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Original Research Article

Lycium barbarum polysaccharide attenuates cisplatin-induced apoptosis in ovary granulosa cells via alleviation of endoplasmic reticulum stress

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Abstract

Purpose: *To investigate the effect of Lycium barbarum polysaccharide (LBP) on apoptosis of ovary granulose cells (GCs), as well as its underlying mechanism.*
Methods: *GCs were isolated from Sprague Dawley (SD) rats and divided into three groups: control group, model group (DDP) and LBP group. Cell morphology was observed by H & E staining under optical microscope. Expression of follicle stimulating hormone receptor (FSHR) was assessed by immunohistochemistry (IHC), while cell viability was assayed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). Apoptosis was determined by flow cytometry. Expressions of glucose-regulated protein 78(GRP78), C/EBP homologous protein (CHOP), caspase-3, Bax protein and B cell lymphoma-2(Bcl-2) were assayed by Western blot and qRT-PCR.*
Results: *Apoptosis index (37.6 ± 2.44 %) was significantly higher ($p > 0.05$) in DDP group than in the control group (14.3 ± 1.09 %), while mRNA levels and expressions of caspase-3, Bcl-2 and Bax increased significantly ($p > 0.05$). Expressions of GRP78 and CHOP in the DDP group were also higher than in the control group ($p > 0.05$). However, these effects were effectively blocked by co-incubation with LBP. Moreover, the DDP-induced increase in apoptosis index was dose-dependently and significantly lowered by LBP ($p > 0.05$).*
Conclusion: *LBP exerts protective effect on cisplatin-induced apoptosis in ovary granulosa cells by alleviating endoplasmic reticulum stress and regulating levels of apoptosis-related proteins. Thus, LBP has the potential for alleviating adverse effects induced by cisplatin in the treatment of ovary granulosa lesions.*

Keywords: *Cis-platin, Ovary granulosa lesions, Lycium barbarum polysaccharide, Endoplasmic reticulum stress, Apoptosis*

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INTRODUCTION

Ovarian cancer, the seventh most common cancer worldwide, is a serious threat to women's health [1]. In the past few decades, improvement of medical science and technology has significantly increased the five-year survival rate of ovarian cancer patients. However, chemotherapy, one of the main therapeutic methods used for various cancers, has serious effects on the life quality of cancer patients. Cisplatin is frequently used in the treatment of ovarian cancer. Unfortunately, the side effects of cisplatin can lead to ovarian lesions [2,3]. Thus, the use of cisplatin necessitates prevention of

chemotherapy-induced premature ovarian failure and sustenance of ovarian function in ovarian cancer survivors.

Granulosa cells (GCs) are one of the major functional groups of cells in the follicle. During oocyte development, each oocyte is usually surrounded by several layers of GCs. They act as mediators of follicular potential by communicating with oocytes via oocyte paracrine signals. Proliferation and apoptosis of GCs are closely associated with ovarian function [4-6].

L. barbarum polysaccharide (LBP), the major active ingredient extracted from *L. barbarum*, has traditionally been used in Chinese medicine for protection of eyesight, regulation of immune system and treatment of cancer. Moreover, it has been reported that LBP is non-toxic to the liver and kidney [7-11]. Pharmacological studies have showed that LBP exhibits protective effects on the reproductive system via regulation of stress response and hormone balance [12,13]. However not much is known about the efficacy of LBP against ovary granulose.

The present study was aimed at investigating the effects of LBP treatment on ovarian damage induced by cisplatin and the likely underlying mechanisms.

EXPERIMENTAL

Isolation and culture of cells

Rats were injected subcutaneously with 40 U of pregnant mare serum gonadotropin (PMSG), and sacrificed 48 hours later. The ovaries were dissected out. The antral follicles were excised under anatomical microscope, and the oocytes and GCs were then separated from the antral follicles. The GCs were suspended in cold DMEM (HyClone Corporation, Utah, USA) and filtered with stainless steel cell cribble (200-mesh sieve). Following brief centrifugation, the cells were re-suspended, and then cultured in DMDM supplemented with 10 % fetal bovine serum (FBS) (HyClone Corporation) and 1 % antibiotics mix (100 U/mL penicillin and 100 µg/mL streptomycin; HyClone Corporation). The second passage of the primary GCs were used for further experiments.

H & E staining

The GCs were seeded and incubated at 37 °C in 24-well plates at a density of 1×10^5 cells/well. At about 70 – 80 % confluency, the cells were collected and fixed in 4 % PFA for 20 min, washed in PBS, and observed after H & E staining.

Immunohistochemistry (IHC)

The cultured GCs were first fixed with 4 % para-formaldehyde, and then blocked with 5 % BSA. They were thereafter incubated, first with anti-FSHR antibody (Abcam, Cambridge, MA) at 1:200 dilutions overnight at 4 °C, and then with labeled secondary antibody for 1 h.

Cell viability assay

The GCs were seeded in 96-well plates at a density of 2×10^4 cells/well. Different concentrations (1.25, 2.5, 5, 10 and 12.5 µg/mL) of cisplatin (DDP, Qilu Pharmaceutical Co., Ltd, Shandong, China) were added in the exponential growth period. The cells were then cultured for another 12, 24 and 48 h. MTT (10 µL) solution was further added to each well, and the cells were incubated for a further 4 h at 37 °C. Optical density (OD) values were read at 570 nm in a microplate reader (Thermo, USA).

Cell treatment and grouping

The cells were divided into three groups: control group, model group (DDP) and LBP group. The LBP group was pre-treated with different concentrations of LBP (80, 160, 240, 320 and 400µg/mL) for 12 h, while the DDP and LBP groups were treated with 5 µg/mL of DDP for 24 h.

Cell apoptosis assay

All three groups of cells were collected by centrifugation (2000x g for 10 min at 4 °C) and fixed with 70 % ethanol+0.5 % Triton X-100. The fixed cells were further harvested by centrifugation (2000x g for 10 min at 4 °C) and re-suspended in 0.1 mL of PBS containing 500 U/mL RNase A. Annexin V (5 µL, conjugated with FITC) and 10 µL propidium iodide were added to the solution and the cells were incubated in the dark at room temperature for 30 min. Thereafter, the cells were sorted by PI and Annexin V by FACs (Becton Dickinson FAC Star Plus flow cytometer).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cultured GCs using TRIzol reagent (Takara Bio Inc, Dalian, China), and reverse-transcribed into cDNA using PrimeScript RT reagent Kit with cDNA Eraser (Takara Bio Inc., Dalian, China). The qRT-PCR

was performed using SYBR Premix Ex Taq (Takara Bio Inc., Dalian, China) according to the manufacturer's instructions. The sequences of primers used are shown in Supplementary Table 1. Real-time PCRs were carried out using Roche LightCycle 480 (Roche, Penzberg, Germany), with GAPDH as internal control to normalize gene expression. The relative level of gene transcripts was calculated on the basis of CP value using the comparative cycle threshold method.

Western blot analysis

After the treatments, GCs were harvested and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of proteins were electrophoresed on 10 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and then transferred to polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked and incubated with primary antibodies. After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies (Zhongshan, Beijing, China) for 1 h. Immunoreactive bands were detected and analyzed with BIO-RAD ChemiDoc MP Imaging System and Image Lab Software. Relative protein levels in each sample were normalized by GAPDH to standardize the loading variations.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from at least three independent experiments, and were analyzed using one-way analysis of variance (ANOVA), followed by Tukey comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Primary culture of ovary granulosa cells

In order to ensure proper growth, the morphology and growth of the primary cells were checked soon after plating. Cells adhered to the surface of culture plates after 4 - 6h, and appeared stellate-shaped or fusiform-shaped under the microscope at 48h. The cells exhibited normal morphology (Figure 1) and normal H & E staining results (Figure 2), and were able to cover almost 90 % of the plate surface at 96 h (Figure 1).

Table 1: Primers used in quantitative Real-time PCR analysis

Gene	Primer sequence	Species
GRP78	Forward:5'-CTCAACATGGATCTGTTCCG-3' Reverse:5'-CCAGTTGCTGAATCTTTGGA-3	Human
CHOP	Forward:5'-CGCATGAAGGAGAAAGAACA-3' Reverse:5'-CACCATTCGGTCAATCAGAG-3	Human
Caspase-3	Forward:5'-AGCACTGGAATGACATCTCG-3' Reverse:5'-CGCATCAATTCCACAATTTC-3'	Human
Bcl-2	Forward:5'-GCCCTGTGGATGACTGAGTA-3' Reverse:5'-CAGCCAGGAGAAATCAAACA-3'	Human
Bax	Forward:5'-TTGCTTCAGGGTTTCATCC-3' Reverse:5'-GACACTCGCTCAGCTTCTTG-3'	Human
GAPDH	Forward:5'-TGGTATCGTGGAAGGACTCA-3' Reverse:5'-CCAGTAGAGGCAGGGATGAT-3'	Human

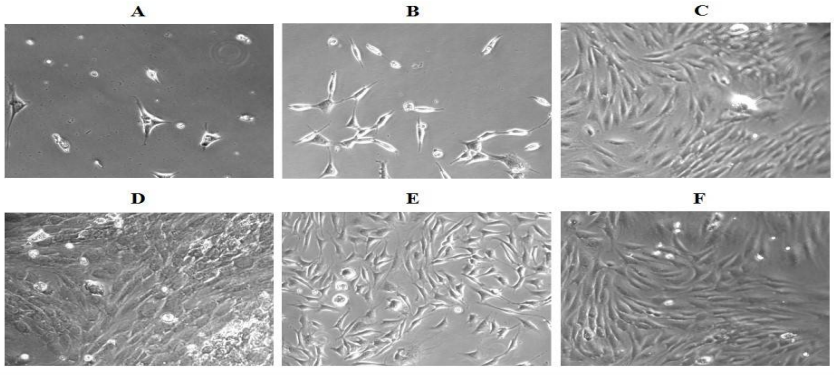

Figure 1: Morphology of the GCs (x200). A: 4 h; B: 6 h; C: 24 h; D 48 h; E: 72 h; F: 96 h after seeding FSHR, which are expressed only in granulosa cells of the ovary, was used as a marker for GCs. More than 90 % of the cells were FSHR-positive according to IHC results (Figure 2), which indicated that GCs were successfully prepared

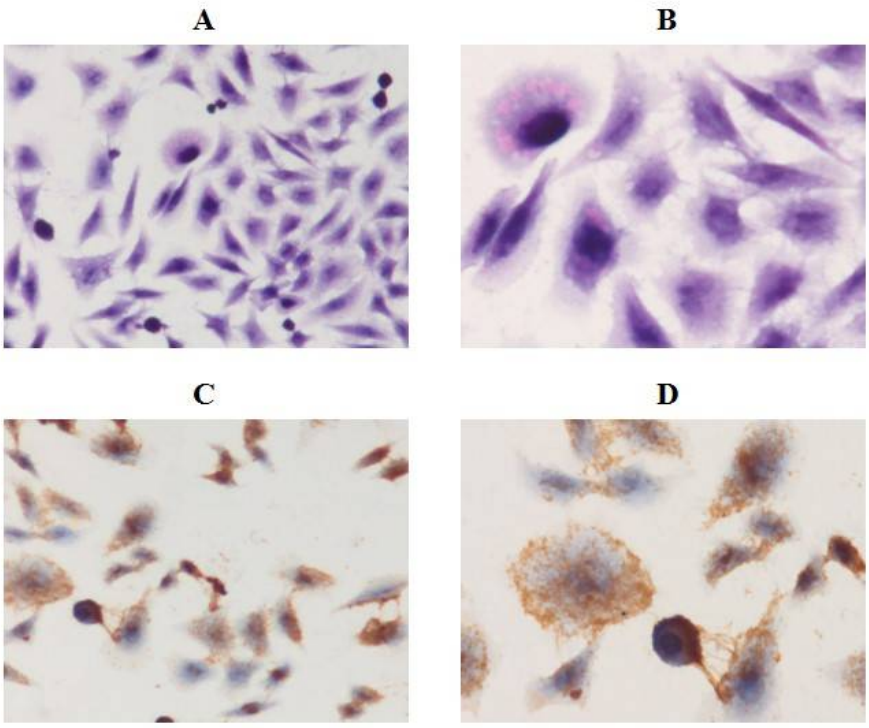

Figure 2: HE staining and IHC (anti-FSHR) images of the ovary GCs. A = H & E stain image of the ovary GCs (x200); B: H & E stain image of the ovary GCs (x1000); C = IHC image of the ovary GCs (x200); D = IHC image of the ovary GCs (x1000)

DDP inhibited viability of the GCs

To investigate the effect of DDP on GC viability, the cells were treated with different concentrations of DDP (1.25, 2.5, 5, 10 and 12.5 μg/mL) for 12, 24 and 48 h and were examined by MTT assay. In general, DDP inhibited GC viability. The inhibition was time-dependent and dose-and-time-dependent (Figure 3). It was observed that GC viability reduced from 100 % to 45 % after treated with 5 μg/mL DDP, which was comparable with the results of 48 h treatment. Thus 5 μg/mL was selected for use in subsequent experiments.

LBP reduced DDP-induced apoptosis in ovary GCs

In order to investigate how DDP and LBP impact GC viability, we examined the effect of DDP on GC apoptosis with and without LBP treatment. The results are shown in Figure 4. The degree of apoptosis in DDP and LBP groups were significantly higher than that in the control group, suggesting that both treatments induced GC apoptosis. The apoptosis index values due to treatment with 80, 160, 240, 320 and 480 μg/mL of LBP were 33.2 ± 2.15, 33.9 ± 2.08, 26.5 ± 1.63, 25.4 ± 1.51 and 16.3 ± 1.16 %, respectively. The results indicated that the apoptosis index in the LBP group decreased in a dose-independent manner when compared with the DDP group (37.6 ± 2.44 %). In addition, there was no significant difference in the apoptosis index between the control group and the 400 μg/mL- LBP group.

Effect of LBP on expression of apoptosis-associated proteins in ovary GCs

To further investigate LBP effect on apoptosis-associated gene expression in ovary granulosa cells, protein and mRNA expression levels of caspase-3, Bcl-2 and Bax were assessed by Western blot and qRT-PCR, respectively. The mRNA and protein expression levels of caspase-3 and Bax in GCs were first increased by DDP treatment (5 μg/mL) and then decreased in a dose-dependent manner, while that of Bcl-2 increased in a dose-dependent manner (Figure 5, $p < 0.05$).

Effect of LBP on ERS-associated protein in ovary GCs

The results in Figure 6 show that mRNA and protein expression levels of GRP78 and CHOP were first elevated by DDP treatment (5 μg/mL), but were decreased by LBP in a dose-dependent manner. Higher expression levels of GRP78 and CHOP were seen in the DDP group. However, the expressions of GRP78 and CHOP decreased significantly after LBP treatment when compared

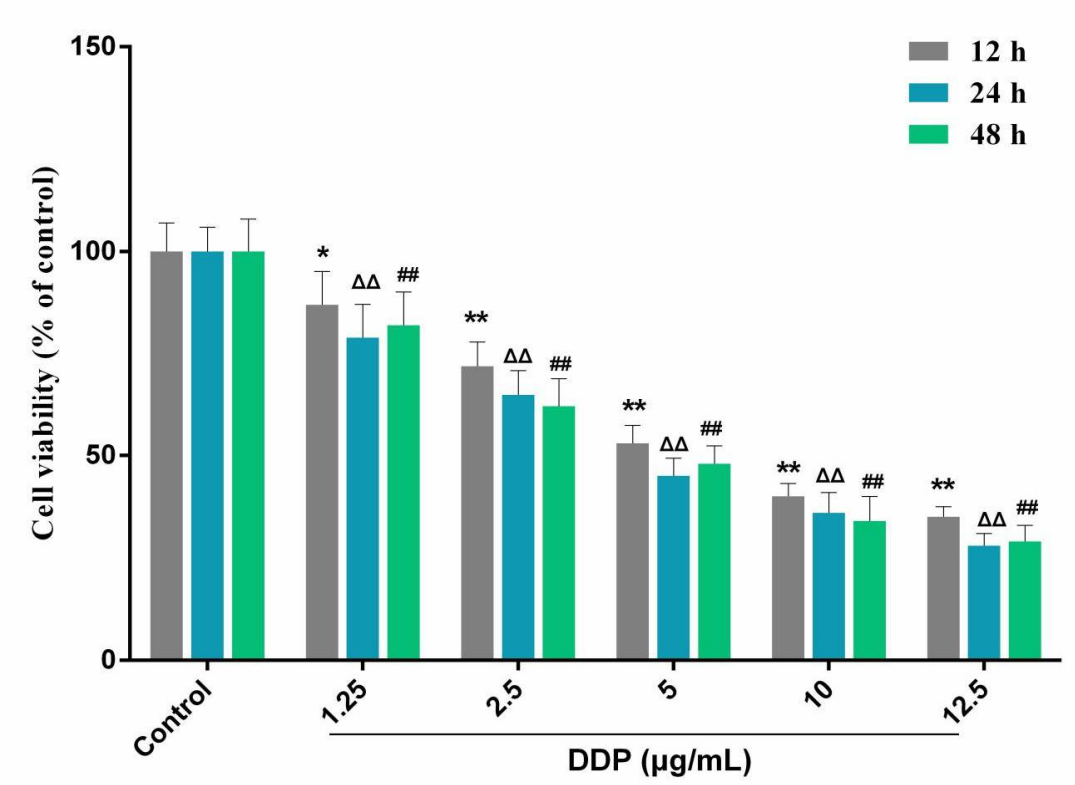

Figure 3: Effects of DDP on proliferation of ovary GCs. Cells were treated with different doses of DDP (1.25, 2.5, 5, 10 and 12.5 μg/mL) for 12, 24 and 48 h. MTT assay was used for evaluation of cell proliferation. Data are presented as mean ± SD, n = 3); $^{**}p<0.05$ and $^{**}p < 0.01$ vs. control, $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control

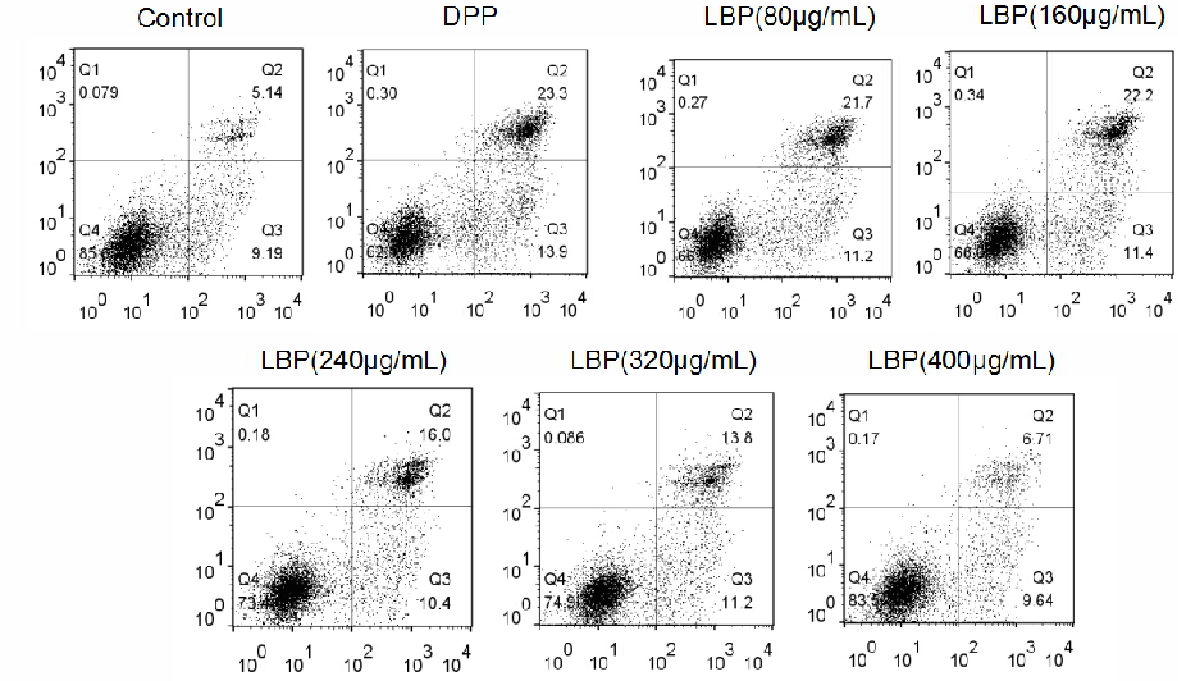

Figure 4: Effects of LBP (80-400 μg/mL) treatment on DDP-induced apoptosis of ovary GCs. Cells were treated with DDP and DDP+LBP (80 - 400 μg/mL) for 24 h, and cell apoptosis was evaluated by Flow cytometry (A). Cell apoptosis index was determined for samples subjected to DDP treatment and DDP/LBP co-treatment (B). Data are presented as mean ± SD (n = 3)

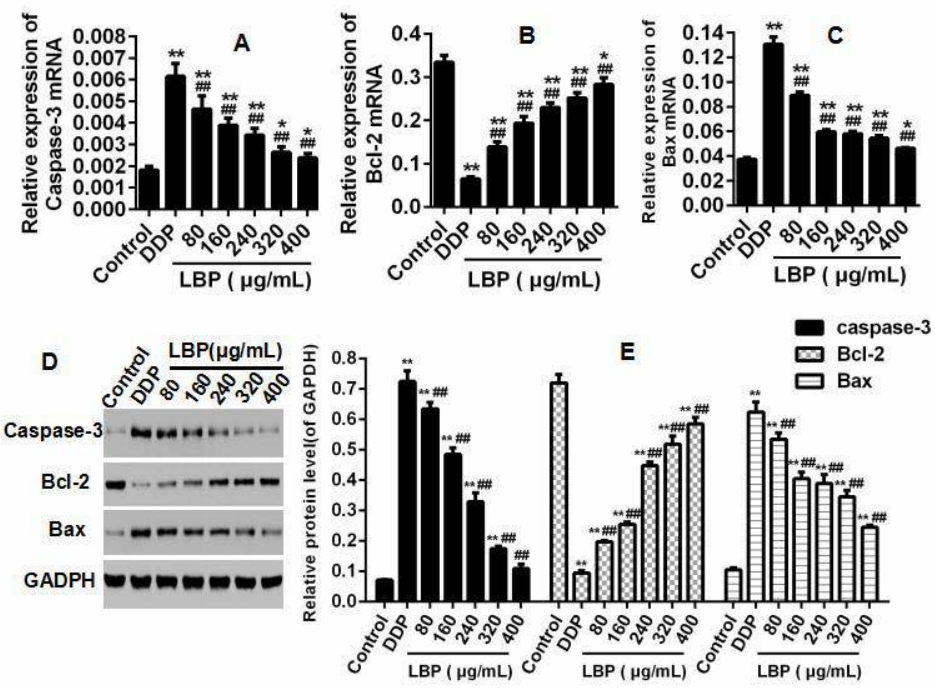

Figure 5: Effect of LBP on expressions of caspase-3, Bcl-2 and Bax. Cells were treated with different doses of LBP (80-400μg/mL) for 24 h and qRT-PCR was performed for mRNA expression level determination (A, B & C). Western blot analysis was performed using antibodies against caspase-3, Bcl-2 and Bax proteins (D & E). GAPDH was used as internal control. Data are presented as mean ± SD (n=6); **p < 0.05 and **p < 0.01 vs. control, $^{\#}p < 0.05$, $^{\#\#}p$ < 0.01 vs. DDP*

with similar expressions in the DDP group ($p < 0.05$).

DISCUSSION

The follicle is the fundamental structure and functional unit of the ovary. It is composed of oocyte, granulosa and thecal cells. Studies have shown that GC apoptosis plays an important role in regulation of ovulation and atresia in the follicle [18-20]. In the present study, we have revealed that viability of GCs was significantly decreased after treatment with different concentrations of DDP for 12, 24 and 48 h, while GC apoptosis was significantly increased when treated with 5μg/mL DDP for 24 h. The endoplasmic reticulum (ER) is an important intracellular sub-cellular organelle for synthesis, post-translational modification, and proper folding of proteins. ER homeostasis can be compromised by various external stress factors, which results in ER stress. DDP induces cell apoptosis through ERS [21].

The expression levels of GRP78 and CHOP (marker proteins of ERS) have been shown to be up-regulated during ERS, which is involved in protein folding and activation of the apoptotic pathways [22,23]. Moreover, the expression levels of Bcl-2 and Bax are associated with cell apoptosis. Bcl-2 has been shown to inhibit cell apoptosis by interfering with the release of cytochrome C which could block the activation of caspase, while Bax could induce cell apoptosis through facilitation of cytochrome C across the mitochondrial membrane. It has been reported that Bcl-2 and Bax affect the activation of caspase-3/9 [24-27]. In the present study, expression of these apoptosis-related proteins (caspase-3, Bcl-2 and Bax) were regulated by DDP treatment and DDP/LBP co-treatment. After LBP treatment, Bcl-2 expression was increased while caspase-3 and Bax expressions decreased. GRP78 and CHOP expressions in DDP group were much higher than that of control group, suggesting that DDP could induce ERS, which could further trigger GC apoptosis. These results are consistent with those obtained in previous studies [21].

The findings of the study also showed that LBP was able to reverse the DDP effect at various levels including the effects on GC viability, apoptosis and expression of GRP78 and CHOP. This was correlated with ERS regulation, suggesting that LBP exerted its protective effect on GCs via regulation of ERS and cell apoptosis.

CONCLUSION

DDP induces GC apoptosis and causes excessive ERS. However, LBP effectively reduces DDP-induced GS apoptosis and protects ovarian functionality via regulation of expressions

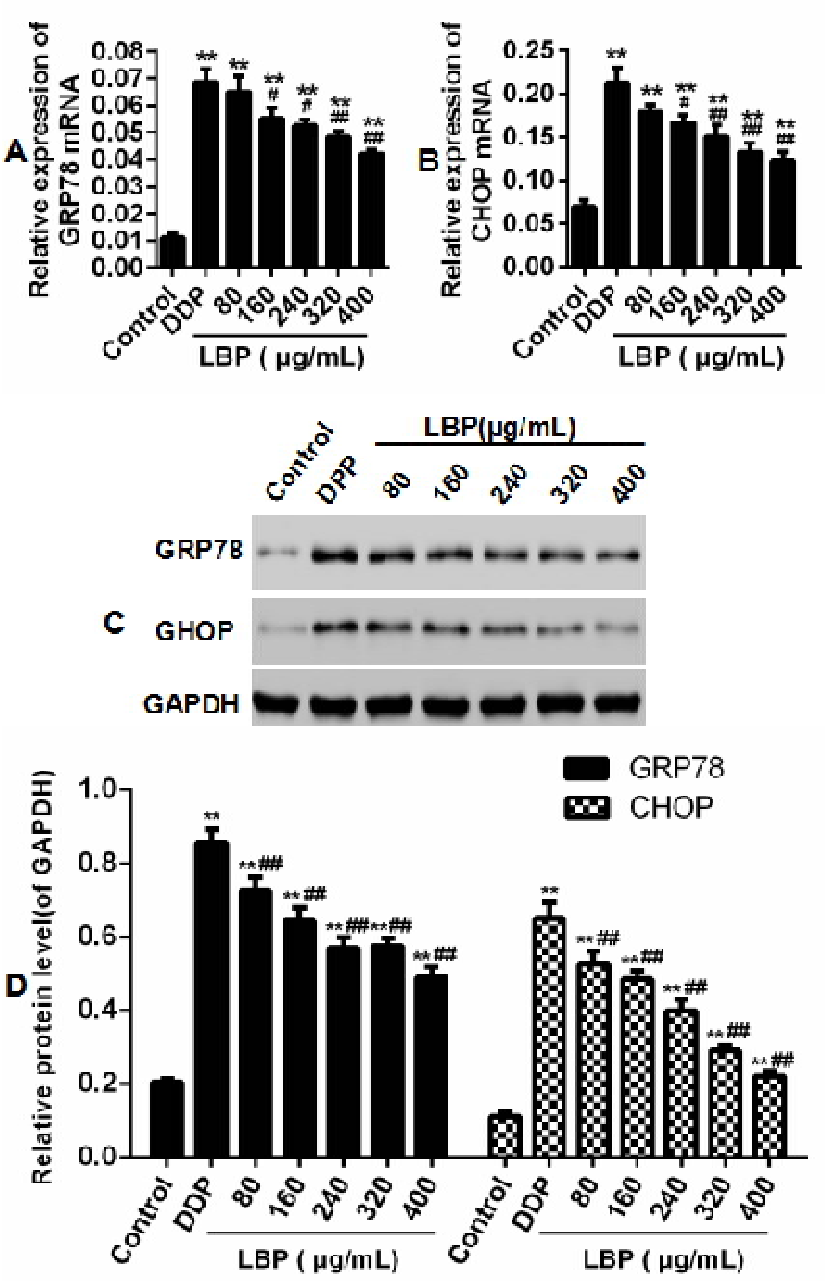

Figure 6: Effect of LBP on expressions of GRP78 and CHOP in GCs. Cells were treated with different doses of LBP (80-400μg/mL) for 24 h and qRT-PCR was performed for mRNA expression level determination (A & B). Western blot analysis was performed using antibodies against GRP78 and CHOP proteins (C & D). GAPDH was also detected as the control of sample loading. Data are presented as mean ± SD (n = 6); $^{**}p < 0.05$, $^{**}p < 0.01$ vs. control, $^{\#\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DDP

of apoptosis-related proteins and alleviation of excessive or aberrant ERS.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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