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Research Article
Chemoprotective Efficacy of Salvianolic Acid B via Triggering Apoptosis in MCF-7 Human Breast Cancer Cells

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Abstract

Background and Objective: Salvianolic acid B (SB) is one of the active components of a popular Chinese herb called *Salvia miltiorrhiza*, which possess numerous biological activities including anti-tumor. Current study was framed to evaluate the chemoprotective efficiacy of SB on human breast cancer MCF-7 cells. **Materials and Methods:** MCF-7 cells were exposed to dimethyl sulfoxide (DMSO-Control) as well as treated with an increased concentration of SB (25, 50, 100 and 200 μm) to explore the anti-proliferative (Cell viability), pro-oxidative and pro-apoptotic activities. Statistical analysis was carried out by student t-test using SPSS software. **Results:** MCF-7 cells treated with an increased concentration of SB after 24 and 48 h showed a sharp decline in the count of viable MCF-7cells and thus showcased its potent anti-proliferative and toxicity property. A pronounced increase in ROS generation was noted in MCF-7 cells co-cultured with SB in dose and time-dependent fashion. Likewise, DNA fragmentation and apoptotic markers like caspase 3 and 9 were also significantly increased upon treatment with SB in a dose-dependent manner to display its pro-oxidative and pro-apoptotic activities. Moreover, the protein expression of Bcl2 and Bax were notably downregulated and upregulated respectively after administration with increased concentration of SB than DMSO treated MCF-7 cells. **Conclusion:** Taking together, that increased concentration of SB could show better chemoprotective activity on human breast cancer MCF-7 cells by exhibiting anti-proliferative, pro-oxidative and pro-apoptotic activities in a dose-dependent manner.

Key words: Breast cancer, salvianolic acid B, DNA fragmentation, pro-oxidative, pro-apoptotic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is the commonest female cancer and also the leading cause of mortality of all type of cancer death particularly in Northern America, Europe and South-East Asia and Africa[1]. The prevalence rate of breast cancer in China (urban) has been escalated in the last decade owing to a modified life pattern and unique reproductive pattern (one child policy). China accounts for 12.2% of newly diagnosed breast cancer with 9.6% of all death breast cancer globally[2]. Conventional breast cancer treatment regimens are the administration of chemotherapy like paclitaxel, topotecan, tamoxifen and docetaxel, etc., surgery (mastectomy), radiotherapy or combination (adjuvant chemotherapy with radiation and surgery) of various procedures[3]. However, chemotherapy and radiotherapy results in various undesirable events, non-responsive or resistance (a specific type of patients) as well as expensive as the patients continue for several days to years[4,5]. The surges for the alternative natural medicine with chemotherapeutic property (anti-apoptotic and anti-proliferative/cytotoxic activities) are of high need to develop a novel safer and effective anti-cancer agent which targets cancer cells.

Salvianolic acid B (SB) is an abundant hydrophilic di-terpenoids (active phytocomponent) of a popular Chinese herb called *Salvia miltiorrhiza* (Danshen-dried roots). Traditionally Danshen has been recommended to treat myocardial infarction, angina pectoris, stroke, Parkinson disease and cancer[6,7]. Accumulating evidence indicated that SB possesses multiple biological functions including antioxidant, anti-inflammation, anti-coagulant, anti-angiogenic and anti-diabetic, immuno-modulatory, modulate apoptosis as well as hepatoprotective, neuroprotective and cardioprotective[8-10]. Moreover, the anti-cancer or tumor activity of salvianolic acid B has been reported in different cell line model[11-13], however no studies have been conducted on human breast cancer MCF-7 cells. Hence, the current study was undertaken to investigate the chemoprotective (anti-breast cancer) activity of SB on human breast cancer MCF-7 cells by assessing the cytotoxicity or anti-proliferative, DNA fragmentation, inflammatory and apoptotic markers.

MATERIALS AND METHODS

This cell line (*in vitro*) study was conducted at Department of Breast Surgery of The First Hospital of Nanping Affiliated to Fujian Medical University between September-October, 2017.

Chemicals: All the chemicals used for this study are of either analytical or HPLC grade.

Cell culture: MCF-7 human breast cancer cells is purchased from the American Type Culture Collection (ATTC; MD, USA) and was cultured as monolayer maintained in Dulbecco Modified Eagle Medium (DMEM) which were supplemented with 10% Fetal Bovine Serum (FBS), 2 mM l-glutamine, 100 U mL^{-1} penicillin and streptomycin at 37°C under 5% CO_2.

MTT assay: The 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to assess the anti-proliferative activity by Van Meerloo *et al.*[14] method. Briefly, MCF-7 cells were placed in a 96 well microplate at a density of 1×10^5 cells mL^{-1} and supplemented with increasing concentration of SB (25, 50, 100 and 200 μm) and DMSO (control) for 24 and 48 h at 37°C. Followed by the incubation of MTT at a concentration of 500 μg mL^{-1} (dissolving with DMSO) for 4 h at 37°C and finally treated with DMSO to dissolve the formazan crystals (solubilization). The viable cells were counted by recording the Optical Density (OD) at 490 nm wavelength using an ELISA reader (iMark; 680; Bio rad Laboratories., Inc, CA, USA).

Assessment of Reactive Oxygen Species (ROS) generation: The intracellular ROS generation on MCF-7 cells were quantified by the method of Shashi *et al.*[15]. In short, MCF-7 cells were seeded in a 96 well microplate at a density of 1×10^5 cells mL^{-1} and cultured with different concentrations of SB (25, 50, 100 and 200 μm) and DMSO (control) for 24 at 37°C. Then, the cells were incubated with 1 mM N-acetylcysteine (NAC) and followed by the addition of 100 M 2,7-dichlorofluorescein diacetate (DCF-DA) for 45 min at 37°C in the dark and washed with Phosphate Buffered Solution (PBS). The ROS production (stained cells by DCF-DA) was detected by a fluorescence COULTAREPICS XL (ALTRA) flow cytometer purchased from Beckman Coulter Inc., (CA, USA).

Detection of DNA fragmentation: DNA fragmentation of cell lysate (cytosolic fraction) after treatment with DMSO or 25, 50 or 100 μm SB was detected by ELISA DNA fragmentation kit provided by Roche Diagnostics (Risch-Rotkreuz, Switzerland) based on manufacturers protocol.

Apoptotic assay: Both caspase 3 and 9 activities were assayed in cell lysates (of MCF-7) after DMSO or 25, 50 or 100 μm SB treatments using Caspase-Glo 3 and 9 assay kits provided by Promega (WI, USA) based on supplier's instruction.

Western blot: Protein expression of Bcl2 and Bax were quantified by western blot technique using PAGE apparatus as described previously by Silva *et al.*[16]. The SB incubated MCF-7 cells (as mentioned in MTT assay) were harvested and lysed with lytic enzymes (RIPA enzymes) and the protein levels was estimated using BCA protein assay kit from Pierce (IL, USA). Fifty grams of protein from each group cells were resolved on 10% SDS-PAGE geland then transferred on to a PVDF membrane. Then the membrane was blocked with Tris-buffered solution (with Tween 20, skimmed milk and sodium chloride) and incubated with primary antibodies such as mouse anti-Bax antibody (1:1000), mouse anti-Bcl2 antibody (1:800) and mouse anti-beta actin (1:1200) at 4°C for 10 h (from Santa Cruz Biotechnology; CA, USA). Excessive antibodies are removed by tris buffered solution and finally incubated with secondary antibody-like HRP anti-mouse antibody for 1 h at 4°C. The respective conjugated antibodies bands (equivalent to protein expression) were quantified by Bio-radChemiDoc imagingSystem (CA, USA).

Data analysis: All the experiments were performed as triplicates. Student t-test was used for this study to analyze the difference between the groups using SPSS software (ver. 21 from IBM, NY, USA). A probability value (p-value) less than 0.05 was deemed as statistically significant. Values were expressed as the Mean±standard deviation.

RESULTS

The MTT assay (cell viability) was carried out to assess the anti-proliferative/cytotoxicity effect of SB on MCF-7 cells. The Fig. 1 showed that administration with different concentration of SB (25, 50, 100 and 200 µm) on MCF-7 cells for 24 and 48 h showed significant ($p<0.05$ for 25 and 50 µm; $p<0.01$ for 100 and 200 µm) inhibition of cell growth or proliferation with less viable cells count in dose and time-dependent fashion as compared with DMSO treated cells. Hence, it was cleared from the MTT assay that SB endorse its potent anti-proliferative and cytotoxicity properties.

As Fig. 2 illustrated the efficacy of SB on ROS generation on MCF-7 cells. To prove ROS induced apoptosis by SB, the intra-cellular ROS generation levels on MCF-7 cells was quantified. Co-culturing of MCF-7 cells with increasing concentration of SB showed an exponential increase ($p<0.05$ for 25 and 50 µm; $p<0.01$ for 100 and 200 µm)

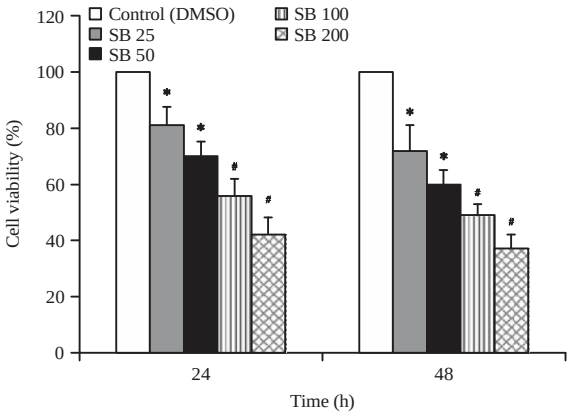

Fig. 1: Efficacy of SB in cell viability on MCF-7 cells by MTT assay

Values were expressed as the Mean±standard deviation, #p<0.01 and p<0.05: Control vs. SB treated group (different dose)

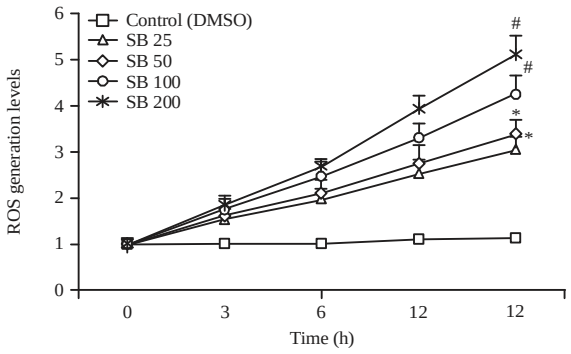

Fig. 2: Efficacy of SB on ROS generation on MCF-7 cells

Values were expressed as the Mean±standard deviation, #p<0.01 and *p<0.05: Control vs. SB treated group (different dose)

in ROS generation (dose and time-dependent fashion) and thus conferring that ROS production by SB on MCF-7 cells might be the reason behind the cytotoxicity or apoptosis property.

Efficacy of SB on DNA fragmentation on MCF-7 cells was cross-checked whether the DNA damage induced apoptosis on MCF-7 cells (Fig. 3). An exponential increase ($p<0.05$ for 50 and 100 µm; $p<0.01$ for 200 µm) in the levels of DNA fragmentation was noted in SB treated MCF-7 cells as equivalence to DMSO treated MCF-7 cells. As the concentration of SB increased the levels of DNA damage were also increased respectively. Results of DNA fragmentation/damage inferred that SB induced oxidative stress (ROS generation) and results in DNA damage/fragmentation.

Efficacy of SB on apoptotic markers like caspase 3 and 9 on MCF-7 cells was presented in Fig. 4. The activities of

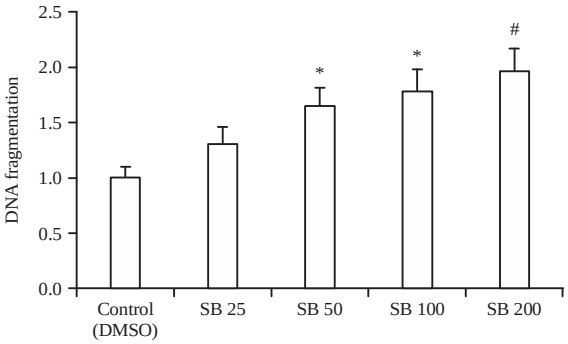

Fig. 3: Efficacy of SB on DNA fragmentation on MCF-7 cells

Values were expressed as the Mean±standard deviation, #p<0.01 and *p<0.05: Control vs. SB treated group (different dose)

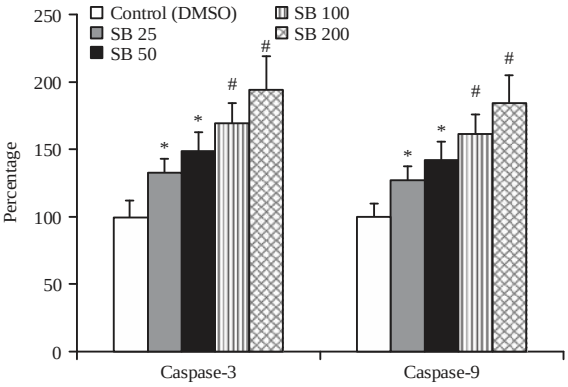

Fig. 4: Efficacy of SB on apoptotic markers on MCF-7 cells

Values were expressed as the Mean±standard deviation, #p<0.01 and *p<0.05: Control vs. SB treated group (different dose)

caspase 3 and 9 were examined MCF-7 cell line to explore which kind of apoptotic pathway was favored by SB to exhibit apoptosis. The caspase 3 and 9 activities were significantly improved (p<0.05 for 25 and 50 µm; p<0.01 for 100 and 200 µm) upon co-culturing with different concentration of SB on MCF-7 cells than DMSO (control) treated MCF-7 breast cancer cells. The outcome proved that SB highly favored the intrinsic apoptotic pathway by upregulating caspase 9 and subsequently activate caspase 3 to exhibit apoptosis on MCF-7 cells.

Furthermore, the protein expression of major pro and anti-apoptotic factors like Bcl2 and Bax were assessed by western blot technique. The data in Fig. 5 portraited the efficacy of SB on the protein expression of Bcl2 and Bax (apoptotic factors) on MCF-7 cells. A marked regulation in the protein expression of pro-apoptotic factors like Bcl2 (downregulation) and anti-apoptotic factors like Bax (upregulation) was noted after incubation with increased concentration of SB on MCF-7 cells than that of DMSO treated

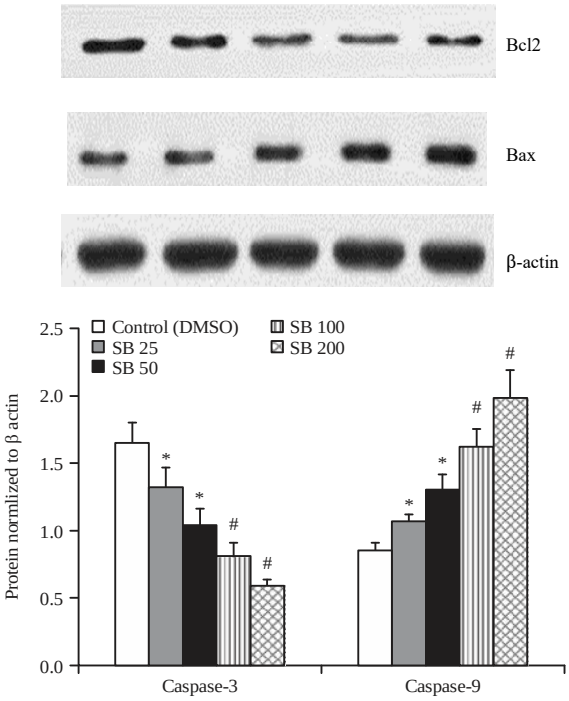

Fig. 5: Efficacy of SB on protein expression of Bcl2 and Bax (apoptotic cascade) on MCF-7 cells by western blot analysis

Values were expressed as the Mean±standard deviation, #p<0.01 and *p<0.05: Control vs. SB treated group (different dose). Lane 1: Control (DMSO) treated, Lane 2-5: Different SB treated (25, 50, 100 and 200)

MCF-7 cells. Hence, proving that SB elicited pro-apoptotic activity on MCF-7 breast cancer cells and thus showed its chemoprotective activity.

DISCUSSION

Current experiment was designed to access the chemoprotective efficacy of SB on human breast cancer MCF-7 cell line. Results showed that MCF-7 cells added to DMSO (control) as well as treated with an increased concentration of SB (25, 50, 100 and 200 µm) showed potent anti-proliferative (decreased viable cell count), pro-oxidative (increased ROS generation), pro-apoptotic activities (increased activity of caspase-3/9 and pro-apoptotic factor like Bcl2. Cell viability or cytotoxicity study by MTT assay showed that supplementation with increasing doses of SB on MCF-7 cells for 24 and 48 h showed a sharp decline in the number of viable MCF-7 cells in a time and dose-dependent fashion. The above results were accounted for the anti-proliferative/cytotoxicity property of SB in MCF-7 breast cancer cell line. Present results were in corroboration with the results of Jing *et al.*[9], who also indicated that treatment with salvianolic acid B decreased the viable cell count (colorectal cancer cells).

Sample amount of studies demonstrated that ROS (free radical) induced apoptosis plays a protective or therapeutic role against various cancer cells[17,18]. Hence, to examine whether SB induced apoptosis via ROS generation, the intracellular ROS generation levels on MCF-7 cells was quantified upon supplementation with different concentration of SB. As expected administration of SB showed an exponential increase in ROS generation on MCF-7 cancer cells. Likewise, Zeng *et al.*[19] concluded that treatment with salvianolic acid B could inhibit cell proliferation and favor anti-tumor activity by inducing apoptosis through ROS generation in osteocytes. Thus, conferring that ROS production by SB on MCF-7 cells might be the reason behind the cytotoxicity or apoptosis property.

As a continuation of the previous study to explore the mechanism behind the cytotoxicity property of SB, authors cross-checked whether the DNA damage induces apoptosis on MCF-7 cells. As the concentration of SB increased the levels of DNA damage were also increased respectively and showed that SB induces oxidative stress (ROS generation) and results in DNA damage/fragmentation. The previous study conducted by Wang *et al.*[20] highlighted that salvianolic acid could induce apoptosis by damaging DNA (mito-DNA) in lung cancer cells.

Apoptosis (programmed cell death) is a crucial factor in maintaining balance in the cell count and evading of apoptosis is one of the hallmarks of cancer cells[21]. Hence, for this research, study focussed on apoptotic markers (Caspase cascade) and apoptotic factor (execute apoptosis). Caspases (cysteine-aspartic proteases) are the set (family) of protease (enzyme) that play a key role in triggering apoptosis. Caspase 3 (effector) and 9 (initiator) were the two main caspase enzymes involved in executing apoptosis by sequential reaction[22]. Upon co-culturing with different concentration of SB on MCF-7 cells the activities of caspase 3 and 9 were significantly improved. The SB treatment could highly favored the intrinsic apoptotic pathway by upregulating caspase 9 and subsequently activate caspase 3 to exhibit apoptosis on MCF-7 cells. Also, salvianolic acid B induced apoptosis through upregulating various caspase enzymes in human glioma U87 cells[11].

Apoptosis is tightly controlled by pro and anti-apoptotic factors like Bcl2 (inhibit apoptosis) and Bax (trigger apoptosis). Both these factors execute apoptosis via various caspase cascade especially in breast cancer MCF-7 cells[23]. The protein expression of Bcl2 (anti-apoptotic factors) and Bax (pro-apoptotic factors) were notably downregulated and upregulated respectively after administration with increased concentration of SB than DMSO treated MCF7 cells. This outcome was in agreement with the outcome of Guo *et al.*[13], who also hinted that salvianolic acid B addition on acute promyelocytic leukemia cells (HL-60) could notably upregulate Bax and down regulated Bcl2. This study has few limitations including the avoidance of cell cycle analysis as well as autophagy. However, in the future will give a special attention on both the cell cycle and autophagy to prove the in-depth chemoprotective mechanism of SB on MCF-7 breast cancer cells.

CONCLUSION AND FUTURE RECOMMENDATIONS

Taking together, that different concentration of SB showed better chemoprotective activity on MCF-7 cells by exhibiting anti-proliferative, pro-oxidative and pro-apoptotic activities in time and dose-dependent fashion. Nevertheless, further experiments are required to prove the precise signaling pathway involved in the chemoprotective mechanism of SB on MCF-7 breast cancer cells.

SIGNIFICANCE STATEMENT

Results from this study discovered that SB could act as better chemoprotective activity on breast cancer MCF-7 cell line by exhibiting anti-proliferative, pro-oxidative and pro-apoptotic activities. Hence, SB can be combined with other standard chemoprotective agent to produce a novel pharmacological agent to combat breast cancer and its related complications.

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