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Citrus aurantium Peel Extract Combined with Doxorubicin-Induced ROS-driven Cell Cycle Arrest and Apoptosis of Triple-Negative Breast Cancer Cells

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Abstract

BACKGROUND: The current approach to breast cancer has problems with the emergence of resistance, side effects, and even the emergence of post-therapy relapses. One of the reasons is that the available chemotherapy is still based on cytotoxicity through cell cycle inhibition and apoptosis induction. In fact, there are still several mechanisms for the direction of cytotoxicity to become more prospective targets of chemotherapy action, such as reactive oxygen species (ROS) leading to cell death induction. One prospective candidate from natural ingredients is Citrus aurantium peel extract (CSP).

AIM: This study aims to develop a CSP as a cochemotherapy candidate that leads to aging induction and ROS modulation in breast cancer cells.

METHODS: The breast cancer cell model used is triple-negative breast cancer cells, which is a highly metastatic cell model. Apoptosis and cell cycle modulation profile analyzed under PI-Annexin and PI-flow cytometry, respectively. The ROS level evaluated under DCFDA flow cytometry.

RESULTS: The combination of CSP and Dox induces oxidative stress with ROS levels up to 3.5 times. The increase in ROS levels was in line with the dose-dependent induction of apoptosis and induced G2/M phase cell cycle arrest.

CONCLUSION: Taken together, CSP is potentially induced Dox effect on MDA-MB-231 cells which may be mediated by the elevation of the ROS levels leading to cell death induction.

Introduction

Breast cancer is one of the most complex cancers. One type of breast cancer is triple-negative breast cancer (TNBC). TNBC is cancer cells that do not express estrogen, progesterone, and human epidermal growth factor receptor (HER2) receptors. TNBC represents aggressive breast cancer cells, with more frequent recurrence and metastasis rates than other breast cancer cells [1]. Breast cancer with this type of TNBC is difficult to treat with surgery, radiotherapy, or chemotherapy especially targeted therapy [2]. The current chemotherapy approaches are based on apoptotic mechanisms [3]. Apoptosis in cancer cells can occur as a result of cell senescence and can also be triggered by increased levels of cellular reactive oxygen species (ROS) [4], [5], [6]. Senescence that is often used with premature senescence is a physiological process that occurs at the cellular level involving complex signaling pathways that are generally triggered by increased ROS in cells. In general, the accumulation of ROS levels above the threshold can cause DNA damage that causes cell proliferation and causes cell death [7]. Therefore, the exploration of agents that can induce ROS levels is expected to increase cell death, especially in cancer cells.

Potential therapeutic candidates or even acting as new therapies based on natural compounds can be obtained from the active compound of *Citrus aurantium peel* extract (CSP). The flavonoid groups of CSP have antioxidant, anticancer, and anti-inflammatory activities [8]. The previous studies reported that hesperidin from citrus peel extract has a cytotoxic effect on cancer cells resistant to doxorubicin (Dox) MCF-7, MCF-7 HER, MDA-MB-231, and ovarian cancer cells [9], [10]. The use of herbal compounds in the form of extracts, fractions, or pure compounds has been shown to reduce side effects caused by active activities [11]. Clinical and preclinical studies show that the use of natural products can reduce neurotoxicity side effects, neurotoxicity side effects, and nephrotoxicity due to chemotherapy and radiotherapy [12]. Dox chemotherapy has been shown to cause the formation of E-cadherin, which triggers the migration and metastasis of breast cancer cells [13], [14], [15]. Recently, the effect of CSP as

cochemotherapy to prevent side effect of Dox is still unclear. Therefore, this study aims to evaluated the effect of CSP in combination with Dox on the cell death mechanism through ROS elevation on TNBC cells.

Materials and Methods

Preparation of crude extracts of C. aurantium

CSPs were successively extracted using ethanol. Three hundred grams of dry peels pieces mixed with ethanol 96% and macerated for 3 days. The filtrate was filtered with Whatman filter paper and concentrated under reduced pressure on rotary vacuum evaporator (IKA RV10®) at 50°C. The crude extracts were dried in a vacuum freeze dryer and preserved at −20°C for subsequent analysis [15].

Cell cycle analysis using flow cytometry

The flow cytometer measures the cell cycle distribution. The MDA-MB-231 cells were treated with CSP and Dox on several doses for 24 h and collected, washed twice with PBS, and then suspended in 500 μL of binding buffer. Five microliters of Annexin V-fluorescein isothiocyanate and 5 μL of propidium iodide (PI) were added for apoptosis measurement, and 500 μL of PBS containing 50 μg/mL PI were added for cell cycle measurement, and then in the dark at room temperature incubate for 15 min and detect the expression of fluorescence with a flow cytometer [16].

Apoptosis analysis

Apoptosis assay was performed using Annexin V-FITC/PI using previously described protocol with some modification [17], [18]. Briefly, 2×10^5 cells per well were seeded in six well-plates overnight. Cells were, then, treated with CSP and Dox at various concentrations for 24 h. Live cells were stained with FITC-annexin V (BD Biosciences, San Jose, CA) for 15 min in 1x biding buffer and then 5 μL of PI (Himedia) for 15 min at room temperature in the dark. Cells were washed 3 times with PBS and percent apoptosis cells were acquired using BD Accuri C6 flow cytometer (BD, Singapore) and data were analyzed using BD Accuri C6 Plus software (BD Biosciences, CA).

ROS level analysis

For all ROS experiments, MDA-MB-231 cells were collected by centrifugation, washed with PBS, and incubated with 2.5 μM DCFDA in supplemented buffer (10% FBS in PBS) for 30 min in the dark at 37°C. Each cell was treated with CSP (86 and 172 μg/mL), Dox 10 nM, and combination thereof then incubated for 4 h in 37°C CO_2 5%. Intracellular ROS was determined by flow cytometry (DB Accury C6 plus, BD Biosciences, USA) [19].

Statistical analysis

All data were presented as mean ± standard deviation (SD). All calculations were carried out using SPSS 23.0 (IBM Corp., Armonk, NY, USA). Analysis of variance (ANOVA) followed by a Tukey *post hoc* was used for multiple comparisons. $p < 0.05$ was considered to be statistically significant.

Results

The previous study reported that CSP extract has mild cytotoxic effect on several breast cancer cells. However, the mechanism underlying the effect of CSP on triple-negative breast cancer cells (TNBCs) still unclear. Therefore, this study investigated the mechanism of the cytotoxic effect of CSP on TNBC cells. First, we evaluated the cell cycle analysis on several doses of CSP for 24 h. The doses of this study depend on the our previous study, IC50 of CSP on MDAMB-231 cells, and the IC50 value was 344 μg/mL. In addition, Dox reported several side effects such as chemoresistance and metastasis induction. In this study, we design to combinated doxorubicin and CSP as a cochemotherapy agent to reduce the side effect of Dox.

The combination of CSP- and Dox-induced G2/M cell cycle arrest

CSP at a single dose was shown to induce a dose-dependent S phase cell cycle arrest. Dox singly induces the cell cycle in the G2/M phase. These results support previous studies which stated that Dox induces cell cycle arrest in breast cancer cells in the G2/M phase in a dose- and time-dependent manner. The combination of CSP and Dox also induces cell cycle arrest in the G2/M phase. Interestingly, the combination of the two induces an increase in the Sub G1 phase, indicating cell death (Figure 1). This phenomenon was confirmed by testing for apoptosis by flow cytometry.

The combination of CSP- and Dox-induced apoptosis

Low-dose CSP was not significantly different in inducing apoptosis compared to controls. However, high doses of CSP induced cell death of up to 13.8%.

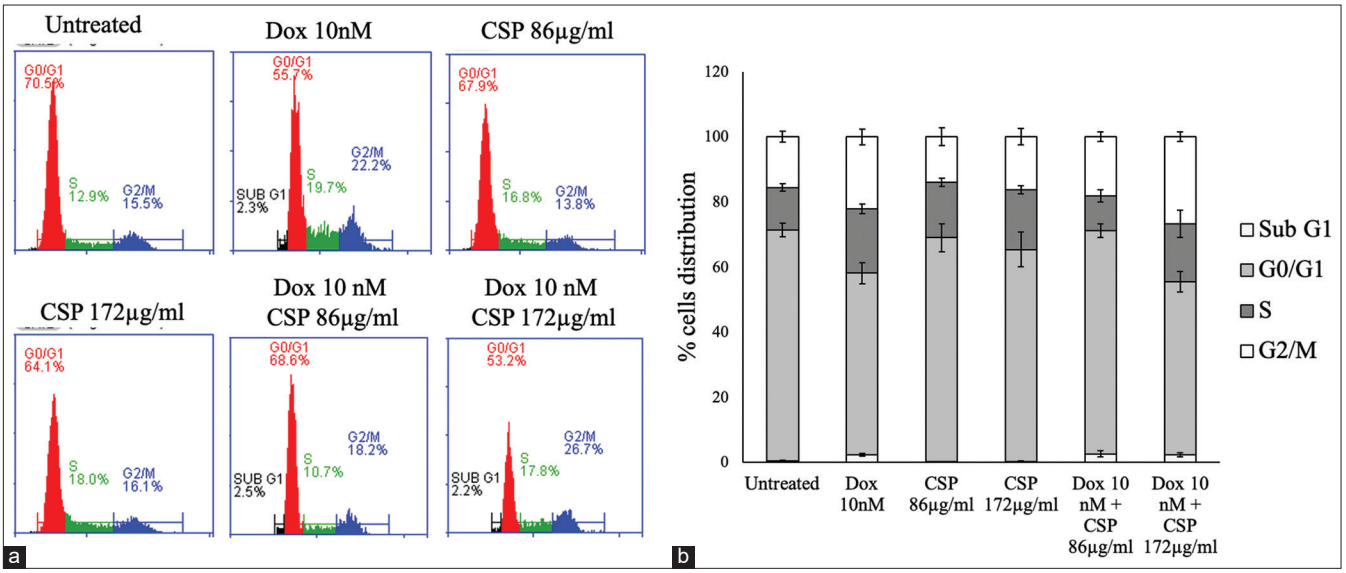

*Figure 1: Cell cycle distribution of Citrus aurantium peel, Dox, and combination thereof on MDA-MB-231 cells. The assay was conducted based on flow cytometry as described in the method. (a) The flow cytometry profile of the treatments. (b) The percentage cell cycle distribution of each phase under different concentration treatments for 24 h on MDA-MB-231 cells. The columns represent the mean ± SD of three independent trials with at least three replicates. Statistical difference was analyzed using two-way ANOVA; *p < 0.05*

Combination with Dox significantly induced cell death up to 67.4% (Figure 2).

The combination of CSP- and Dox-elevated ROS level

Single treatment of CSP and DOX in combination had a synergistic cytotoxic effect after 24 h of treatment. The combination of the two induces cell death, and one of the mechanisms of inducing death is through increased levels of ROS. CSP-induced ROS up to 1.5 times greater than control cells. Interestingly, when combined with Dox, high CSP doses induced ROS levels up to 3.5 times (Figure 3). Increased levels of ROS above the threshold lead to the induction of cancer cell death and suppress cancer cell metastasis.

Discussion

Breast cancer as a cancer disorder is characterized by the increase of an immature, abnormally differentiated cell population in the breast and peripheral blood [2], [20]. About one-third of the breast cancer patients do not attain a complete remission with conventional chemotherapy treatment developed drug-resistant, which is considered a highly

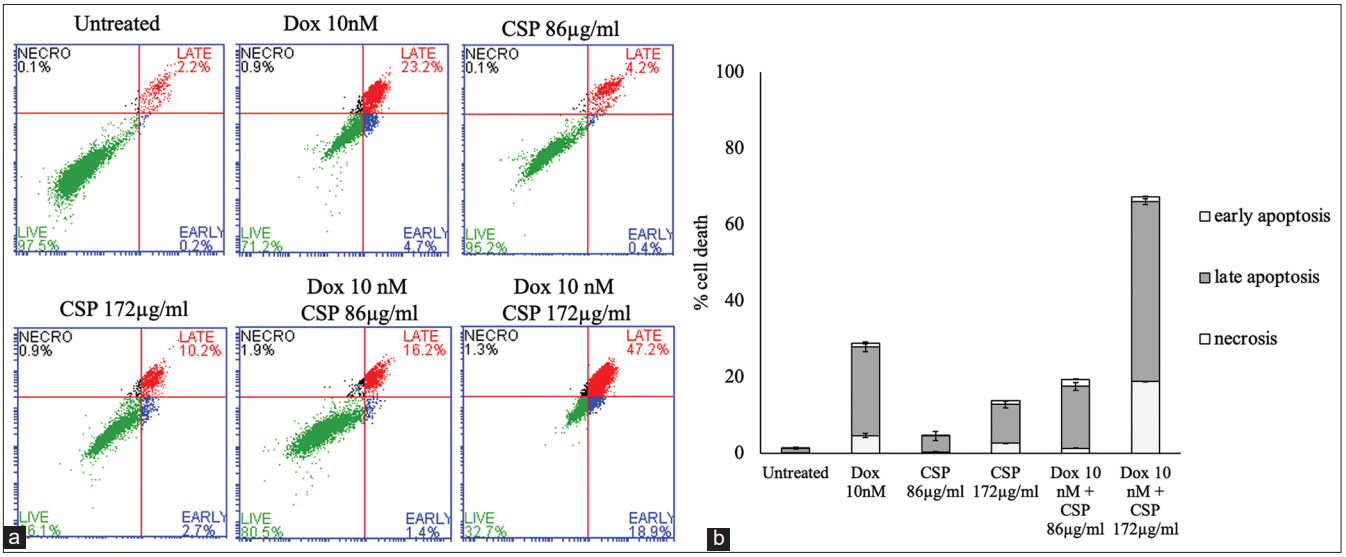

*Figure 2: Citrus aurantium peel (CSP) and the combination with Dox-induced apoptosis of MBAMB-231 cells. (a) The flow cytometry-based assay was performed on 24 h treated cells. (b) The quantification of percentage apoptosis cells under CSP and the combination with Dox treatment for 24 h on MDA-MB-231 cells. The columns represent the mean ± SD of three independent trials with at least three replicates. Statistical difference was analyzed using two-way ANOVA; *p < 0.05*

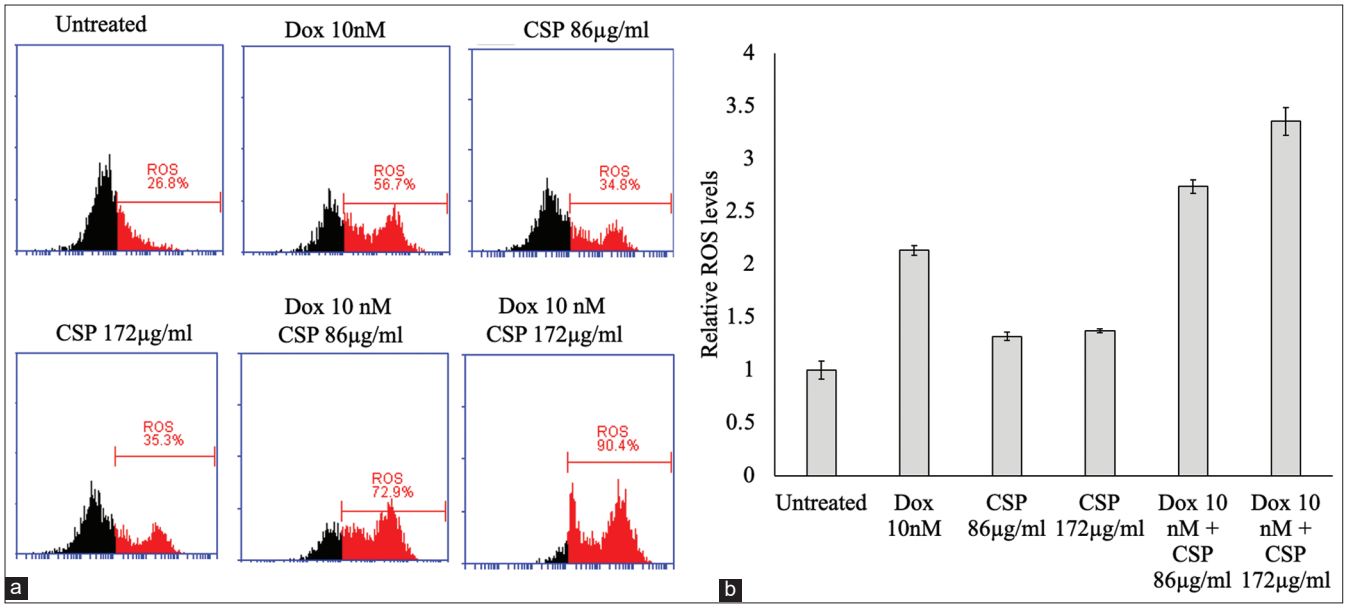

*Figure 3: The effect of combination Citrus aurantium peel and DOx-induced reactive oxygen species (ROS) generation in MDA-MB-231 cells. (a) ROS generation was determined by fluorescence microscopy and FACS flow cytometer after DCFDA staining in MDA-MB-231. (b) The percentage of DCFDA stained cell population converted to fold of change in MDA-MB-231 and given as a bar graph. The columns represent the mean ± SD of three independent trials with at least three replicates. Statistical difference was analyzed using two-way ANOVA; *p < 0.05.*

heterogeneous disease showing aggressive behavior toward all of the normal strategies of treatment therapy [21], [22], [23]. Natural compounds and their derivatives as enhancer drugs play an important role in cancer treatment. In addition, chemotherapy such as Doxorubicin has cytotoxic effects on normal cells, causes drug resistance, and induces migration. This is a major obstacle in cancer treatment with chemotherapeutic agents. To mitigate the side effects and drug resistance, modified therapeutic regimens such as cochemotherapy have been introduced. Therefore, our study aims to evaluate the effect of combination CSP extract and Dox on MDA-MB-231 cells.

Our study found that under PI staining of cell cycle distribution, the combination of CSP, and Dox-induced G2/M and Sub G1 cell cycle arrest. The elevation of Sub G1 marked apoptotic cell death. This findings consistent with the previous study that several citrus species including *Citrus sunki, C. aurantium,* and *Citrus plantymamma* induced G2/M cells cycle arrest on HL-60, AGS, and A549 cancer cells [24], [25]. The citrus species induced G2/M cell cycle arrest by increasing the expression of p21 and decreasing the expression of cyclin B1, cell division cycle 25C (CDC25C), and cyclin dependent kinase 1 (CDC2) [26]. Furthermore, the induction of sub G1 cell cycle arrest indicated the debris cells that correlated with apoptosis cells. Therefore, in this study, we also evaluated the apoptosis effect of combination CSP and Dox. We found that combination of CSP- and Dox-induced apoptosis on doses-dependent manner up to 67.4%. This mechanism might be CSP induce sensitivity of Dox tolerance MDA-MB-231 cells. In addition, this approach might lead toward reduced or minimal toxicity of the chemotherapeutic agent. In addition, apoptosis induction by citrus extract was reported due to its ability to caused cell cycle arrest in G1 phase. The previous study stated that *Citrus unshiu* extract induces apoptosis mainly through intrinsic pathway by reducing anti-apoptosis B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-XL) expression and increasing proapoptotic proteins [Bcl2 associated X protein (Bax), proapoptotic Bcl-2 protein (Bid), Bcl2 homologous antagonist killer (Bak), and Bcl-2-associated death promoter (Bad) in different cancer cell lines [26], [27], [28], [29].

To characterize the mechanism underlying CSP-elicited apoptosis, we have shown that CSP can increase the level of intracellular ROS production in MDA-MB-231 cell lines. It has been shown that ROS accumulation results in impairment of some cellular functions and leads to the promotion of apoptosis [30]. Our data are consistent with the data presented in the study of Monti *et al* that sodium butyrate induced apoptosis through oxidative stress [31]. Interestingly, the CSP single treatment possess antioxidant effect; however, in combination treatment with Dox, CSP induced sensitivity of Dox to increased ROS levels.

Conclusion

Taken together, we concluded that CSP potential natural chemotherapeutic agent may be used to reduce the side effect of Dox-induced cancer resistance.

Authors' Contributions

NDA and MZ: Conception, design, and manuscript writing; NDA, MZ, and BC: Provision of study material and data analysis; NDA: Provision of data interpretation; NDA: Conduct experiment. All authors read and approved the final manuscript.

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