

Dihydromyricetin induces apoptosis in a human choriocarcinoma cell line

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Abstract. Choriocarcinoma is a malignant trophoblastic tumor. The development of novel drugs is required to reduce the toxicity of current multi-agent chemotherapy and to successfully treat chemoresistant cases of the disease. The purpose of the present study was to investigate the effect of dihydromyricetin (DMY) on the human choriocarcinoma cell line, JAr, to identify a novel drug for the treatment of choriocarcinoma. An MTT assay was performed to determine the effects of DMY at different concentrations and for different exposure durations. Flow cytometry and TUNEL assays were performed to detect apoptosis, and western blotting was utilized to investigate the underlying mechanism. The results revealed that DMY significantly inhibited JAr cell viability in a time- and dose-dependent manner. The flow cytometry and TUNEL assays demonstrated that DMY inhibited proliferation by inducing apoptosis. Further analysis by western blotting indicated that the protein expression level of BCL-2 associated X, associated protein increased, while the protein expression levels of BCL-2 and pro-caspase-3 decreased. These findings suggest that DMY induced apoptosis in human choriocarcinoma JAr cells, through a mitochondrially mediated apoptotic pathway.

Introduction

Choriocarcinoma is a highly malignant trophoblastic pregnancy-associated tumor that often occurs with complete hydatidiform mole (1,2). It grows rapidly and is able to metastasize widely to other organs or tissues through the venous and lymphatic system. Although the complete recovery rate has improved owing to advances in chemotherapy, the toxicity

and side effects of the drugs used are poorly tolerated (3). Furthermore, ~7% of low-risk patients and 27% of high-risk patients may exhibit an incomplete response to first-line, single-agent or multi-agent chemotherapy, and may relapse from remission (4). Thus, it remains necessary to develop novel, specific and low-toxicity drugs for the treatment of choriocarcinoma.

Dihydromyricetin (DMY; C₁₅H₁₂O₈), a natural flavonoid, is an active component of extracts from *Ampelopsis grossedentata* (5). Numerous pharmacological functions of DMY have been reported, including antioxidant, antibacterial, anti-inflammatory, antihypertensive, hepatoprotective and anticancer effects (6-10). The potent *in vitro* antitumor activity of DMY has also been revealed through the induction of apoptosis in various cell lines, including HepG2 cells, head and neck squamous cell carcinoma, human non-small cell lung cancer and gastric cancer cells (11-14). DMY has been indicated to have antitumor effects in nude mice inoculated with GLC-82 lung cancer cells, as well as nude mice inoculated with Bel-7402 hepatocellular carcinoma cells (15,16). Furthermore, DMY could suppress distant pulmonary metastasis of 4T1 mouse breast carcinoma (17). DMY has been demonstrated to exert a strong antitumor effect with low toxicity (18,19) with a maximum tolerated dose of 5.0 g/kg in Wistar mice (20). DMY has been indicated to exhibit antitumor activity *in vitro* and *in vivo* without evident toxicity. However, the effects of DMY on human choriocarcinoma remain to be described. In the present study it was revealed that DMY inhibited JAr cell viability *in vitro*, which indicated that DMY may be a novel drug for the treatment of choriocarcinoma. Subsequently, the antitumor activity of DMY in human choriocarcinoma was determined.

Materials and methods

Reagents. DMY (>99% purity) was purchased from the Beijing Hengyuan Qitian Research Institute of Chemical Technology (Beijing, China) and dissolved in DMSO (<0.05%, v/v, without detectable effects) for all study experiments.

Cell culture. Human fetally derived trophoblast choriocarcinoma JAr cells were obtained from the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy

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of Sciences (Shanghai, China). JAr cells were cultured at 37°C in 5% CO₂ in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were then passaged using 0.25% trypsin and 0.02% EDTA (Gibco; Thermo Fisher Scientific, Inc.) when the confluency reached 70-80%.

MTT assay. JAr cells were seeded in 96-well plates and allowed to adhere overnight. When the confluency reached 30-40%, the cells were incubated for 24 or 48 h with 0, 20, 40, 80 or 100 mg/l of DMY in 200 μ l. Each treatment was performed in 6 wells. The cell viability was determined using MTT reagent (Gibco; Thermo Fisher Scientific, Inc.), according the manufacturer's protocol, and the absorbance was determined at a wavelength of 492 nm by using a Multiskan MK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The experiment was repeated three times.

Flow cytometry assay. JAr cells were incubated for 48 h with DMY at the designated concentrations (0, 40, 60 and 100 mg/l) and then processed with an AV-FITC kit (BioBox, Ba11100, Nanjing, China), in accordance with the manufacturer's protocol. The samples were analyzed by a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at 488 nm, in order to quantify the apoptotic rate.

TUNEL assay. Apoptosis of JAr cells was determined using a TUNEL detection kit (Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer's protocol. The JAr cells were incubated for 48 h with DMY at the designated concentrations (0, 40, 60 and 100 mg/l), fixed with 4% paraformaldehyde in PBS for 1 h at room temperature and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. According to the manufacturer's protocol, a positive control was permeabilized with DNase I recombinant for 10 min at 15-25°C to induce DNA strand breaks. Then the cells were washed in PBS, incubated with the TUNEL reaction mixture for 1 h at 37°C (a negative control incubated with label solution instead of TUNEL reaction mixture), washed again with PBS, and incubated with 0.1 μ g/ml DAPI in PBS at 30°C for 15-30 min. The samples were analyzed, following a final wash with PBS, under a fluorescence microscope (IX73; Olympus, Tokyo, Japan). The apoptotic rate was quantified by counting the apoptotic cells in six random fields.

Western blot analysis. The JAr cells were incubated for 48 h with DMY at the designated concentrations (0, 40, 60 and 100 mg/l), collected, and lysed on ice with radioimmuno-precipitation assay buffer (Thermo Fisher Scientific, Inc.). The cell lysates were centrifuged at 14,000 \times g for 10 min at 4°C. The protein concentration was quantified by the bicinchoninic acid assay (BCA; Pierce; Thermo Fisher Scientific, Inc.). Equal amounts (30 μ g) of protein were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk and incubated with the following primary antibodies (diluted 1:1,000) overnight at 4°C: β -actin (mouse anti-human monoclonal; cat. no. sc-130065; Santa Cruz Biotechnology, Inc., Dallas, TX, USA),

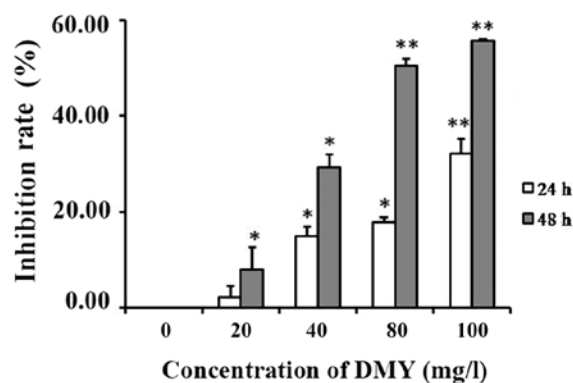


Figure 1. The effects of DMY on the viability of JAr cells. The cell viability was measured by MTT assay and the experiment was repeated three times. The cells were incubated with 0, 40, 80 or 100 mg/l DMY for 24 and 48 h. The viability of JAr cells was time- and dose-dependently reduced. *P<0.05 and **P<0.01, vs. 0 mg/l DMY group. DMY, dihydromyricetin.

Bax (mouse anti-human monoclonal; cat. no. ab77566; Abcam, Cambridge, UK), Bcl-2 (rabbit anti-human polyclonal; cat. no. E1A6139; Enogene, Nanjing, China), poly (ADP-ribose) polymerase (Parp), pro-caspase-3 and cleaved caspase-3 (rabbit anti-human polyclonal; cat. nos. 9661s and 14220s, respectively; Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were then washed in TBS-Tween (0.05%) buffer and incubated with a secondary antibody (peroxidase-conjugated Affinipure goat anti-mouse IgG, cat. no. ab6728; peroxidase-conjugated Affinipure goat anti-rabbit IgG, cat. no. ab6721; dilution 1:5,000; Abcam) for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) and imaged using the Tanon-6100 Chemiluminescent Imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China). The band densities were calculated with Quantity One software 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). All data are expressed as the mean \pm standard deviation. One-way analysis of variance was used to make comparisons between groups. The pairwise comparison of means among groups was performed using the Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

DMY inhibits the viability of JAr cells. An MTT proliferation assay was performed to evaluate the influence of 0, 20, 40, 80 and 100 mg/l DMY on the cellular viability of JAr cells at 24 and 48 h (Fig. 1). As indicated, the viability of JAr cells was time- and dose-dependently reduced following treatment with DMY in comparison with the control cells (P<0.05; Fig. 1).

DMY induced JAr cell apoptosis. The present study investigated apoptosis following incubation with different concentrations of DMY for 48 h. The quantitative analysis of apoptosis by flow cytometry using Annexin V/PI dual staining revealed that the apoptotic rate increased in a dose-dependent manner (Fig. 2A).

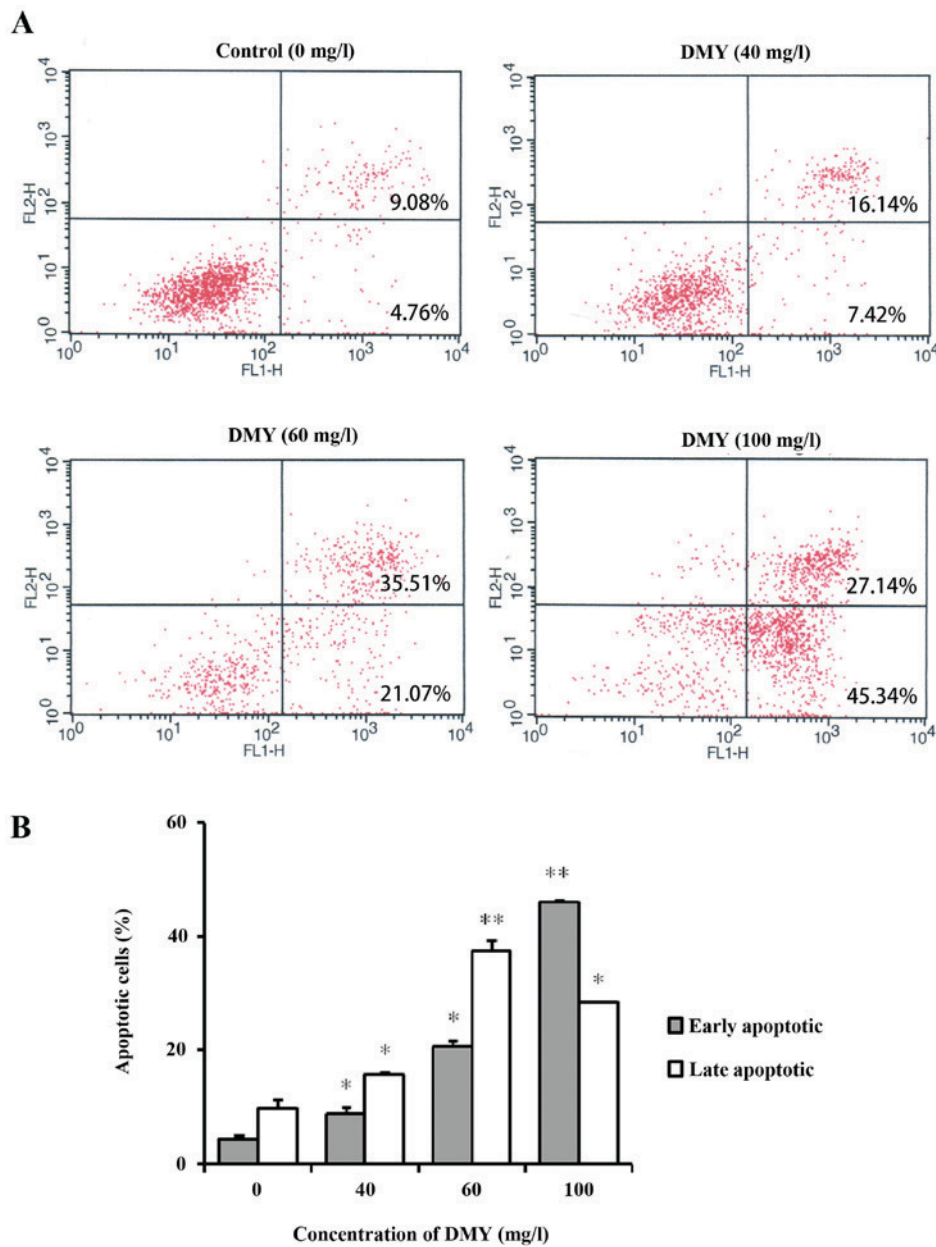


Figure 2. The cells were incubated with 0, 40, 60 or 100 mg/l DMY for 48 h. (A) Apoptosis was detected using an FITC Annexin V-PI Apoptosis detection kit and measured using flow cytometry (B). The data revealed that DMY induced JAR cell apoptosis in a dose-dependent manner. * $P < 0.05$ and ** $P < 0.01$ vs. 0 mg/l DMY group. DMY, dihydromyricetin.

At 0, 40, 60 and 100 mg/l of DMY the proportion of apoptotic cells was 14.2 ± 1.69 , 24.43 ± 1.72 , 58 ± 2.08 and $74.42 \pm 0.41\%$, respectively ($P < 0.05$; Fig. 2B).

The DMY-induced apoptosis of JAR cells was also confirmed by TUNEL staining. In the control group, few apoptotic JAR cells were observed, but DMY treatment resulted in a dose-dependent increase in TUNEL-positive JAR cells ($P < 0.05$; Fig. 3).

DMY induced apoptosis increases the Bax/Bcl-2 ratio and decreases pro-caspase-3 expression. With DMY treatment, the protein expression level of Bax increased, whereas the expression level of Bcl-2 decreased in a dose-dependent manner (Fig. 4A). The Bax/Bcl-2 ratio significantly increased with DMY treatment in comparison with that of the control group ($P < 0.05$), particularly in the 100 mg/l group ($P < 0.01$; Fig. 4B).

The expression of pro-caspase-3 decreased with DMY treatment, ($P < 0.05$) and cleaved caspase-3 was not detected (Fig. 4B).

Discussion

In the current study, an MTT proliferation assay was performed to determine the effect of DMY treatments at different concentrations and for different time periods. The results revealed that DMY reduced JAR cell viability in a time- and dose-dependent manner. Two distinct modes of cell death, apoptosis and necrosis, can be distinguished from the differences in the morphological, biochemical and molecular changes of cells. Flow cytometry was used to detect phosphatidylserine ectropion and the TUNEL assay was used to detect DNA fragmentation of apoptosis. The two methods detect the morphological and the biochemical processes of

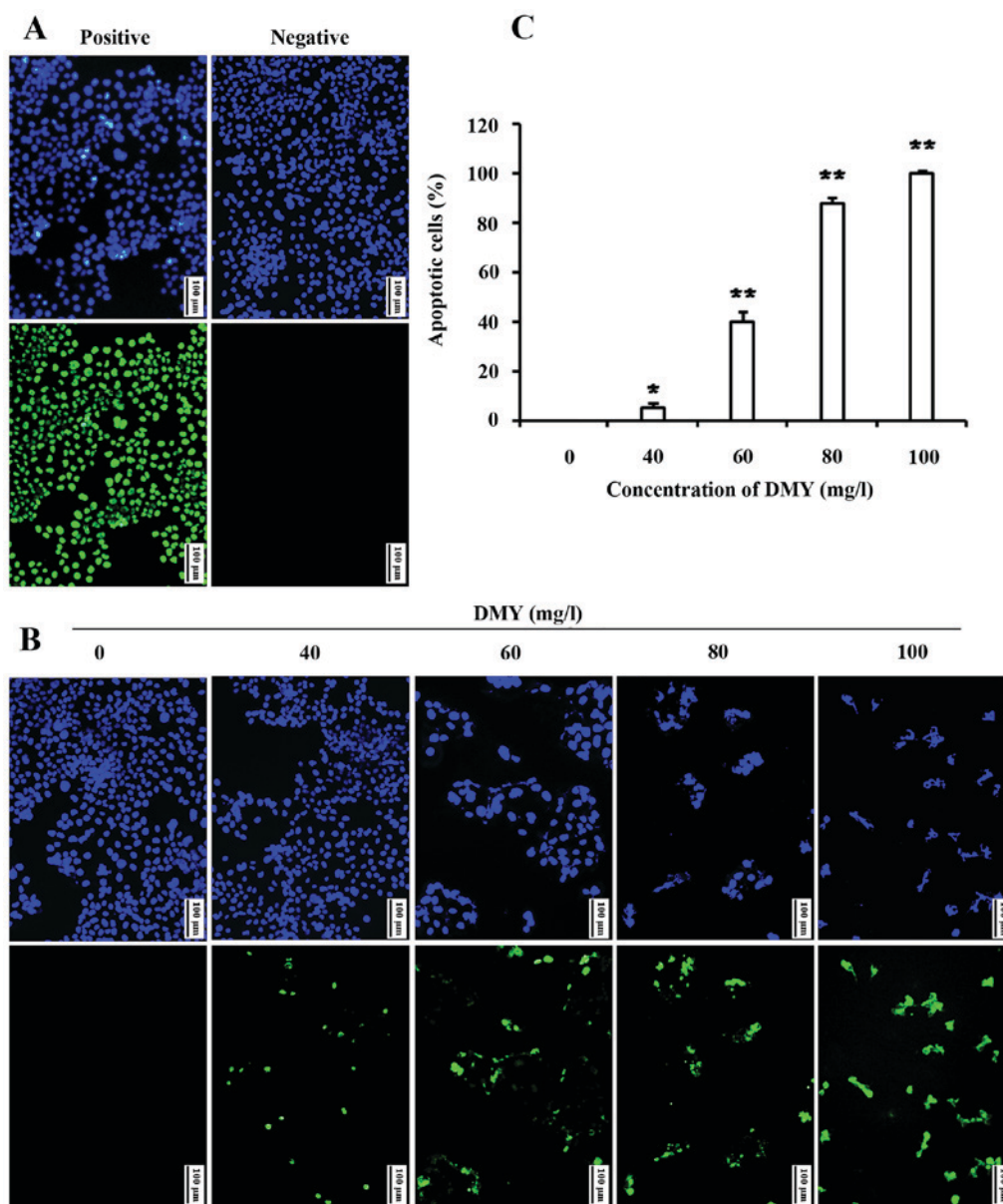


Figure 3. The effects of DMY on JAR cells apoptosis. The exposure time to TUNEL was the same for all groups. Apoptosis was determined by TUNEL staining (green staining) and cell total number was determined by DAPI staining (blue staining). (A) Positive and negative groups. In accordance with the TUNEL manufacturer's protocol, for the negative control Label solution was added instead of TUNEL reaction mixture, so the negative control group exhibits no green staining, with the aim of eliminating false-positives. A positive control was permeabilized with DNase I recombinant for 10 min at 15-25°C to induce DNA strand breaks. The positive control group thus exhibits all green staining, with the aim of eliminating false-negatives. (B) Changes in the nuclear chromatin were observed in JAR cells using TUNEL/DAPI staining. (C) Quantification of TUNEL-positive cells following treatment with 0, 40, 60, 80 or 100 mg/l DMY. TUNEL staining indicated that the proportion of apoptotic JAR cells increased in a dose-dependent manner with DMY treatment. * $P < 0.05$ and ** $P < 0.01$ vs. 0 mg/l group. Scale bar, 100 μ m. DMY, dihydromyricetin.

apoptosis and were performed at 48 h of DMY treatment, the time point at which the inhibitory effect was most significant. The results revealed that DMY induced the apoptosis of JAR cells in a dose-dependent manner, suggesting that DMY reduced proliferation through the induction of apoptosis.

Apoptosis is a form of programmed cell death that serves an important role in the development and treatment of tumors (21). The regulatory pathways of apoptosis, which include a number of gene families, orchestrate the specific morphological and biochemical changes that occur during the apoptotic process (22). In the mitochondrial pathway to cell death, the apoptotic threshold is set by interactions on the mitochondrial outer membrane between three functionally

and structurally distinct subgroups of the Bcl-2 protein family: Bcl-2 homology 3 (BH3)-only proteins, which convey signals to initiate apoptosis, pro-survival cell guardians, including Bcl-2 itself, and pro-apoptotic effector proteins, including Bax and Bcl-2 antagonist/killer (23). Bax forms oligomers, which permeabilize the mitochondrial outer membrane, releasing cytochrome c into the cytosol and activating the effector caspases (24-26). Bcl-2 binds to Bax and inhibits its pro-apoptotic activity (27). To investigate the mechanism by which DMY triggers apoptosis, western blot analysis was performed to analyze the potential proteins involved. The results revealed that DMY treatment increased the protein expression level of Bax and decreased that of Bcl-2. Although

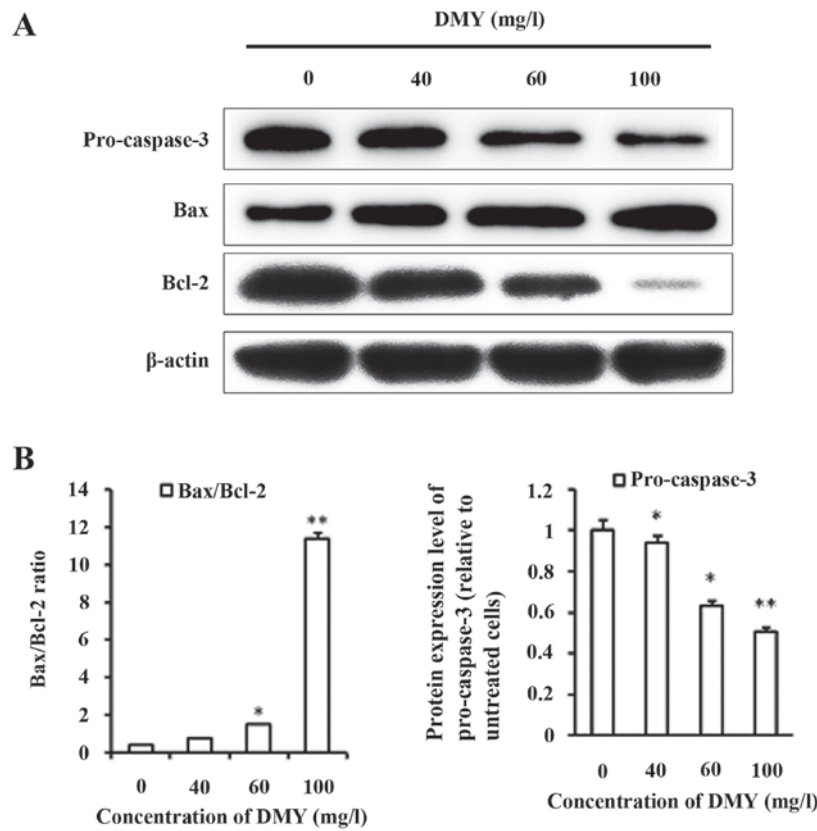


Figure 4. Western blot analysis of the effects of DMY on protein expression levels in JAr cells. (A) Western blot analysis of cells treated 0, 40, 60 or 100 mg/l DMY for 48 h. (B) Bar charts indicating DMY increased the expression level of Bax and decreased that of Bcl-2 (arbitrary units). The protein expression level of pro-caspase-3 decreased, which was indicative of its cleavage and activation. The experiment was performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. 0 mg/l group. DMY, dihydromyricetin; Bax, Bcl-2 associated X.

cleaved caspase-3 was not detected, the expression level of pro-caspase-3 decreased, which indicated that it was cleaved and activated. Pro-caspase-3 is processed by autoproteolytic cleavage (or by one or more other proteases in transit) that leads to the assembly of the active heterotetrameric enzyme (28). The activation of caspase-3 raises the question of whether this protease is required for cell death (28).

It was concluded that DMY induced apoptosis in the JAr cell line and it was confirmed that the apoptosis was mitochondrially mediated by changes in the expression level of the Bax/Bcl-2 protein ratio and decreased protein expression of pro-caspase-3. To determine whether DMY could be a novel therapeutic drug for choriocarcinoma, further studies are being performed, in which the promising prospects of the present study will be reported in detail.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZZ designed the study, performed the experiment, analyzed and interpreted the data and prepared the first draft of the manuscript. QX, YJL and DYS contributed to the design, data analysis and revision of the manuscript. KW and YTL performed the experiment. XJL contributed to flow cytometry assay. YHL developed the concept of the study, performed the data analysis and revised the manuscript. All authors read and approved the final manuscript.

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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