

Protective Effects of Aspirin Supplemented With Quercetin in L-NAME-Induced Preeclampsia-Like Rats

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Summary

Aspirin supplemented with quercetin was reported to enhance the therapeutic effects of aspirin in a rat model of preeclampsia. In this study, the underlying mechanisms were further explored. Preeclampsia was induced by L-NAME (50 mg/kg/day) *via* oral gavage from gestation day (GD)14 to GD19. Aspirin (1.5 mg/kg/day) administration was performed using aspirin mixed with rodent dough from GD0 to GD19. The administration of quercetin (2 mg/kg/day) was performed by intraperitoneal infusion from GD0 to GD19. Protein levels were evaluated using ELISA or Western blot, and microRNA (miRNA) level was evaluated by RT-PCR. Aspirin supplemented with quercetin ameliorated the increase of systolic blood pressure (SBP), proteinuria, tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) levels, and improved the pregnancy outcomes in preeclampsia rats. Aspirin supplemented with quercetin inhibited miR-155 expression in preeclampsia rats. The decreased miR-155 level in placenta further increased the protein level of SOCS1 and inhibited the phosphorylation of p65. In this study, we demonstrated that aspirin supplemented with quercetin enhanced the effects of aspirin for the treatment of preeclampsia.

Key words

Preeclampsia • microRNA-155 • Aspirin • Quercetin

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Introduction

Preeclampsia manifests as hypertension first detected after 20 weeks of gestation and may progress to eclampsia [1]. Preeclampsia may cause severe damage to multiple organ functions and adverse maternal and perinatal outcomes [2]. The prevalence rate of preeclampsia in the world is 3-10 % [3], and in China is about 2-6 % [4]. The serious complications of preeclampsia include eclampsia, stroke, pulmonary edema, malignant hypertension, myocardial ischemia/infarction, acute kidney injury, liver failure, and stillbirth, which are serious threats to maternal and fetal health [5]. Early-onset severe preeclampsia causes medically induced preterm birth [6]. Although there has been significant clinical progress in the diagnosis and treatment of preeclampsia, the most fundamental treatment for disease remission is still termination of pregnancy [7]. Therefore, it is important to prevent the occurrence of preeclampsia for the health of both mother and child.

Among the measures to prevent preeclampsia, the application of low-dose aspirin is considered effective and recommended [8]. Thromboxane A₂ (TXA₂) is a powerful inducer of platelet aggregation. Aspirin decreases the production of TXA₂ in platelets and thus resists platelet aggregation and thrombosis [9]. However, at high concentrations, aspirin also reduces the synthesis of prostacyclin I₂ (PGI₂), a physiological antagonist of TXA₂, and its reduced synthesis may promote thrombosis [10]. Small doses of aspirin (50-150 mg/d) can effectively inhibit the synthesis of TXA₂ in platelets without affecting the synthesis of PGI₂ in the vascular wall, so that the TXA₂/PGI₂ balance tends to be dominated by PGI₂, thereby inhibiting platelet activity, preventing microthrom-

basis, and improving local blood circulation [11].

Quercetin is a bioflavonoid with antioxidant ability. It has been proven that the supplement of quercetin enhances the therapeutic effects against preeclampsia [12]. In the uterus of preeclampsia rats induced by L-NAME, quercetin together with aspirin decreases vascular endothelial growth factor (VEGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) mRNA levels [13]. Compared with aspirin alone, quercetin together with aspirin enhanced the therapeutic effects of aspirin in preeclampsia rat model [13]. In this study, we investigated the molecular mechanisms underlying the effects of aspirin supplemented with quercetin in preeclampsia rats.

Methods

Animals

Female Sprague-Dawley rats (8-10 weeks old, weighing 210-250 g) were housed under humidity 50-60 %, laboratory temperature 22-24 °C, and illumination between 06:00 am and 06:00 pm. All rats had free access to food and tap water. During the sexual excitement periods, every two female rats were copulated with one weight-matched male rat, and a positive vaginal smear for sperm was defined as day 0 of pregnancy (gestation day 0, GD0). Pregnant Sprague-Dawley rats were randomly divided into the control group (no L-NAME treatment, n=10), the L-NAME group (L-NAME treatment, n=10), the L-NAME + Asp (Asp) group (L-NAME and aspirin treatment, n=10), the L-NAME + Que group (L-NAME and quercetin treatment, n=10), or the L-NAME + Que + Asp group (L-NAME, quercetin, and aspirin treatment, n=10).

Preeclampsia was induced by L-NAME (50 mg/kg/day) (N5751, Sigma, MO, USA) from GD14 to GD19 *via* oral gavage. Low-dose aspirin (1.5 mg/kg/day) administration was performed using aspirin mixed with rodent dough (Bio-Serv, NJ, USA) from GD0 to GD19. Quercetin was first dissolved in DMSO to prepare a store solution, then diluted with saline solution before injection. The control group was treated with a vehicle. The administration of quercetin (2 mg/kg/day) (PHR1488, Sigma, MO, USA) was performed by intraperitoneal injection from GD0 to GD19. Serum and placental tissue samples were collected on GD20. Animal studies were approved by the Cangzhou Central Hospital.

Proteinuria and blood pressure

Female rat systolic blood pressure (SBP) was detected by noninvasive tail-cuff method using the BP-6

noninvasive blood-pressure detector (Bi-Equip, Shanghai, China) on GD20. Before measuring blood pressure, rats were fixed and preheated for 5 min to 38 °C, and 3 measurements were for averaging. The rats were housed in metabolism cages on GD19, and 24 h later, urine was collected. The level of was evaluated through Coomassie brilliant blue kit (A045-2-1, Jiancheng, Nanjing, China).

ELISA

Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in serum and placental samples were detected through ELISA kits (R6000B, RTA00, Bio-Rad, CA, USA). Samples were store at -80 °C.

MicroRNA (miRNA) sequencing

Total RNA was isolated by TRIzol (Invitrogen, CA, USA). Libraries were produced by TruSeq small RNA library prep kit (RS-200, Illumina, CA, USA). These RNA samples were barcoded by ligation with unique adaptor sequences. RNA samples were reverse transcribed, polymerase chain reaction amplified, and size selected using gel electrophoresis. Illumina NextSeq 500 high-output kit and Illumina NextSeq 500 platform were employed for sequencing.

Barcode and adaptor sequences in the raw FASTQ files were removed. The data were analyzed through miRDeep2 algorithm and statistical analysis was performed by DESeq2 package in R. Sequencing results were analyzed using the miRDeep2 program to identify known microRNAs. Raw counts and corresponding microRNAs underwent normalization and statistical analysis. The median ratio method was used for normalization and applies a generalized linear model for differential expression. Statistical analysis and significance were calculated using a Wald test, corrected for multiple testing using the Benjamini and Hochberg procedure.

Real-time (RT)-PCR

Trizol reagent (Invitrogen, CA, USA) was applied to extract the total RNA from the tissues according to the manufacturer's instructions. A reverse transcription kit (Fermentas, St. Leon-Rot, Germany) was used for cDNA synthesis by adding approximately 2 μ g of RNA. Quantitative analysis of various mRNA levels was examined by SYBR-Green Master mix (Life Technologies, Carlsbad, CA, USA). U6 was internal control.

miR-155 forward: CTTAATGCTAATCGTGAT-AGGGGT, miR-155 reverse: CCAGTGCAGGGTCCGA-

GGT, U6 forward: GCTTCGGCAGCACATATACTAA-AAT, U6 reverse: CGCTTCACGAATTTGCGTGTCAT.

Western blot

Western blot was performed through standard protocol. β -actin was loading control. The relative expression levels of protein were expressed as fold of control group. Tissues were homogenized using lysis buffer, followed by determining the concentration using bicinchoninic acid kit. Total of 20 μ g protein was separated by SDS-PAGE and transferred to the PVDF membranes. Then, the membranes were blocked using 5 % non-fat milk for 90 min at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies. Antibodies used in this assay included: anti-suppressors of cytokine signaling 1 (SOCS1) (ab62584; Abcam, MA, USA), anti-NF- κ B p65 (#4764S; Cell Signaling Technology, MA, USA), p-p65 (Ser536) (#3031; Cell Signaling Technology), and anti- β -actin (ab8226; Abcam). Protein samples were store at -80 °C. Three replications were carried out.

Statistical analysis

Statistical analysis was performed by SPSS version 17.0. Data were expressed as mean \pm standard deviation (SD) and analyzed using one-way ANOVA followed with Tukey's *post hoc* test, with $p < 0.05$ considered significant.

Results

Aspirin supplemented with quercetin ameliorated the increase of SBP and proteinuria in preeclampsia rats

In this research, preeclampsia was induced by the administration of L-NAME from GD14 to GD19. The administration of aspirin and quercetin was performed from GD0 to GD19 (Fig. 1a). Compared with rats in the control group, rats with L-NAME treatment showed significantly higher SBP (Fig. 1b). Rats in the L-NAME + Asp group and the L-NAME + Que group had lower SBP than those in the L-NAME group (Fig. 1b). In the L-NAME + Que + Asp group, the level of SBP was significantly lower than in the other L-NAME-treated groups (Fig. 1b). Meanwhile, the level of proteinuria showed the same tendency in these groups (Fig. 1c).

Aspirin supplemented with quercetin attenuated the levels of pro-inflammatory cytokines in preeclampsia rats

The levels of IL-6 and TNF- α in plasma or placenta samples were evaluated by ELISA. In Figure 2a,

b, L-NAME significantly elevated IL-6 and TNF- α levels in plasma. In the L-NAME + Asp group and the L-NAME + Que group, IL-6 and TNF- α levels in plasma were lower than in the L-NAME group (Fig. 2a, b). Rats in the L-NAME + Que + Asp group had significantly lower IL-6 and TNF- α levels than the other preeclampsia rats (Fig. 2a, b). IL-6 and TNF- α levels in placenta showed the same tendency in these groups (Fig. 2c, d).

Aspirin supplemented with quercetin improved the pregnancy outcomes of preeclampsia rats

L-NAME significantly declined fetal survival ratio, fetal weight, and the ratio of pups to placenta weights (Fig. 3a, b, d). Rats in the L-NAME + Asp group and the L-NAME + Que group had higher fetal survival ratio and the ratio of pups to placenta weights than those in the L-NAME group (Fig. 3a, d). In the L-NAME + Que + Asp group, these pregnancy outcomes were significantly higher than in the other L-NAME-treated groups (Fig. 3a, b, d). There was no significant difference of placenta weight between these groups (Fig. 3c).

Aspirin supplemented with quercetin inhibited the expression of miR-155 in preeclampsia rats

Statistical analysis of miRNA levels between different groups was performed and displayed as a heat map (Fig. 4a). Based on the heat map, the administration of L-NAME significantly enhanced miR-155 expression in placenta (Fig. 4a). In the L-NAME + Asp group and the L-NAME + Que group, the levels of miR-155 were lower than in the L-NAME group (Fig. 4a, b). Compared with other preeclampsia rats, those in the L-NAME + Que + Asp group had significantly lower level of miR-155 in placenta (Fig. 4a, b).

Aspirin supplemented with quercetin decreased inflammation in L-NAME-induced preeclampsia rats via repression of miR-155/NF- κ B

In Figure 5a, miR-155 targets to the mRNA of SOCS1, enhances NF- κ B activation, and induces TNF- α and IL-6 production. The administration of L-NAME significantly decreased SOCS1 level in placenta (Fig. 5b, c). When compared with the other preeclampsia rats, those in the L-NAME + Que + Asp group had dramatically elevated SOCS1 protein level in placenta (Fig. 5b, c). The phosphorylation of p65 was enhanced by L-NAME (Fig. 5b, d). Aspirin supplemented with quercetin further inhibited the phosphorylation of p65 when compared with the administration of quercetin or aspirin alone (Fig. 5b, d).

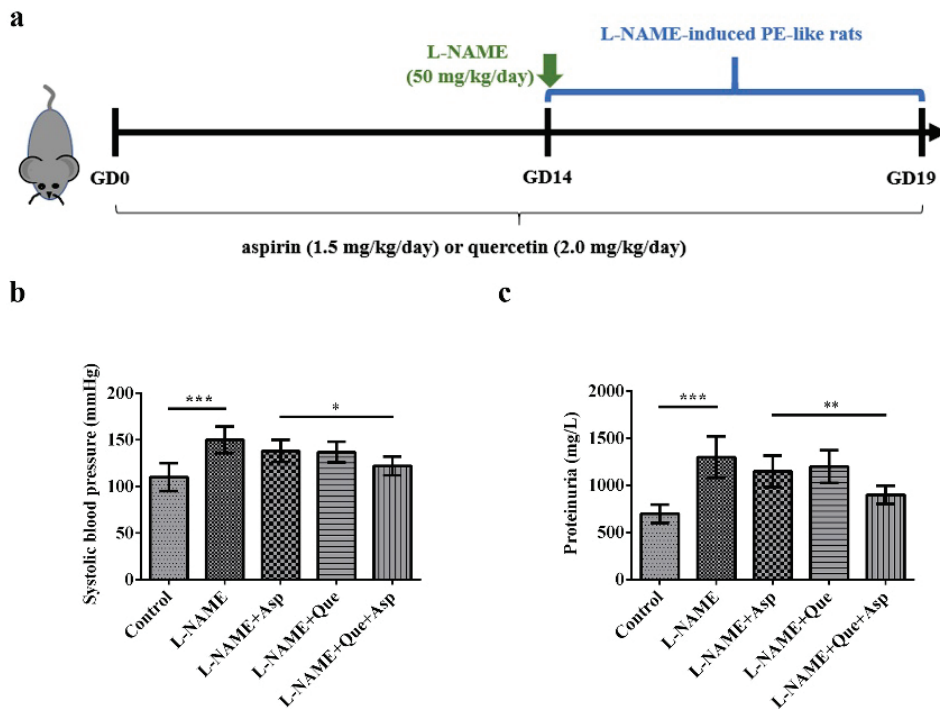


Fig. 1. Aspirin (Asp) supplemented with quercetin (Que) ameliorated the increase of SBP and proteinuria in L-NAME induced preeclampsia rats. **(a)** The scheme of the animal experiment. N=10 rats per group. **(b)** SBP of each indicated group was measured non-invasively using the tail-cuff method on GD20. **(c)** The proteinuria in each group was detected using CBB kits on GD20. Data were represented as mean ± SD. ANOVA was performed; * p<0.05; ** p<0.01; *** p<0.001 between the groups as indicated by the lines.

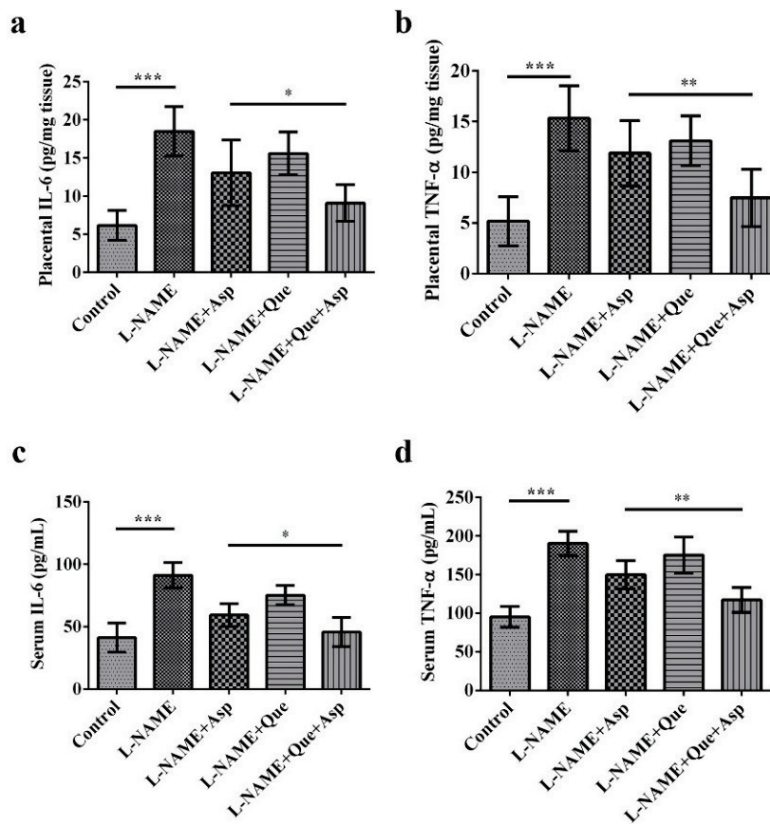


Fig. 2. Aspirin (Asp) supplemented with quercetin (Que) attenuated the expression levels of pro-inflammatory cytokines in both plasma and placenta of L-NAME induced preeclampsia rats. Plasma IL-6 **(a)** and TNF-α **(b)**, placental IL-6 **(c)** and TNF-α **(d)** were measured in different group on GD 20 by ELISA. Data were represented as mean ± SD. N=10 rats per group. ANOVA was performed; * p<0.05; ** p<0.01; *** p<0.001 between the groups as indicated by the lines.

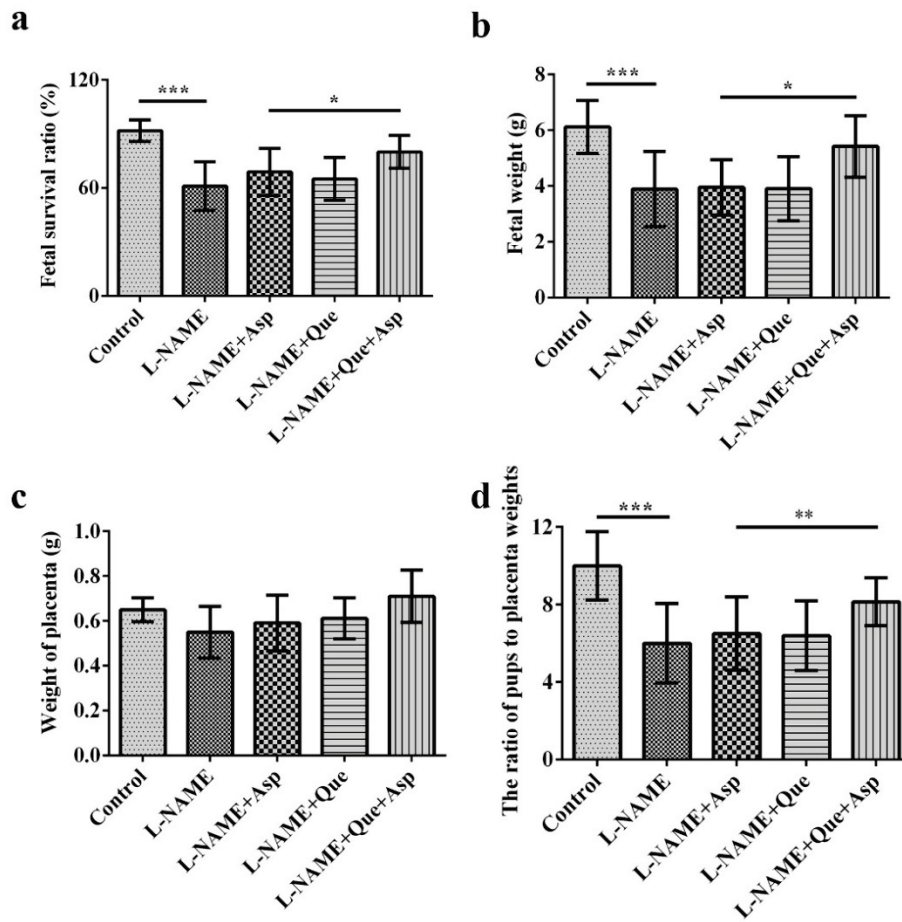


Fig. 3. Aspirin (Asp) supplemented with quercetin (Que) improved the pregnancy outcomes of L-NAME induced preeclampsia rats. Fetal survival ratio (a), fetal weight (b), weight of placenta (c), and the ratio of pups to placenta weights (d) were compared. Data were represented as mean ± SD. ANOVA was performed; * p<0.05; ** p<0.01; *** p<0.001 between the groups as indicated by the lines.

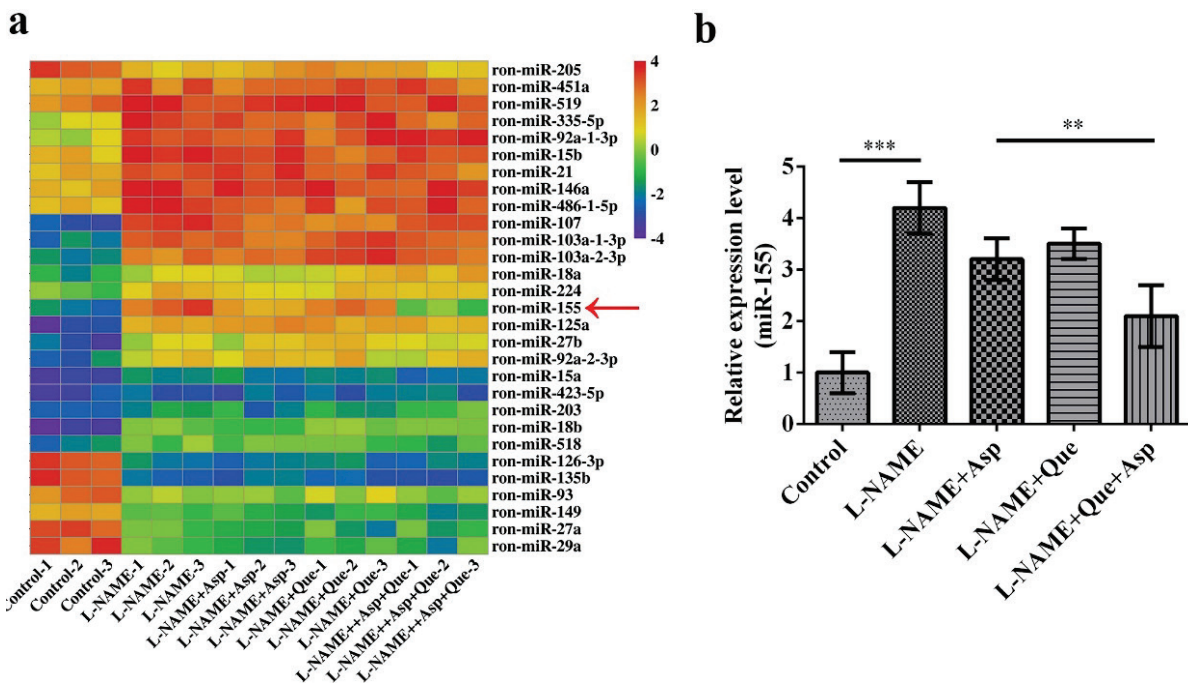


Fig. 4. The differentially expressed miRNAs in the placenta. (a) Heat maps. Red represents high levels of expression and purple represents low levels of expression. (b) The expression levels of miR-155 in the placenta were measured by qPCR. Data were represented as mean ± SD. N=3. ANOVA was performed; ** p<0.01; *** p<0.001 between the groups as indicated by the lines.

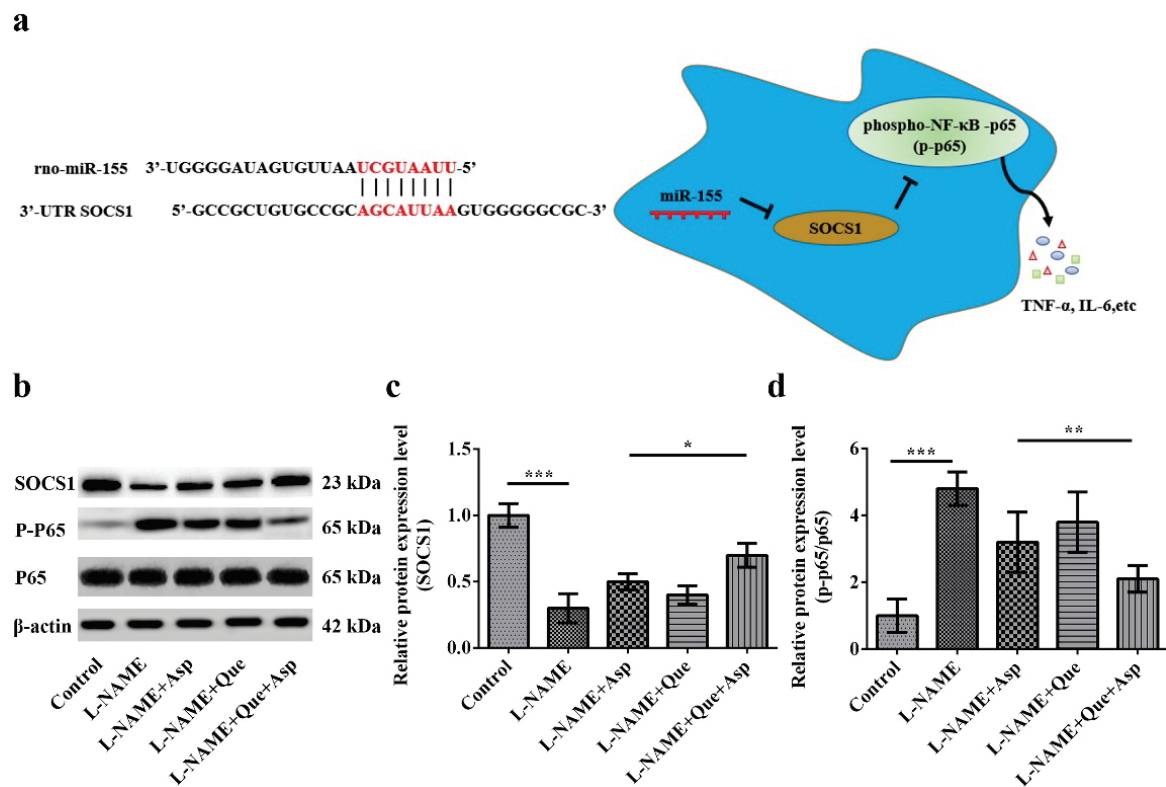


Fig. 5. Aspirin (Asp) supplemented with quercetin (Que) ameliorates inflammation of L-NAME-induced preeclampsia rats *via* repression of miR-155/NF- κ B. **(a)** MiR-155 induced macrophage inflammation by targeting SOCS1, stimulated NF- κ B activation, and induced the production of TNF- α and IL-6. **(b)** The protein levels of SOCS1, p-p65, and p65 in the placenta were determined by Western blot. β -actin was used as a loading control. The quantified relative band intensity was determined by ImageJ **(c and d)**. Data are represented as mean \pm SD. N=3. ANOVA was performed; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between the groups as indicated by the lines.

Discussion

Preeclampsia is a condition caused by hypertensive disorders during pregnancy. It is a pregnancy-specific disorder occurring after 20 weeks of gestation, with proteinuria, hypertension, and other systemic disorders as the main clinical manifestation [14]. Placental hypoxia and extensive damage to the vascular endothelium may be the pathophysiological basis for the pathogenesis of preeclampsia [15]. The mechanism of preeclampsia development remains unclear. Therefore, many studies have focused on the prediction and pathogenesis of preeclampsia.

Altered coagulation is an important part of the pathogenesis of preeclampsia, and patients with preeclampsia have a greater propensity for thrombosis because of the relatively hypercoagulable state in which normal pregnant women are [16]. This process involves the activation of platelets. Aspirin is an antiplatelet drug with antipyretic, analgesic, and anti-inflammatory effects [17]. Aspirin is used for the prevention and treatment of preeclampsia. Several studies have shown that the prophylactic use of low-dose aspirin reduces

preeclampsia incidence in high-risk groups without harming the perinatal infant [18,19]. It has been reported that in LPS-induced preeclampsia-like rats, aspirin assuaged LPS-induced proteinuria excretion and hypertension and decreased levels of IL-6, IL-1 β , TNF- α , and IFN- γ in serum and placenta tissue that were elevated by LPS [13]. In this study, preeclampsia was induced by L-NAME from GD14 to GD19 *via* oral gavage. In L-NAME-induced preeclampsia rats, the administration of aspirin had no significant influence on SBP, proteinuria, or the expression levels of proinflammatory cytokines in plasma and placenta.

Because pregnancy causes a mild systemic inflammatory response, preeclampsia is strongly correlated with excessive maternal systemic inflammatory response [20]. Clinical studies have demonstrated that serum and placental inflammatory cytokine levels are higher in patients with preeclampsia [21]. Quercetin is a major flavonoid, and its antioxidant capacity makes quercetin a powerful free radical scavenger [22]. Quercetin has various biological functions such as antioxidant, antiviral, antiproliferative, antiallergic, antitumor, and anti-inflammatory activities [23].

It has been reported that quercetin could only regulate proteinuria but not SBP in pre-eclampsia rat model [13]. The effects of aspirin on the regulation of SBP and proteinuria were both enhanced by quercetin supplementation [13]. In this study, quercetin or aspirin treatment alone had no significant effects on ameliorating SBP and proteinuria in preeclampsia rats, but quercetin supplement to aspirin ameliorated SBP and proteinuria.

In both serum and placenta from preeclampsia rats, the upregulated levels of IL-6 and TNF- α were declined by aspirin. Quercetin treatment in L-NAME-induced preeclampsia rats only down-regulated the serum IL-6 level, but serum TNF- α level and the placental levels of IL-6 and TNF- α were not influenced [13]. In this study, quercetin or aspirin treatment alone had no significant influence on the levels of IL-6 and TNF- α in both plasma and placenta of L-NAME induced preeclampsia rats. When quercetin was supplied with aspirin together, the levels of IL-6 and TNF- α were significantly downregulated in both serum and placental tissues. Meanwhile, the supplement of quercetin to aspirin also ameliorated the pregnancy outcomes for preeclampsia rats.

The relationship between miRNAs and pregnancy is gaining attention. Many miRNAs are fully expressed in the placenta and plasma during pregnancy [24]. Studies on pregnancy-related diseases have shown that miRNAs are promising markers for the diagnosis of pregnancy-related diseases [25]. Many research groups have attempted to explain the development of preeclampsia by analyzing the differences in miRNAs in the placenta of preeclampsia patients and healthy women [26].

We also analyzed the expression of miRNAs in the placenta of rats. Based on these results, miR-155 was highly expressed in the placenta of L-NAME-induced preeclampsia rats. miR-155 is an inflammation-associated miRNA. miR-155 is differentially expressed in preeclampsia placentas [27]. There is a negative correlation between high miR-155 expression and low cysteine-rich

protein 61 (CYR61) expression in placentas with severe preeclampsia [28]. CYR61 is an important angiogenic factor in early pregnancy and induces the expression of VEGF. Therefore, overexpression of miR-155 in the placenta may be associated with preeclampsia through the downregulation of CYR61 and thus VEGF levels [28]. Another research has proved that miR-155 targets the mRNA of SOCS1, stimulates NF- κ B activation, and induced the production of IL-6 and TNF- α [29].

We demonstrated that the supplement of quercetin to aspirin declined the expression of miR-155 in L-NAME-induced preeclampsia rats. The decreased miR-155 level in placenta further increased the protein level of SOCS1 and inhibited the phosphorylation of p65. Through this signaling pathway, the supplement of quercetin to aspirin inhibited the excessive systemic inflammatory response in L-NAME-induced preeclampsia rats.

Conclusions

In conclusion, we demonstrated that the aspirin supplemented with quercetin showed the effects in the therapy of preeclampsia in a rat model. Aspirin supplemented with quercetin inhibited the expression of miR-155 to suppress the excessive systemic inflammatory response in L-NAME-induced preeclampsia rats.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

IL-6, interleukin-6; PE, Preeclampsia; Que, Quercetin; SBP, systolic blood pressures; TNF- α , tumor necrosis factor- α .

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