaging System was used for imaging the cells (Thermo Fisher Scientific).

Statistical analysis

All statistical analyses were assessed with a significance level of 0.05 and analyzed with SPSS version 22 (IBM Corp, Armonk, New York, USA). The statistical comparison of viability and apoptotic cell death was evaluated by one-way ANOVA analysis with *post hoc* tests.

RESULTS

The cytotoxic effects of rutaecarpine on breast cancer cell viability

We evaluated the cytotoxic effects of rutaecarpine on the proliferation of both breast cancer cell lines by WST-1 analysis. According to our results, rutaecarpine inhibited the cell viability of MCF-7 and MDA-MB-231 in a time and dose dependent manner [Figure 1]. After 48 h incubation with 20, 40, 80, and 160 µM rutaecarpine, the growth of MCF-7 cells significantly reduced to 75.45 ± 2.85%, 62.36 ± 2.01%, 50.70 ± 2.04%, and 67.83 ± 2.41%, respectively [$^*P < 0.05$, $^{**}P < 0.01$, Figure 1a]. In addition, the viability of MDA-MB-231 cells were significantly reduced to 95.25 ± 0.63%, 82.20 ± 2.12%, 65.60 ± 1.55%, and 70.55 ± 2.41%, respectively, for 48 h [$^*P < 0.05$, $^{**}P < 0.01$, Figure 1b]. Therefore, 20 and 40 µM rutaecarpine treatment did not decrease cell viability as we expected for 48 h in both breast cancer cells. Thus, 80 and 160 µM rutaecarpine were selected as suitable doses for further analysis to evaluate the effects of rutaecarpine on both breast cancer cells.

The effect of rutaecarpine on apoptotic cell death

To determine the apoptotic effects of rutaecarpine on breast cancer cells, we performed the Annexin V assay [Figure 2]. According to our results, rutaecarpine significantly induced apoptotic cell number of both breast cancer cells for

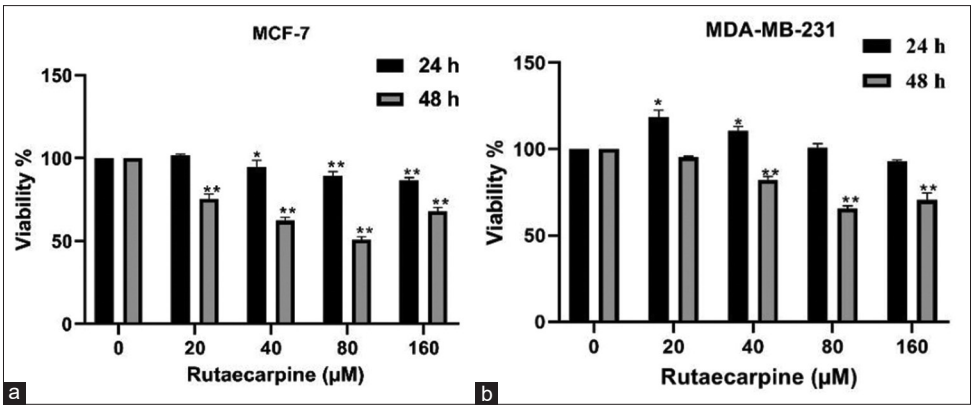

Figure 1: The cytotoxic effects of rutaecarpine on (a) MCF-7 and (b) MDA-MB-237 cells (*$P < 0.05$, **$P < 0.01$)

48 h [**$P < 0.01$, Figure 2]. Following the treatment of 80 and 160 μM rutaecarpine, a significant increase (5.05 ± 0.92% to 48.44 ± 1.25% and 22.03 ± 1.87%, respectively) was detected in the number of total apoptotic cells in MCF-7 cells for 48 h [**$P < 0.01$, Figure 2A]. However, 80 and 160 μM rutaecarpine treatment resulted in a lower total apoptotic cells (from 0.65 ± 1.25% to 36.29 ± 0.65%, and 25.14 ± 1.75%), respectively, in MDA-MB-231 cells [**$P < 0.01$, Figure 2A]. As a result of Annexin V analysis, it was concluded that rutaecarpine caused apoptotic cell death in both breast cancer cells. However, the apoptotic effect of rutaecarpine was higher in MCF-7 cells than MDA-MB-231 cells [Figure 2B].

Evaluation of cell cycle arrest following incubation with rutaecarpine

Our results demonstrated that rutaecarpine treatment resulted in G0/G1 arrest for 48 h in both breast cancer cells [Figure 3]. The accumulation of MCF-7 cells in the G0/G1 phase increased significantly from 75.80 ± 1.58% to 80.20 ± 1.87% and 76.60 ± 1.86% at 80 and 160 μM rutaecarpine for 48 h, respectively [**$P < 0.01$, Figure 3A]. However, a small increase was observed in the percentage of MDA-MB-231 cells at G0/G1 phase (from 61.80 ± 1.45% to 63.70 ± 1.33% and 63.30 ± 2,14%, for 48 h, respectively) [Figure 3A]. Therefore, G0/G1 phase arrest was significantly increased after 80 μM rutaecarpine treatment, especially in MCF-7 cells. However, a higher concentration (160 μM) of rutaecarpine showed a lower effect on both breast cancer cells.

Evaluation of morphological changes after treatment with rutaecarpine

To observe rutaecarpine-induced apoptosis in breast cancer cells, AO staining was performed [Figure 4]. Compared to control cells, 80 μM rutaecarpine treatment induced nuclear blebbing, nuclear fragmentation, and cell contraction in MCF-7 cells [Figure 4a]. In addition, our results showed that chromatin condensation and some vacuolar formation were occurred in MDA-MB-231 cells at 80 μM rutaecarpine for 48 h [Figure 4b]. On the other hand, the effectiveness of rutaecarpine was lower at the higher concentration (160 μM) in both breast cancer cells. Consequently, rutaecarpine caused apoptotic cell death in these cells. Nevertheless, more apoptotic cell death morphology was observed in MCF-7 cells than MDA-MB-231 cells.

DISCUSSION

For the first time, we compared the potential therapeutic effect of rutaecarpine on different breast cancer cells and our findings demonstrated that hormone-sensitive cells (MCF-7 ER+, progesterone receptor positive (PR+), and human epidermal growth factor receptor 2 negative [HER2−]) were much more sensitive to rutaecarpine than triple negative breast cancer cells (MDA-MB-231 [estrogen receptor negative (ER−), progesterone receptor negative (PR−), and HER2−]). Furthermore, rutaecarpine caused apoptotic cell death and arrested accumulation of cells at G0/G1 phase.

Several *in vitro* studies show that COX-2 expression is not found in ER+ MCF-7 breast cancer cell lines, and moderate expression is detected in MDA-MB-231 cells.[17-20] However, Thill *et al*.[21] state that the expression of COX-2 is a 2-fold higher in MCF-7 cells compared to MCF-10F normal breast. Furthermore, Singh and Lucci.[22] detect an increase of COX-2 protein expression in hormone receptor-negative breast cancer. The difference in the mRNA and protein expression levels of COX-2 is likely to be based on posttranscription or posttranslational processing.[20] On the other hand, several studies report that COX-2 expression is correlated with ER−, PR−, and HER2+ status in breast cancer patients and indicates poor prognosis such as large tumor size, higher grade, and lymph node metastasis.[23,24] In the current study, our preliminary findings demonstrated that rutaecarpine was more effective in MCF-7 cells than MDA-MB-231 breast cancer cells. These effects could be associated with the inhibition of COX-2 by rutaecarpine. However, further studies should be evaluated to clarify the underlying molecular mechanism of COX-2 and the association of the efficacy of rutaecarpine with COX-2 inhibition in accordance with the subtype of breast cancer.

In the literature, many studies have focused on the comparison of evodiamine and rutaecarpine in breast (MCF-7 and

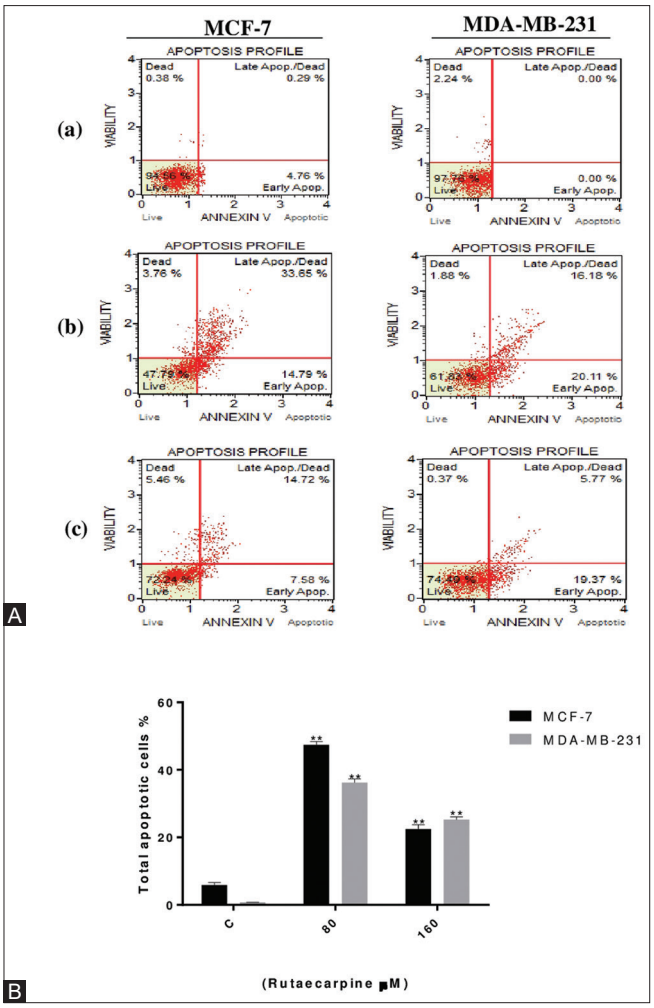

Figure 2: The apoptotic effects of rutaecarpine determined by Annexin V analysis. (A) MCF-7 and MDA-MB-231 cells treated with (a) control, (b) 80 μM, and (c) 160 μM rutaecarpine for 48 h. (B) Statistical comparison of the percentage of rutaecarpine-induced apoptotic cell death (**$P < 0.01$)

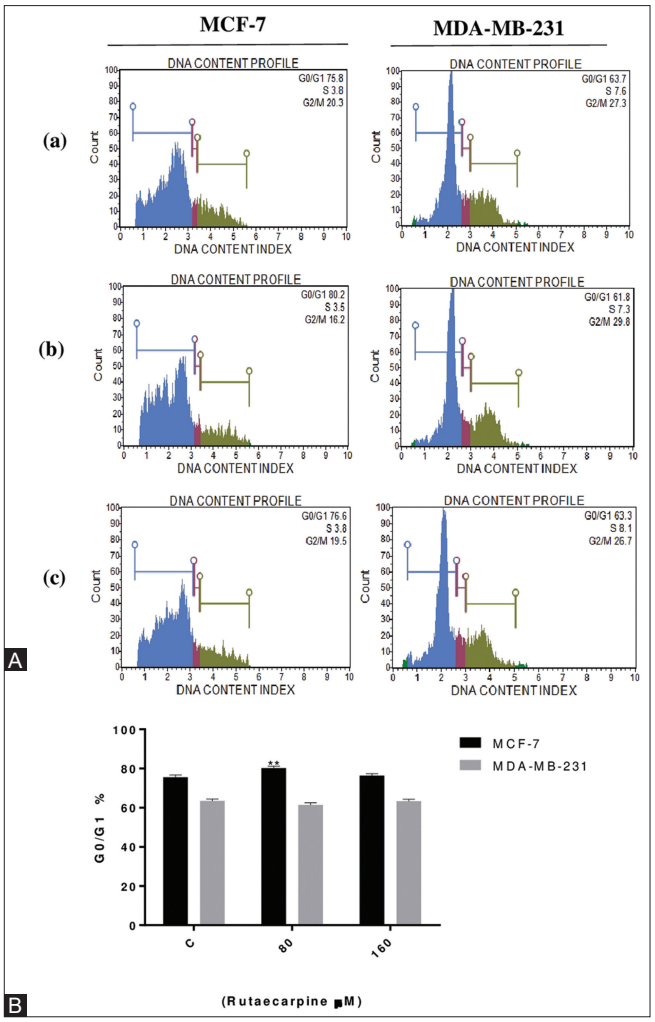

Figure 3: The effects of rutaecarpine on cell cycle distributions. (A) MCF-7 and MDA-MB-231 cells treated with (a) control, (b) 80 μM, and (c) 160 μM rutaecarpine for 48 h. (B) Statistical comparison of the accumulation of the cells in G0/G1 phase in breast cancer cells (**$P < 0.01$)

SMMC-7721) and ovarian cancer cells (SKOV3).[25,26] In addition, the antiproliferative effects of rutaecarpine have evaluated on many types of cancer including colon, leukemia, lung, renal, ovarian, and breast cancer cells.[16] *In vitro* studies show that the 50% growth inhibition concentration (IC50) changes based on the type of cancer (GI50: A549 human lung adenocarcinoma: 14.5 μM; HT-29 human colon carcinoma: 31.6 μM, OVCAR-4 ovarian cancer: 18.9 μM, and HS-578T breast cancer: 22.6 μM).[8,16] In addition, Guo *et al*.[26] investigate the cytotoxic effects of rutaecarpine (5–20 μM) and evodiamine (5–20 μM) for 48 h on MCF-7 and SMMC-7721 cells through 2D and 3D model. They found that the IC50 values of rutaecarpine are 44.1 μM and 24.2 μM for MCF-7 breast cancer and SMMC-7721 endocervical adenocarcinoma cells, respectively.[26] Therefore, we selected higher concentrations (0–160 μM) of rutaecarpine in our study, and we found that the IC50 concentration of rutaecarpine was 74.5 and 117.6 μM for MCF-7 and MDA-MB-231 cells, respectively. This difference could be derived from the selection of concentrations analyzed by WST-1 assay. Furthermore, the study of Zhang *et al*.[27] states that evodiamine is more potent than rutaecarpine on different cancer cell lines and evodiamine causes apoptotic cell death through apoptotic bodies and G1 arrest in L929 murine fibrosarcoma cells. Chen *et al*.[28] show that evodiamine treatment can result in the G2/M arrest and DNA fragmentation, and the activation of different caspase levels (3, 8, and 9) in ARO thyroid cancer cells. However, the underlying mechanism for the efficacy of rutaecarpine has not yet been studied. Our findings showed that rutaecarpine caused apoptotic cell death in these cells through G0/G1 arrest and nuclear blebbing and chromatin condensation. However, more detailed studies are needed to assess the molecular mechanism of rutaecarpine-induced apoptotic cell death in these cells.

CONCLUSION

Herein, we showed that rutaecarpine had more potentially cytotoxic and apoptotic effect on hormone-sensitive breast

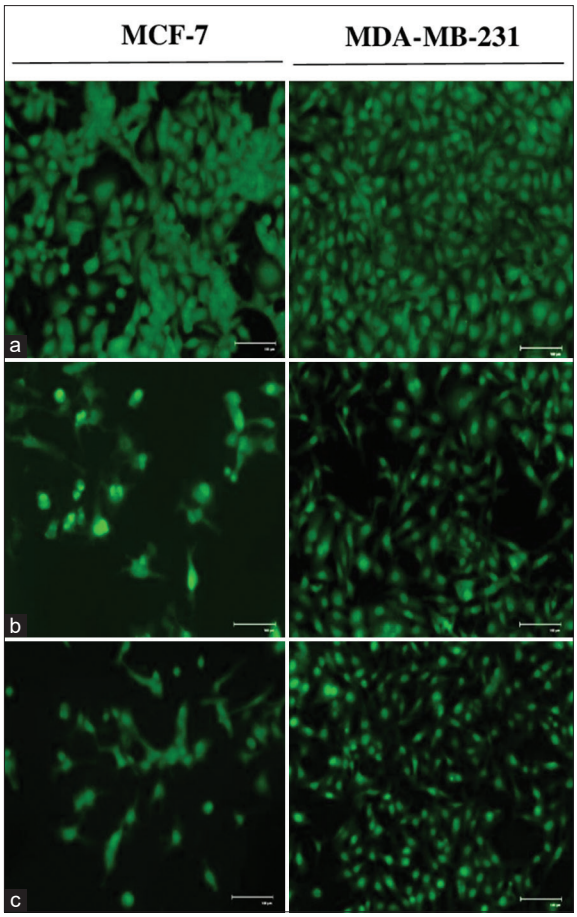

Figure 4: The effects of rutaecarpine on the morphology of MCF-7 and MDA-MB-231 cells determined by Acridine orange staining. MCF-7 and MDA-MB-231 cells treated with (a) control, (b) 80 μM, and (c) 160 μM rutaecarpine for 48 h

cancer cells than triple negative breast cancer cells. However, further studies are needed to assess the relationship COX-2 inhibition and other molecular mechanisms, inflammation, and apoptotic cell death between the efficacy of rutaecarpine on each subtype of breast cancer, *in vitro* and *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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