**Lycium barbarum** polysaccharides protect human trophoblast HTR8/SVneo cells from hydrogen peroxide-induced oxidative stress and apoptosis

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Abstract. Pregnancy complications are associated with abnormal cytотrophoblast differentiation and invasion. Hydrogen peroxide (H2O2) is an important mediator of oxidative ischemia/reperfusion stress in the placenta. **Lycium barbarum** polysaccharides (LBP) have been demonstrated to counteract oxidative free radicals. The effects of LBP in trophoblast HTR8/SVneo cells injured with H2O2 were examined. A cell counting kit-8 assay was performed to detect the effect of LBP at different concentrations on the proliferative ability of H2O2 injured trophoblast cells. Flow cytometry was used to determine the levels of reactive oxygen species (ROS), mitochondria membrane potential (MMP) disruption and apoptosis. Superoxide dismutase (SOD) activity and lactate dehydrogenase (LDH) leakage into the supernatant was detected by ultraviolet spectrophotometry. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to detect the expression of apoptosis-associated factors, including survivin, hypoxia inducible factor 1-α (HIF1-α), Bcl-2 apoptosis regulator (Bcl-2), Bcl-2 associated X apoptosis regulator (Bax). The results revealed that LBP protected the proliferative ability of trophoblast cells injured with H2O2 in a dose-dependent manner. LBP inhibited the oxidative stress induced by H2O2, by reducing ROS and LDH levels and increasing SOD activity. Additionally, LBP decreased MMP disruption and cell apoptosis induced by H2O2, by increasing the mRNA and protein expression of survivin, HIF1-α and Bcl-2 and decreasing Bax expression. Therefore, it was concluded that LBP protected human trophoblast cells from H2O2-induced oxidative stress and cell apoptosis via regulation of apoptosis-associated factor expression. It will provide a novel strategy for the treatment of pregnancy complications.

Introduction

Preeclampsia (PE), preterm labor and intrauterine growth retardation (IUGR) are detrimental pregnancy complications that result in significant perinatal morbidity and mortality. Normal placental development is associated with the differentiation and invasion of trophoblasts, the predominant cellular component of the placenta. PE is one of the most common and serious pregnancy complications characterized by maternal endothelial dysfunction (1). PE pathogenesis originates from abnormal cytotrophoblast differentiation, shallow cytotrophoblast invasion of the uterus and decreased maternal blood flow to the placenta (2). In addition, it is associated with future development of cardiovascular disease in the mother and child (3).

The molecular mechanism of PE remains unclear, but oxidative stress is considered to have an important role in the endothelial dysfunction and systemic vasoconstriction associated with PE (4,5). Hydrogen peroxide (H2O2), a key factor in the cellular oxidative stress cascade, is also reported as an important component in placental oxidative ischemia/reperfusion stress (6,7). Previous study has demonstrated that more H2O2 is produced in the maternal circulation of patients with PE in the stage of early pregnancy (8).

Apoptosis is critical for normal placental development and removes superfluous or dysfunctional cells to maintain normal tissue functions. However, apoptosis also participates in the pathophysiology of pregnancy complications (9). In addition, apoptosis is important in maintaining maternal immune tolerance to the antigens expressed on trophoblasts (10,11). Increased trophoblast apoptosis has been observed in pregnancy complications, including PE and IUGR (12-14), indicating that an alteration in trophoblast apoptosis may result in these diseases (15-17).

**Lycium barbarum** polysaccharides (LBP) is the active ingredient extracted from **Lycium barbarum** is, a plant species...
which produces the wolfberry and a traditional Chinese medicine, and has beneficial effects, particularly in the liver, kidney and eyes (18,19). Recent reports have demonstrated that LBP effectively improves immune function, resists oxidative free radicals and protects the testes from high-temperature injury (20). In China, Lycium barbarum is often used to treat male and female infertility in conjunction with other medicines. However, whether LBP repairs \( \text{H} \text{O}_2 \)-induced injury in trophoblast cells remains unknown.

In the present study, an oxidative injury model of trophoblast cells damaged by \( \text{H} \text{O}_2 \) was established in order to determine the protective effect of LBP on \( \text{H} \text{O}_2 \)-induced injury in trophoblast cells, and whether thus is mediated via apoptosis pathway regulation. The results of the present study may provide a novel strategy for the treatment of pregnancy complications, including PE and IUGR.

**Materials and methods**

**Cell culture and treatment.** Human trophoblast HTR8/SVneo cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM)/F12 nutrient mixture (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin in a humidified incubator containing 5% \( \text{CO}_2 \) at 37°C. Cells in the logarithmic growth phase were used in subsequent experimentation.

LBP is the main active component of the Chinese wolfberry (21). LBP was purchased from Qinghai General Health Bio-science Co., LLC (Xining, China) and the purity of LBP was >50%. HTR8/SVneo cells were treated with different concentrations (100, 200 and 400 \( \mu \text{g/ml} \)) of LBP dissolved in PBS for 6 h. \( \text{H} \text{O}_2 \) (250 \( \mu \text{mol/l} \); Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was subsequently added to treat cells for 6 h. There were five experimental groups in total: LBP1 + \( \text{H} \text{O}_2 \) (cells treated with 100 \( \mu \text{g/ml} \) LBP and 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \)), LBP2 + \( \text{H} \text{O}_2 \) (cells treated with 200 \( \mu \text{g/ml} \) LBP and 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \)), LBP3 + \( \text{H} \text{O}_2 \) (cells treated with 400 \( \mu \text{g/ml} \) LBP and 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \)), \( \text{H} \text{O}_2 \) (cells treated with 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \)) and control (without treatment) (22-24).

**Cell viability assay.** HTR8/SVneo cell viability was determined following treatment with LBP and \( \text{H} \text{O}_2 \), with Cell Counting kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Haimen, China). Cells were treated with 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \) for 1, 4 and 6 h. Briefly, following cells treatments, cells were seeded into a 96-well plate at an initial density of 5x10\(^4\) cells/well and incubated in DMEM media with PBS for 24 h at 37°C. Then cells were treated with 100 \( \mu \text{l} \) 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \) for 1, 4 and 6 h. At total of 20 \( \mu \text{l} \) CCK-8 reagent was added in and incubated for 1 h in a 5% \( \text{CO}_2 \) incubator at 37°C. Finally, the optical density values were acquired with a microplate reader at 450 nm.

**Reactive oxygen species (ROS) detection.** ROS were detected in different groups (Control, \( \text{H} \text{O}_2 \), LBP1 + \( \text{H} \text{O}_2 \), LBP2 + \( \text{H} \text{O}_2 \), LBP3 + \( \text{H} \text{O}_2 \)) with a 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay (Beyotime Institute of Biotechnology). After 6 h of 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \) treatment, cells, treated with different concentrations (100, 200 and 400 \( \mu \text{g/ml} \)) of LBP for 6 h, were seeded into wells of 6-well plate. DCFH-DA (10 \( \mu \text{mol/l} \)) was subsequently added into each well. After incubation for 20 min at 37°C, cells were rinsed with PBS and analyzed by flow cytometry. ROS levels were analyzed by CellQuest software version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA) and results were calculated relative to the control group.

**Mitochondria membrane potential (MMP) detection.** Alterations in the MMP of each group treated with LBP (100, 200 and 400 \( \mu \text{g/ml} \)) for 6 h and 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \) for another 6 h were determined by aJC-1 assay (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), using the cationic dye to detect potential-dependent accumulation in mitochondria. Briefly, 5x10\(^5\) cells were collected and resuspended in 500 \( \mu \text{l} \) PBS with JC-1 (10 \( \mu \text{mol/l} \)) for 20 min at 37°C. MMP alterations were reflected by a fluorescence emission shift from 550 nm (red) to 525 nm (green). Cells were analyzed by flow cytometry with Cell Quest software version 5.1 (BD Biosciences).

**Apoptosis detection.** The cell apoptosis proportion in each group treated with LBP (100, 200 and 400 \( \mu \text{g/ml} \)) for 6 h and 250 \( \mu \text{mol/l} \) \( \text{H} \text{O}_2 \) for another 6 h were determined by Annexin-V/propidium iodide (PI) double-stain assay, according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN, USA). Briefly, both floating and trypsinized adherent cells (5x10\(^5\)) of the five experimental groups were collected and resuspended in 500 \( \mu \text{l} \) PBS containing 0.5 \( \mu \text{g/ml} \) Annexin-V-fluorescein isothiocyanate for 20 min. Subsequently, 400 \( \mu \text{l} \) PBS with 50 \( \mu \text{g/ml} \) PI was added to cells for 5 min at room temperature in the dark. The analysis of cell apoptosis rate was immediately conducted with a flow cytometer and CellQuest software version 5.1 (BD Biosciences).

**Superoxide dismutase (SOD) and lactate dehydrogenase (LDH) detection.** The cell supernatant of each group was collected. The activity of the antioxidant enzyme SOD was determined using the total SOD assay kit with WST-8 (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. During the reaction process of SOD detection, 2-iophenyl-3-nitrophenyl tetrazolium chloride was catalyzed to formazin, which could be detected by a microplate reader. The activity of LDH was detected with a LDH assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The mRNA expression levels of survivin, hypoxia inducible factor 1-\( \alpha \) (HIF1-\( \alpha \)) and Bcl-2 apoptosis regulator (Bcl-2), Bcl-2 associated X apoptosis regulator (Bax) were reverse transcribed to cDNA using a QuantiScript Reverse Transcriptase kit (both Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. PCR amplification was performed for 15 sec at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 25 sec in an ABI 7300 Thermocycler (Applied Biosystems;
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Thermo Fisher Scientific, Inc.) with Fast SYBR-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used as the reference gene; primer sequences are displayed in Table I. The quantification was identified by \(2^{-\Delta\Delta C_q}\) method (25).

**Western blot analysis.** Cells were harvested, washed twice with PBS, lysed using lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% Na2, 100 µg/ml phenylmethanesulfonyl fluoride, 1 µg/ml aprotinin, and 1% Triton X-100) and centrifuged at a speed of 12,000 x g for 30 min at 4˚C. The supernatant was subsequently collected from the lysate and protein concentration was determined by a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). A total of 10 µg proteins were boiled and separated by 10% SDS-PAGE, followed by transfer onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk in PBS for 1 h at 37˚C and incubated with the following primary antibodies at 4˚C overnight: Anti-survivin (cat. no. ab76721; 1:5,000; Abcam). PVDF membranes were exposed to X-ray film and immunoreactive bands were detected with an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences). Finally, protein density was detected by Bio-Rad ChemiDoc XRS+ System with Image Lab Software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### Statistical analysis.** Data is expressed as the mean ± standard deviation of three independent experiments. Statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL., USA) and significance was calculated with one-way analysis of variance followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

### Results

**LBP protects the proliferative ability of HTR8/SVneo cells injured with H2O2.** To identify the effects of LBP on the proliferation of HTR8/SVneo cells injured by H2O2, a CCK8 assay was conducted in each experimental group (control, H2O2, LBP1 + H2O2, LBP2 + H2O2 and LBP3 + H2O2 groups). The results revealed that the cell proliferation ability of the H2O2 group was significantly reduced by 250 µmol/l H2O2 in a time-dependent manner, compared with the control group (P<0.01). When pre-treated with LBP for 6 h prior to the addition of H2O2, proliferation in the LBP1 + H2O2, LBP2 + H2O2 and LBP3 + H2O2 groups significantly increased in a dose-dependent manner, compared with the H2O2 group (P<0.05), indicating that LBP may protect the cell proliferation ability of HTR8/SVneo cells injured by H2O2 (Fig. 1).

**LBP reduces ROS levels in HTR8/SVneo cells injured by H2O2.** Intracellular ROS levels were measured using the oxygen-sensitive fluorescent dye DCTH-DA. The assay results revealed that the accumulation of ROS in HTR8/SVneo cells was significantly increased in the H2O2 group, compared with the control (P<0.01). Furthermore, a significant decrease in ROS formation was detected in...
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the LBP1 + H₂O₂, LBP2 + H₂O₂ and LBP3 + H₂O₂ groups, in a dose-dependent manner, compared with the H₂O₂ group (P<0.05; Fig. 2A).

**LBP increases SOD levels and decreases LDH levels in HTR8/SVneo cells injured by H₂O₂.** LDH leakage reflects cytotoxicity and mitochondrial damage (26). Levels of antioxidant enzyme SOD and leaked LDH in the cell supernatant were determined by the corresponding assay kits. Decreased SOD activity and increased LDH activity was detected in the H₂O₂ group, compared with the control group (P<0.01). LBP increased SOD activity and decreased LDH activity in dose-dependent manner, compared with H₂O₂ group (P<0.05; Fig. 2B and C).

**LBP reduces the percentage of cells with MMP disruption in HTR8/SVneo cells injured by H₂O₂.** The percentage of cells with MMP disruption was measured by JC-1 assay. The percentage of cells with MMP disruption in the H₂O₂ group was significantly increased compared with the control group (P<0.01). The percentage of cells with MMP disruption in the LBP1 + H₂O₂, LBP2 + H₂O₂ and LBP3 + H₂O₂ groups decreased significantly in a dose-dependent manner, when pretreated with different concentrations of LBP (100, 200 and 400 µg/ml) prior to H₂O₂ injury, compared with the H₂O₂ group (P<0.05; Fig. 3A). These results indicate that LBP may reduce MMP disruption in HTR8/SVneo cells with H₂O₂-induced injury.

**LBP inhibits cell apoptosis induced by H₂O₂ in HTR8/SVneo cells.** An Annexin-V/PI double-stain assay was performed to detect the apoptosis status of each group. The apoptosis rate of the H₂O₂ group significantly increased compared with control group (P<0.01); apoptosis in the LBP1 + H₂O₂, LBP2 + H₂O₂ and LBP3 + H₂O₂ groups was significantly decreased in a dose-dependent manner, compared with the H₂O₂ group (P<0.05; Fig. 3B). This indicated that LBP may inhibit H₂O₂-induced apoptosis in HTR8/SVneo cells.

Figure 2. LBP decreases ROS and LDH levels, and increases SOD activity in HTR8/SVneo cells injured by H₂O₂. (A) Levels of ROS were detected by a 2',7'-dichlorodihydrofluorescein diacetate assay. (B) The activity of the antioxidant enzyme SOD and (C) LDH leakage in the cell supernatant were determined. *P<0.05 vs. control group; **P<0.01 vs. control group; ***P<0.001 vs. control group. H₂O₂, hydrogen peroxide; LBP, Lycium barbarum polysaccharide; ROS, reactive oxygen species; SOD, superoxide dismutase; LDH, lactate dehydrogenase.
LBP regulates the expression of apoptosis-associated factors in HTR8/SVneo cells injured by H$_2$O$_2$. To detect whether the protective function of LBP on HTR8/SVneo cells injured by H$_2$O$_2$ was via regulation of apoptosis-associated factors, RT-qPCR (Fig. 4A) and western blot analysis (Fig. 4B and C) was performed to detect the expression of survivin, HIF1-α, Bax and Bcl-2 in TR8/SVneo cells treated with H$_2$O$_2$ and/or LBP. The results revealed that the mRNA and protein levels of survivin, HIF1-α and Bcl-2 decreased significantly in H$_2$O$_2$ group compared with control group, and significantly increased in the LBP treated groups in a dose dependent manner (P<0.05). The mRNA and protein levels of Bax exhibited the opposite trend (P<0.05).
Discussion

Pregnancy complications, including PE, cause marked damage to normal fetal development, which is associated with abnormal cytotrophoblast differentiation and invasion. As a key factor of the cellular oxidative stress cascade, \( \text{H}_2\text{O}_2 \) is considered an important component in placental oxidative ischemia/reperfusion stress. LBP has been reported to protect the testes from high-temperature injury, and improve immune ability and oxidative free radical resistance (27). However, whether LBP reduces \( \text{H}_2\text{O}_2 \)-induced injury in trophoblast cells remains unclear.

LBP are efficient free radical scavengers in vivo and may be useful in counteracting oxidative stress associated with aging (28,29). Therefore, the function of LBP on oxidative injury in trophoblast cells in vitro was investigated in the present study. At present, \( \text{H}_2\text{O}_2 \) is the most commonly used substance to construct a cell model of oxidative stress in various research fields (30,31). In the present study, an oxidative injury model was established in trophoblast cells injured by \( \text{H}_2\text{O}_2 \) and the effects of LBP were examined, including those on oxidative stress, MMP and cell apoptosis. The proliferative ability of human trophoblast HTR8/SVneo cells following various treatments was evaluated with aCCK-8 assay and revealed that proliferation was reduced by \( \text{H}_2\text{O}_2 \) and LBP reduced the damage caused by \( \text{H}_2\text{O}_2 \) in a dose-dependent manner.

Oxidative stress is the imbalance of oxidative and anti-oxidative factors in cells, resulting in more ROS production than ROS elimination, causing cell injury and tissue damage (32). Abundant ROS accumulate when the placenta is under conditions of ischemia hypoxia, and trophoblast cells suffer from oxidative stress when pregnancy complications exist (33-36). The present study demonstrated that \( \text{H}_2\text{O}_2 \) treatment of cells resulted in abundant ROS accumulation in trophoblast cells, and LBP decreased the levels of ROS and oxidative stress induced by \( \text{H}_2\text{O}_2 \). Following this, the potential effect of LBP on antioxidant levels in \( \text{H}_2\text{O}_2 \) injured trophoblast HTR8/SVneo cells was also investigated. SOD is the most important antioxidant in the defense system of antioxidative injury (37). The latest LBP chemical component analysis demonstrated that all glycopeptides in LBP act as the eliminators of lipid peroxide active components (38-40). Thus, it was speculated that as an anti-oxidant, LBP may decrease trophoblast cell injury induced by oxygen free radicals and protect cell development and differentiation. The results of the present study demonstrated that LBP significantly increased SOD activity and reduced ROS levels simultaneously, to protect cells from oxidative injury. Disruption of the cell membrane induced by oxidative stress or apoptosis may mediate the release of enzymes from the cytoplasm into the culture media, including LDH, which is relatively stable. By detecting the amount of LDH leakage into culture media, the cytomembrane integrity was measured. The results indicated that LDH leakage was attenuated by LBP treatment in \( \text{H}_2\text{O}_2 \)-injured trophoblast cells.

Oxidative stress is one of the main causes of cell apoptosis, through the mitochondria-dependent or independent pathways (41). Mitochondrial dysfunction caused by damage to structural integrity induces oxidative stress and the release of ROS (42). In addition, the apoptosis rates may reflect the degree of oxidative stress (43-45). The present research demonstrated an increased percentage of cells with MMP disruption and apoptosis in trophoblast cells injured by \( \text{H}_2\text{O}_2 \), whereas LBP could markedly alleviate these effects. Furthermore, it was revealed that the function of LBP in protecting trophoblast cells from \( \text{H}_2\text{O}_2 \)-induced injury may be via regulation of apoptosis-associated factors, including survivin, HIF1-\( \alpha \), Bax and Bcl-2.

Survivin is an important member of the inhibitor of apoptosis protein family that regulates apoptosis and cell division (46). Previous research has demonstrated that the expression of survivin in embryonic development promotes tissue stability and differentiation (47,48). The inhibition of survivin expression in the early stage of embryonic development results in embryonic deformities (49). HIF1-\( \alpha \) is highly expressed in anaerobic conditions, and promotes cell proliferation and tumor angiogenesis in cancer (50,51). Previous studies demonstrated that HIF1-\( \alpha \) overexpression is associated with tumor aggressiveness in human neoplasms (52,53). HIF1-\( \alpha \) knockdown promotes cell apoptosis.
by downregulating surviving, and upregulating caspase-3 and other apoptosis-promoting factors (54). Bcl-2 family members directly regulate death signals, or act indirectly through the intrinsic pathways of apoptosis, including regulation of pro-apoptotic factor release from the mitochondria. Abrupt excess expression of Bcl-2 inhibits cell apoptosis and induces tumor development, including in gastric, lung, papillary thyroid and ovarian cancer (55-58). Bax interacts with Bcl-2 to suppress its apoptosis-inhibiting effects. The data of the present study indicated that \( \text{H}_2\text{O}_2 \) may suppress the mRNA and protein expression of survivin, HIF1-\( \alpha \) and Bcl-2, and facilitate Bax expression to promote apoptosis and oxidative stress. LBP pre-treatment promoted the expression of survivin, HIF1-\( \alpha \) and Bcl-2, and decreased Bax expression to inhibit apoptosis and oxidative stress. Taken together, the results of the present study suggest that LBP alleviated \( \text{H}_2\text{O}_2 \)-induced oxidative stress through altering MMP, inhibiting cell apoptosis and regulating the expression of apoptosis-associated factors in HTR8/SVneo cells.

In summary, an \( \text{H}_2\text{O}_2 \)-injured HTR8/SVneo trophoblast cell model was constructed and LBP was demonstrated to reduce oxidative stress and apoptosis via regulating the expression of apoptosis-associated factors. Future research should focus on elucidating the mechanism of LBP in oxidative stress alleviation in trophoblast cells. Increased trophoblast cell protection from oxidative stress and apoptosis in pregnancy may provide improved treatment outcomes for individuals with pregnancy complications. In the future, the authors of the present study will examine the effects of LBP from a mechanistic view, in order to identify the receptors and downstream signals involved.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL designed the study; JL and ZD performed the flow cytometry to investigate the role of LBP on ROS, mitochondria and apoptosis regulation; YY detected cell viability; BM and YW did the RT-qPCR and western blotting assays; XX analyzed and interpreted all the experiments' data, drafted the manuscript and revised it.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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