



## Original Article

# Qianliening Capsule Promotes Mitochondrial Pathway Mediated the Apoptosis of Benign Prostatic Hyperplasia Epithelial-1 Cells by Regulating the miRNA-181a



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## SUMMARY

**Background:** Our previous studies reported that Qianliening capsule (QC) has a significant therapeutic effect on BPH. Therefore, we investigated the effect QC on apoptosis of human prostatic hyperplasia epithelial-1 cells (BPH-1).

**Methods:** The BPH-1 cells were treated with various concentrations of QC *in vitro*. Morphology of BPH-1 cell was observed, and the cell viability was determined by the 3-(4,5)-dimethylthiazoliumromide (MTT) assay. The levels of Cytochrome C, caspase-9 and caspase-3 were detected using the flow cytometry and colorimetric assay respectively. The Bax mRNA and the miRNA-221, -222, -15a, -16, -181a was determine by Real-time PCR analysis.

**Results:** The apoptosis of BPH-1 cells treated with QC increased than that of untreated cells, as evidenced by loss of plasma membrane asymmetry, the nuclear condensation and fragmentation, collapse of mitochondrial membrane potential in a dose depended manner. The levels of Cytochrome C and caspase-9, caspase-3 in the cells treated with QC increased using the flow cytometry and colorimetric assay respectively. The mRNA and protein expression of Bax and the expression of miRNA-181a in the cells treated with QC increased in a dose dependent manner.

**Conclusion:** QC could induce BPH-1 cells apoptosis by regulating miRNA-181a mediated mitochondrial dependent apoptosis pathway, which may be one of the important mechanisms that QC treated benign prostatic hyperplasia.

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## 1. Introduction

Multiple factors contribute to the pathogenesis of benign prostatic hyperplasia (BPH).<sup>1–4</sup> Proliferation and apoptosis of prostatic cells play an important role in the processes of BHP.<sup>5</sup> The caspase cascading pathways are very important in regulating the apoptosis of prostatic cells.<sup>6–9</sup> Mitochondria mediated apoptosis pathway

play an important role in the internal pathway of cell apoptosis, which involves the release of cytochrome C, the activation of caspase-3/-9 and the abnormal expression of Bcl-2 and Bax, etc. Furthermore, many miRNAs, such as miR-15a, -16, -221, -222, -181a regulate the activation of mitochondrial signaling pathway in prostatic cells.<sup>10–14</sup> Qianliening capsule (QC) as a traditional Chinese medicine from the People's Hospital of Fujian University of Traditional Chinese Medicine was revealed that QC could inhibit the proliferation of prostate and suppress the EGF/EGFR signaling pathway and regulate the expression of sex hormones as well as their receptors to treat the BPH *in vivo*,<sup>15,17–19</sup> meanwhile QC could promote the apoptosis of BPH-1 cells *in vivo* and *in vitro*.<sup>15,16</sup> However, the precise mechanism of QC on the apoptosis has remained to be fully elucidated. In this study, we

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investigated whether promotes mitochondrial pathway mediated the apoptosis of BPH-1 cells by regulating the miRNA-181a.

## 2. Materials and methods

**Drugs preparation.** QC (Food and Drug Administration approval no. Z20110009), consisting of 5 herbs: *Radix et Rhizoma Rhei*, *Hirudo*, *Radix Astragali*, *Radix Achyranthis Bidentatae* and *Semen Cuscutae* in a 5:1:4:3:2 ratio, was provided by the Academy of Pharmacology of Fujian University of Traditional Chinese Medicine (Fujian, China).<sup>14,15</sup> The culture medium diluted into 10 mg/ml filtered and then matched into the final concentration of 0, 1.25, 2.5, 5 mg/ml when used. All materials and methods were performed in compliance with international ethical guidelines and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

### 2.1. Cell line

The BPH-1 cell line was provided by the Institute for Molecular Biology, College of Life Sciences, Nankai University, Tianjin, China.

### 2.2. Culture of BPH-1 cells

The BPH-1 cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone, SV30010; GE Healthcare Life Sciences, Little Chalfont, UK), at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. The cells were subcultured at 80–90% confluency.

### 2.3. Determination of cell viability by MTT assay

BPH-1 cells seeded into 96-well plates at a density of  $1 \times 10^5$  cells/ml in 100 µl medium. The cells were treated with various concentrations of QC (0, 1.25, 2.5, 5 mg/ml) for 24 h, and then incubated with 100 µl 0.5 mg/ml methyl thiazolyl tetrazolium (MTT) at 37 °C for 4 h. The medium in each well was removed, and 100 µl dimethyl sulfoxide (Sigma-Aldrich) to resolve the crystals prior to incubation at room temperature for 10 min. The absorbance (A) was determined using a microplate reader (Model ELX800; BioTek, Winooski, VT, USA) at 570 nm. The survival rate was calculated as follows: Survival rate (%) = A experiment/A control × 100%.

### 2.4. Cell morphology observation

BPH-1 cells seeded into 6-well plates at a density of  $2 \times 10^5$  cells/ml in 2 ml medium. The cells were treated with various concentrations of QC for 24 h and cell morphology was observed using a DP70 phase-contrast microscope (Olympus Corporation, Tokyo, Japan). Images were captured at a magnification of × 100.

### 2.5. Detection of cell apoptosis

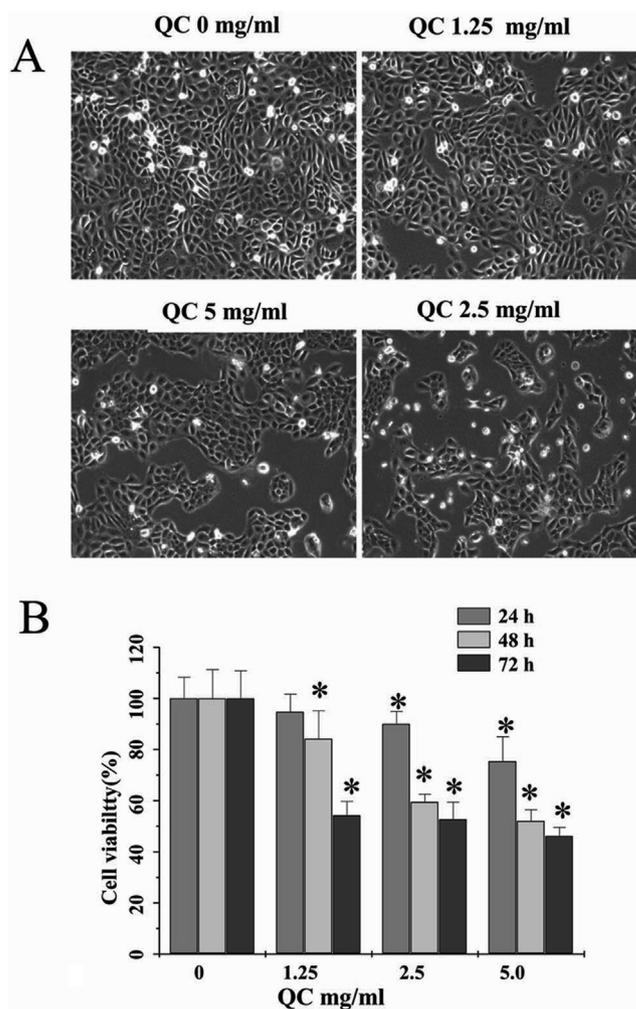
Apoptotic cell rates were determined by flow cytometry, the cells were trypsinized (0.25% trypsin without EDTA) and a cell suspension in RPMI-1640 was prepared. Aliquots (1 ml) of the cell suspension were washed three times in PBS at 4 °C. Following the final wash, the cells were resuspended in 500 µl binding buffer (Beyotime Institute of Biotechnology, Shanghai, China). Subsequently, 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) were added and the cells were incubated at room temperature for 15 min. The cells were then analyzed using a FACScalibur flow cytometer (BD Biosciences).

### 2.6. Detection of cytochrome C by flow cytometry

BPH-1 cells seeded into 6-well plates at a density of  $1 \times 10^6$  cells/ml in 2 ml medium, and treated with various concentrations of QC for 24 h. The supernatant was removed, add 2 ml clear liquid GENMED (Reagent A), and removed Reagent A, added Trypsin EDTA mixture (GMS12024) at 37 °C incubated for 1 min, added 3 ml complete cell culture medium (GMS12052) and followed by the following operation according to the manufacturer's instructions (GENMED SCIENTIFICS INC. U.S.A). In brief, added Clear liquid, Blocking fluid, Cytochrome C antibody, Staining Solution, counter stain respectively. Finally, Cytochrome C was analyzed by flow cytometry (FACSAria).

### 2.7. Detection of mitochondrial membrane potential by flow cytometry

Changes in mitochondrial membrane potential (MMP) were measured using the JC-1 Mitochondrial Potential Assay Kit (Nanjing KeyGen Biotech. Inc. Jiangshu, China). BPH-1 cells were seeded into 6-well plates at a density of  $2 \times 10^5$  cells/ml. After cultured for 24 h, and treated with various concentrations of QC for 24 h. The cells were



**Fig. 1.** Effect of QC on the morphology and viability of the BPH-1 cells. A. The cells were observed under a phase-contrast microscope. Images are representative photographs captured at a magnification of × 100; The BPH-1 cells were treated with various concentrations (0, 1.25, 2.5, 5 mg/ml). B. The cells were treated with various concentrations (0, 1.25, 2.5, 5 mg/ml) and the cell detected viability by MTT assay.

harvested, and resuspended in 500  $\mu$ l JC-1 binding buffer (Nanjing KeyGen Biotech. Inc. Jiangshu China). Subsequently, 500  $\mu$ l Incubation Buffer (Nanjing KeyGen Biotech. Inc. Jiangshu, China) were added, the cells were resuspended and analyzed by flow cytometry. JC-1 monomers and J-aggregates were evaluated separately in the FL 1 and FL 2 channels, respectively, and variations in the red/green fluorescence intensity ratio represent changes in MMP.

### 2.8. Determination of Caspase-9 and Caspase-3 viability by colorimetric assay

The cells were treated with various concentrations of QC for 24 h, and suspended in chilled cell lysis buffer (50  $\mu$ l) for 10 min, the supernatant was incubated with the substrates. Dilute 100  $\mu$ g protein to 50  $\mu$ l cell lysis Buffer for each assay, add 50  $\mu$ l of 2 $\times$  Rezdtion Buffer (containing 10 mM DTT) to each sample. Add 5  $\mu$ l of the 4 mM DEVD-pNA (Caspase-3) or LEHD-pNA (Caspase-9) substrate (200  $\mu$ M final conc.) and incubate at 37  $^{\circ}$ C for 2 h. Substrate hydrolysis was measured at 405 nm with a microplate reader (SpectraMax<sup>®</sup> i3, Austria).

### 2.9. Detection of the mRNA expression of Bax by real-time PCR

The total RNA was extracted from the BPH-1 cells treated with various concentrations of QC for 24 h using TRIzol reagent. Oligo (dT)-primed RNA (1  $\mu$ g) was reverse-transcribed using SuperScript II Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The cDNA was used to determine the mRNA expression levels of Bax by Real time PCR.

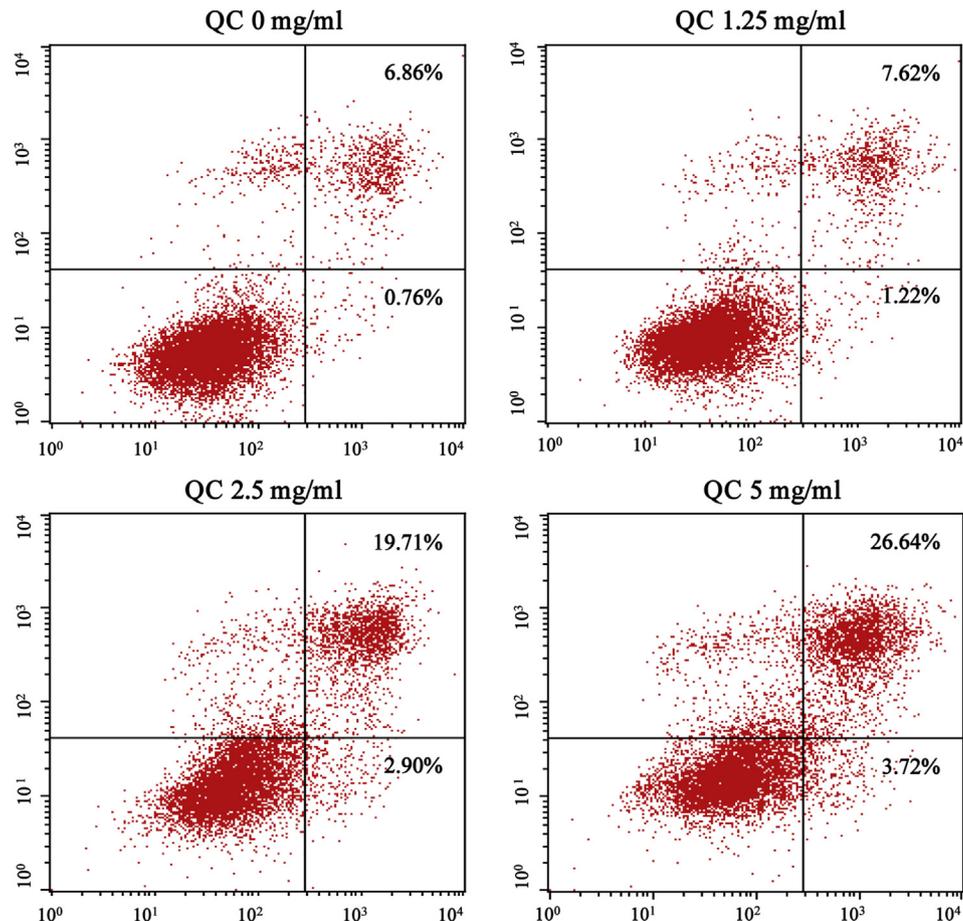
GAPDH was used as an internal control. The sequences of the primers used for amplification were as follows: for GAPDH, Forward primer: 5'-CAT CAG CAA TGC CTC CTG CAC-3' and Reverse primer: 5'-TGA GTC CTT CCA CGA TAC CAA AGT T-3' [annealing Temperature (Tm) = 62  $^{\circ}$ C; 86 bp]; Bax, Forward primer: 5'-GGA TCC AAG ACC AGG GTG GTT G-3' and Reverse primer: 5'-TCT GAA GAT GGG GAG AGG GCA C-3' [annealing temperature (Tm) = 63  $^{\circ}$ C; 164 bp]; The samples were analyzed using 1.5% agarose gel electrophoresis (HyClone). The DNA bands were visualized using a Gel Documentation system (Model Gel Doc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### 2.10. Quantitative real-time PCR analysis for miRNA

Total miRNA were extracted and purified using Trizol reagent for small RNA (TaKaRa, Japan) according to the user's guide. One micrograms of small RNA was tailed and reverse transcribed by SYBR<sup>®</sup> PrimeScript<sup>™</sup> miRNA RT-PCR Kit (TaKaRa, Japan) according to the user's manual. Quantitative real-time PCR was performed by miRNA specific primers purchased from Dalian Takara Biotechnology Co., Ltd (TaKaRa, Japan). All Ct values of miRNAs were normalized to small nuclear RNA U6. The  $2^{-\Delta\Delta Ct}$  method was used to calculate relative expression level of miRNAs.

### 2.11. Detection of the protein levels of Bax by western blot

The cells were treated with QC for 24 h, the total protein were extracted lysed in lysis buffer (50 mM Tris-Cl, pH 6.8; 15 mM NaCl,



**Fig. 2.** Effect of QC on apoptosis of BPH-1 cells. The rate of apoptosis in the BPH-1 cells induced by QC. Apoptosis was analyzed by AnnexinV-FITC/PI staining at 24 h after treatment, the apoptotic rate of the cells increased significantly compared with untreated group.

5 mM EDTA, 0.5% NP-40 and 1 mM PMSF; Sigma-Aldrich), and protein degeneration by boiling at 100 °C for 5 min, and the quantify of protein was used Bicinchoninic Acid (BCA) measured. The lysates were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked using 1% non-fat milk for 2 h, and incubation overnight at 4 °C with the primary antibodies (1:1000) against Bax and  $\beta$ -actin (1:1,000, Cell Signaling Technology, Inc.). The membrane was then incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 h. The membrane was washed in TBS containing 0.25% Tween-20 and then incubated with enhanced chemiluminescence solution (1:1; Technology Co., Ltd, Shanghai, China) at 25 °C for 5 min, followed by film exposure using a Bio-Rad Chemi Doc XRS + system (Bio-Rad Laboratories, Inc.).

### 2.12. Statistical analysis

Statistical analysis was performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean  $\pm$  SD deviation. Group data were compared using one-way ANOVA with Newman-Keuls tests.  $P < 0.05$  was considered to indicate a statistically significant difference.

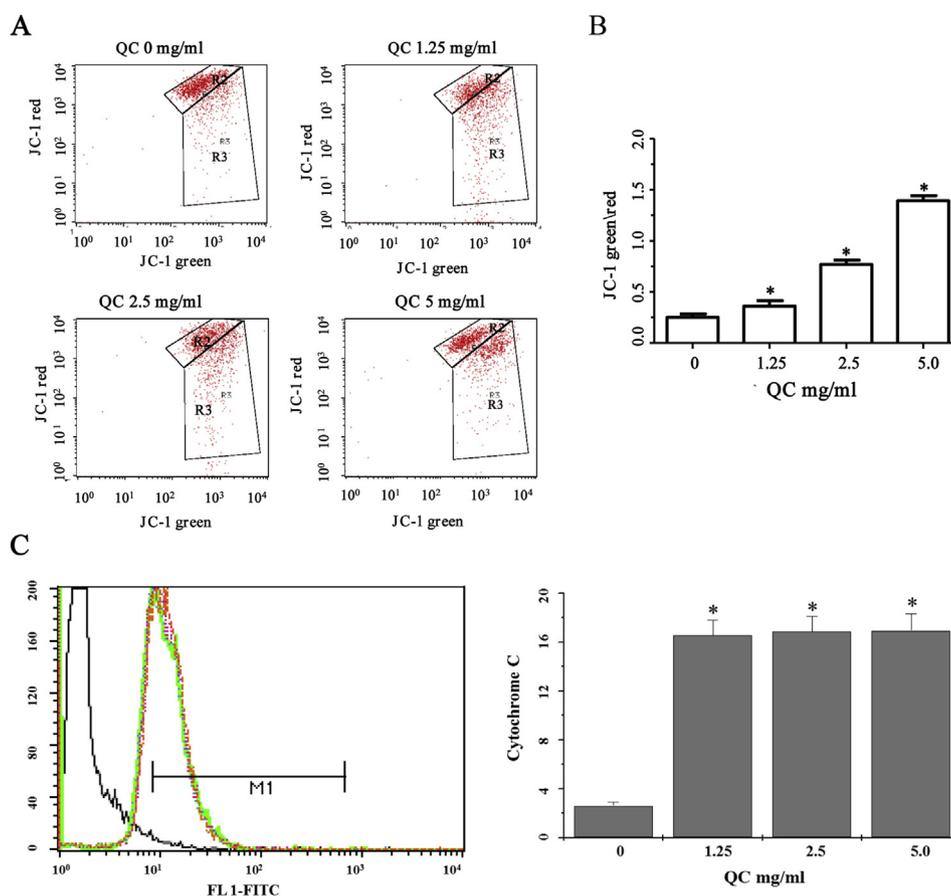
## 3. Results

### 3.1. Effect of QC on the morphology and viability of the BPH-1 cells

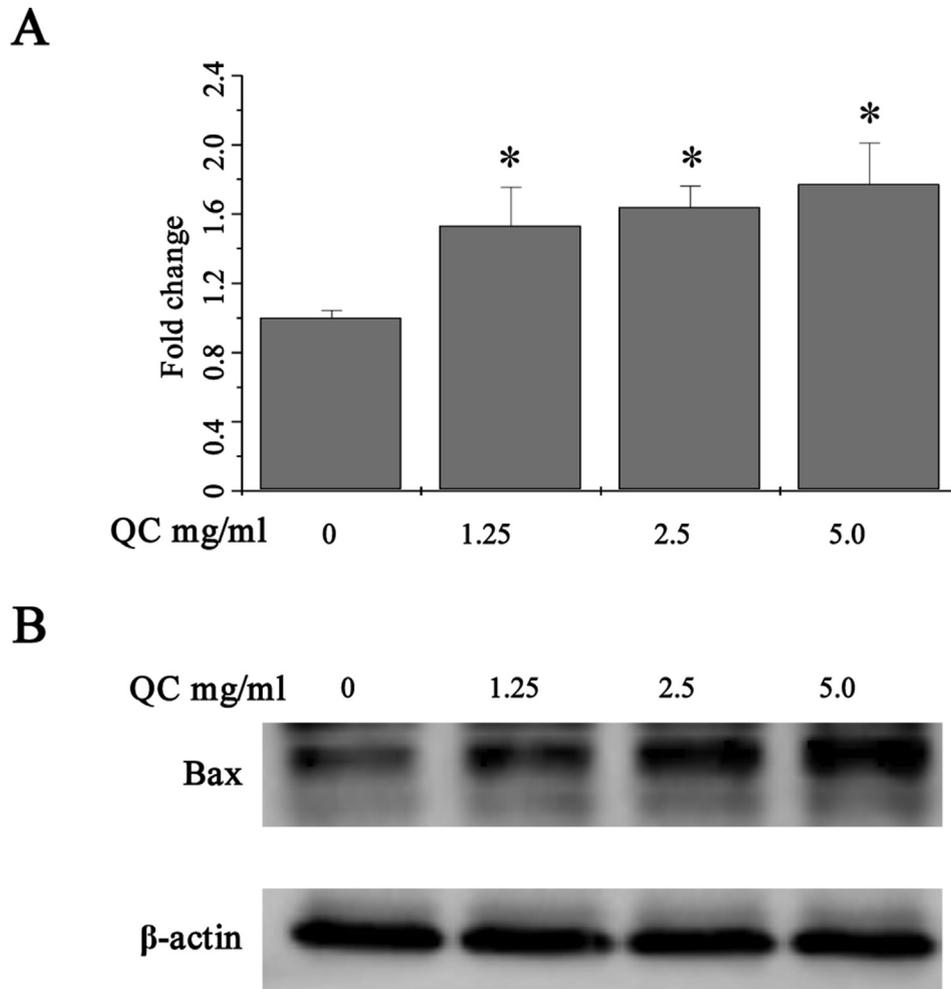
As shown in Fig. 1, the morphology of cells gradually changed, such as, the cells suspension were more and more, and volume of cells decreased gradually. The number of cells obviously decreased dose depended manner of QC compared with that of the untreated cells (Fig. 1A). The MTT assay showed that the viability of the BPH-1 cells were significantly inhibited in a time-dependent manner after treating with 1.25–5 mg/ml QC, and in 24 h and 48 h, cell viability was suppressed with dose-dependent, but not in 72 h ( $P < 0.05$ ; Fig. 1B). Taken together, these results implied that QC inhibited BPH-1 cells growth.

### 3.2. Effect of QC on apoptosis and mitochondrial membrane potential of BPH-1 cells

The annexin V-FITC staining indicated that in groups treated with various concentrations (0, 1.25, 2.5 or 5 mg/ml) of QC, the apoptotic index of the cells increased significantly compared with untreated group. In each group, the apoptotic index increased in dose depended manner of QC (Fig. 2). JC-1 detection results showed that BPH-1 cells treated with QC mitochondrial membrane



**Fig. 3.** Effect of QC on mitochondrial membrane potential of BPH-1 cells and QC induces the release of Cytochrome C from Mitochondria. The mitochondrial membrane potential of BPH-1 cells were detected by JC-1, the BPH-1 cells treated with various concentrations (0, 1.25, 2.5, 5 mg/ml) mitochondrial membrane potential decreased significantly; A. The image of mitochondrial membrane potential of BPH-1 cells. B. Mitochondrial membrane potential of BPH-1 cells decreased in a dose-dependent manner in response to QC; \* $P < 0.05$ , compared with standard. C The Cytochrome C was analyzed by flow cytometry, after treatment with various concentrations of QC for 24 h, as shown in Figure-3C, the expression of the Cytochrome C significantly increased in cytoplasm compared with that of untreated cells ( $P < 0.05$ ).



**Fig. 4.** Effect of QC on the mRNA and protein of Bax in BPH-1 cells. A. The mRNA expression of Bax was determined by Real time-PCR. GAPDH was used as an internal control. Treated with QC, the mRNA expression of Bax increased in BPH-1 cells. B. QC treatment profoundly increased protein level of Bax in BPH-1 cells  $\beta$ -actin were used as the internal controls for Western blotting assays.

potential decreased significantly. As shown in Fig. 3A, Compared with the untreated cells (0 mg/ml), the percentage of JC-1 accumulation in the mitochondrial membrane, the percentage of cells in JC-1 release as a monomer (Fig. 3A); Our results suggested that the mitochondrial membrane potential of QC treatment group decreased significantly, and apoptosis of the cells showed a significant increase trend (Fig. 3B).

### 3.3. QC induces cytochrome C released from mitochondria to cytoplasm

After treatment with various concentrations of QC for 24 h, as shown in Fig. 3C, the expression of the Cytochrome C significantly increased than that of untreated cells ( $P < 0.05$ ), suggesting QC induced the release of cytochrome C from the mitochondria to the cytoplasm in BPH-1 cells.

### 3.4. Effects of QC on the Bax mRNA and protein expression in BPH-1 cells

The mRNA and protein expression of bax was examined in BPH-1 cells treated with various concentrations of QC for 24 h by Real time-PCR and Western blot respectively, The results showed that the

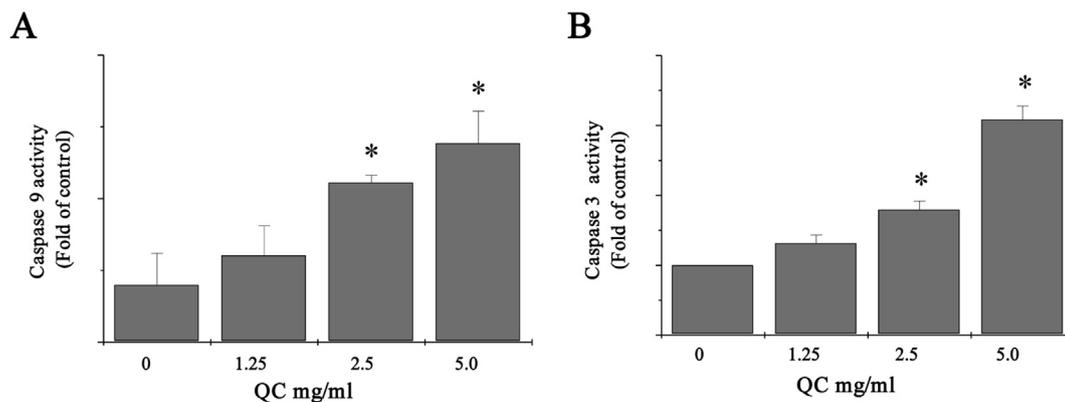
mRNA and protein expression of Bax in the BPH-1 cells increased significantly compared with untreated cells (Fig. 4A and Fig. 4B).

### 3.5. QC treatment activates the caspase-9 and -3 of BPH-1 cells

Caspase-9 and caspase-3 are able to induce changes in the intracellular biochemical and morphological properties. The results showed that the levels of caspase-9 and caspase-3 in the QC treated for 24 h significantly increased compared with untreated cells ( $P < 0.05$ ); the caspase-9 and caspase-3 increased as the concentration of QC increased (Fig. 5A and Fig. 5B).

### 3.6. Effect of QC on miRNA181a of BPH-1 cell

To study the mechanism of QC on BPH-1 cell apoptosis, the expressions of miR-221, -222, -181a, -15a, -16 and miRNA-181a were examined by Real time-PCR. As shown in Fig. 6, there was no alteration that the expressions of miR-15a, -16, -221, -222. However, the expression of miR-181a is up-regulated in BPH-1 cells with QC ( $P < 0.05$ ) (Fig. 6). According to the previous reports<sup>24,25</sup>, the miR-181a is an important factor in the regulation of the intrinsic apoptotic pathway, our results suggested that QC could regulate the expression of miRNA-181a.

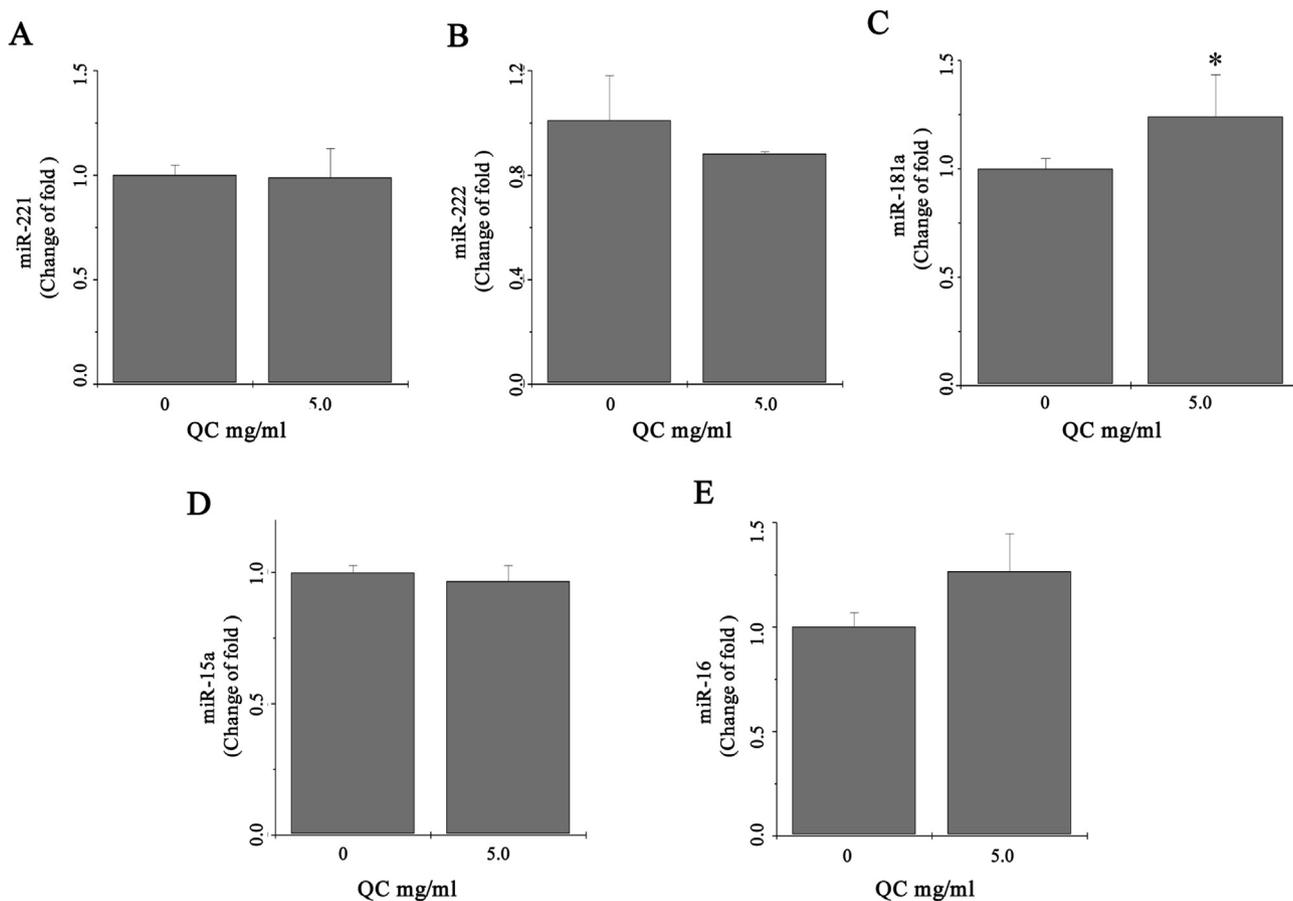


**Fig. 5.** Effect of QC on the activity of the caspase-9 and caspase-3 in BPH-1 cells. The caspase-9 and caspase-3 viability were detected by colorimetric assay; A. Effect of QC on the activity of the caspase-9 in BPH-1 cells. B. Effect of QC on the activity of the caspase-3 in BPH-1 cells.

**4. Discussion**

Apoptosis, namely programmed cell death. In the process of apoptosis, the morphological integrity and the number and the viability of cells will decrease. In our study, the morphology change and MTT assay showed that the density and cell viability were significantly decreased in QC treated BPH-1 cells, which indicated that QC could inhibit the proliferation of BPH-1 cells. In apoptotic

cells, the membrane phospholipid phosphatidylserine (PS) is flipped from the inner to the outer leaflet of the plasma membrane, exposing PS to the external cellular environment. Therefore, the phospholipid binding protein Annexin V can recognize apoptotic cells with exposed PS. Moreover, apoptotic cells display condensed chromatin and fragmented nuclear morphology using Annexin-V/PI staining followed by FACS analysis, we demonstrated that QC treatment could significantly induce the apoptosis of BPH-1 cells.



**Fig. 6.** Effect of QC on the expression of miRNAs in BPH-1 cells. The levels of apoptosis-related miR- 15a, miR-16, miR- 221, miR-222, miR181a were detected by RealtimePCR. A. Effect of QC on the levels of miR-221; B. Effect of QC on the expression of miR-222; C. Effect of QC on the levels of miR-181a; D. Effect of QC on the levels of miR-15a; E. Effect of QC on the levels of miR-16.

The results of FACS analysis with JC-1 staining clearly showed that BPH-1 cells treated with QC led to a loss of plasma membrane asymmetry, the nuclear condensation and fragmentation, collapse of mitochondrial membrane potential.

In the early stage of apoptosis observed a decrease of mitochondrial membrane potential, membrane permeability transition pore (PTP) opening in the mitochondrial, and cytochrome C, calcium ions from mitochondria released into the cytoplasm, which will trigger apoptotic cascade.<sup>20</sup> The detection of cytochrome C showed that the cytochrome C in treated cells were significantly increased compared with that of the untreated cells. Our mRNA and protein detection also suggested that the expression of Bax in BPH-1 cells with QC were significantly increased.

Intracellular death signals can be induced by the release of Cyt-C and Caspase-9 in mitochondrial, subsequently Caspase-9 and Caspase-3 is released and activated, which can trigger apoptosis process and the substrate degradation.<sup>21,22</sup> Caspases are the key proteins that modulate the apoptotic response, and Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9 during apoptosis. In this study, we found that QC induces the activation of both caspase-9 and caspase-3 in BPH-1 cells in a dose-dependent manner. Thus, QC-induced BPH-1 cell death is accompanied by an increase in the activities of caspases-9 and caspase-3, which then stimulates the molecular cascade for apoptosis.

The present study showed miRNAs play an important role in the cell apoptosis by Caspase signaling pathway.<sup>23,24</sup> miR-15a, -16 inhibit the activity of Bcl-2 and promote the activity of caspase-9 and induce the apoptosis of the cells.<sup>23</sup> miR-221 and miR-222 down-regulate the activity of caspase-8, which is an important factor of the extrinsic apoptosis pathway. The activated caspase-8 can activate caspase-3 and trigger cell apoptosis process. miR-181a promotes the aggregation of Bax, and release of cytochrome C and then activate the caspase-9 to push the progress of apoptosis.<sup>25</sup> The BPH-1 cells as BPH model observe the effect of apoptosis-related miR-15a, -16, -221, -222, -181a on apoptosis after treated by QC. Our results showed that QC had no effects on miR-15a and miR-16, while QC can down-regulate the expression of bcl-2 in BPH rats.<sup>15,16</sup> That is related the lack of QC dose and need further research and exploration. In addition, this study showed QC had no effect on miRNA-221 and miRNA-222, which showed that QC could not activate the extrinsic apoptosis pathway. Finally, this study showed QC could up-regulate miR-181a, suggesting that QC could enhance miRNA-181a to promote the apoptosis of BPH cells.

Our results showed QC exert a significant therapeutic effect on BPH, its therapeutic mechanism are probably regulated by the expression of MiR-181a and then stimulates the molecular cascade for apoptosis. This might be one of the mechanisms by which QC treats BPH.

#### Competing interests

The authors declare that they have no competing interests.

#### Conflict of interest

None.

#### Acknowledgements

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijge.2018.04.002>.

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