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Research Article
Protective Effect of Obovatol Against MCF-7 Human Breast Adenocarcinoma Cells via Inducing Apoptosis and Cell Cycle Arrest

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Abstract

Background and Objective: Breast cancer are a frequently diagnosed cancer in a female with a high mortality. This cell line study was planned to investigate the chemo protective efficiency of obovatol (OBO) in MCF-7 human breast adenocarcinoma cells. **Materials and Methods:** The MCF-7 cell treated with various concentrations of OBO (10, 25, 50 and 100 μM) to evaluate the anti-proliferative/ cytotoxicity study by MTT assay, pro-apoptotic activity by assessing caspase-3/9 (apoptotic markers) and protein expression of Bcl2 and Bax as well as cell cycle arresting property (cycle phases) by flow cytometry. **Results:** The MCF-7 cells supplemented with increased concentration of OBO displayed significant cytotoxicity/anti-proliferative activity with increased DNA fragmentation. Whereas, cell cycle phase analysis revealed considerable cell arrest after the addition of OBO by improving G0/G1and S phase. Moreover, the activities of caspase-3 and 9 were notably increased after treatment with OBO as well as the protein expression of Bax (pro-apoptotic protein) were markedly upregulated with a suppressed protein expression of Bcl2 (anti-apoptotic protein) were noted in MCF-7 administered with OBO in a dose-dependent manner. **Conclusion:** Overall, OBO showed potent chemopreventive activity by demonstrating its anti-proliferative, apoptosis and cell cycle arrest properties in a concentration-dependent fashion.

Key words: Obovatol, chemoprotective, cell cycle, anti-proliferative, breast cancer, adenocarcinoma cells

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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 one of the commonly diagnosed cancer globally among female with the highest mortality rate, especially in developed and less developed countries. Breast cancer alone contributes about 1/4th of all cancer cases worldwide[1]. Even the prevalence of breast cancer in China is quite high particularly in urban areas owing to a sedentary lifestyle (less physical activity), high obesity rate, modified sleeping pattern (hormonal imbalance), as well as the modified reproductive pattern. Recent studies (statistical report) indicated that almost 11% of the global breast cancer occur in China and the prevalence was alarming increasing in recent times[2,3]. Current conventional breast cancer treatment, including surgery (mastectomy-removal of breast), radiotherapy (radiation treatment) used to suppress the proliferation and migration of cancer cells, chemotherapy (tamoxifen, paclitaxel, topotecan etc), hormonal therapy, especially for post-menopausal patients (Estrogen, progesterone), targeted therapy (nanotechnology-active/passive targeting) or combination of various above-mentioned therapies (adjuvant therapy) are used now a days[4,5]. Nevertheless, usage of above-indicated therapies might result in numerous adverse effects including air loss, loss of appetite, modified sleeping pattern, gastrointestinal discomfort and non-responsive to certain breast cancer patients as well as expensive. Therefore, the search for alternative or complementary therapies using natural medicine (herbs) with potent chemotherapeutic property (anti-proliferative, pro-apoptotic, anti-invasive properties) would be the best option[6,7].

Obovatol (OBO; $C_{18}H_{18}O_3$) is one of the major bi-phenolic component of bark and leaves of *Magnolia officinalis* and *Magnolia obovata* which has been widely used as a folk medicine for treating various ailments including anxiety, allergy, gastrointestinal problems[8]. The OBO possess a broad range of biological activities including anti-inflammation, anti-oxidant, anti-thrombotic, antiproliferative, apoptotic properties as well as neuroprotective and osteoprotective function[9,10]. Previously, many studies have reported that obovatol could considerably inhibit cell proliferation/growth by enhancing apoptosis in various solid cancer cells by modulating various signaling pathways[9,11,12]. However, no experiment was conducted with obovatol against breast cancer cells like MCF-7. Therefore, the current experiment was framed to investigate the chemoprotective activity of obovatol against breast cancer MCF-7 cells by evaluating cell toxicity or anti-cell proliferative, DNA fragmentation, apoptotic markers as well as cell cycle phase.

MATERIALS AND METHODS

Chemicals and reagents: Obovatol (OBO; 98% HPLC grade) was purchased from Herbest (Shaanxi, China). Whereas ethanol (75%), 3-(4,5dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), RNAases, phosphate buffered saline (PBS) solution, Tween 20 were bought from Sigma Aldrich (MO, USA).

Cell culture: The MCF-7 human breast carcinoma cell line was received from ATTC (MD, USA). The MCF-7 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μg mL^{-1} of streptomycin, 1% penicillin and maintained at 37°C in a CO_2 incubator under 5% CO_2. All the experiments were conducted at Chung Shan Medical University from March-April, 2018.

MTT assay: The MCF-7 cells were plated in 96 well plates at a density of 1×10^6 cells/well and cultured with different concentration (10-100 μM) of OPO and DMSO for 24 and 48 h at 37°C. To each well add 0.5 mg mL^{-1} of MTT solution and incubated for another 4 h at 37°C and followed by addition of isopropanol and shaken well to dissolve the formazan dye/crystal. The absorbance (OD_{540}-OD_{630}) were measured using SpectraMax M5 Microplate Reader (Molecular Devices; CA, USA) which indicate the percentage of cell viability.

DNA fragmentation: The DNA fragmentation of MCF-7 cells co-cultured with increased concentration of OBO (10, 25, 50, 100 μM) was determined using ELISA DNA fragmentation commercial kit bought from Roche Diagnostics (Risch-Rotkreuz, Switzerland) in accordance to supplier procedure.

Apoptotic markers: The activities of both Caspase 3 and Caspase 9 were assayed in MCF-7 cell lysate (supernatant) which were supplemented with various concentration of OBO and DMSO (control) using Caspase-Glo 3 and Caspase-Glo 9 commercial assay kits purchased from Promega Corporation (WI, USA) based on manufacturers protocol.

Cell cycle analysis: For cell cycle phase analysis, MCF-7 cells (1×10^6 cells/well) were plated in a 96 well plate and administrated with different concentration of OBO and DMSO (control) and resuspended with 1 mL of 75% ethanol (fixing agent) at 37°C for overnight. After incubation, MCF-7 cells were washed with phosphate buffered saline (PBS) solution (remove unfixed cells) and treated with RNAase solution for

20 min in the dark. Then, the cells were incubated with 100 µL of propidium iodide (PI) at 37°C in the dark condition. Finally, the MCF-7 cells cycle phases were analyzed using flow cytometer (BD FACSVerse) bought from BD Biosciences (NJ, USA). The percentage of MCF-7 cells in each cell cycle phase was determined using Modfit LTLT software bought from Verity Software House (Topsham, UK).

Western blot: The MCF-7 cell (2×10^6 cells/well) exposed to different concentration of OBO (10, 25, 50, 100 µM) and lysed using RIPA lysis buffered solution and the protein concentration was estimated using Pierce BCA protein assay kit from Thermo Fisher Scientific (MA, USA). Forty micrograms of protein were separated using 8% SDS-PAGE apparatus and electro-transferred onto polyvinylidene difluoride (PVDF) membrane[12]. The PVDF membrane was blocked with Tween 20, 5% skimmed milk and PBS solution and incubated with primary antibodies including anti-rabbit monoclonal anti-Bcl2 antibody (1:800 dilution), anti-rabbit monoclonal anti-Bax antibody (1:1000 dilution) and β-actin (Santa Cruz Biotechnology; CA, USA) for overnight at 4°C. Again, the PVDF membrane was incubated with secondary antibody (HRP anti-rabbit IgG) for 1 h at 37°C. Finally, the formed protein band in the PVDF membrane were developed using the ECL detection system with Pierce ECL kit from Thermo Fisher Scientific (MA, USA). The protein bands were captured and quantified using image analyzing software (Image Master Elite software; V3.1) from the Amersham Pharmacia Biotech (Little Chalfont, UK).

Data evaluation: Data were evaluated by Student t-test for the comparison between control and different OBO group using Statistical Package for Social Sciences (SPSS) Software (v 23; IBM, NY, USA). Values are expressed as mean±standard deviation (SD). All the experiments were conducted as triplicate and probability value (p-value) less than 0.05 is considered as statically significant between the groups (OBO Vs control).

RESULTS

Effect of OBO on cytotoxicity: Figure 1 represents the cytotoxicity or anti-proliferative effect of OBO in MCF-7 cells using the MTT assay. Multiple concentration of OBO (10, 25, 50, 100 µM) administration in MCF-7 cells for 24 and 48 h displayed significant cell death (cytotoxicity) as well as inhibit cell growth (anti-proliferative activity) in a dose-dependent fashion as compared with DMSO alone (control) treated MCF-7 cells.

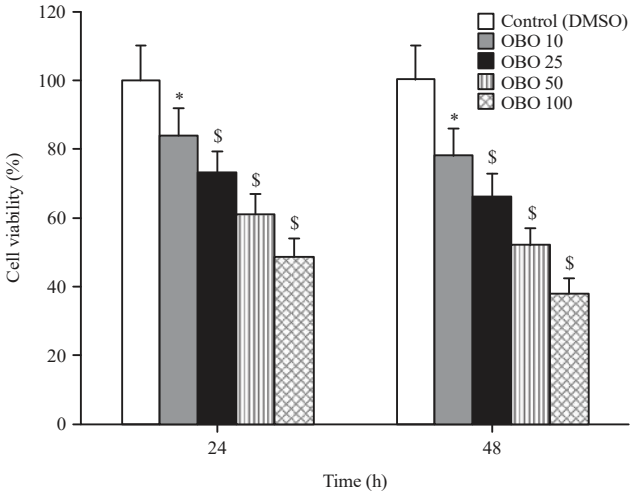

Fig. 1: Effect of OBO on cell viability or cytotoxicity in MCF-7 cells
Values are expressed as Mean±Standard deviation (SD), * and $ indicated a significant of p<0.05 and p<0.01 as compared with the control dimethyl sulfoxide (DMSO) group

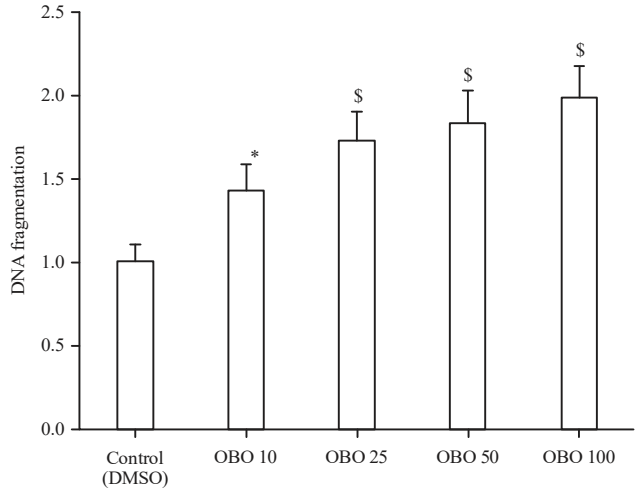

Fig. 2: Effect of OBO on DNA fragmentation in MCF-7 cells
Values are expressed as Mean±Standard deviation (SD), * and $ indicated a significant of p<0.05 and p<0.01 as compared with the control dimethyl sulfoxide (DMSO) group

Effect of OBO on DNA fragmentation: A commercial ELISA kit was used to determine the effect of OBO on DNA fragmentation in MCF-7 cells (Fig. 2). A pronounced increased in the levels of DNA fragmentation were observed in MCF-7 cells treated with different concentration of OBO (concentration depended manner) on comparison with control (DMSO) cells.

Effect of OBO on cell cycle phases: The MCF-7 cells are co-cultured for 24 h with an increased concentration of OBO

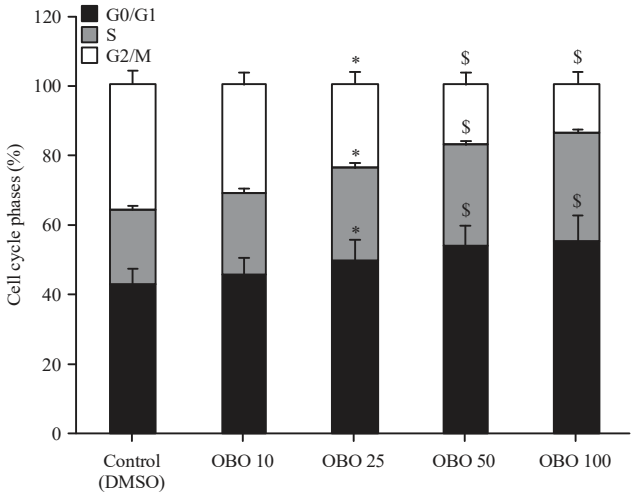

Fig. 3: Effect of OBO on cell cycle phases in MCF-7 cells
Values are expressed as Mean±Standard deviation (SD), * and $ indicated a significant of p<0.05 and p<0.01 as compared with the control dimethyl sulfoxide (DMSO) group

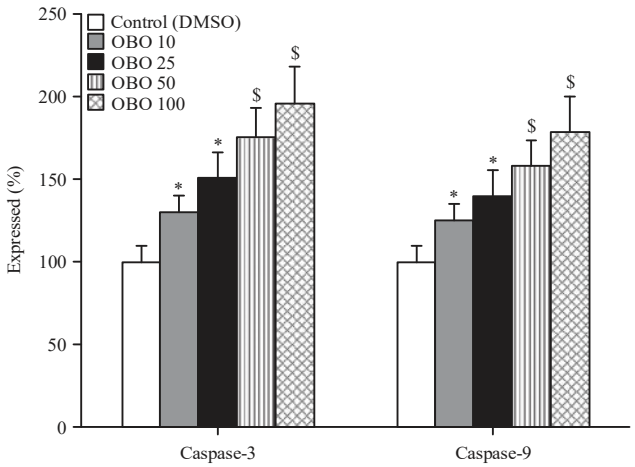

Fig. 4: Effect of OBO on apoptotic markers like caspase 3 and 9 in MCF-7 cells
Values are expressed as Mean±Standard deviation (SD), * and $ indicated a significant of p<0.05 and p<0.01 as compared with the control dimethyl sulfoxide (DMSO) group

(10-100 μM) exhibit strong cell arrest by decreasing the cell population percentage in G2/M phase with increased G0/G1and S phase (Fig. 3).

Effect of OBO on apoptotic markers: As showed in Fig. 4 the activities of apoptotic markers like caspase 3 and 9 were substantially elevated in OBO (10, 25, 50, 100 μM) exposed MCF-7 cells as compare to the control MCF-7 cells.

Effect of OBO on the protein expression: The protein expression of Bax (pro-apoptotic protein) and Bcl2

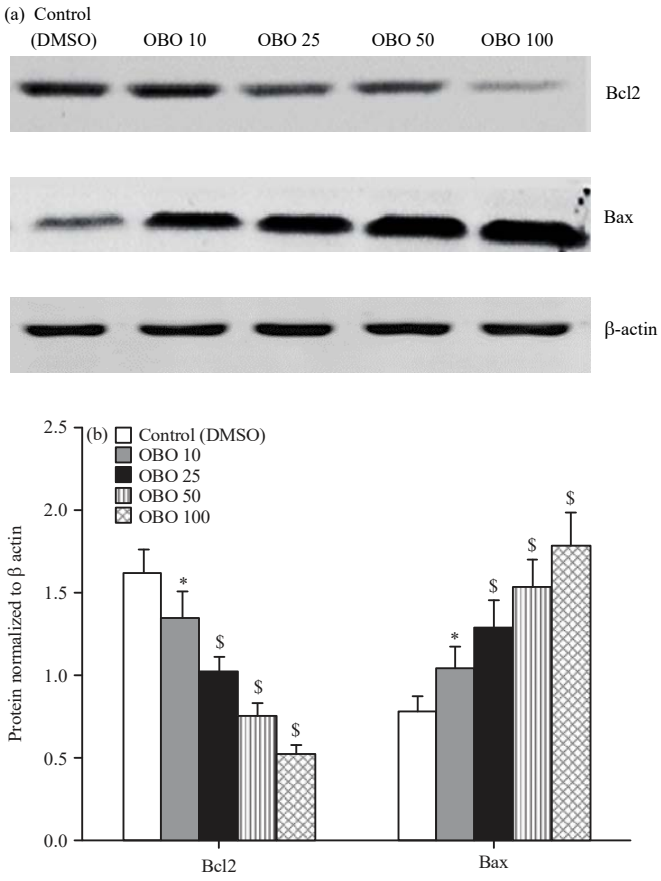

Fig. 5(a-b): Effect of OBO on the protein expression of Bax and Bcl2 in MCF-7 cells
Values are expressed as Mean±Standard deviation (SD), * and $ indicated a significant of p<0.05 and p<0.01 as compared with the control dimethyl sulfoxide (DMSO) group

(anti-apoptotic protein) were investigated using western blot technique using SDS-PAGE apparatus (Fig. 5). The protein expression of Bax was markedly upregulated with suppressed protein expression of Bcl2 (anti-apoptotic protein) were noted in MCF-7 cells administered with OBO in a dose-dependent fashion in comparison with control (DMSO) treated MCF-7 cells.

DISCUSSION

The outcome of this present cell line experiments clearly showed that treatment with an increased concentration of OBO in MCF-7 cells elicit potent apoptotic activity as well as inhibit cell proliferation and differentiation/growth (cell cycle arrest). The major hallmark of the chemotherapeutic agent is cytotoxicity and anti-proliferative activity[9]. Therefore, for the present study, the cytotoxicity or anti-proliferative effect of

OBO in MCF-7 cells was evaluated using the MTT assay. Increased concentration of OBO administration in MCF-7 showed a significant increase in cell death which reflects in decreased viable cells in a dose-dependent fashion. Likewise, many researchers indicated that obovatol could induced cell cytotoxicity only in cancer cells via triggering apoptosis and necrosis[8,13]. From the above MTT assay it's clear that OBO induce cytotoxicity in MCF-7 cells but the reason behind the cytotoxicity might be the induction of DNA fragmentation. Since many studies, the chemoprotective agent showed their cytotoxicity effect via triggering DNA fragmentation[14,15]. Herein, the effect of OBO on DNA fragmentation in MCF-7 cells was assessed using ELISA kit. The levels of DNA fragmentation were considerably increased in OBO treated MCF-7 cells than control cells in a concentration-dependent manner. This result is in agreement with the outcome of Lee and his co-workers[11].

The cell proliferation rate of any cell is tightly regulated by various cell cycle proteins which regulate various cell cycle phase including G0, G1, S, G2 and M phases[16]. G1 phase is a crucial phase where the cells can prepare for proliferation (preceding S or replication phase) or revert to resting phase (G0) or trigger apoptosis based on various signal transduction. If any dysfunction or regulation in the cell cycle phases might result in a cancer-uncontrolled proliferation of cells[16,17]. Hence, the various cell cycle phases in MCF-7 cells were measured using flow cytometry. The MCF-7 cells co-cultured with increased concentration of OBO (10-100 μM) for 24 h showed strong cell arrest by decreasing the cell population percentage in G2/M phase with increased G0/G1 and S phase. Thus, inferring that OBO treatment directed MCF-7 breast cancer cells to undergo resting phase (G0) and halt the progression of cell division at S phase and thereby inhibiting the cell progress to M phase (important for proliferation and division). Similarly, Lee and his colleagues also demonstrated that treatment with obovatol in SW620 colorectal carcinoma cells considerably halt cell cycle progression (cell proliferation) by increasing the percentage of G0 and S phase cells[11].

The hallmark of any cancer cell is evading apoptosis and therefore the activity of caspase 3 and 9 as well as the protein expression of pro and anti-apoptotic proteins like Bax and Bcl2. Results have shown that MCF-7 cells exposed with various concentration of OBO considerably enhanced the activity of caspase 3 and 9 as well as upregulate the protein expression of Bax (pro-apoptotic protein) along with downregulation of Bcl2 (anti-apoptotic protein). The above result is in agreement with the results of Kim and others[8]. Recently, Duan and his colleagues, also highlighted that obovatol treatment in tongue squamous carcinoma SCC9 cells triggers apoptosis by increasing caspase 3 and 9 as well as modulated various proteins involved in apoptosis via regulating various signaling pathways[9]. This study has few limitations including the lack of evaluation of protein involved in cell cycle regulation like cyclin D, E and CDK2/4 as well as the in-depth mechanism of chemoprotective activity by exploring various signaling pathway (MAPK, JNK or ERK).

CONCLUSION

Taking together, MCF-7 cells treated with an increased concentration of OBO could trigger apoptosis via increasing the activity of caspase 3 and 9 as well as protein expression of pro-apoptotic proteins like Bax and suppress the Bcl2. Moreover, OBO supplementation significantly arrests cell cycle progression or proliferation. Thus, OBO showed potent chemo preventive activity in MCF-7 cells by showing anti-proliferative, apoptosis and cell cycle arrest properties in a concentration-dependent fashion. However, detailed mechanism underpinning anti-breast cancer activity related to signaling pathway as to be explored in the future.

SIGNIFICANCE STATEMENT

Outcome of this cell line study clearly showcase that OBO can display potent chemoprotective activity against human breast carcinoma cell line (MCF-7) by exerting cytotoxicity/anti-proliferative, pro-apoptotic and cell cycle arrest activities. Therefore, OBO could be recommended for treating breast cancer along with standard chemotherapeutic drugs to lower the mortality and morbidity related to breast cancer.

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