

# Chinese herbal medicine *Yougui* Pill reduces exogenous glucocorticoid-induced apoptosis in anterior pituitary cells

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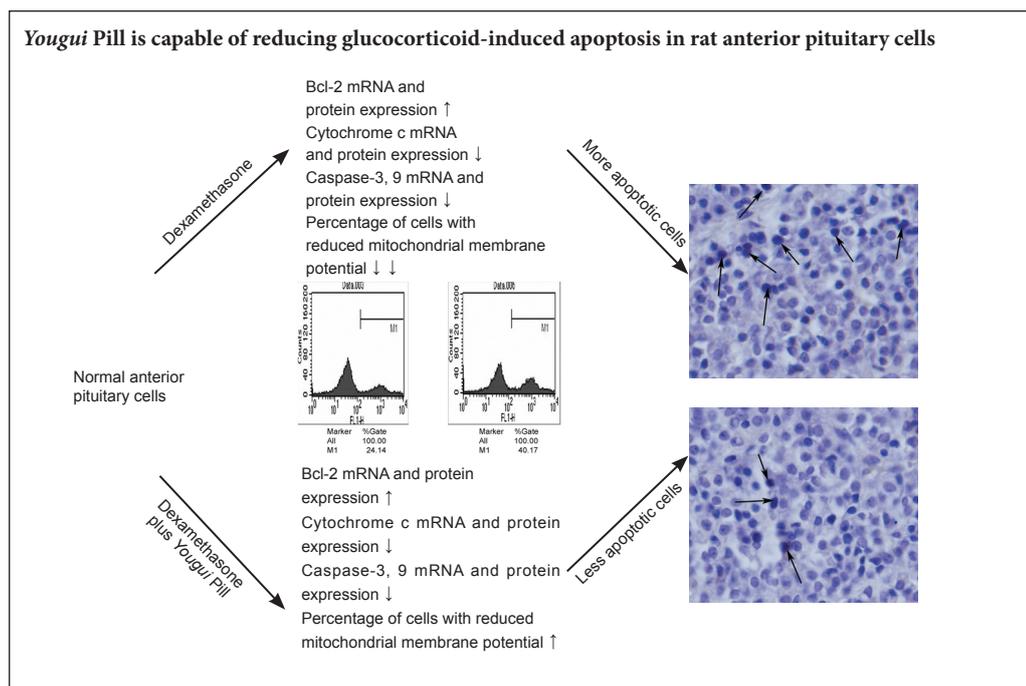
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## Graphical Abstract



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## Abstract

Long-term glucocorticoid use may result in sustained suppression of one or more secreted components from the hypothalamo-pituitary-adrenal axis, and often results in apoptosis. *Yougui* Pill (YGP), a 10-component traditional Chinese herbal medicine, has been shown to be clinically effective for glucocorticoid-induced suppression of the hypothalamo-pituitary-adrenal axis. However, the pharmacological and molecular mechanisms remain unclear. We hypothesized that YGP would exert an anti-apoptosis effect on dexamethasone-treated anterior pituitary cells. *In vivo* experiments showed that YGP significantly reduced the number of apoptotic cells, down-regulated mRNA expression of cytochrome c, caspase-3, and caspase-9, and up-regulated mRNA expression of Bcl-2. These findings suggest that YGP reduced glucocorticoid-induced apoptosis in rat anterior pituitary cells by regulating the mitochondria-mediated apoptosis pathway.

**Key Words:** nerve regeneration; *Yougui* Pill; glucocorticoid; apoptosis; Bcl-2; cytochrome c; caspase-3; caspase-9; mitochondrial membrane potential; neural regeneration

## Introduction

Glucocorticoids have been widely applied to treat autoimmune-related diseases, such as systemic lupus erythematosus, scleroderma, and rheumatoid arthritis (Buttgereit et al., 2002; Bijlsma et al., 2005). Long-term glucocorticoid use prohibits secretion of compounds from the hypothalamo-pituitary-adrenal (HPA) axis, which results in a series of adverse reactions. Previous studies have shown that glucocorticoid use results in sustained suppression of one or more secreted components from the HPA axis, both in humans (Lamberts et al., 1997; Richter et al., 2002) and in rats (Nicholson et al., 1984; Calogero et al., 1990; Huang, 1994). Apoptosis is thought to play an important role in the negative effects of glucocorticoids (Nolan et al., 1998; Nolan and Levy, 2001).

*Yougui* Pill (YGP), a 10-component Chinese herbal mixture (Radix Rehmanniae Preparata, Radix Aconiti Lateralis Preparata, cinnamon, yam, *Macrocarpium officinale*, *Cuscuta reflexa*, *Lycium barbarum*, antler glue, Cortex *Eucommiae*, and Radix *Angelicae Sinensis*), has been used to improve symptoms caused by glucocorticoids, although the underlying mechanisms remain unknown. The present study aimed to explore the anti-apoptotic effects of YGP on rat anterior pituitary cells treated by dexamethasone.

## Material and Methods

### Experimental animals

Male, specific pathogen-free, 3-month-old, Sprague-Dawley rats weighing 180–200 g, were purchased from the Department of Laboratory Animal of China Medical University (Shenyang, Liaoning Province, China). Ten animals per cage were housed in temperature- and humidity-controlled rooms, and were maintained on a 12-hlight/dark cycle and allowed free access to water and food. The experimental protocol was approved by the committee for experimental animals of China Medical University Laboratory Animal Center (Approval No. CMUEC2008035) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

### YGP preparation

The ten YGP components were purchased from Tongrentang Pharmaceutical Group (Beijing, China). Briefly, all YGP components (24 g Radix Rehmanniae Preparata, 6 g Radix Aconiti Lateralis Preparata, 6 g cinnamon, 12 g yam, 6 g *Macrocarpium officinale*, 12 g *Cuscuta reflexa*, 9 g *Lycium barbarum*, 12 g antler glue, 12 g Cortex *Eucommiae*, and 9 g Radix *Angelicae Sinensis*) were respectively broken down into a coarse powder, and then mixed together. The mixture was mixed for 1 hour with distilled water to a final concentration of 10%, heated to the boiling point, and then decocted at a simmer for 1 hour. After filtering out the crude herbs, the residuals were decocted twice using the same method. The filtrates were then collected and mixed, then centrifuged at 400× g for 5 minutes to remove residual sediment. The

extracting solution was concentrated to 0.45 g/mL in a 90°C water bath. Finally, the drug-containing mixture was cooled down, and stored at 4°C until further use.

### Treatment

A total of 80 male rats were randomly assigned to four groups. For the dexamethasone group, the rats were administered an intramuscular injection of 1 mg/kg dexamethasone (Tiantai Mountain Chengdu Pharmaceutical Co., Ltd., Chengdu, Sichuan Province, China) once daily for 15 days, followed by 0.5 mg/kg dexamethasone for 15 days. For the dexamethasone plus YGP group, the rats were intramuscularly injected with 0.5 mg/kg dexamethasone once daily for 15 days, followed by intragastric administration of 10 mL/kg YGP once daily for another 15 days. For the YGP group, the rats were intramuscularly injected with 1 mL/kg normal saline once daily for 15 days, followed by a half dosage of normal saline and intragastric administration of 10 mL/kg YGP once daily for another 15 days. For the control group, rats were intramuscularly injected with normal saline once daily for 15 days, followed by intragastric administration of normal saline (10 mL/kg) once daily for another 15 days. All treatments were performed from 8:00 a.m. to 9:00 a.m. The rats were sacrificed by decapitation at day 31, and the anterior pituitaries were dissected for observation.

### Preparation of tissue sections

Immediately after decapitation, the anterior pituitary glands were carefully removed and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 48 hours, washed twice with fresh PBS, and embedded in paraffin wax. A series of 5- $\mu$ m-thick axial sections was cut from each pituitary for immunohistochemistry and terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay.

### Immunohistochemical staining

Immunohistochemistry was performed to analyze expressions of various apoptotic regulatory factors on paraffin sections of pituitary glands from all groups, as previously described (Gu et al., 2012). Antigen retrieval was performed by incubating for 10 minutes in a 700-W microwave oven in 10 mM citric acid, pH = 6.0, to increase sensitivity of antigen detection. The rabbit anti-rat primary antibodies (Boster, Wuhan, China) used in this study included Bcl-2 (1:80), cytochrome c (1:80), caspase-3 (1:80), and caspase-9 (1:80) at 37°C for 20 minutes. All sections were stained using the streptavidin-biotin peroxidase complex (SABC) method with goat anti-rabbit antibodies (Boster, Wuhan, China) as the secondary antibody at 37°C for 30 minutes, and then 4°C overnight. Brown-yellow granules in the cytoplasm represented a positive staining. Six views were randomly quantified from each section under a microscope (Olympus CX31-LV320 Olympus (China) Co., Ltd., Shanghai, China). The results are expressed as a percentage of positive cells.

### TUNEL assay

Apoptotic activity was analyzed by TUNEL staining using

a commercial kit (Boster) on paraffin sections of pituitary tissue. Briefly, anterior pituitary sections were treated with proteinase K (1:200 in Tris-HCl buffered saline (TBS)) for 15 minutes at 37°C prior to the TUNEL reaction. After incubation with DIG-dUTP and TdT enzyme for 2 hours at 37°C, the sections were incubated with anti-DIG-biotin (1:100) for 30 minutes at 37°C, and then developed with SABC and diaminobenzidine (DAB) for 10 minutes. Apoptosis was evaluated by randomly quantifying six views from each section under a microscope (Olympus (China) Co., LTD., Shanghai, China). The results are expressed as a percentage of apoptosis.

### Real-time quantitative PCR

Real-time quantitative PCR was performed to identify differentially expressed apoptosis-related genes. Total RNA was extracted from anterior pituitaries as described in the TRIZOL Reagent protocol (Invitrogen, Shanghai, China) with some modifications (Zhang et al., 2014). Total RNA was treated with deoxyribonuclease I (DNase I; Sigma) and reverse-transcribed using Prime Script™ reverse-transcriptase (Takara, Dalian, China). Real-time quantitative PCR reactions were performed using a Light Cycler (Roche, Shanghai, China) and the fluorescent reporter dye SYBR Green I (Molecular Probes, Takara, Dalian, Liaoning Province, China). Primers (designed and compounded by Takara Japan) were as follows: Bcl-2, upstream primer: 5'-TGA ACC GGC ATC TGC ACA C-3', downstream primer: 5'-CGT CTT CAG AGA CAG CCA GGA G-3'; cytochrome c, upstream primer: 5'-GGA GGC AAG CAT AAG CAT GG-3', downstream primer: 5'-CTG CCC TTT CTC CCT TCT TC-3'; caspase-3, upstream primer: 5'-GCA GCA GCC TCA AAT TGT TGA CTA-3', downstream primer: 5'-TGC TCC GGC TCA AAC CAT C-3'; caspase-9, upstream primer: 5'-AGG AAC TCA CAG CTC CAT TAC-3', downstream primer: 5'-CAG CAT TAG CGA CCC TAA GCA-3'; GAPDH, upstream primer 5'-AGA AGG CTG GGG CTC ATT TG-3', downstream primer 5'-AGG GGC CAT CCA CAG TCT TC-3'. The relative amount of mRNA for a particular sample was represented by the threshold cycle (Ct) of amplification. The relative quantitation of transcripts was calculated using the comparative Ct method (Livak and Schmittgen, 2001). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The GAPDH Ct value was subtracted from the target gene Ct value to obtain a  $\Delta$ Ct. Likewise, the difference between the  $\Delta$ Ct values of samples for the target gene and the  $\Delta$ Ct values of the calibrator (untreated control) were determined. The relative and normalized quantitative apoptosis-related gene expression levels ( $2^{-\Delta\Delta Ct}$ ) were calculated.

### Preparation of YGP-containing serum

Twenty rats were equivalently randomized into two groups, followed by daily administration of either distilled water or YGP (10 mL/kg) by gavage for 11 days. Before the last administration, the rats were fasted all night, and blood

samples were collected 3 hours after the final administration. Serum from YGP-treated and distilled water-treated rats were respectively obtained by centrifugation at  $800 \times g$  for 10 minutes and then filtered through a 0.22- $\mu$ m filter membrane. All samples were stored at  $-20^\circ\text{C}$ .

### Anterior pituitary cell culture and treatment

Six normal rats were sacrificed by decapitation. The anterior pituitary glands were removed, washed several times with low-glucose-DMEM (HyClone, Beijing, China), and then cut into small fragments. The cells obtained from the gland fragments by enzymatic dispersion (trypsin/deoxyribonuclease I; Solarbio, Beijing, China) were washed twice and suspended in low-glucose DMEM. Cell viability assessed by trypan blue exclusion was  $> 90\%$ . All cells were seeded onto 96-well tissue culture plates ( $1 \times 10^6$  cells/well) for 3 days ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in air) in low-glucose DMEM supplemented with 100 U/mL streptomycin (Solarbio), 100 U/mL penicillin (Solarbio), and 10% FBS (HyClone). The cultured cells were then divided into three groups: dexamethasone, dexamethasone plus YGP, and control. There were four samples in each group. The medium was replaced with fresh low-glucose DMEM plus test substances and the cells were incubated for an additional 3 days. The test substances were dexamethasone ( $1 \times 10^{-4}$  M) plus 10% serum from distilled water-treated rats, dexamethasone ( $1 \times 10^{-4}$  M) plus 10% serum from YGP-treated rats, and 10% serum from those controls, respectively. Afterwards, the cells were assayed using flow cytometry (BD Biosciences, San Diego, CA, USA).

### Detection of changes in mitochondrial membrane potential according to flow cytometry

The mitochondrial membrane potential ( $\Delta\psi_m$ ) was assessed using rhodamine 123, a mitochondrial potential sensor (Molecular Probes), as previously described (Mondal et al., 2014). Trypsin/EDTA was added to the cells and cell suspensions ( $1 \times 10^6$  cells/mL, 500  $\mu$ L/well) were obtained. The medium in each well was discarded and treated with 500  $\mu$ L of medium containing rhodamine 123 at 10  $\mu$ g/mL for 30 minutes at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in the dark. The cells were washed three times in PBS, and then analyzed using flow cytometry (BD Biosciences).

### Detection of cell apoptosis by flow cytometry

The cells were harvested, then rinsed with ice-cold PBS and re-suspended in 100  $\mu$ L of reaction buffer. Annexin V-FITC stock solution (5  $\mu$ L, Boster, Wuhan, China) and 5  $\mu$ L propidium iodide (PI) were added to the cells and incubated for 15 minutes at  $25^\circ\text{C}$  in the dark, as previously described (Lin et al., 2013). Subsequently, 400  $\mu$ L of reaction buffer was added to the cells and immediately analyzed on a FACSort equipped with CellQuest software (BD Biosciences).

### Statistical analysis

All data are expressed as the mean  $\pm$  SD. Statistical analyses were evaluated by one-way analysis of variance and *post-hoc*

Student-Newman-Keuls with SPSS 13.0 (SPSS, Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

## Results

### YGP reduced cell apoptosis in the dexamethasone-stimulated anterior pituitary

TUNEL staining revealed a significantly increased number of apoptotic cells in the dexamethasone group compared with the control group ( $11.58 \pm 0.95\%$  vs.  $0.03 \pm 0.01\%$ ;  $P < 0.001$ ). There were less TUNEL-positive cells in the dexamethasone plus YGP group ( $7.60 \pm 0.48\%$ ) than in the dexamethasone group ( $P < 0.001$ ). There were no visible TUNEL-positive cells in the YGP and control groups (Figure 1).

### Effect of YGP on apoptosis-related factors in the dexamethasone-stimulated anterior pituitary

Compared with the dexamethasone group, expression of the anti-apoptosis protein Bcl-2 was significantly increased, while expressions of the pro-apoptosis proteins cytochrome c, caspase-3, and caspase-9 were significantly decreased in the dexamethasone plus YGP group ( $P < 0.01$ ). There were no significant differences in pro-apoptotic protein expression between the YGP and control groups ( $P > 0.05$ ) (Table 1, Figure 2).

### Effect of YGP on apoptosis-related mRNA expressions in the dexamethasone-stimulated anterior pituitary

Apoptosis-related mRNA expression trends were almost consistent with their immunoreactivities in the corresponding groups. There was a significant increase in Bcl-2 mRNA expression, and a significant decrease in mRNA expressions of the pro-apoptosis factors cytochrome c, caspase-3, and caspase-9 in the dexamethasone plus YGP group compared with the dexamethasone group ( $P < 0.01$ ). The above indices show no significant differences between the YGP and control groups ( $P < 0.01$ ) (Table 2).

### YGP reduced apoptosis in cultured anterior pituitary cells treated with dexamethasone

Flow cytometry showed that a greater number of Annexin V<sup>+</sup>/PI<sup>-</sup> cells (early-stage apoptotic cells) in the dexamethasone group than in the control and dexamethasone plus YGP groups (Figure 3).

### Effect of YGP on changes in mitochondrial membrane potential of cultured anterior pituitary cells treated with dexamethasone

Flow cytometry revealed a significantly increased percentage of cells with reduced mitochondrial membrane potential in the dexamethasone group than in the control and dexamethasone plus YGP groups ( $P < 0.01$ ) (Figure 4).

## Discussion

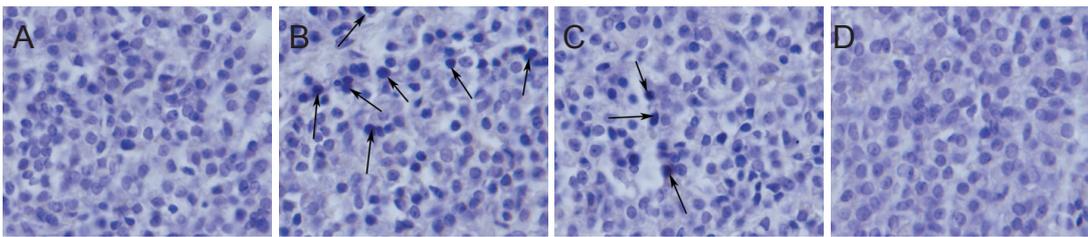
Because of the potential health benefits, Chinese herbal medicines have long been used as an alternative therapy in

China, Japan, and Korea, and are even popular in Western Society (No authors listed, 1996; Eisenberg et al., 1998). One advantage of Chinese herbal medicines over Western medicine has been attributed to the interactions among the herbal components (Ikegami et al., 2006), which are thought to be synergistic, enhancing, and immuno-suppressive. Unlike modern Western pharmaceutical agents, traditional herbal medicines have an overall combined effect that is greater than the effect of each individual component (Zhang et al., 2014). Herbal medicines have been extensively used for preventing and treating many diseases, including diarrhea (Li et al., 2013), diabetes (Ghorbani, 2014), and various types of cancers (Ling et al., 2014a; Safarzadeh et al., 2014). Interestingly, traditional Chinese medicine has been reported to play an important regulatory role in gene therapy (Ling et al., 2014b). In particular, YGP has been commonly used, although the mechanisms of action remain unclear. Our study specifically focused on the anti-apoptotic effect of these herbs on dexamethasone-stimulated anterior pituitary cells.

Apoptosis constitutes a highly regulated mechanism for removal of old, damaged, or mutated cells through two distinct molecular mechanisms (Galluzzi et al., 2007). On one hand, the extrinsic apoptotic pathway mediates cell death in response to extracellular stimuli, which occurs either through ligand-induced activation of death receptors at the plasma membrane (Wajant, 2002; Peter and Kramer, 2003), or *via* signaling cascades emanating from dependency receptors in the absence of their ligands (Bredesen et al., 2005). On the other hand, intrinsic apoptosis is regulated by mitochondria, which integrate lethal and pro-survival signals, eventually deciding the fate of a cell (Green and Kroemer, 1998).

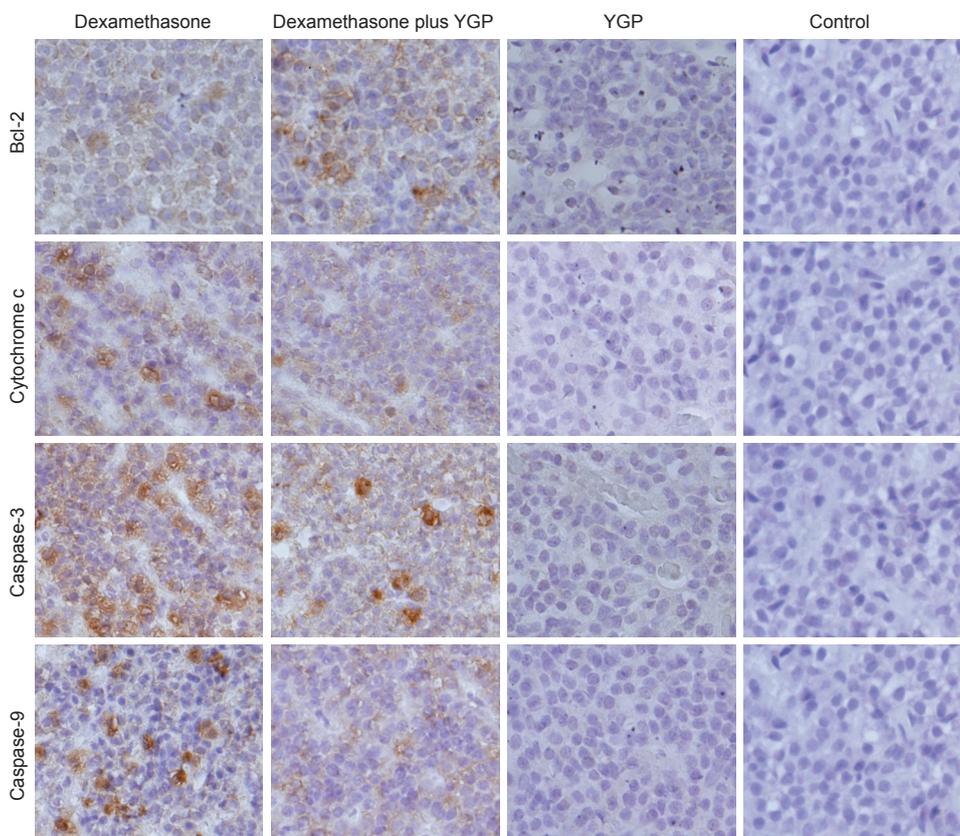
In vertebrate animals, the mitochondrial pathway is the major pathway for apoptosis, and the critical event responsible for caspase activation is mitochondrial outer membrane permeabilization (MOMP), which has been referred to as the “point of no return” of cell death (Green and Kroemer, 2004). In the mitochondria, death signals lead to changes in mitochondrial membrane permeability and subsequent release of pro-apoptotic factors, including cytochrome c, apoptosis-inducing factor, second mitochondria-derived activator of caspase (Smac/DIABLO), and endonuclease G. This release leads to the cytoplasmic assembly of procaspase-9, cytochrome c, and apoptosis protease-activating factor 1 into an initiation complex known as the apoptosome (Ghosh et al., 2002; Düsselmann et al., 2003; Delivani and Martin, 2006), which results in activation of caspase-9 and subsequent activation of “executioner” caspases, such as caspase-3.

The key regulatory proteins of mitochondria-mediated apoptosis belong to the Bcl-2 family and contain both pro-apoptotic (Bax, Bad, Bak, Bim) and anti-apoptotic (Bcl-2, Bcl-xL) members. Pro-apoptotic proteins normally reside in the cytosol (Wolter et al., 1997), but target the mitochondria during apoptotic signaling, causing the release of apoptotic signaling molecules, such as cytochrome c, SMAC/DIAB-



**Figure 1** Effect of YGP on cell apoptosis in the dexamethasone-stimulated anterior pituitary (TUNEL staining, × 200).

(A) Control group: rats were intramuscularly injected with normal saline, and intragastrically administered normal saline; (B) dexamethasone group: rats were injected intramuscularly with dexamethasone; (C) dexamethasone plus YGP group: rats were intramuscularly injected with dexamethasone, and intragastrically administered YGP; (D) YGP groups: rats were intramuscularly injected with normal saline, and intragastrically administered YGP. Arrows indicate apoptotic cells. There are less TUNEL-positive cells in the dexamethasone plus YGP group than in the dexamethasone group. There are no visible TUNEL-positive cells in the YGP and control groups. YGP: *Yougui* Pill; TUNEL: terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-and labeling.



**Figure 2** Effect of YGP on apoptosis-related factor expression in the dexamethasone-stimulated anterior pituitary (immunohistochemical staining, × 200).

Control group: rats were intramuscularly injected with normal saline, and intragastrically administered normal saline; dexamethasone group: rats were intramuscularly injected with dexamethasone; dexamethasone plus YGP group: rats were intramuscularly injected with dexamethasone, and intragastrically administered YGP; YGP group: rats were intramuscularly injected with normal saline, and intragastrically administered YGP. Brown staining represents a positive immunoreaction. Bcl-2 expression increased in the dexamethasone plus YGP group compared with the dexamethasone group. However, expressions of cytochrome c, caspase-3, and caspase-9 were opposite. Expression of the pro-apoptotic factors did not significantly differ between the YGP and control groups. YGP: *Yougui* Pill.

LO, and apoptosis-inducing factor (Arden and Betenbaugh, 2004). Conversely, anti-apoptotic members of the Bcl-2 family maintain mitochondrial membrane potential and prevent the release of cytochrome c during apoptotic insults (Kluck et al., 1997).

In the current study, we demonstrated that intrinsic apoptosis pathways were involved in the anti-apoptotic effects of YGP on dexamethasone-treated anterior pituitary cells. The anti-apoptotic protein Bcl-2 was up-regulated, whereas the pro-apoptotic proteins cytochrome c, caspase-3, and caspase-9 were down-regulated, further potentiating the process. This herbal medicine concoction could act by regulating gene expression of mitochondrial pathways.

In conclusion, our study demonstrated that long-term dexamethasone use leads to apoptosis of anterior pituitary cells,

and YGP administration reduces the number of apoptotic cells, suggesting that YGP exhibits anti-apoptosis effects by regulating mitochondrial function in apoptosis pathways. Further studies are needed to identify the types of anterior pituitary cells that are sensitive to YGP therapy, as well as to provide a better understanding of the molecular mechanisms involved.

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**Author contributions:** LG conceived and designed the study. YZJ and HBZ performed the experiments. YZJ and LG wrote the paper. HCW and HDC reviewed and edited the manuscript. All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

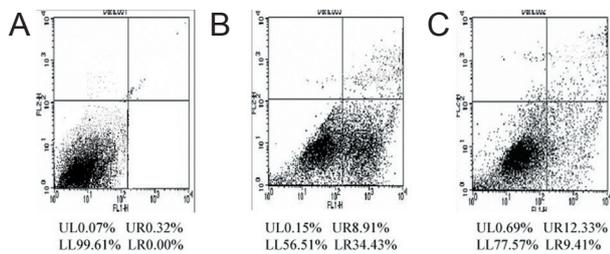
**Plagiarism check:** This paper was screened twice using CrossCheck to verify originality before publication.

**Peer review:** This paper was double-blinded and stringently reviewed by international expert reviewers.

**Table 1 Effect of YGP on the percentage of cells expressing apoptosis-related factors in the dexamethasone-stimulated anterior pituitary**

	Dexamethasone	Dexamethasone plus YGP	YGP	Control
Cytochrome c	7.64±0.14	4.55±0.73**	0.01±0.03	0.01±0.02
Caspase-3	12.04±1.02	8.27±0.45**	0.01±0.01	0.01±0.03
Caspase-9	10.74±0.14	9.26±1.22**	0.01±0.02	0.01±0.01
Bcl-2	1.27±0.09	2.01±0.11**	0.02±0.01	0.01±0.01

Control group: rats were intramuscularly injected with normal saline, and intragastrically administered normal saline. Dexamethasone group: rats were intramuscularly injected with dexamethasone. Dexamethasone plus YGP group: rats were intramuscularly injected with dexamethasone, and intragastrically administered YGP. YGP groups: rats were intramuscularly injected with normal saline, and intragastrically administered YGP. All data are expressed as the mean ± SD, *n* = 10 rats in each group. Statistical analyses were evaluated by one-way analysis of variance and *post-hoc* Student-Newman-Keuls. \*\**P* < 0.01, vs. dexamethasone group. YGP: *Yougui* Pill.



**Figure 3 Effect of YGP on apoptosis in cultured anterior pituitary cells treated with dexamethasone.**

(A) Control group: anterior pituitary cells were cultured with 10% serum from distilled water-treated rats; (B) dexamethasone group: anterior pituitary cells were cultured with dexamethasone and 10% serum from the distilled water-treated rats; (C) dexamethasone plus YGP group: anterior pituitary cells were cultured with dexamethasone and 10% serum from YGP-treated rats. There were more Annexin V<sup>+</sup>/PI<sup>+</sup> cells (in right lower quadrant) in the dexamethasone group than in the dexamethasone plus YGP and control groups (detected by flow cytometry, stained with Annexin V-FITC and PI). The experiments were repeated at least three times. YGP: *Yougui* Pill; PI: propidium iodine; V-FITC: Annexin V-fluorescein isothiocyanate.

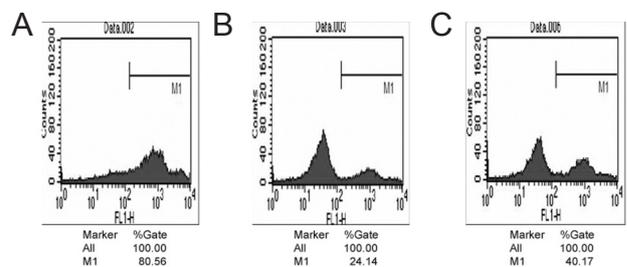
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**Table 2 Effect of YGP on apoptosis-related mRNA expressions in the dexamethasone-stimulated anterior pituitary**

	Dexamethasone	Dexamethasone plus YGP	YGP	Control
Cytochrome c	2.96±0.11	2.76±0.11***	1.01±0.04	1.00
Caspase-3	2.43±0.17	2.23±0.12***	1.00±0.01	1.00
Caspase-9	3.42±0.14	3.22±0.07***	1.00±0.01	1.00
Bcl-2	2.16±0.12	2.44±0.11***	0.90±0.08	1.00

Control group: rats were intramuscularly injected with normal saline, and intragastrically administered normal saline. Dexamethasone group: rats were intramuscularly injected with dexamethasone. Dexamethasone plus YGP group: rats were intramuscularly injected with dexamethasone, and intragastrically administered YGP. YGP groups: rats were intramuscularly injected with normal saline, and intragastrically administered YGP. The mRNA relative expression is expressed as 2<sup>-ΔΔCt</sup>. All data are expressed as the mean ± SD, *n* = 10 rats in each group. Statistical analyses were evaluated by one-way analysis of variance and *post-hoc* Student-Newman-Keuls. \**P* < 0.05, \*\**P* < 0.01, vs. dexamethasone group; \*\*\**P* < 0.01, vs. YGP group. YGP: *Yougui* Pill.



**Figure 4 Effect of YGP on changes in mitochondrial membrane potential of cultured anterior pituitary cells treated with dexamethasone.**

(A) Control group: anterior pituitary cells were cultured with 10% serum from distilled water-treated rats; (B) dexamethasone group: anterior pituitary cells were cultured with dexamethasone and 10% serum from the distilled water-treated rats; (C) dexamethasone plus YGP group: anterior pituitary cells were cultured with dexamethasone and 10% serum from YGP-treated rats. The percentage of cells with reduced mitochondrial membrane potential was less in the dexamethasone plus YGP group than in the dexamethasone group (determined by flow cytometry, stained with Rhodamine 123). The experiments were repeated at least three times. YGP: *Yougui* Pill.

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