

Hesperidin inhibits ovarian cancer cell viability through endoplasmic reticulum stress signaling pathways

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Abstract. Hesperidin is a vitamin P flavonoid compound primarily present in citrus fruits. The aim of the present study was to investigate whether hesperidin inhibits ovarian cancer cell viability via endoplasmic reticulum stress signaling pathways. A2780 cells were treated with various doses of hesperidin for 6, 12 or 24 h, and the viability of A2780 cells was assessed using the MTT assay. Hesperidin decreased the viability of A2780 cells and increased cytotoxicity in a dose- and time-dependent manner. In addition, hesperidin induced apoptosis and increased cleaved caspase-3 protein expression levels in A2780 cells. Furthermore, hesperidin markedly increased the protein expression of anti-growth arrest- and DNA damage-inducible gene 153, anti-CCAAT'enhancer-binding protein homologous protein, glucose-regulated protein 78 and cytochrome *c* in A2780 cells. The results of the present study indicated that hesperidin inhibits cell viability and induces apoptosis in ovarian cancer cells via endoplasmic reticulum stress signaling pathways. Thus, hesperidin may offer a novel therapeutic tool for ovarian carcinoma.

Introduction

Ovarian carcinoma is one of the most common types of malignant tumors in gynecology: Annually, there are ~204,449 novel cases of ovarian carcinoma worldwide and ~124,860 cases of patient mortality due to associated diseases (1). The early lesion may not be identified easily since the ovaries are located deep in the pelvic cavity. The onset of symptoms is delayed which means that when a patient presents with symptoms, the patient is typically already in end-stage disease, particularly for patients with epithelial ovarian carcinoma (2,3). Ovarian carcinoma exhibits multi-drug resistance, recurrence and

metastasis which causes it to be the most fatal type of gynecological malignant tumor (2).

The endoplasmic reticulum (ER) is an important organelle which has an important role in protein conformation and post-translational modification, enfoldment and oligomerization (4,5). In addition, ER serves a role in lipid metabolism, steroid hormone synthesis and storage. Under a variety of conditions (including anoxia, alimentary deficiency, glycosylation, oxidative stress, metabolic disorders and mutant protein expression) cells cause non-folding or misfolding protein aggregation in the ER and result in the expression of the ER stress protein glucose-regulated protein 78 (GRP78). GRP78 is a primary molecular chaperone in the ER and is a member of the heat-shock protein-70 family (5). Multiple stimuli may disturb ER functions and induce GRP78 expression (6), which serves a protective role and enables the survival of tumor cells (7). A previous study demonstrated that GPR78 is expressed in a number of types of tumor including breast, liver, lung, gastric, esophageal and skin cancer. Furthermore, by conducting studies involving overexpression and short interfering RNA knockouts, the tolerance, invasion and irradiation of cancer cells may be determined (8). GRP78 serves an important role in tumor survival which indicates a novel target for antineoplastic drugs (9).

The observed failure of chemotherapy in patients with ovarian carcinoma is attributed to the ability of ovarian carcinoma cells to exhibit drug resistance (10) by mechanisms including unregulated apoptosis (11). A previous study indicated that administration of anticancer drugs, including cisplatin, results in tumor cells releasing cytochrome *c*, activating caspase-3 and undergoing apoptosis (12). Abnormal expression of genes which regulate apoptosis cause caspase-3 to become dependent on abnormal apoptotic conduction and therefore alter the sensitivity of chemotherapeutics (13).

Sources of citrus, a generic term for the retaceous plant citrus, are abundant in China and hesperidin (chemical structure presented in Fig. 1) is an important factor in processing the by-product pomace (14). Hesperidin functions in cancer prevention, decreasing cholesterol, and serves a role in anti-anaphylaxis, antihypertension and as an antioxidant (15,16). A previous study has demonstrated that hesperidin has broad-spectrum bacteriostatic actions on *Bacillus subtilis*, *Salmonella*, *Shigella* and *Streptococcus haemolyticus* (17). Hesperidin is therefore widely applied as a food additive and

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in food processing. The aim of the present study was to investigate whether hesperidin exhibited an effect on ovarian cancer cell viability through endoplasmic reticulum stress signaling pathways.

Materials and methods

Cell culture and treatment. Human ovarian cancer cell line A2780 was obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). A2780 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin at 37°C in a humidified incubator containing 5% CO_2 .

Cell viability and cytotoxicity assay. A2780 cells were seeded in 96-well plates at 1×10^4 cells/well for 6 h and subsequently treated with various concentrations of hesperidin (0, 0.1, 1 and 10 μM for 6, 12 and 24 h). Subsequently, 5 mg/ml MTT (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was added to the cells prior to incubation at 37°C for 4 h. MTT solution was removed and 150 μl dimethyl sulfoxide was added (Sigma Aldrich; Merck KGaA). Cell viability was measured at 490 nm using a microplate reader (Tecan Group Ltd., Zurich, Switzerland) and cells treated with 0 μM were used as a control for comparison. Next, cytotoxicity was evaluated by the lactate dehydrogenase (LDH) assay according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Jiangsu, China). Cytotoxicity was measured at 490 nm using the aforementioned microplate reader.

Apoptosis analysis. A2780 cells in 6-well plates were treated with various concentrations of hesperidin (0, 0.1, 1 and 10 μM) for 48 h following seeding at 1×10^6 cells/well for 12 h. Following treatment with hesperidin, A2780 cells were suspended with 100 μl binding buffer (IMGENEX; Novus Biologicals, LLC, Littleton, CO, USA). A total of 10 μl Annexin V-fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ, USA) was added to the cells prior to incubation for 30 min in the dark at room temperature. Subsequently, 5 μl propidium iodide was added to the cells prior to incubation for 5 min in the dark. Apoptosis was analyzed using a flow cytometer (C6; BD Biosciences).

Western blot analysis. A2780 cells in 6-well plates were treated with various concentrations of hesperidin (0, 0.1, 1 and 10 μM) for 48 h following seeding at 1×10^6 cells/well for 12 h. Subsequently, A2780 cells were lysed with lysis buffer (radioimmunoprecipitation assay buffer) containing protease inhibitor cocktail (phenylmethanesulfonyl fluoride) and EDTA, at 4°C for 30 min. Cell lysates were centrifuged at 12,000 \times g for 30 min at 4°C. Total protein was extracted and protein concentration was determined using the Bradford assay. Extracted proteins (40 $\mu\text{g}/\text{lane}$) were subjected to SDS-PAGE (10–12% gel) and subsequently transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 5% non-fat milk, in Tris-buffered saline containing Tween-20 for 1 h at 37°C and subsequently incubated with the

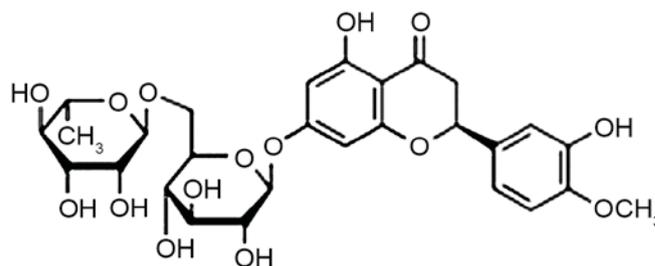


Figure 1. Molecular structure of hesperidin.

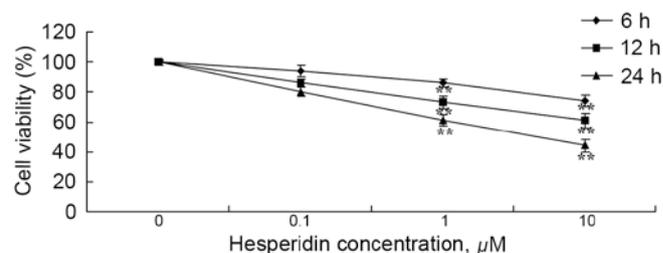


Figure 2. Hesperidin inhibits ovarian cancer cell proliferation. ** $P < 0.01$ vs. the control (0 μM hesperidin).

following primary antibodies: Anti-cleaved caspase-3 (dilution, 1:500; cat. no. sc-98785), anti-growth arrest- and DNA damage-inducible gene (GADD) 153 (dilution, 1:500; cat. no. sc-575), anti-78 kDa glucose-regulated protein (GRP-78; dilution, 1:500; cat. no. sc-13968), anti-cytochrome *c* (dilution, 1:500; cat. no. sc-7159) and anti- β -actin (dilution, 1:2,000; cat. no. A2780; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-CCAAT'enhancer-binding protein homologous protein (CHOP; dilution, 1:2,000; cat. no. 2895; Cell Signaling Technology, Inc.) overnight at 4°C. Following this, membranes were incubated with the anti-mouse or anti-rabbit immunoglobulin G secondary antibody (dilution, 1:5,000; catalog no. 14709 and 14708, respectively; Cell Signaling Technology, Inc.) at 37°C for 2 h. Western blots were developed using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Western blots were quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and Bio-Rad Laboratories Quantity One software (version 3.0; Bio-Rad Laboratories, Inc.).

Statistical analysis. Results are presented as the mean \pm standard error of the mean of at least three independent experiments. Statistical analyses were carried out using Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hesperidin inhibits ovarian cancer cell proliferation. Human ovarian cancer A2780 cells were incubated with various concentrations of hesperidin (0, 0.1, 1 and 10 μM) for 6, 12 and 24 h. As presented in Fig. 2, treatment with hesperidin decreased the viability of A2780 cells in a time- and dose-dependent manner. At hesperidin concentrations of 1 and 10 μM , A2780 cell viability was significantly decreased (Fig. 2).

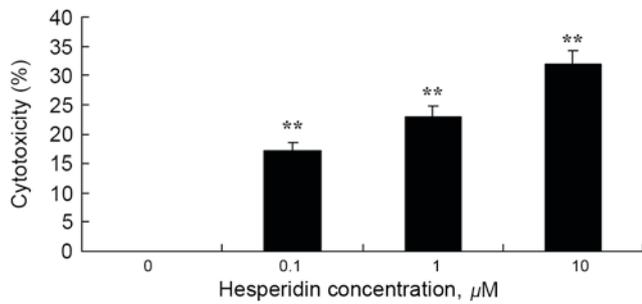


Figure 3. Hesperidin exhibits cytotoxicity towards ovarian cancer cells. **P<0.01 vs. the control (0 μM hesperidin).

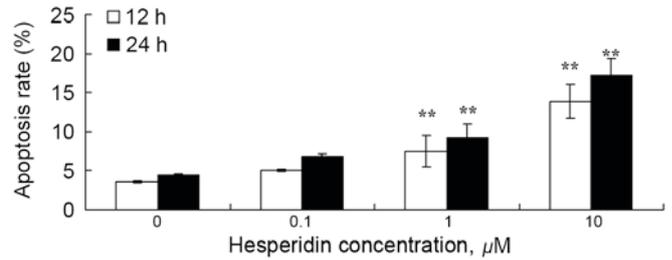


Figure 4. Hesperidin induces ovarian cancer cell apoptosis. **P<0.01 vs. the control (0 μM hesperidin).

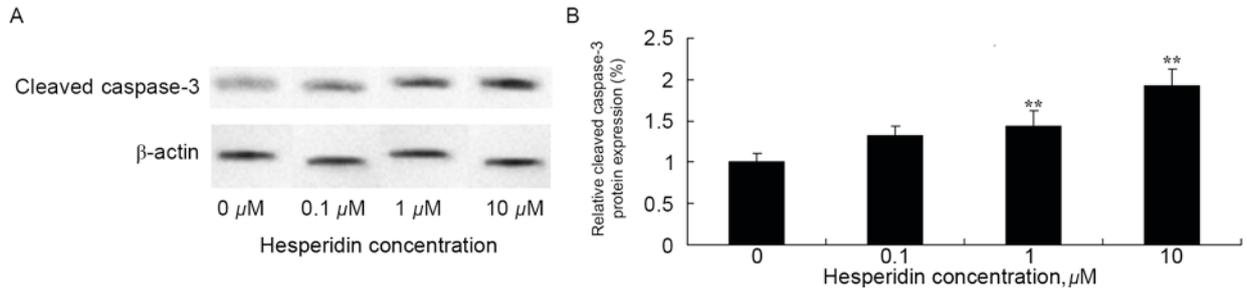


Figure 5. Hesperidin induces cleaved caspase-3 protein expression in ovarian cancer cells. (A) Western blot analysis of cleaved caspase-3 protein expression. (B) Quantification of cleaved caspase-3 protein expression in ovarian cancer cells relative to the control. **P<0.01 vs. the control (0 μM hesperidin).

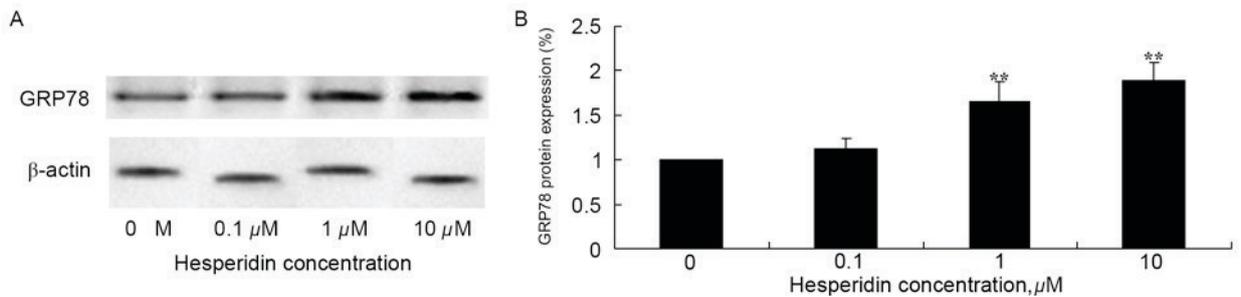


Figure 6. Hesperidin induces GRP78 protein expression in ovarian cancer cells. (A) Western blot analysis of GRP78 protein expression levels. (B) Quantification of GRP78 protein expression in ovarian cancer cells vs. the control. **P<0.01 relative to the control (0 μM hesperidin). GRP78, glucose-regulated protein 78.

Hesperidin induces ovarian cancer cytotoxicity. To investigate whether hesperidin exhibits cytotoxic effects on ovarian cancer A2780 cells using an LDH assay. As presented in Fig. 3, 1 and 10 μM hesperidin exhibited significantly increased cytotoxicity in A2780 cells.

Hesperidin induces ovarian cancer cell apoptosis. To examine the effect of hesperidin on ovarian cancer cell apoptosis, the apoptotic rate was analyzed using a flow cytometer. As presented in Fig. 4, 1 and 10 μM hesperidin significantly induced apoptosis in A2780 cells.

Hesperidin induces cleaved caspase-3 protein expression in ovarian cancer cells. To investigate whether cleaved caspase-3 may be involved in the anticancer effect of hesperidin, expression levels of cleaved caspase-3 protein was determined using western blot analysis. The results of the western blot analysis identified that 1 and 10 μM hesperidin significantly increased the protein expression levels of cleaved caspase-3 in A2780 cells (Fig. 5).

Hesperidin induces GRP78 protein expression in ovarian cancer cells. To investigate the effect of hesperidin on protein expression levels of GRP78 in ovarian cancer cells, western blot analysis of A2780 cells was performed. As presented in Fig. 6, GRP78 protein expression levels in A2780 cells was significantly increased following treatment with 1 and 10 μM hesperidin, compared with the control (0 μM hesperidin).

Hesperidin induces GADD153 protein expression in ovarian cancer cells. The effect of hesperidin on GADD153 protein expression levels in ovarian cancer cells was investigated using western blot analysis. Hesperidin concentrations of 1 and 10 μM significantly increased the protein expression levels of GADD153 in A2780 cells, compared with the control (0 μM hesperidin; Fig. 7).

Hesperidin induces CHOP protein expression in ovarian cancer cells. The effect of hesperidin on CHOP protein expression levels in ovarian cancer cell was determined using western blot analysis.

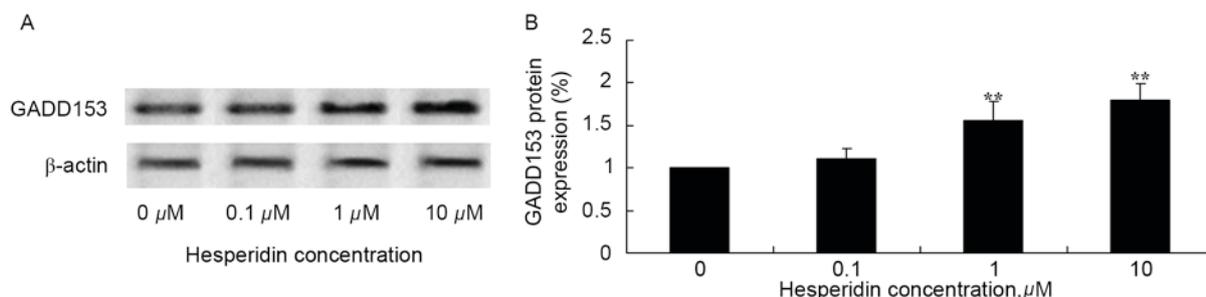


Figure 7. Hesperidin induces GADD153 protein expression in ovarian cancer cells. (A) Western blot analysis of GADD153 expression levels. (B) Quantification of GADD153 protein expression in ovarian cancer cells relative to the control. *** $P < 0.01$ vs. the control (0 μ M hesperidin). GADD153, growth arrest- and DNA damage-inducible gene 153.

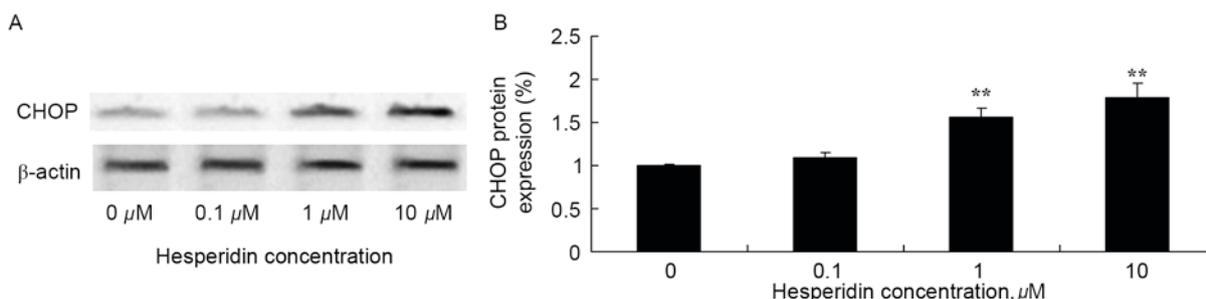


Figure 8. Hesperidin induces CHOP protein expression in ovarian cancer cells. (A) Western blot analysis of CHOP protein expression. (B) Quantification of CHOP protein expression in ovarian cancer cells relative to the control. ** $P < 0.01$ vs. the control (0 μ M hesperidin). CHOP, CCAAT⁺enhancer-binding protein homologous protein.

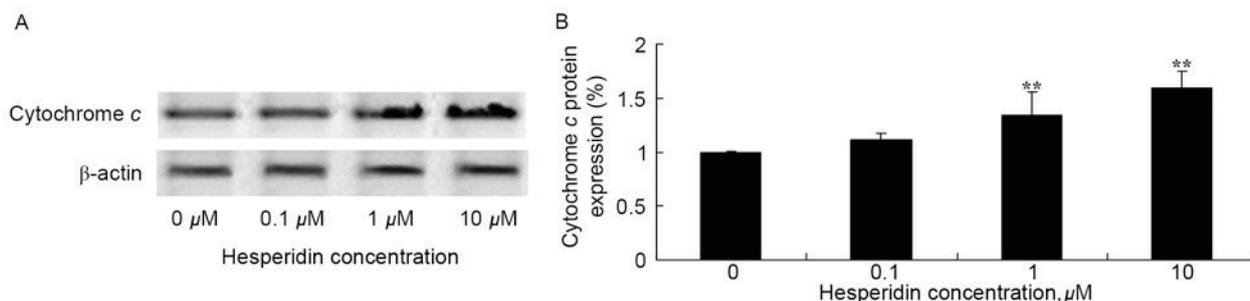


Figure 9. Hesperidin induces cytochrome *c* protein expression in ovarian cancer cells. (A) Western blot analysis of cytochrome *c* protein expression. (B) Quantification of cytochrome *c* protein expression in ovarian cancer cells relative to the control. ** $P < 0.01$ vs. the control (0 μ M hesperidin).

As presented in Fig. 8, treatment with 1 and 10 μ M hesperidin significantly increased expression levels of CHOP protein in A2780 cells, compared with the control (0 μ M hesperidin).

Hesperidin induces cytochrome c protein expression in ovarian cancer cells. To investigate the effect of hesperidin on cytochrome *c* protein expression in ovarian cancer cells, levels of cytochrome *c* protein expression were determined using western blot analysis. As presented in Fig. 9, cytochrome *c* protein expression levels were significantly increased by treatment with hesperidin (1 and 10 μ M) in A2780 cells, compared with the control (0 μ M hesperidin).

Discussion

Ovarian carcinoma is the most fatal type of malignant gynaecological tumor. If epithelial ovarian carcinoma is identified

to be in phase II or phase III of the disease, the mortality rate within 5 years is estimated to be ~70% (18). Currently, the standard treatment for patients with later-stage ovarian carcinoma, is cytoreductive surgery and postoperative combined chemotherapy (19). There are successful treatments; however, chemotherapy is not well tolerated which restricts the efficacy and prognosis of patients with ovarian carcinomas (20). The results of the present study identified that hesperidin significantly decreased cell viability and increased ovarian cancer cytotoxicity, apoptosis and protein expression levels of cleaved caspase-3 in A2780 cells. Birsu Cincin *et al* (21) indicated that hesperidin exhibits anti-proliferative, apoptotic and signal transduction effects in non-small cell lung cancer cells through the activation of caspase-3. Ghorbani *et al* (14) demonstrated that hesperidin induces apoptosis in acute lymphoblastic leukemia NALM-6 cells through the activation of p53 and suppression of nuclear factor κ B.

The abnormal viability and uncontrolled apoptosis of cells are important factors which enable the development of malignant tumors. GADD153 is a DNA damage protein which inhibits cell viability and apoptosis (22). Following DNA damage, GADD153 expression is increased, and cells are unable to progress through the cell cycle and exhibit decreased viability. If damage continues, GADD153 may induce apoptosis (23). GADD153 has increased expression and is accompanied by inhibiting the viability of the human ovarian cancer cell line CAOV3. Cell cycle arrest, triggered by GADD153, prevents cells progressing from G₁ to S phase and occurs in numerous apoptotic cells (23). This indicates that long-term starvation results in damage to DNA, inducing increased expression of GADD153 and results in decreased cell viability (24). The results of the present study indicated that hesperidin induces GADD153 protein expression in ovarian cancer cells.

Protein kinase R-like endoplasmic reticulum kinase (PERK)-mediated apoptosis depends primarily on the activation of the downstream apoptosis-promoting transcription factor CHOP (25). Activation of CHOP may be induced by downstream transcription factors of PERK including activating transcription factor (ATF)4, ATF6 and X-box-binding protein 1. PERK-eukaryotic initiation factor (eIF)2-ATF4 serves an important role in the decreased expression of CHOP (26). Under the condition of ER stress, for CHOP to be expressed, PERK must be present and ATF4 absent, by phosphorylation of ⁵¹Ser of eIF2 α . CHOP may be regulated at the mRNA level by post-transcription modifications including phosphorylation by p38 mitogen-activation protein kinase (MAPK) (27), which may increase the apoptosis-promoting activity of CHOP. The downstream target of the serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme (IRE)1-tumor necrosis factor receptor-associated factor 2/apoptosis signal-regulating kinase 1 signaling pathway is p38 MAPK; therefore, p38 MAPK phosphorylation of CHOP may link the two signaling pathways of PERK and IRE1 (28). A previous study identified that the loss of CHOP function protected cells, whereas the acquisition of CHOP increased the sensitivity of cells to external stress which caused more disturbance of the ER (29). The results of the present study demonstrated that hesperidin induces CHOP protein expression in ovarian cancer cell.

Compared with normal tissues, development of tumors results in insufficient blood supply and alimentary deficiency, causing the establishment of an anoxic and acidotic microenvironment low in sugar (6). The tumor microenvironment may result in ER stress, activation of the unfolded protein response and induction of increased GRP78 expression. Increased GRP78 expression is an important protein marker of ER stress. A previous study indicated that GRP78 expression was increased in a number of tumors including breast, gastric, prostate, liver and colon cancer (30). In the aforementioned types of tumors, increased expression of GRP78 serves an important role in tumor cell survival, apoptosis, tumor lymphatic metastasis and chemotherapy resistance (31). The results of the present study demonstrated that hesperidin induces GRP78 protein expression in ovarian cancer cells. Wang *et al* (32) revealed that hesperidin inhibits HeLa cell viability via the promotion of GADD153, CHOP, GRP78 and cytochrome *c*.

Apoptosis-associated genes may regulate the accumulation of cytochrome *c* in the cytoplasm by altering the permeability of the mitochondrial membrane to affect mitochondrial contents including caspase-3 (33). B-cell lymphoma (Bcl)-2-associated X protein, B-cell lymphoma-2-antagonist'killer 1 and Bcl-2 homology 3 domain-interacting death agonist proteins may induce mitochondria to release cytochrome *c*, whereas Bcl-2 and Bcl-extra large (xL) inhibit the release of cytochrome *c* (34). Activation of caspase-3 results in the release of cytochrome *c* from mitochondria (35). When cells are stimulated by chemotherapeutics, apoptotic factors, including Bcl-xL, may inhibit mitochondria to release cytochrome *c* to prevent apoptosis (36). The apoptotic signal conduction induced caspase-3 through the promotion of cytochrome *c*, which caused a tumor-cytotoxicity effect (37). Wang *et al* (32) identified that hesperidin inhibits HeLa cell viability by increasing cytochrome *c* protein. Additionally, Saiprasad *et al* (38) demonstrated that hesperidin induces apoptosis in colon carcinogenesis via the phosphoinositide 3-kinase/protein kinase B/glycogen synthase kinase-3 β and cytochrome *c* signaling pathways (38). The results of the present study identified that hesperidin induces cytochrome *c* protein expression in ovarian cancer cells.

From the results of the present study, it may be concluded that hesperidin markedly decreases A2780 cell viability, and induces tumor cytotoxicity and apoptosis. Additionally, hesperidin activated protein expression of cleaved caspase-3 in ovarian cancer cells, through GADD153'CHOP'GRP78 and cytochrome *c* signaling pathways in A2780 cell. Further studies are required.

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