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Quercetin inhibits the expression of miRNA-155 and improves the functions of lipopolysaccharide-induced human extravillous

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Abstract

To investigate the effects of quercetin on the expression of microRNA-155 (miR-155) and the functions of lipopolysaccharide (LPS)-treated extravillous trophoblast cells. Cell apoptosis significantly increased and invasion cell number and wound healing rate significantly decreased in the model group compared with the NC group ($P < 0.001$). Quercetin supplement significantly improved the cell apoptosis rate, invasion cell number and wound healing rate ($P < 0.05$, respectively) compared with the model group. However, after miR-155 transfection, the cell apoptosis rate, invasion cell number and wound healing rate were similar to those in the model group. RT-qPCR and WB assays showed that quercetin decreased miR-155 gene expression and p-JunB and p-FosB protein expression levels. Quercetin pretreatment can protect extravillous trophoblast cells from apoptosis and loss of migratory and invasive abilities through down-regulating the expression of miR-155, which may be a mechanism that inhibits the development of preeclampsia by in vitro study.

Keywords: Quercetin; Preeclampsia; trophoblast; microRNAs; cell movement.

Practical Application: Quercetin had effects to improve Preeclampsia.

1 Introduction

Preeclampsia (PE) is a common clinical complication during pregnancy that seriously threatens the health of pregnant women and perinatal infants. PE is primarily characterised by hypertension and proteinuria in pregnant women after 20 weeks of gestation, accompanied by frequent functional damage to the kidney, heart, liver, brain and other organs (Henderson et al., 2017; Chen & Chen, 2020). The pathogenesis of PE is not completely understood at present. However, a previous study confirmed that superficial implantation of the placenta results from inflammatory immune hyperactivity at the maternal foetal interface, and the disturbance of uterine spiral artery remodelling constitutes the basic pathological feature of PE (Fisher, 2015). It can be explained by the decreased migration and invasion of trophoblasts mediated by pro-inflammatory factors.

miRNA-155 is a small molecule that promotes inflammatory immunity and exhibits an intimate relationship with PE progression (Warrington et al., 2013). MiRNA-155 is highly expressed in the placenta of patients with severe PE, which inhibits the production of angiogenic growth factors by natural killer cells in the basal decidua of human placenta adhesiva (Zhang et al., 2010; El-Ahwany et al., 2019). In addition, miRNA-155 can inhibit cultured endothelial cells to produce endothelial nitric oxide synthetase, thus reducing nitric oxide (NO) release and promoting vasoconstriction, similar to the stimulating effect of tumour necrosis factor-α, an inflammatory factor (Sun et al., 2012). In lipopolysaccharide (LPS)-induced extravillous trophoblasts, activator protein 1 (AP-1) is highly expressed and bound to the promoter region of the B-cell integration cluster gene, causing the up-regulation of miRNA-155 expression, decrease in trophoblast migration and invasion (Dai et al., 2011), and increase in trophoblast apoptosis (Xue et al., 2020). This result indicates that the functional change of trophoblasts is related to the pathogenesis of PE.

Quercetin (QC) is a polyhydroxy-flavonoid found extensively in many plants. At present, QC has been detected in over 100 kinds of Chinese herbal medicines, such as Herba patriniae, Houttuynia cordata, Inula flower, seed of Asiatic plantain and sophora flower. It exhibits various biological activities, including anti-tumour, anti-oxidation, anti-inflammatory, cardiovascular protection and blood pressure reduction (Chen et al., 2018; Men et al., 2014; Li et al., 2018). Abundant evidence supports that QC can inhibit the intracellular activation of mitogen-activated protein kinase cascade (Song et al., 2013) and the abnormal up-regulation of inducible NO synthase (Sekhar et al., 2015) and cyclooxygenase-2 (Carlsen et al., 2015). However, the role of QC in PE remains to be clarified. In the present study, a cell inflammation model was established by inducing the human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cell) with LPS. This study aimed to analyse the effect of QC on the expression of miR-155 and on the functions (migration, invasion and apoptosis) of trophoblast cells. This study is expected to lay a foundation for the study of PE prevention by QC.

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2 Materials and methods

2.1 Experimental materials

The cell line used in this study was the immortalised human extravillous trophoblast line HTR-8/SVneo purchased from Nanjing KeyGen Biotech Co., Ltd. LPS and QC (Sigma, USA); RPMI 1640 medium and foetal bovine serum (Gibco, USA); RNA extraction reagent Trizol (Sangon Biotech Co., Ltd., Shanghai); artificial basement membrane matrix gel (matrigel), and Annexin V-FITC Apoptosis Detection Kit (BD, USA); Transwell chamber (Millipore, USA); enhanced plasmid with green fluorescent protein (pEGFP)-C1 and pEGFP-miRNA-155 plasmid preparation (Nanjing KeyGen Biotech Co., Ltd.).

2.2 Cell grouping

The cells were divided into seven groups according to different treatments: NC group; model group (treatment of HTR-8/SVneo cells with 100 nM LPS); QC-L group (pretreatment of HTR-8/SVneo cells with 10 nM LPS combined with co-culture with 100 nM LPS for 48 h); QC-M group (pretreatment of HTR-8/SVneo cells with 20 μM LPS combined with co-culture with 100 nM LPS for 48 h); QC-H (pretreatment of HTR-8/SVneo cells with 100 μM LPS combined with co-culture with 100 nM LPS for 48 h); QC+miRNA group (treatment of HTR-8/SVneo cells with 100 μM QC combined with transfection of pEGFP-miRNA-155 for 48 h of culture).

2.3 Experimental methods

Culture of extravillous trophoblasts

HTR-8/SVneo cells were cultured in RPMI 1640 culture medium containing 10% foetal bovine serum, 100 U/mL penicillin and 100 mM streptomycin in a 5% CO_2 incubator at 37 °C for 1–2 days. The cells were subcultured for a subsequent experiment after digestion with 0.25% trypsin-EDTA. The cells were inoculated in a 12-well plate at the cell density of 1×10^5 cells per well when the cell confluence reached 70%–90%.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The total RNA of each group was extracted using Trizol reagent in accordance with the manufacturer's instructions, followed by the detection of RNA quality and concentration. The cDNA of miRNA-l55 was synthesised with stem-loop primer 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCCCCTA-3′. Primer sequences of miRNA-155: upstream: 5′-CGTTAATGCTAATCGTGATAG-3′; downstream: 5′-GCAGGGTCCGAGGT-3′. U6 was used as the internal reference (upstream: 5′-ATTGGAACGATACAGAGAAGATT-3′; downstream: 5′-GGAACGCTTCACGAATTTG-3′). The relative expression level of miRNA-155 was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot (WB) assay

AP-1 is an important regulatory molecule of miRNA-155 (Dai et al., 2011). JunB and FosB are the subunits of AP-1, an important transcription factor in the LPS toll-like receptor 4 signalling pathway. The phosphorylation levels of JunB and FosB can predict the activation degree of the inflammatory pathway directly. In this experiment, the HTR-8/SVneo cells of each group were rinsed three times with pre-cooled phosphate buffer and then added with 200 mL of lysis buffer for cell lysis at 4 °C for 20 min. After centrifugation at 12,000 r/min for 20 min with a centrifugation radius of 8 cm, the supernatant was collected for the determination of protein concentration by the bicinchoninic acid method. Afterward, 30 mg protein samples were added per well for sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membrane and then sealed with 5% skim milk for 1 h. Then, the primary antibodies of phosphorylated JunB and FosB antibodies (dilution, 1:1000) and the internal reference of β-actin (dilution, 1:1,000) were added and incubated overnight at 4 °C. Goat anti-rabbit and goat anti-mouse lgG secondary antibodies were added to incubate in the dark for 40–60 min, followed by the detection of protein expression after development with enhanced chemiluminescence. The grey-scale of protein bands in the gel map were analysed by Image J software.

Wound healing assay

When the cells covered the bottom of the 6-well plate after 12 h of different treatments, a scratch perpendicular to the culture plate was made at the centre of each well of the 6-well plate by using the head of a 1 mL pipette. The scratch should be perpendicular to the horizontal line on the back of the 6-well plate as much as possible to keep the cell scratch width of each well the same. The cells were rinsed three times with phosphate buffer to remove the scratched cells. The cells were added with RPMI 1640 medium containing 10% foetal bovine serum and then placed in a 5% CO_2 incubator at 37 °C for further culture. Finally, cell migration was observed and recorded at 0, 24 and 48 h after the scratch.

Transwell assay

A 50 mg/L matrigel was diluted with serum-free RPMI 1640 medium at the ratio of 1:5, coated in the Transwell chamber (50 μL/well) and then cultured in the incubator at 37 °C to make the matrigel coagulate. The HTR-8/SVneo cells collected from the different groups after 24 h of treatment were digested with 0.25% trypsin and re-suspended in RPMI 1640 medium containing 1% BSA. Then, 200 μL of the suspension containing 1 × 105 cells was added to the upper chamber of Transwell, with 800 μL RPMI-1640 medium containing 10% foetal bovine serum added to the lower chamber. Following 24 h of culture at 37 °C, the Transwell chambers were taken out. The cells were placed in 4% paraformaldehyde for fixation at room temperature for 10 min, stained with 0.2% crystal violet for 15 min and then observed under an inverted microscope. In the final step, five fields of vision (200×) were randomly selected for cell counting (penetrated cells).

Cell apoptosis experiment

After cell treatment according to the different groups, the cells were washed once with pre-cooled phosphate buffer and retrieved to a 5 mL flow cytometry tube. Then, 100 μL of trypsin without ethylenediaminetetraacetic acid was added into each well for about 30 s of digestion, and 1 mL of RPMI 1640 complete medium was added to terminate the digestion. Subsequently, the culture medium was retrieved to a 5 mL flow cytometry tube and then washed once with pre-cooled phosphate buffer before retrieval. After centrifugation at 1500 r/min for 5 min (centrifugation radius of 12 cm), the cells were re-suspended with 1 mL of 1 × binding buffer and then centrifuged again at 1500 r/min for 5 min (centrifugation radius of 14 cm) three times. The supernatant was discarded at the last centrifugation, and each tube was added with 100 μL of 1 × binding buffer. With gentle mixing, each tube was added with 5 μL of fluorescein isothiocyanate-labelled Annexin V for culture in the dark at room temperature for 15 min. Another 5 μL propidium iodide was added into each tube prior to detection in the flow cytometer.

2.4 Statistical analysis

Data analysis in this study was completed by using SPSS 21.0. The measurement data of normal distribution were expressed as mean±SD and analysed by t test. Statistical significance was considered at $P < 0.05$.

3 Results

3.1 QC inhibited LPS-induced cell apoptosis

The apoptosis rate increased significantly in model group compared with the NC group ($P < 0.001$, Figure 1), suggesting the LPS-induced apoptosis of HTR-8/SVneo cells. Furthermore, the apoptosis rate was obviously inhibited in the QC treatment groups compared with the model group ($P < 0.05$, respectively, Figure 1). The effect of QC on apoptosis indicated a dose-dependent relationship ($P < 0.05$, respectively, Figure 1).

3.2 QC improved LPS-induced HTR-8/SVneo cell invasion impairment

Compared with the NC group, the number of invasive HTR-8/SVneo cells was significantly inhibited after LPS induction ($P < 0.001$, Figure 2). However, the number of invasive HTR-8/SVneo cells increased evidently in the QC treatment groups compared with the model group ($P < 0.05$, respectively, Figure 2). Meanwhile, an evident dose-dependent relationship was found

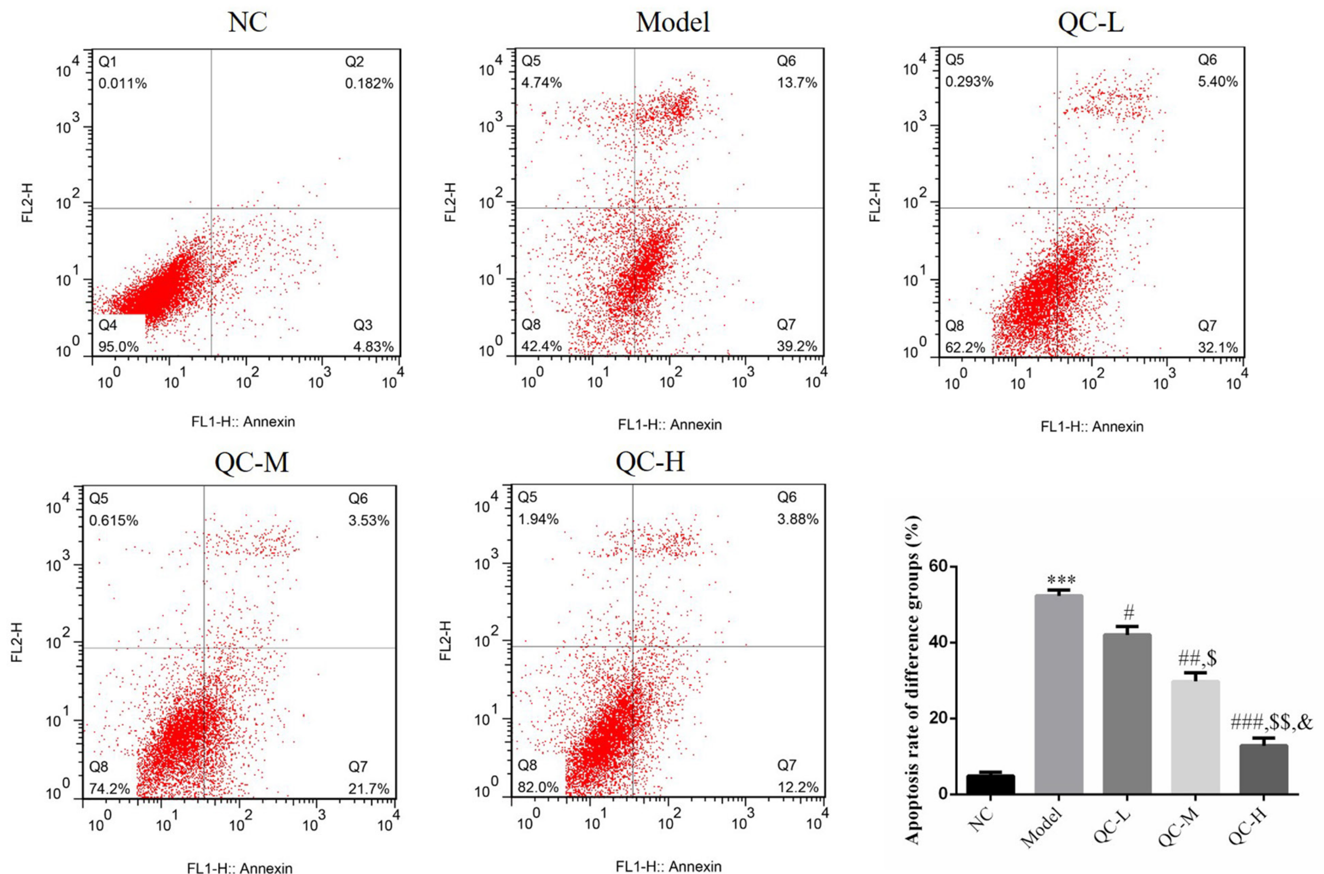

Figure 1. QC inhibited LPS-induced cell apoptosis. NC: The cell were treated with normal; Model: The cell were treated with LPS (100nM); QC-L: The cell were treated with 10 μM QC and 100 nM LPS; QC-M: The cell were treated with 20 μM QC and 100 nM LPS; QC-H: The cell were treated with 100 μM QC and 100 nM LPS. ***: $P < 0.001$, compared with NC group; #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$, compared with Model group; $: $P < 0.05$, $$: $P < 0.01$, compared with QC-L group; &: $P < 0.05$, compared with QC-M.

concerning the role of QC in improving the invasion of HTR-8/SVneo cells (P < 0.05, respectively, Figure 2).

3.3 QC improved LPS-induced HTR-8/SVneo cell migration impairment

The wound healing rate of the HTR-8/SVneo cells was obviously reduced in the model group 24 and 48 h after LPS treatment compared with the NC group (P < 0.001, Figure 3). However, the wound healing rate of the HTR-8/SVneo cells in the QC treatment groups significantly increased (P < 0.05, respectively, Figure 3) in an obvious dose-dependent manner compared with that of the cells in the model group (P < 0.05, respectively, Figure 3).

3.4 Effect of QC on miRNA-155 gene and related protein expression

The results of RT-qPCR showed that the expression of miRNA-155 remarkably increased in the model group compared with the NC group (P < 0.001, Figure 4A). However, this expression was notably suppressed in the QC treatment groups compared with the model group (P < 0.05, Figure 4A) in an obvious dose-dependent manner (P < 0.05, Figure 4A). Simultaneously, WB detection results indicated that the protein expression levels of p-JunB and p-FosB significantly increased in the model group compared with the NC group (P < 0.001, Figure 4B). However, the protein expression levels of p-JunB and p-FosB were significantly inhibited in the QC treatment groups compared with the model group (P < 0.05, Figure 4B), displaying a notable dose-dependent relationship (P < 0.05, Figure 4B). However, no significant differences in JunB and FosB protein expression were found among the groups.

3.5 Role of miRNA-155 in the inhibition of LPS-induced apoptosis by QC

Cell apoptosis obviously increased in the model group compared with the NC group (P < 0.001, Figure 5) but apparently decreased in the QC treatment groups compared with the model group (P < 0.001, Figure 5). Meanwhile, following the simultaneous transfection of miRNA-155, the QC+miRNA group exhibited a distinct increase in cell apoptosis compared with the QC group (P < 0.001, Figure 5).

3.6 Role of miRNA-155 in QC improving LPS-induced HTR-8/SVneo cell invasion impairment

LPS induction remarkably suppressed the number of invasive HTR-8/SVneo cells compared with the NC group (P < 0.001, Figure 6). The number of invasive cells obviously increased in the QC group compared with the model group (P < 0.001, Figure 6). However, the number of invasive cells decreased

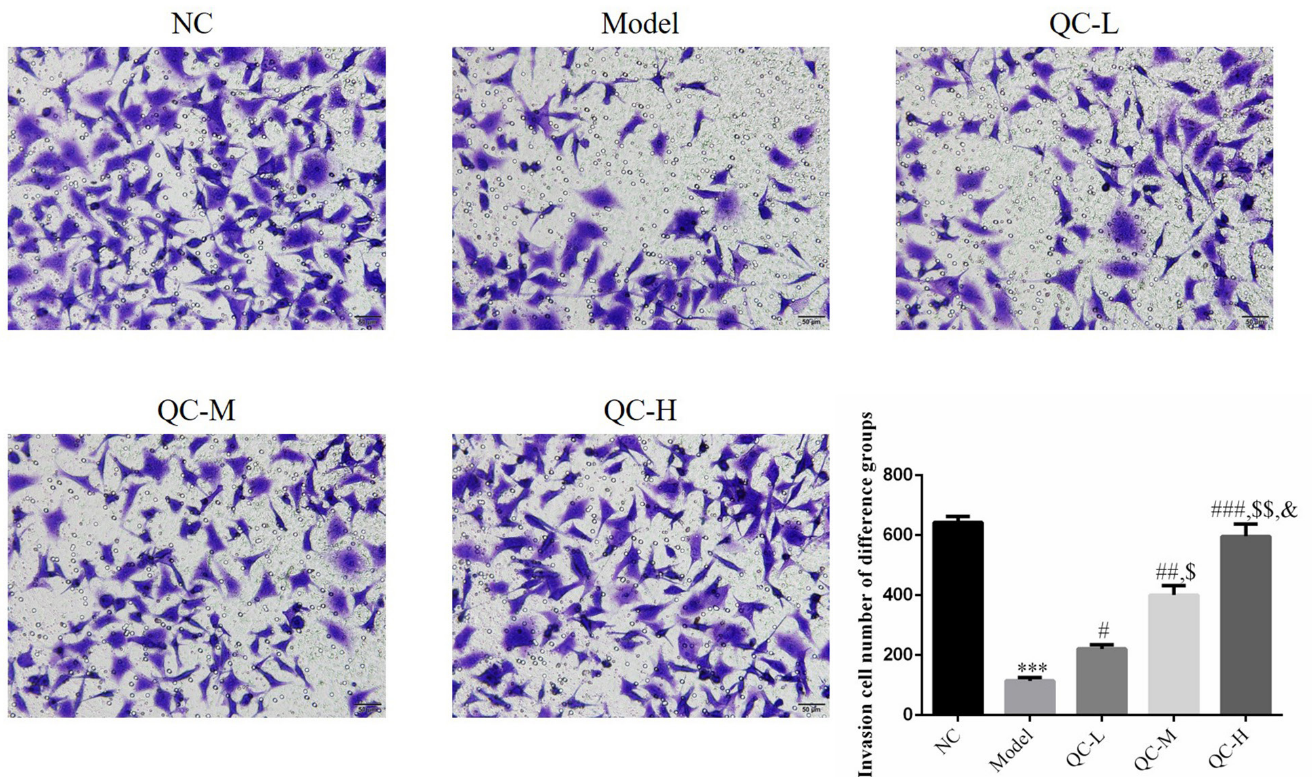

Figure 2. QC improved LPS-induced HTR-8/SVneo cell invasion impairment. NC: The cell were treated with normal; Model: The cell were treated with LPS (100nM); QC-L: The cell were treated with 10 μM QC and 100 nM LPS; QC-M: The cell were treated with 20 μM QC and 100 nM LPS; QC-H: The cell were treated with 100 μM QC and 100 nM LPS. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with QC-L group; &: P < 0.05, compared with QC-M.

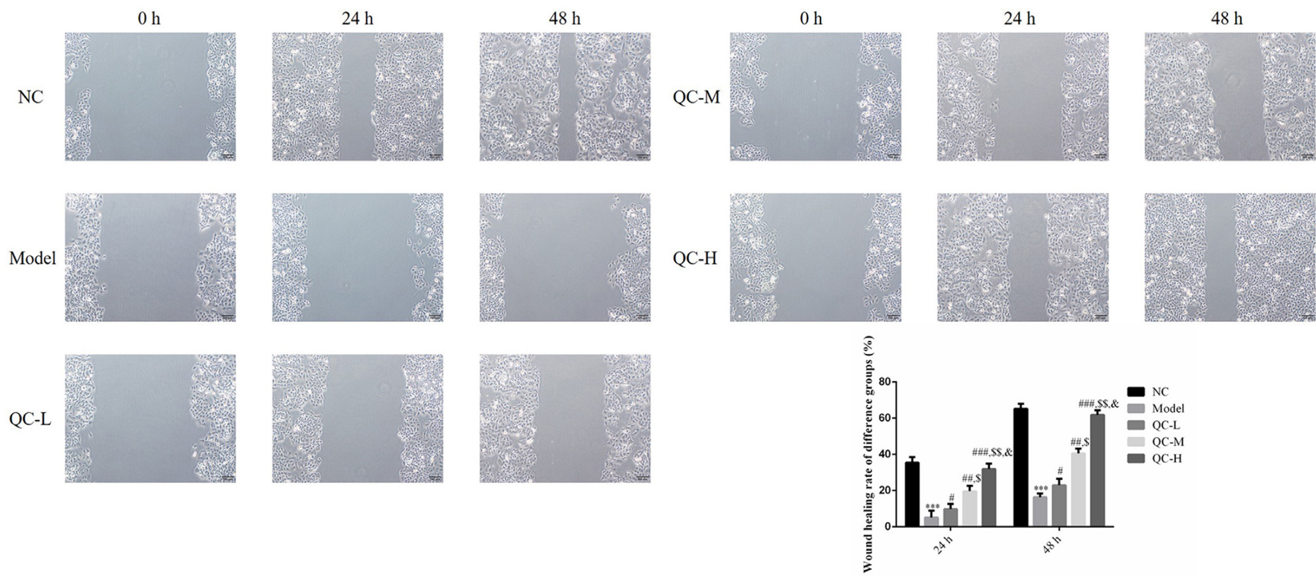

Figure 3. QC improved LPS-induced HTR-8/SVneo cell migration impairment. NC: The cell were treated with normal; Model: The cell were treated with LPS (100 nM); QC-L: The cell were treated with 10 μM QC and 100 nM LPS; QC-M: The cell were treated with 20 μM QC and 100 nM LPS; QC-H: The cell were treated with 100 μM QC and 100 nM LPS. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with QC-L group; &: P < 0.05, compared with QC-M.

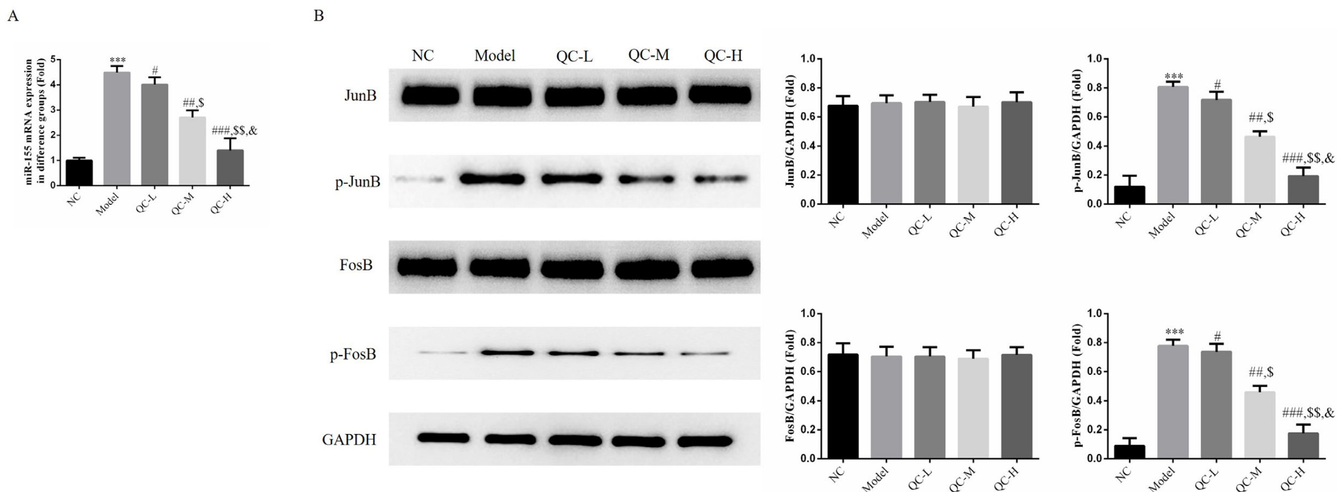

Figure 4. Effect of QC on miRNA-155 gene and related protein expression. NC: The cell were treated with normal; Model: The cell were treated with LPS (100 nM); QC-L: The cell were treated with 10 μM QC and 100 nM LPS; QC-M: The cell were treated with 20 μM QC and 100 nM LPS; QC-H: The cell were treated with 100 μM QC and 100 nM LPS. A. miRNA-155 gene expression in difference groups by RT-qPCR assay. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with QC-L group; &: P < 0.05, compared with QC-M. B. Relative proteins expressions by WB assay. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with QC-L group; &: P < 0.05, compared with QC-M.

evidently in the QC+miRNA group compared with the QC group (P < 0.001, Figure 6).

3.7 Role of miRNA-155 in QC improving LPS-induced HTR-8/SVneo cell migration impairment

The wound healing rate of the HTR-8/SVneo cells obviously decreased in the model group 24 and 48 h after LPS induction compared with the NC group (P < 0.001, Figure 7). Meanwhile, the wound healing rate of the HTR-8/SVneo cells notably recovered in the QC group compared with the model group 24 and 48 h after LPS induction (P < 0.05, Figure 7). Furthermore, after miRNA-155 transfection, the wound healing rate in the QC+miRNA group significantly reduced at 24 and 48 h compared with that in the QC group (P < 0.001, Figure 7).

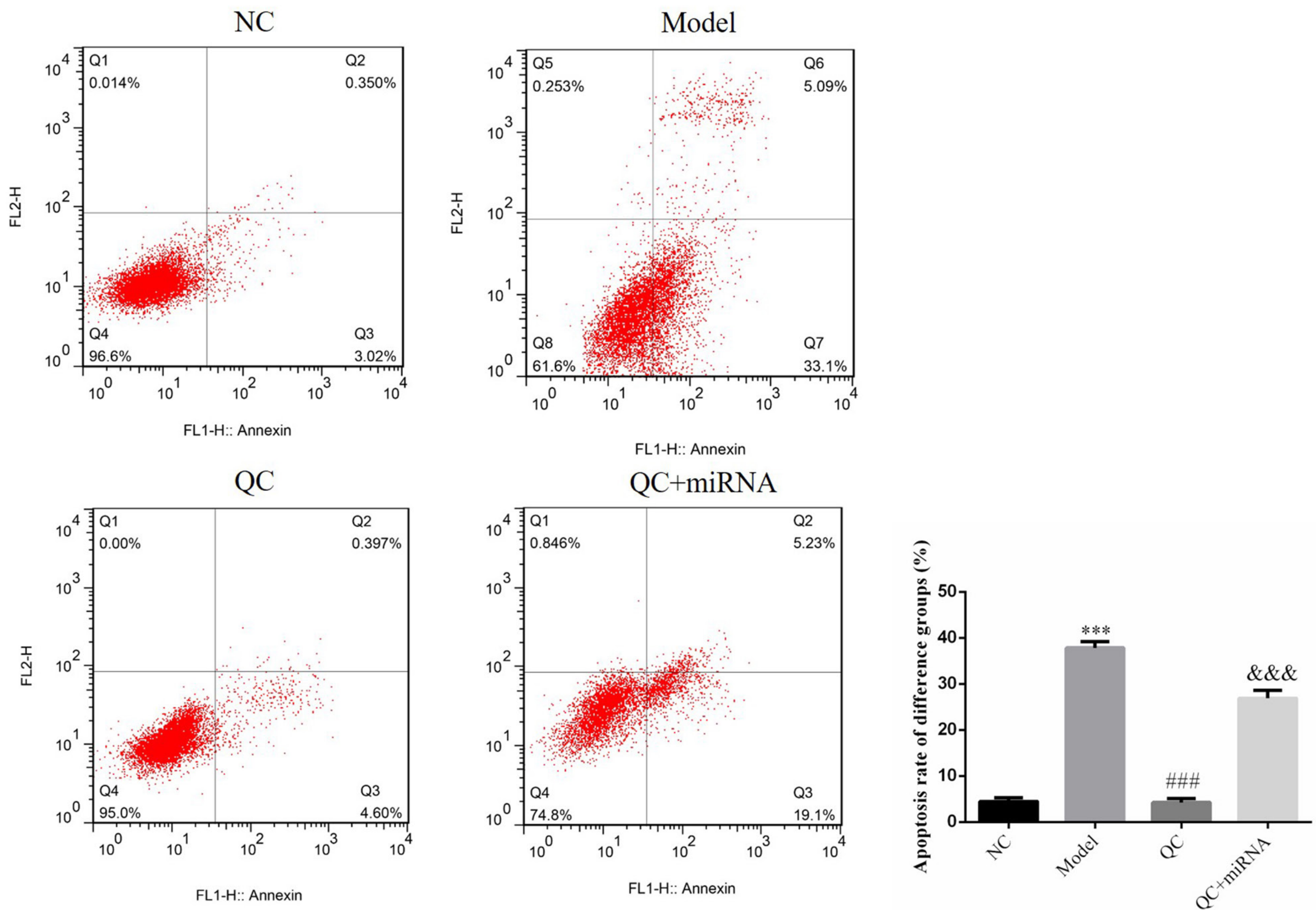

Figure 5. Role of miRNA-155 in the inhibition of LPS-induced apoptosis by QC. NC: The cell were treated with normal; Model: The cell were treated with LPS (100 nM); QC: The cell were treated with 100 μM QC and 100 nM LPS; QC+miRNA: The cell were transfected with miRNA-155 and treated with 100 μM QC and 100 nM LPS. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; $$$: P < 0.001, compared with QC group.

3.8 miR-155 gene and related protein expression

RT-qPCR results showed that miRNA-155 gene expression significantly increased in the model group compared with the NC group (P < 0.001, Figure 8A), but its expression decreased in the QC group compared with the model group (P < 0.001, Figure 8A). Meanwhile, after simultaneous transfection of miRNA-155, miRNA-155 gene expression was remarkably increased in the QC+miRNA group compared with the QC group (P < 0.001, Figure 8A). The results of WB showed that the protein expression levels of p-JunB and p-FosB were obviously up-regulated in the model group compared with the NC group (P < 0.001, Figure 8B). After QC supplementation, the protein expression levels of p-JunB and p-FosB were significantly down-regulated compared with those in the model group (P < 0.001, Figure 8B). With QC and miRNA-155 transfection, the protein expression levels of p-JunB and p-FosB were significantly up-regulated compared with those in the QC group (P < 0.001, Figure 8B). However, no significant differences in JunB and FosB protein expression were found among the groups.

4 Discussion

During normal pregnancy, extravillous trophoblasts invade the maternal decidua and remodel the uterine spiral arteries. These invasive trophoblasts then replace vascular endothelial cells of the maternal spiral arteries, disintegrate vascular smooth muscle cells, destroy the vascular wall structure and transform the narrowed high-resistance elastic blood vessels into low-resistance, inelastic and dilated uteroplacental blood vessels. These vessels lose the sensitivity to vasoactive substances to increase the blood flow into the intervillous space significantly (Shen et al., 2017). Normal migration and invasion of extravillous trophoblasts are critical processes in successful remodelling of uterine spiral arterioles. Inhibited extravillous trophoblast migration and invasion, together with increased cell apoptosis, can contribute to the blocked remodelling of uterine spiral artery, resulting in superficial implantation of the placenta (Warrington et al., 2013).

MiRNA-155 is a multifunctional miRNA related to inflammation, tumour and immune regulation. The gene encoding miRNA-155 is located in the third exon of the B-cell integration cluster gene

on chromosome 21. The expression levels of inflammatory factors and miRNA-155 significantly increase in the placenta of PE patients; miRNA-155 negatively regulates angiogenic factor cysteine-rich 61 to promote the pathophysiological changes in PE (Zhang et al., 2010). Meanwhile, the expression of miRNA-155 is obviously up-regulated by the activation of AP-1 in LPS-treated

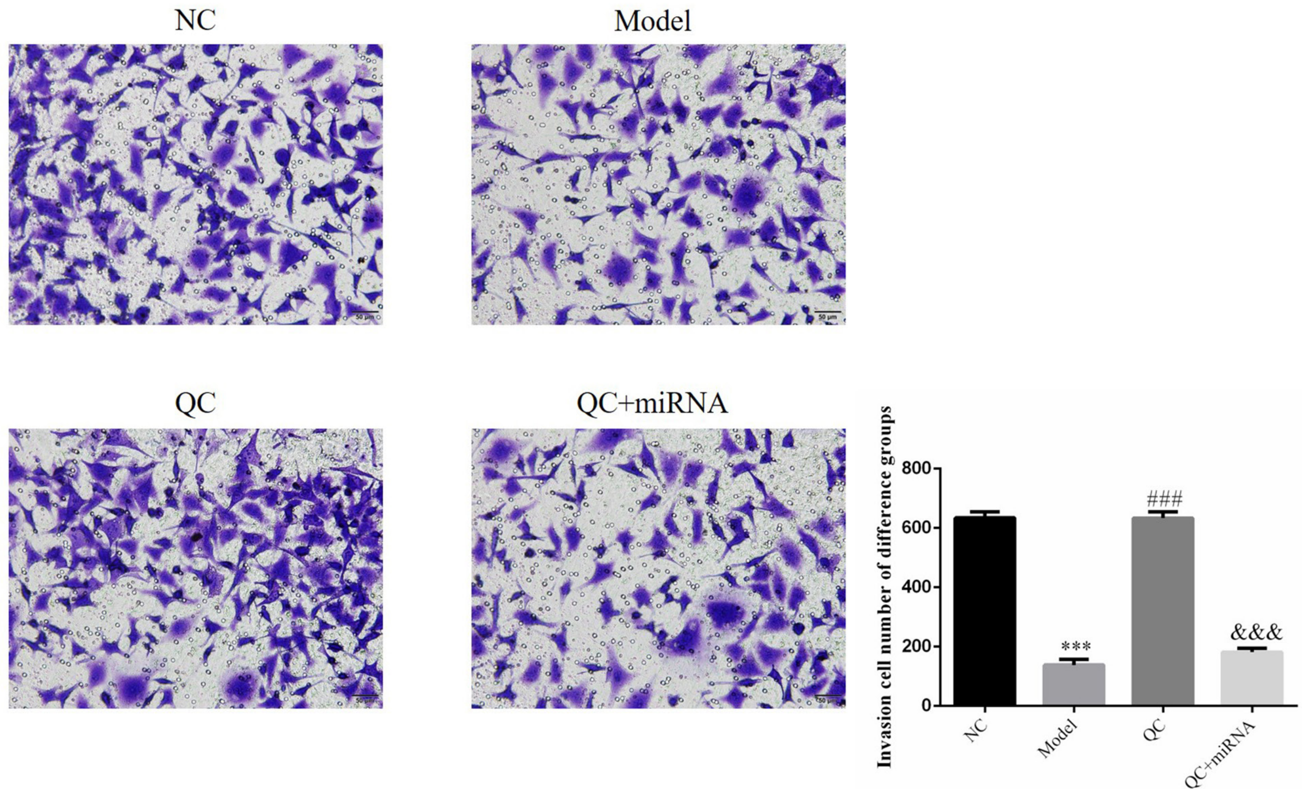

Figure 6. Role of miRNA-155 in QC improving LPS-induced HTR-8/SVneo cell invasion impairment. NC: The cell were treated with normal; Model: The cell were treated with LPS (100 nM); QC: The cell were treated with 100 μM QC and 100 nM LPS; QC+miRNA: The cell were transfected with miRNA-155 and treated with 100 μM QC and 100 nM LPS. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; $$$: P < 0.001, compared with QC group.

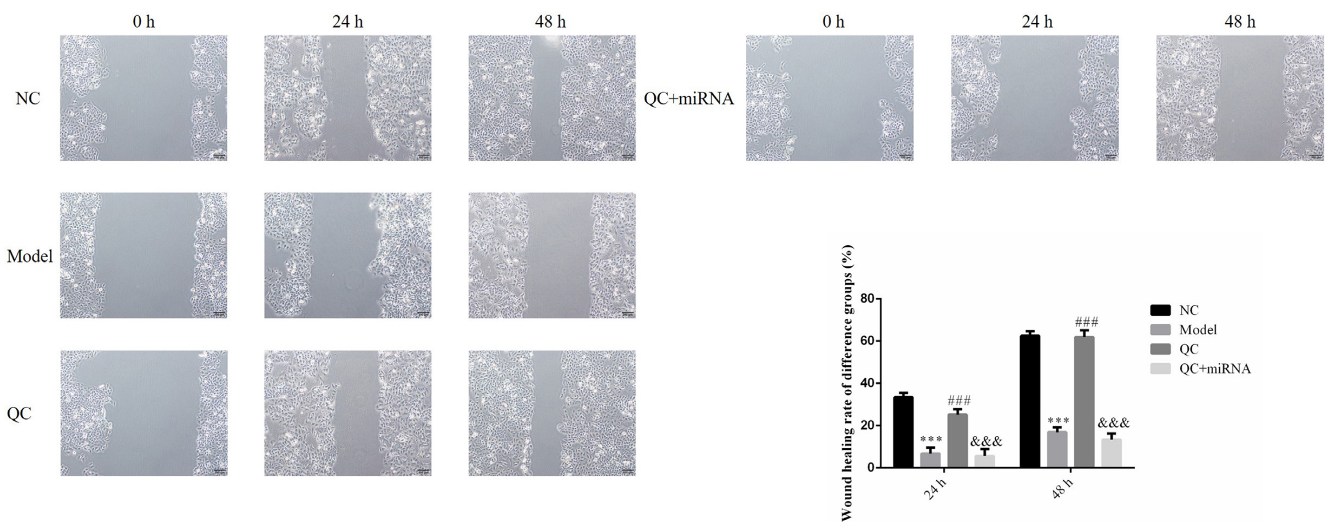

Figure 7. Role of miRNA-155 in QC improving LPS-induced HTR-8/SVneo cell migration impairment. NC: The cell were treated with normal; Model: The cell were treated with LPS (100nM); QC: The cell were treated with 100 μM QC and 100 nM LPS; QC+miRNA: The cell were transfected with miRNA-155 and treated with 100 μM QC and 100 nM LPS. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; $$$: P < 0.001, compared with QC group.

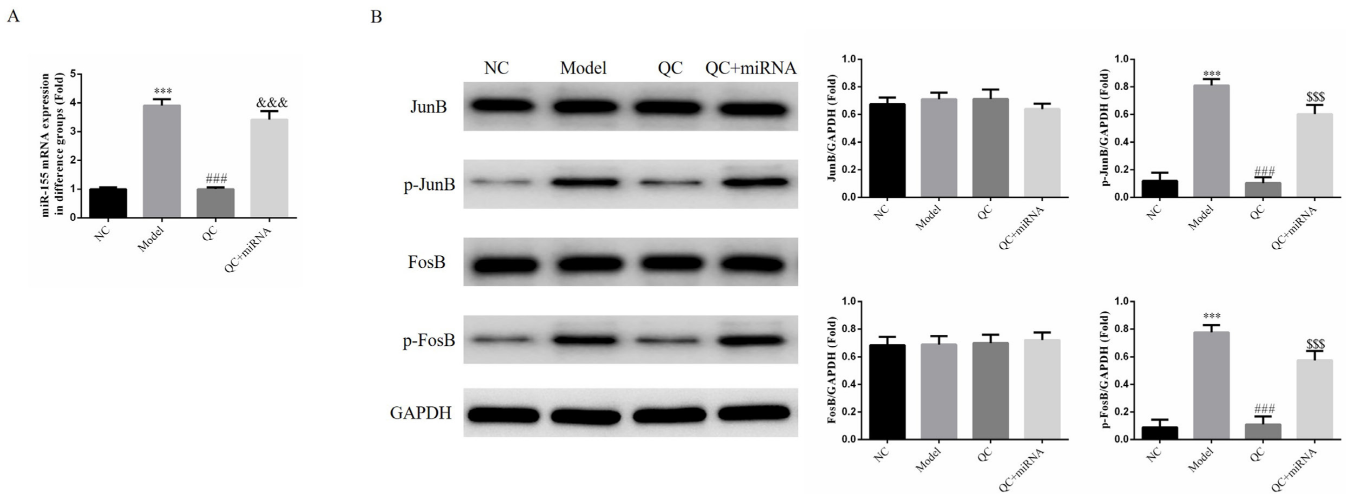

Figure 8. miRNA-155 gene and related protein expression. NC: The cell were treated with normal; Model: The cell were treated with LPS (100 nM); QC: The cell were treated with 100 μM QC and 100 nM LPS; QC+miRNA: The cell were transfected with miRNA-155 and treated with 100 μM QC and 100 nM LPS. A. miRNA-155 gene expression in difference groups by RT-qPCR assay. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; $$$: P < 0.001, compared with QC group. B. p-JunB and p-FosB proteins expressions by WB assay. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model group; $$$: P < 0.001, compared with QC group.

extravillous trophoblasts (Dai et al., 2011). Prior research reported that the increased expression of miRNA-155 can enhance the inflammatory immune response of cells (O'Connell et al., 2010). In addition, miRNA-155 can inhibit the invasion of trophoblast and promote its apoptosis (Xue et al., 2020). In this regard, we hypothesised that an up-regulated expression of miRNA-155 in extravillous trophoblasts is related to the pathogenesis of PE. In our study, the LPS stimulation of extravillous trophoblasts and transfection with pEGFP-miRNA-155 can decrease trophoblast migration and invasion while increase its apoptosis.

LPS-induced PE in rats is a classic model of PE. Hence, in the present study, LPS was used to stimulate human extravillous trophoblasts to establish the inflammatory cell model. Results showed that LPS can activate the expression of AP-1 subunits (p-JunB and p-FosB) in trophoblasts and promote the expression of miRNA-155. After the transfection of pEGFP-miRNA-155 alone, changes in the function of human extravillous trophoblasts were similar to those induced by LPS, exhibiting impaired cell migration and invasion but increased cell apoptosis. However, the migration and invasion of human extravillous trophoblasts increased and apoptosis decreased after the transfection of miRNA-155 inhibitor prior to LPS induction compared with LPS treatment alone. These results suggest that miRNA-155 exerts an important role in LPS-induced trophoblast dysfunction. In our subsequent exploration, QC treatment not only down-regulated the expression of miRNA-155 induced by LPS but also restored the impaired migration and invasion of trophoblasts resulting from miRNA-155 transfection alone and inhibited the effect of miRNA-155 on trophoblast apoptosis. The above results suggest that QC can effectively control the over-activation of the inflammatory signalling pathway, inhibit the excessive apoptosis of extravillous trophoblasts and promote the recovery of migration and invasion abilities of extravillous trophoblasts through the inhibition of miRNA-155 expression.

5 Conclusion

QC can inhibit the migration and invasion of extravillous trophoblasts caused by the increased expression of miRNA-155, highlighting an inhibitory effect of QC in the key process of PE. However, further animal experiments and clinical studies are needed to verify the role of QC as a potential therapeutic agent in the prevention and treatment of PE in clinical practice.

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