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INTERNSHIP ARTICLE

Beet Root Juice Promotes Apoptosis in Oncogenic MDA-MB-231 Cells While Protecting Cardiomyocytes Under Doxorubicin Treatment

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Abstract

Doxorubicin (DOX) is a well-established chemotherapeutic drug widely used to treat a variety of cancers, though its clinical use is restricted by irreversible cardiotoxicity. Earlier studies show beetroot juice (BRJ) as a potent chemopreventive agent; however its cardioprotective function is yet to be established. The purpose of this experiment is to determine the protective effect of BRJ against DOX-induced cardiotoxicity. It was hypothesized that higher concentrations of BRJ, applied to cardiomyocytes, will provide better cardioprotection against DOX, and simultaneously increase cell death of MDA-MB-231, breast cancer cells, in combination with DOX. Adult rat cardiomyocytes and MDA-MB-231 cells were exposed to different concentrations (0.5, 5, 50, 250, 500 µg/ml) of BRJ with or without DOX, with a control of growth medium. Cell death, measured by trypan blue staining, was significantly reduced in cardiomyocytes but increased in MDA-MB-231 following 24 hours of co-treatment with BRJ and DOX. Cell viability was also significantly reduced after BRJ and DOX co-treatment in MDA-MB-231 cells. Similarly, DOX-induced-apoptosis (programmed cell death), as determined by TUNEL assay, was significantly reduced with BRJ treatment for 48 hours in cardiomyocytes. In contrast, BRJ significantly increased DOX-mediated apoptosis in cancer cells. In conclusion, lower concentrations of BRJ with DOX represented the most effective combination of cardioprotection and chemoprevention. These findings provide insight on the possible cardioprotective ability of BRJ in cancer patients treated with anthracycline chemotherapeutic drugs.

Introduction

Doxorubicin (DOX), a quinone-containing anthracycline antibiotic, is a well-known chemotherapeutic drug widely used to treat a variety of human carcinomas[1] including breast cancer, ovarian cancer, and solid tumors. The clinical use is restricted by its severe side effects, especially cardiotoxicity, leading to cardiomyocyte death[2,3]. Despite the side effects, DOX still remains a top choice for chemotherapy for its superior chemotherapeutic effectiveness.

A mechanism of cardiotoxicity of DOX is its redox-activation of reactive oxygen species (ROS) that ultimately results in myocyte apoptosis[4]. ROS are chemically-reactive molecules containing oxygens that take electrons from other molecules and become electrically charged, which damages DNA, RNA, proteins, and lipids. Tumor formations are associated with inflammation due to oxidative stress and ROS generation[5,6]. Natural antioxidants play a critical role in human health by averting vital biological molecules from oxidative damage[7]. Antioxidants are substances found in fruits, nuts, grains and vegetables that inhibit the oxidation process of other molecules and reduce inflammatory properties. Currently, studies show that compounds with antioxidant activity may protect against DOX-induced cardiotoxicity[8,9].

Few table foods contain betacyanins and other betalains, important anticancer constituents for their high radical scavenging activity[10]. Beetroot juice (BRJ) contains colorants that can be divided into two categories of betalains: the red betacyanins and the yellow betaxathines. Betalain is important for cardiovascular health. Betanin, which makes up 95% of the total betacyanins, is more stable in the beetroot extract than in pure chemical form[11,12]. Red beetroot (Beta vulgaris) is a potent chemopreventive agent which reduces cell proliferation, angiogenesis, inflammation and stimulates apoptosis in skin, liver, lung, and esophageal cancer[10,13,14]. However, due to its well recognized antioxidant properties, BRJ may possess an alleviating effect on DOX-induced cardiotoxicity. Based on this compelling background information, it was hypothesized that the combination of DOX and BRJ can enhance the chemotherapeutic efficacy of DOX while reducing the cardiotoxic side effects. The use of BRJ will be safer and more cost effective and could save cancer patients and improve their quality of life by reducing harsh side effects of DOX such as diarrhea, constipation, hair loss and vomiting.

The purpose of this study is to determine the effect of different concentrations and combinations of BRJ and DOX on adult rat cardiomyocytes and a human breast cancer cell line (MDA-MB-231) to demonstrate the possible cardioprotective role of BRJ against DOX, as well as chemotherapeutic ability. Based on numerous studies discussing the antioxidant potency and chemotherapeutic effects of BRJ, in this study it is hypothesized that BRJ will protect cardiomyocytes against DOX-toxicity; as well as, BRJ will enhance DOX-induced cancer cell death. A null hypothesis states that BRJ will have no significant effect on DOX-induced cardiotoxicity or cancer cell death.

Materials and Methods

Adult male Wistar rats (300 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Ventricular cardiomyocytes were isolated using an enzymatic technique as

previously reported[15,16]. In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg intraperitoneally), and the heart was quickly removed from the chest. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system, and the heart was retrogradely perfused (37 °C) at a constant pressure of 55 mm Hg for ~5 min with a Ca2+-free bicarbonate-based buffer containing 120 mm NaCl, 5.4 mm KCl, 1.2 mm $MgSO_4$, 1.2 mm NaH_2PO_4, 5.6 mm glucose, 20 mm $NaHCO_3$, 10 mm 2,3-butanedione monoxime, and 5 mm taurine (Sigma-Aldrich Co., St. Louis, MO) that was continuously gassed with 95% O_2 + 5% CO_2. The enzymatic digestion was commenced by adding collagenase type II (Worthington, Lakewood, NJ; 0.5 mg/ml each) and protease type XIV (0.02 mg/ml) to the perfusion buffer and continued for ~15 min. 50 µm Ca2+ was then added in to the enzyme solution for perfusing the heart for another 10–15 min. The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a three-step Ca2+ restoration procedure (i.e. 125, 250, and 500 µm Ca2+). The freshly isolated cardiomyocytes were plated on 4-chamber slides (for cell death and apoptosis assays) with Medium 199 (Invitrogen Corp., Grand Island, NY) containing 2 mm l-carnitine, 5 mm creatine, 5 mm taurine, 5 mm glucose, 0.1 µm insulin (Sigma-Aldrich Co., St. Louis, MO), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) (Invitrogen Corp., Grand Island, NY). Cells were placed in a 37°C incubator with 5% carbon dioxide. BRJ extract, bought from a local vitamin store, was dissolved in growth medium to prepare a stock solution of 10 mg/mL. Ten milliliters of 0, 0.5, 5, 50, 250, and 500 µg/mL BRJ were prepared by adding 0, 0.5, 5, 50, 250, and 500 µL BRJ (10 mg/mL) in 10 mL medium. DOX (molecular weight 579.98, Sigma-Aldrich Co., St. Louis, MO) was dissolved in water to prepare 1 mg/mL solution. Ten milliliters of 2 µM DOX was prepared by adding 11.6 µL of DOX (1 mg/mL) to 10 mL of medium. An hour after plating, the cells were treated with 2 µM DOX with/without the different concentrations of BRJ. After 24 hours, the cell death was measured by adding 15 µL Trypan blue solution (0.4% solution, Sigma-Aldrich Co., St. Louis, MO). The dead cells, stained blue, were counted under a microscope. The percentage of cell death was determined and recorded. For apoptosis, cells were fixed with 4% paraformaldehyde solution after 48 hours of the treatments and stained using a Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) kit (BD Bioscience, San Jose, CA). The apoptotic cells (fluorescent green) and total cells were counted under a fluorescence microscope and the data were recorded. To examine the chemotherapeutic effects of BRJ, human breast cancer cells, MDA-MB-231 cells (ATCC#: HTB-26, Manassas, VA) were plated on 6-well plates (for cell death assay), chamber slides (for apoptosis assay), and 96-well plates (for cell viability assay) with RPMI medium containing 10% FBS and 1% PS. After 24 hours, cells were treated with 1 µM DOX with/without the different concentrations of BRJ. After 24 hours, the cells were collected following trypsinization and centrifugation at 4000g, and re-suspended in 2 mL phosphate buffer saline (pH 7.4) with 20 µL of Trypan blue solution. The dead cells, which were stained blue, and live cells, not stained, were counted on a hemacytometer under a microscope. The percentage of dead cells was determined and recorded. Apoptosis were determined by TUNEL staining after 48 hours of treatment, as described previously. The cell viability assay (after 24 hours of treatment) was performed using CellTiter96@Aqueous One Solution Cell Proliferation Kit (Promega Corp., Madison, WI) according to manufacturer's protocol. After treatment, 20 µL of reagent with 100 µL medium was added in each well and incubated at 37°C for an hour. On a microplate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Device), absorbance was recorded at 490 nm. For the cell death and cell viability, 8 repeated trials and for apoptosis, 4 repeated trials were conducted for both cardiomyocyte and MDA-MB-231 cells. Data were analyzed using an ANOVA test. This experiment was classified under Biosafety Level 1 containment, because of the cells and DOX.

Results

The effects of BRJ on DOX-induced cardiotoxicity were studied and the results are displayed in Figures 1-7 and Supplementary Data Tables 1-7. The mean was determined for each level of the independent variable. The variance, standard deviation, and ANOVA test at a 0.001 level of significance were calculated and analyzed.

In cardiomyocytes, DOX (2 µM) significantly increased cell death (35.390±2.998%) compared to the control (7.893±1.522%) after 24 hour treatment (n=8, p<0.001; Figure 1, Supplementary Data Table 1). Remarkably, most BRJ treatment with DOX significantly reduced DOX toxicity to cardiomyocytes (n=8, p<0.001), except 500 µg/mL of BRJ+DOX (38.950±3.756%). However, from Figure 1, it can be noted that with increasing concentrations of BRJ alone, cell death increased compared to the control (n=8, p<0.001); though it is lower compared to DOX alone (n=8, p<0.001).

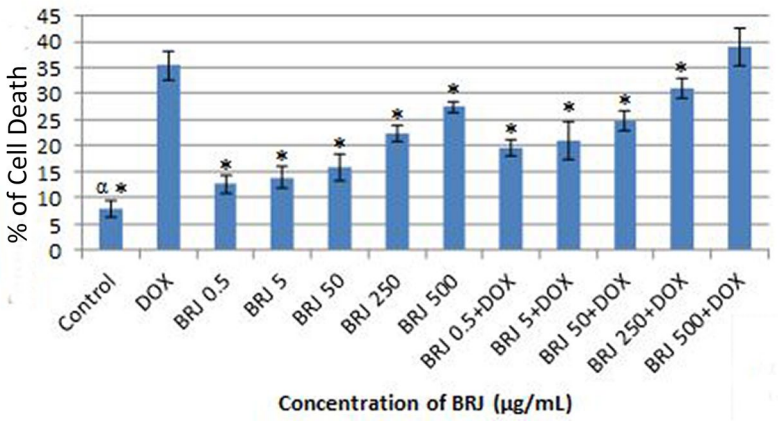

Figure 1. Percentage of rat cardiomyocyte cell death after 24 hour treatment with DOX (2µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Significance α to all p<0.001, to DOX p<0.001.

Cardiomyocyte apoptosis was significantly increased with DOX (23.190±4.068%) when compared to the control (1.969±0.753%) after 48 hour treatment (n=4, p<0.001; Figure 2, 3; Supplementary Data Table 2). BRJ treatment with DOX significantly reduced cell apoptosis compared to DOX alone (n=4, p<0.001), except for 500 µg/mL of BRJ+DOX (17.22±1.422%; p<0.01). Though the apoptosis of BRJ+DOX treatment was significantly lower than DOX alone, it was significantly higher than the control (n=4, p<0.001). BRJ alone

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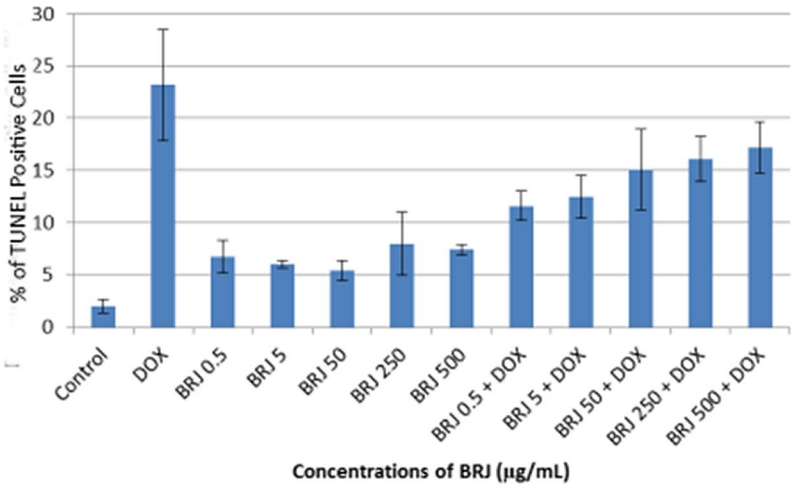

Figure 2. Percentage of rat cardiomyocyte apoptosis after 48 hour treatment with DOX (2µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Significance α to control p<0.001, to DOX p<0.001.

did not significantly increase apoptosis compared to the control.

MDA-MB-231 cells were studied to understand the role of BRJ on cancer cells in addition to cardiomyocytes. DOX increased cell death (21.010±2.223%) as compared to the control (10.090±6.771%), although not significantly (n=8, p<0.01) (Figure 4, Supplementary Data Table 3). However, all concentrations of BRJ with DOX significantly increased cell death with respect to the control and DOX alone (n=8, p<0.001). In order to confirm cell death results, a cell viability assay was performed on the MDA-MB-231 cells following 24 hours of treatment. DOX significantly reduced the viability (72.05±6.774%) compared to the control (100.0±8.537%; n=8, p<0.001; Figure 5, Supplementary Data Table 4). Lower concentrations of BRJ alone (0.5, 5, 50 µg/mL) didn't not significantly alter the cell viability compared to the control (n=12, p>0.05). However, higher concentrations of BRJ (250, 500 µg/mL) significantly reduced viability (n=8, p<0.001). All concentrations of BRJ treatment with DOX significantly reduced cell viability compared to the control and DOX (n=8, p<0.001).

Apoptosis of MDA-MB-231 was significantly increased after 48 hour treatment with DOX (13.74±3.182) and all concentration of BRJ alone compared to the control (1.683±0.52%) (n=6, p<0.001; Figure 6, 7; Supplementary Data Table 5). Combination treatment of BRJ and DOX further increased (nearly double) the apoptosis compared to DOX alone (n=6, p<0.001) with increasing concentration of BRJ.

Discussion

Due to the fact that DOX has been found to be an effective but cardiotoxic chemotherapeutic drug, the main focus in recent years is to find potential dietary supplements to minimize the detrimental effects of DOX on the heart. DOX-induced cardiotoxicity remains an everlasting struggle in the clinical practice of the drug[17]. The purpose of the present study was to determine the protective effect of BRJ against DOX-induced cardiotoxicity. Earlier studies show that BRJ is a potent chemopreventive agent in mouse models, however its cardioprotective function

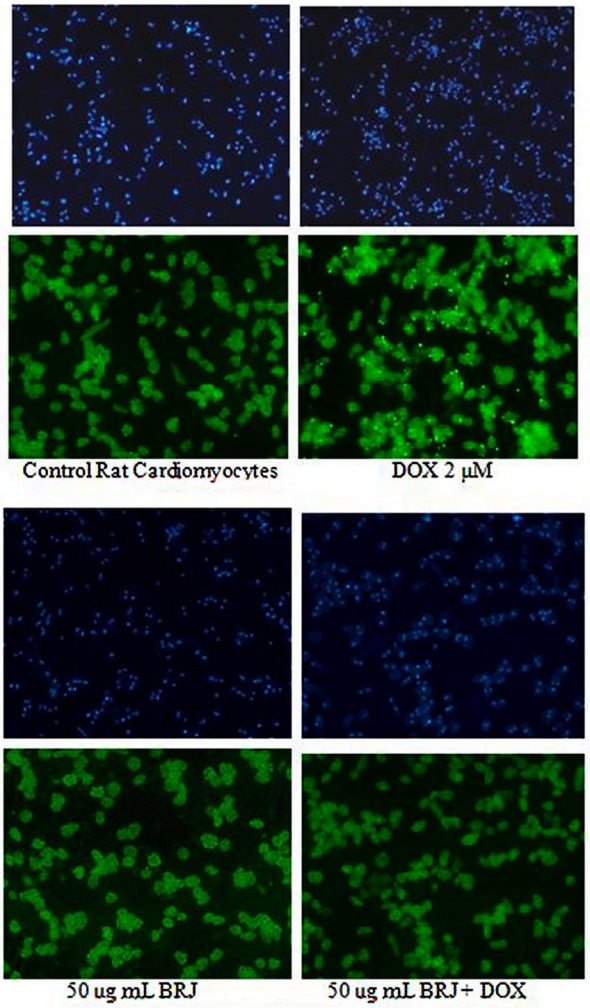

Figure 3. Representative pictures of TUNEL staining in rat cardiomyocyte after 48 hour treatment with DOX (2µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Apoptotic cells have bright, fluorescent green nuclei.

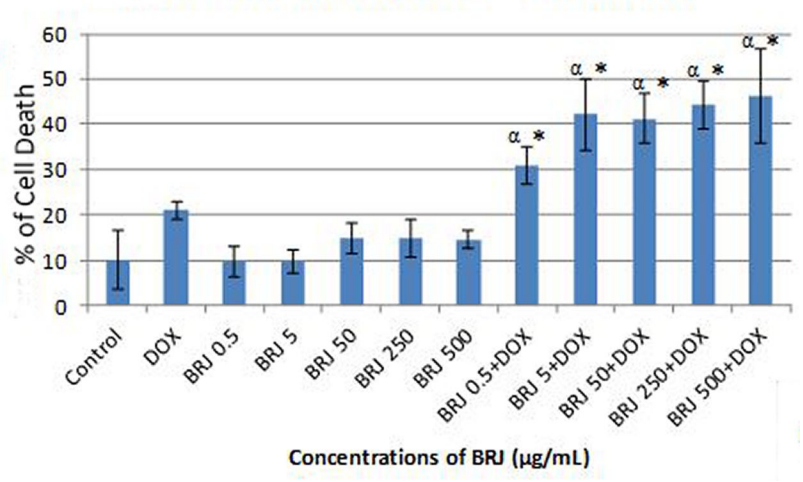

Figure 4. Percentage of MDA-MB-231 cell death after 24 hour treatment with DOX (1µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Significance α to control p<0.001, to DOX p<0.001.

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is unknown[13,14]. In the present study, cardiomyocytes were exposed to different concentrations of BRJ with/without DOX; and the cell death and apoptosis were examined. The BRJ concentrations used in our experiments (i.e., 0.5, 5, 50, 250, 500 µg/mL) were selected according to the previously published study[11] and deemed to be physiologically relevant. To explore whether BRJ could sensitize breast cancer cells to DOX-induced cytotoxicity, the cell death, cell viability and apoptosis of MDA-MB-231 cells were evaluated. The concentration of DOX used in cardiomyocytes (2 µM) was selected according to Ludke et al. (2012)[18]; and in cancer cells (1 µM), according to Di et al. (2010)[19]. It was hypothesized that greater concentrations of BRJ would provide protection from DOX-induced cardiotoxicity in rat cardiomyocytes while inducing further cytoxicity of DOX in MDA-MB-231 cells, based on its antioxidant properties[10]. The present study found that lower concentrations of BRJ protect cardiomyocytes against DOX-induced toxicity, while inducing breast cancer cell death with the aid of DOX. The research hypothesis was partially supported, rejecting the null hypothesis, because BRJ does provide cardioprotection at lower concentrations.

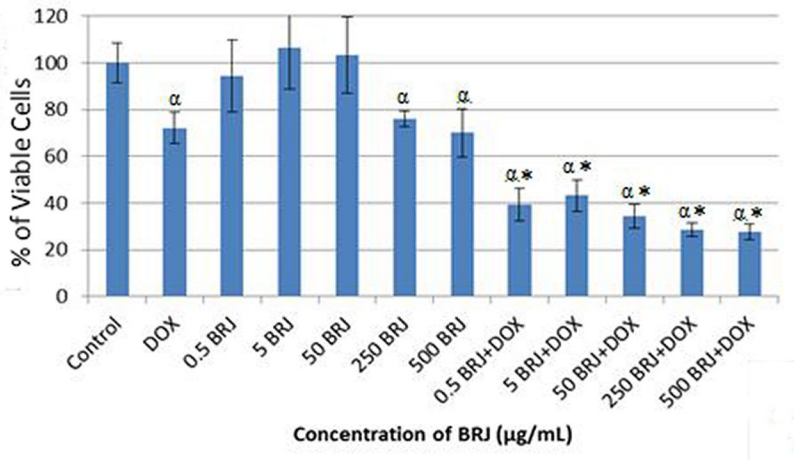

Figure 5. Percentage of MDA-MB-231 cell viability after 24 hour treatment with DOX (1µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Significance α to control p<0.001, to DOX p<0.001.

The cardiotoxicity of DOX involves the redox-activation of ROS in cardiomyocytes, which induced cardiomyocyte cell death[4]. However, the ideal therapeutic approach against DOX cardiotoxicity is yet to be established. The present study indicated that cardiomyocyte cell death increases with DOX and also with increasing concentrations of BRJ alone, compared to control. However, the combination of BRJ with DOX significantly reduced DOX toxicity to cardiomyocytes, except for the higher dose i.e., 500 µg/ml BRJ. In contrast, in breast cancer cells, MDA-MB-231, all concentrations of BRJ in combination with DOX significantly increased cell death with respect to the control and DOX alone. Cell viability also significantly reduced after BRJ and DOX co-treatment compared to DOX in MDA-MB-231 cells.

Apoptosis is an essential process in human development, immunity, and tissue homeostasis[20]. Failure of apoptosis could allow the survival of cancer cells that are prone to undergo further genetic damage and play an important role in cancer growth[21]. Most chemotherapeutic agents, particularly DOX, kill tumor cells by a complex mechanism of action: they intercalate DNA,

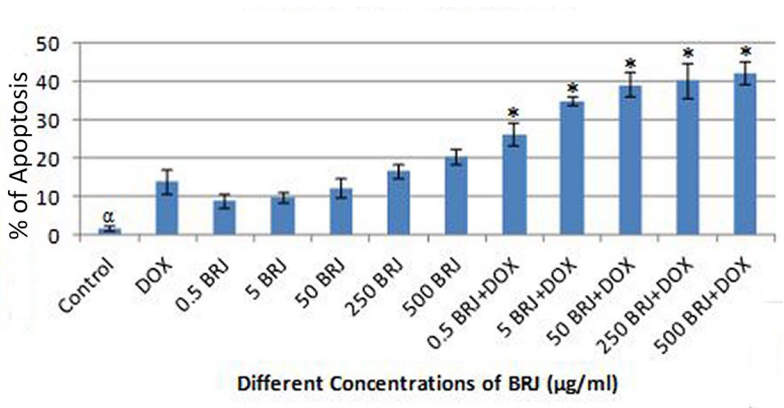

Figure 6. Percentage of MDA-MB-231 apoptosis after 48 hour treatment with DOX (1µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Significance α to all p<0.001, to DOX p<0.001.

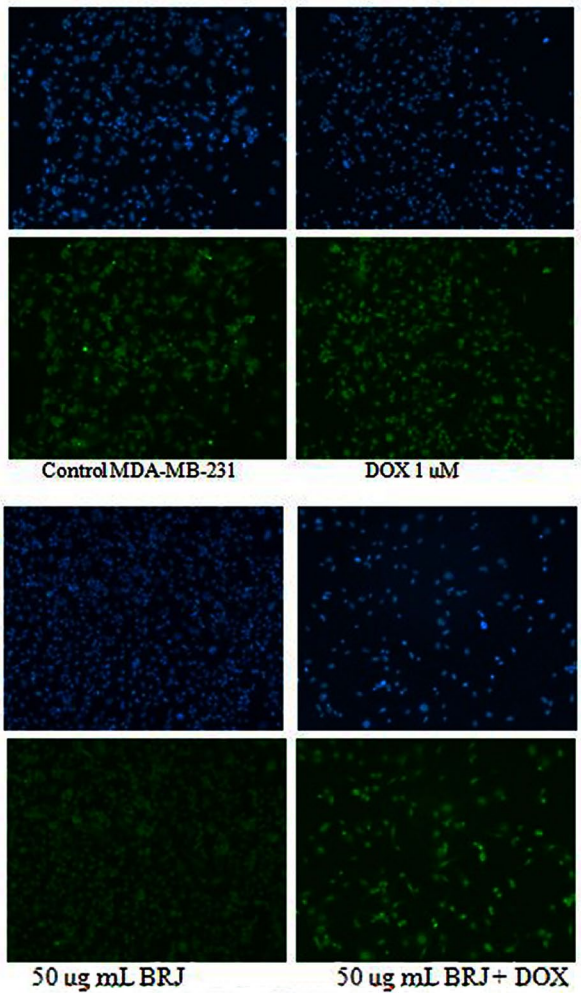

Figure 7. Representative pictures of TUNEL staining in MDA-MB-231 cells after 48 hour treatment with DOX (1µM) and/or BRJ (0.5, 5, 50, 250, 500 µL). Apoptotic cells have bright, fluorescent green nuclei.

generate free radicals and inducing apoptosis[4,22] . In the present study, BRJ significantly protected the cardiomyocytes from DOX-induced apoptosis, though it was significantly higher than the control. In MDA-MB-231 cells, BRJ significantly increased apoptosis in combination with DOX when compared to DOX alone. This data supported the research hypothesis, rejecting the null.

The present study occasionally displayed high values for variance, due to the different isolated batches of cardiomyocytes and passage of MDA-MB-231 cells. It is anticipated that this variability will be reduced by conducting additional trials. A future aspect would be to test other types of cancer, to investigate the mechanism of apoptosis and to measure the generation of reactive oxygen species in both cancer cells and cardiomyocytes following treatment with DOX and/or BRJ. Improving the antioxidant defenses of cardiomyocytes could be one strategy for cardiac protection against oxidative stress-mediated DOX-induced apoptosis[18]. In BRJ, betalains possess severable positive aspects: antioxidant, anti-inflammatory, and antitumor properties[23].

To expand the scope of this work in the future, mechanistic studies on protection of cardiomyocytes with BRJ should be investigated, as well as BRJ's increased toxicity in cancer cells. Since the lowest dose of BRJ shown on Figure 1 resulted in nearly 50% reduction in cardiomyocyte cell death, reducing the concentration of BRJ to determine the EC50 (half maximal effective concentration) for the cardioprotective dose of the juice would be advantageous. These data would help to compare the efficacy of BRJ on different cells with the various doses of DOX. Also, the effect of lower doses of BRJ needs to be determined on apoptosis in cardiomyocytes and on the cell death and viability assays for the cancer cell lines. These studies might make it easier to perform mechanistic investigations without the potential other effects of BRJ occurring at higher doses.

In conclusion, the present experiment provides valuable information for the possible application of the natural and safe food product BRJ to attenuate DOX-induced cardiotoxicity, in addition to preventing breast cancer. While higher concentrations of BRJ induced more cancer cell death, lower concentrations of BRJ in combination with DOX proved to be the most effective combination for both cardioprotection and chemoprevention. These findings are potentially significant in the clinical world to advance in the cardioprotection against DOX in patients with cancer.

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