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Inhibitory and protective effect of obovatol against uterine fibroid (leiomyoma) cells

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Abstract: Uterine fibroids (UF) or leiomyomas can be presented in post-menopausal women. The present study was aimed to examine the inhibitory and protective (anti-proliferative and apoptotic) effect of the obovatol (OB) in human leiomyoma cells (HuLM). The cell proliferative activity was determined by MTT assay and inflammatory markers were measured. Followed by evaluating DNA fragmentation and apoptotic markers using the ELISA kit method. Also, the apoptosis regulatory proteins expressions were determined using the immunoblot technique. Treatment with increasing concentration of OB (25-200 µM) significantly lowered the cell proliferation rate as well as considerably reduced the values of various pro-inflammatory cytokines like IL-1β, TNF-α, IL-6. Whereas, the levels of DNA fragmentation and apoptotic marker like caspase-3 and 9 were considerably elevated after co-culturing HuLM cells with OB. In addition, apoptosis regulatory proteins like Bcl2 and Bax were substantially down and up-regulated respectively, by OB in a dose-dependent fashion. The above data clearly showcase that OB possesses potent anti-proliferative (inhibitory) as well as apoptotic activity and may be recommended as a chemotherapeutic agent against UF and related conditions. However, further studies are required before recommended for treating UF subjects.

Keywords: Leiomyomas, obovatol, apoptotic markers, inflammatory markers, cell proliferation

INTRODUCTION

Uterine fibroids (UF) or leiomyomas is a common form of benign tumors which are developed in the myometrium of uterus especially in post-menopausal women (Bulun, 2013). The incidence of UL is higher in China with the 3rd most commonly diagnosed reproductive disorder/disease in women and directly contributes to hysterectomy (Zhu *et al.,* 2015). However, only 20-50% of women with UF show clinical symptoms like pelvic pain/pressure, prolonged menstrual bleeding, pelvic mass, reproductive dysfunction and thus it is hard to diagnose (Islam *et al.*, 2017). In addition, UF is considered as a hormonal dependent disease, with unclear pathophysiological mechanism makes it a complicated tumor (Draver and Catherino, 2015). The current treatment management protocol includes surgery (expensive and inconvenient-results in infertility), thermal ablation as well as gonadotrophin-releasing hormone (GnRH) and selective progesterone receptor modulators (SPRMs) (high various adverse effects including bone loss and cardiovascular complications). Nevertheless, those above indicated treatment regimen are very much limited as most of them used to but suppress symptoms related to UF (Donnez and Dolmans, 2016; Islam *et al.,* 2013). Therefore, the need for an effective pharmaceutical agent to treat leiomyoma is of high demand especially to preserve fertility (Bao *et al.*, 2018).

Obovatol (OB) is a major phenolic phytocomponent of *Magnolia obovata* and *Magnolia officinalis,* especially from bark and leaves. Traditionally, the bark of *Magnolia obovate* and *Magnolia officinalis* was used to treat anxiety (relieve distension), allergy and digestive problems (Seo *et al.,* 2016; Kim *et al.,* 2014). Ample amount of studies has indicated various biological activities of OB which includes anti-inflammatory, antioxidant, antibacterial, antithrombotic activities (Duan *et al.,* 2018; Choi *et al.,* 2017; Kim *et al.,* 2016) as well as exhibit neuroprotective and gastroprotective properties (Choi *et al.,* 2012). In addition, obovatol reported to display chemoprotective activity in various cell and animal models by effectively inhibiting cell proliferation, inflammatory response and cell arrest as well as trigger apoptosis-related signaling pathway (Kim *et al.*, 2014; Lee *et al.,* 2008; 2009). Hence, we hypothesize that OB would be the better choice to exploring the inhibitory and chemoprotective (anti-proliferative and apoptotic) effect of the obovatol (OB) in human leiomyoma cells (HuLM) through evaluating cell proliferation, DNA fragmentation, inflammatory and apoptotic markers.

MATERIALS AND METHODS

Chemicals

The pure HPLC grade (98%) obovatol (OB; CAS: 83864-78-2) was kindly gifted by Herbest Pharmaceutical Ltd. (Shaanxi, China). Unless otherwise specified, all the materials were purchased from Sigma-Aldrich (MA, USA).

Cell culture

Immortalized human leiomyoma cells (HuLM) were grown in a smooth muscle cell growth media (SmGM)

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with 5% fetal bovine serum (FBS), 0.2% human fibroblast growth factor (hFGF), 0.1% human epidermal growth factor (hEGF), 0.1% insulin, 0.1% gentamycin and maintained at 37° C with 5% CO_2.

MTT assay

The HuLM cell proliferative activity was determined by MTT assay as indicated previously by Kashif and others (2017). HuLM cells were seeded in 48 well plates (1 x 10^4 cells/well) and followed by treatment with increased concentration of OB (25, 50, 100, and 200 µM) and dimethyl sulfoxide (DMSO; only for control cells) for 24 and 48 h at 37° C. Then the HuLM cells were mixed with 0.5 mg/mL of MTT reagents and incubate for 4 h at 37°C. The optical density (OD) at $540_{Exi}/630_{Emi}$ wavelength were measured using Tecan Sunrise Microplate Reader (NC, USA) and is expressed as the percentage of viable cells.

Inflammatory markers

HuLM cells were probed in 96 well plates (1 x 10^6 cells/well) and followed by treatment with increased concentration of OB and DMSO. Then the cells were lysed using the lytic buffered solution and centrifuged at 15,000 for 8 min at 4° C to get a pellet and supernatant. The separated supernatant were used for quantification of various pro-inflammatory cytokines (markers) like interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) using commercial ELISA kit from BD Biosciences (NJ, USA) based on supplier protocol.

DNA fragmentation

HuLM cells were seeded at a concentration of 1 x 10^3/cells and co-cultured with different concentration of OB and the cellular DNA Fragmentation (apoptotic cells) were determined by adding ELISA (Non-radioactive assay) kit reagents (Sigma-Aldrich; MA, USA) based on manufacturer instruction.

Apoptosis

The HuLM cells lysate (prepared as indicated in the inflammatory marker section) was used to measure the activity of various apoptotic markers like caspases 3 and 9 using Caspase-3 and 9 commercial assay kits bought from Abcam (Cambridge, UK) by following the manufacturer's procedure.

Immunoblot

The protein concentration of OB treated HuLM cells lysate were determined and quantified using Protein Assay and quantification kit from Biocompare (CA, USA) based on suppliers' protocol. Equal concentration of proteins was loaded in each well of 10% SDS-PAGE gel and allowed to electro-transferred onto polyvinylidene difluoride (PVDF) membrane and followed by probing with different primary antibodies like anti-rabbit polyclonal anti-Bcl2 (1:1000 dilution) and anti-Bax (1:1200 dilution) and house-keeping anti-β-actin (1:2000 dilution) were purchased from Abcam (Cambridge, UK) for overnight at 4°C. Followed by washing with phosphate buffer and again probed with secondary antibodies like HRP anti-rabbit IgG for 2h at 37°C. Finally, the protein band intensity in the membrane were detected using enhanced chemiluminescence system image analyzer (ChemiDoc-Image lab; Bio-Rad Laboratories, Inc; CA, USA).

STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (SEM) after conducting all experiments as triplicate (n =3). The statistical analysis of data in the present study was measured using a student paired t-test by comparing control (DMSO) against OB using SPSS software (Ver 21: IBM; NY, USA). The p-value less than 0.05 ($p<0.05$) is conceived as statistically significant.

RESULTS

Efficacy of OB on cell viability and inflammatory markers

Fig. 1 represents the changes on cell viability upon treatment with elevated concentration of OB on HuLM cells using MTT assay. A significant decrease ($p<0.01$) in the HuLM cell count (live cells) were observed in OB treated cells in a dose-dependent fashion at both 24 h and 48 h than DMSO treated control HuLM cells. As indicated in the fig. 2, the levels of pro-inflammatory cytokines (markers) like IL-1β, TNF-α, and IL-6 were considerably declined ($p<0.01$) as the concentration of OB increases (treated cells), as compared to control cells. The above data shows that OB showed potent anti-proliferative and anti-inflammatory activity in a dose-dependent manner.

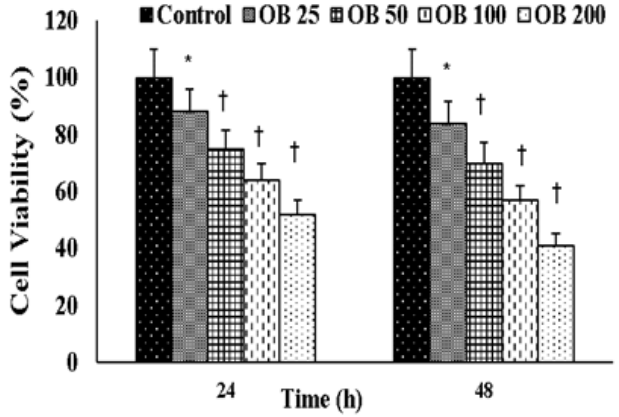

Fig. 1: Represent the changes on cell viability upon treatment with increased concentration of obovatol (OB) on HuLM cells using MTT assay. Values are expressed as the mean ± SEM. Probability value (p value) were indicated with symbol * for $p<0.05$ and † for $p<0.01$,

where the comparison were carried out between control Vs OB (25, 50, 100 and 200µM).

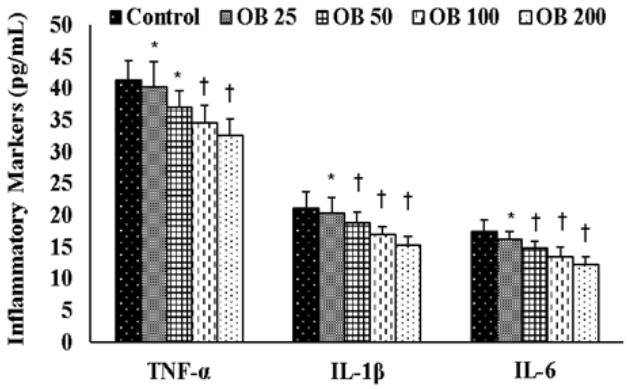

Fig. 2: Represent the changes on inflammatory markers (cytokines) after treatment with an increased concentration of obovatol (OB) on HuLM cells. Values are expressed as the mean ± SEM. Probability value (p value) were indicated with symbol * for p<0.05 and † for p<0.01, where the comparison were carried out between control Vs OB (25, 50, 100 and 200 µM).

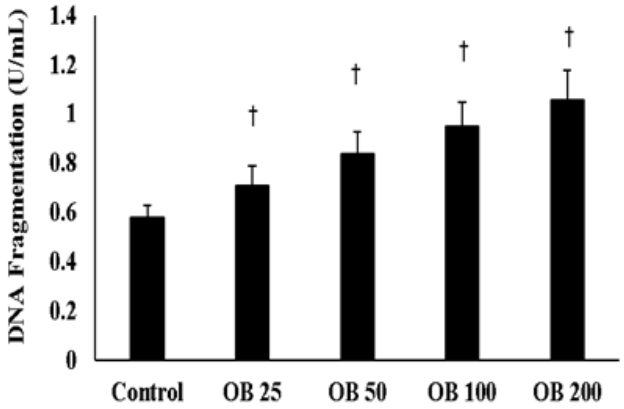

Fig. 3: Represent the changes on DNA fragmentation (apoptotic cells) upon treatment with an increased concentration of obovatol (OB) on HuLM cells. Values are expressed as the mean ± SEM. Probability value (p value) were indicated with symbol * for p<0.05 and † for p<0.01, where the comparison were carried out between control Vs OB (25, 50, 100 and 200 µM).

Effect of OB on DNA fragmentation and apoptotic markers

Fig. 3 shows the levels of DNA fragmentation after treatment with an increased concentration of OB on HuLM cells. On comparison with DMSO (control) treated cells, the OB treated cells showed increased (p<0.01) levels of DNA fragmentation. The levels of DNA fragmentation starts to increase as the concentration of OB start to incline and thus proving that OB induces cell death via damaging DNA in dose-dependent trend. Fig. 4 represent the changes in apoptotic markers (caspase 3 and 9) after treatment with an increased concentration of OB on HuLM cells. A pronounced increased (p<0.01) in the activity of Caspase 3 and 9 were detected in OB (25, 50, 100 and 200µM) treated HuLM cells than DMSO alone treated control cells.

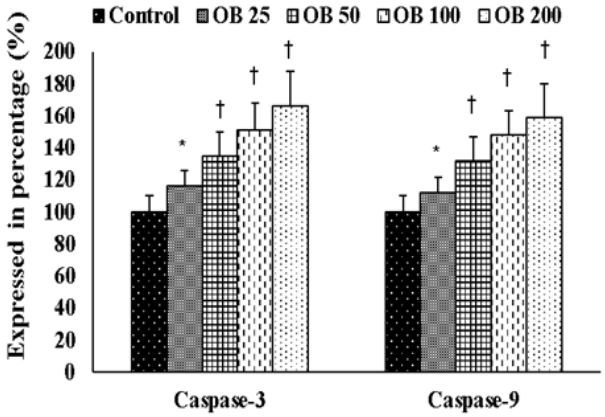

Fig. 4: Represent the changes on apoptotic markers after treatment with increased concentration of obovatol (OB) on HuLM cells. Values are expressed as the mean ± SEM. Probability value (p value) were indicated with symbol * for p<0.05 and † for p<0.01, where the comparison were carried out between control Vs OB (25, 50, 100 and 200 µM).

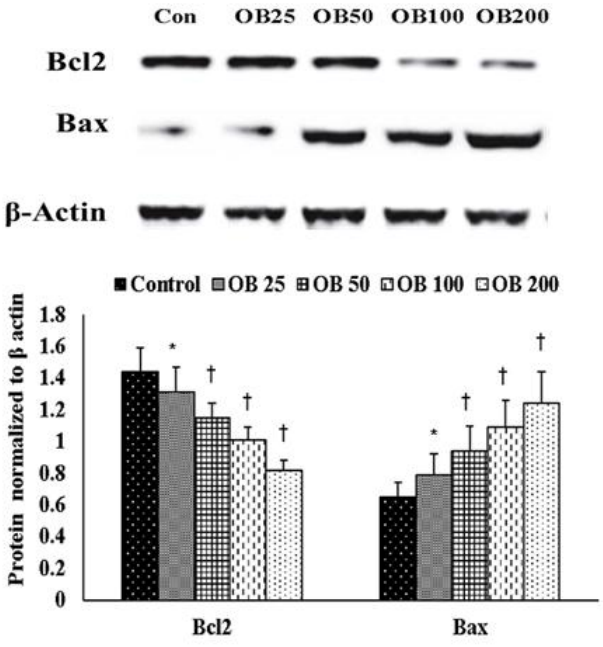

Fig. 5: Represent the changes on protein expression of Bcl2 and Bax upon treatment with increased concentration of obovatol (OB) on HuLM cells. Values are expressed as the mean ± SEM. Probability value (p value) were indicated with symbol * for p<0.05 and † for p<0.01, where the comparison were carried out between control Vs OB (25, 50, 100 and 200 µM).

Effect of OB on the protein expression of Bcl2 and Bax (apoptotic protein)

Fig. 5 illustrates the changes on Bcl2 and Bax protein expressions after treatment with OB on HuLM cells using

immune blot technique. The pro-apoptotic factor-like Bax (protein expression) were markedly up-regulated ($p<0.01$) in OB treated cell. While anti-apoptotic factor-like Bcl2 (protein expression) were markedly down-regulated ($p<0.01$) in OB treated cell on equivalent with control cells. Hence, OB showed potent apoptotic activity by enhancing pro-apoptotic protein like Bax on human leiomyoma cells in a dose-dependent manner.

DISCUSSION

Uterine fibroids (UF) or leiomyomas is non-cancerous tumors of the uterus, but it may result in discomfort and infertility (Bulun, 2013). At present surgery and GnRH, SPRMs are the best option, but with a higher relapse rate and also produce serious adverse effects. Hence, the need for the development of anti-uterine fibroid agents is of high demand (Lee *et al.,* 2014). Previously, OB showed beneficial effects against various cancer cell lines by abolishing cell proliferation and trigger cell arrest and enhance apoptosis (Kim *et al.,* 2014; Lee *et al.*, 2008; 2009). Hence, the author suspects that OB might also display protective activity against HuLM cell line. During this study, the author observes the potent protective activity of OB by inhibiting cell proliferation (cytotoxicity) and suppresses inflammatory markers along with elevated apoptosis, evidenced by increased apoptotic markers/proteins and DNA fragmentation in HuLM UF model cell line.

The anti-proliferative or cytotoxicity of OB was checked by MTT assay, as anti-proliferative activity will be the major criteria for a chemoprotective agent. Cells co-cultured with increasing concentration of OB showed increased cytotoxicity property by decreasing cell count as well as abolish HuLM cell proliferation. The elevated cytotoxicity activity of OB is already demonstrated by several authors in different cell line model (Lee *et al.*, 2008; Duan *et al.,* 2018). To explore the reason for cytotoxicity and anti-proliferative activity of OB, authors also examined inflammatory and apoptotic markers.

Ample amount of studies suggest that inflammation is one of the key factors for the development and growth of UF (Islam *et al.,* 2014 and 2013). Especially pro-inflammatory markers like IL-1β, TNF-α, and IL-6 are highly upregulated via triggering the NF-κB signaling pathway (Protic *et al*., 2016; Plewka *et al*., 2013). During this experiment, we also confer the above statement that the mean value of inflammatory markers like TNF-α, IL-1β, and IL-6 are higher in the control of HuLM cells. However, HuLM cells treated with OB showed decreased levels of the inflammatory response by abolishing the synthesis of numerous pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6. Previously, Choi and his colleagues (2012), demonstrated that obovatol can inhibit the production of various pro-inflammatory activity in the LPS model and thus displaying its potent anti-inflammatory activity.

Apoptosis is a programmed cell death, which is a crucial process/mechanism to eliminate uncontrolled or abnormal cells (cancerous cells). But the dysfunction of this apoptosis mechanism would lead to abnormal cells and subsequently results in various pathogenesis and thus contributes to various diseases including cancer, auto-immune disorders (He *et al*., 2016). Elevated DNA fragmentation is mainly found after apoptosis and most of the research focuses on DNA fragmentation to indirectly quantify the apoptosis. The DNA fragmentation levels were considerably higher in OB treated HuLM cells and thus endorsing that OB induces cell death via damaging DNA. Similarly, Lee and others (2008) also inferred that obovatol can induce chromosomal DNA fragmentation and thus trigger the apoptosis process to inhibit colorectal cancer growth and proliferation. Moreover, the apoptotic markers like caspase 3/9, as well as pro-apoptotic and anti-apoptotic proteins (intrinsic apoptotic pathway), play a crucial role in the development of growth of uterine fibroid (Chen *et al*., 2019; Lee *et al*., 2014). During this study, the activity of caspase-3 and 9 were evaluated. A pronounced increase in the activities of Caspase 3 and 9 were noted in OB added HuLM cells and thus proving that OB favors intrinsic apoptosis signaling pathway to kill HuLM cells. This outcome is in correspondence with the results of Kim and others (2016).

The anti-apoptotic (Bcl2) and pro-apoptotic (Bax) protein expressions were examined using the western blot technique. Results showed that treatment with OB notably up-regulated the protein expression of Bcl2 with significant down-regulation of Bax protein expression. Overall, OB showed potent apoptotic activity by enhancing pro-apoptotic protein like Bax as well as increasing the activation of caspase-3 and 9 to favor caspase cascade to induce apoptosis on human leiomyoma cells in a dose-dependent manner. Likewise, Kim and his co-workers (2014), also indicated that obovatol can increase the protein expression of Bax as well as activate caspase cascade to exhibit apoptosis via altering various signaling pathway. The major limitation of this cell line study is not to explore the anti-uterine fibrosis activity of OB by checking various signaling pathways includes NF-κB, PI3K/Akt or ERK1/2 and Smad-3/4.

CONCLUSION

Overall, OB could prevent uterine tumor initiation by abolishing cell proliferation (induce cytotoxicity) and halt the further development of uterine tumor by inhibiting inflammatory cascade, but triggering apoptosis. However, in the near future, the author would like to explore the underpinning mechanism for anti-uterine fibrosis by checking various signaling pathways.

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