Protective effect of quercetin on lipopolysaccharide-induced miscarriage based on animal experiments and network pharmacology

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Abstract. Spontaneous abortion (SA) occurs in woman of child-bearing age, jeopardizing their physical and mental health. Quercetin is a natural flavonoid, which exhibits a variety of pharmacological activities. However, the role and mechanisms of quercetin in SA still need to be further explored. Animal experiments were performed to examine the effect of quercetin in treating SA. Institute of Cancer Research mice were injected with lipopolysaccharide into the tail vein on the 7th day of gestation to establish a SA model. Gavage was performed during days 3-8 of gestation with high-, medium- and low-dose of quercetin. Then the effect of quercetin on embryos was evaluated. Animal experiment showed that quercetin could remarkably reduce the embryo loss rate and increase the mean weight of surviving embryos to some degree. Furthermore, network pharmacology was employed to explore the underlying mechanisms of quercetin in the treatment of SA. Several databases were used to collect the targets of SA and quercetin. Protein-protein interaction network, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis were performed to elucidate the interactions between SA and quercetin. The relative mRNA expressions of several targets in uterine were detected by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Network pharmacology indicated that the effects of quercetin in treating SA were mainly related to hormone response and the modulation of defense response and inflammatory response, involving signaling pathways such as PI3K-Akt, VEGF, MAPK and core targets such as AKT1, albumin, caspase-3. RT-qPCR showed that quercetin could up-regulate AKT1, MAPK1, PGR, SGK1 and down-regulate ESR1, MAPK3. The results showed that quercetin may modulate multiple signaling pathways by targeting core targets to prevent and treat SA.

Introduction

In China, spontaneous abortion (SA), commonly occurs in women of child-bearing age. SA is defined as the termination of a pregnancy before 28 weeks of gestation with a fetal weight of <1,000 g (1). Of clinically diagnosed pregnancies ~15% undergo SA and ~11% of women with a history of one abortion experience SA (2). SA not only jeopardizes the physical and mental health of patients, but also imposes a serious burden on families and society (2). The causes of SA are complex, including chromosomal defects in the embryo, abnormalities in the decidualization process, maternal immunological abnormalities, thrombotic tendencies, endocrine abnormalities, anatomical abnormalities of the female reproductive tract and infections (2-4). Modern medical treatment mainly involves correcting endocrine abnormalities, improving luteal function, balancing immunity, anticoagulant therapy and vitamin supplementation. However, the effectiveness and safety of these treatments are still under investigation.

Chinese traditional medicine has a unique theoretical basis and experience in tranquilizing fetus and is effective in SA treatment with fewer toxic and side effects (5-7). *Cuscuta* and *Herba Taxilli*, as the main medicines in the basic formula of Shoutai Pill, are commonly used in Chinese traditional medicine to what is termed 'tonifying the kidneys' and 'stabilizing the fetus'. Modern pharmacological studies have revealed that quercetin is one of the primary active ingredients of *Cuscuta* and *Herba Taxilli* (8-10). As a natural polyphenol, quercetin is found in a number of edible and medicinal plants and exhibits a diverse array of pharmacological activities such as anti-inflammatory, antioxidant, antiviral, antitumor, immunomodulatory

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and estrogen-like functions (11-16). However, current research on quercetin is mainly focused on cardiovascular, endocrine, tumor and chronic pain areas (17-20) and the specific role of quercetin in preventing and treating SA and its related mechanisms still need to be further investigated.

Network pharmacology emphasizes that the development of disease is a long-term and complex dynamic process and suggests that the essence of disease is the imbalance of complex biological networks (21). Network pharmacology breaks the traditional thinking paradigm of 'one disease, one target, one drug' and elaborates the pathogenesis of complex diseases through multi-targets and multi-pathways, which has a positive predictive role in exploring the unknown pharmacological effects of multi-target natural products (22,23). The present study used a bacterial lipopolysaccharide (LPS)-induced abortion model in pregnant mice to simulate the pathology of maternal-fetal immune imbalance caused by increased blood endotoxin levels due to reproductive tract infection and intestinal inflammation (24). The effect of quercetin on the embryonic loss rate was evaluated. The relevant signaling pathways of quercetin for SA treatment were analyzed by network pharmacology methods, which provides ideas for further application of quercetin in reproductive medicine.

Materials and methods

Experimental animals. A total of 78 specific pathogen free (SPF) grade female Institute of Cancer Research (ICR) mice (6-8 weeks old, body mass 25-35 g) and 30 SPF grade male ICR mice (8-10 weeks old, body mass 30-35 g) were purchased from Shanghai Sippe-Bk Lab Animal Co., Ltd. [Laboratory Animal License No. SCXK (Shanghai) 2018-0006]. The mice were housed in Zhejiang Chinese Medical University Laboratory Animal Research Center, SPF grade animal laboratory: 22-24˚C, relative humidity 55-65%, 12-h light/dark cycle and food and drink supplied *ad libitum*.

The present study was approved by the Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University (ethics approval no. IACUC-20220919-25).

Main drugs and reagents. Lipopolysaccharide (LPS; MilliporeSigma; cat. no. L2630) was diluted using pH 7.2-7.4 phosphate-buffered saline (PBS), which had been filtered through a membrane to remove bacteria. The LPS solution was prepared at a concentration of 50 μg/ml. Quercetin (MilliporeSigma; cat. no. Q4951; purity ≥95%) was initially dissolved in a small quantity of anhydrous ethanol. It was subsequently diluted with PBS to create concentrations of 0.25, 1.25 and 2.5 mg/ml (25,26).

Animal modeling, grouping and drug administration. Mice were acclimated for 1 week and were included in the experiment after observing a normal estrous cycle. Females and males were mated 2:1 and cages were combined at 17:00 pm. Females were checked for vaginal plugs at 8:00 am the following morning. Those without vaginal plugs were recorded and continued to mate with males, while those with vaginal plugs were designated as the 1st day of gestation (D1). Pregnant mice confirmed by vaginal plugs were randomly divided into 6 groups, including control group, model group, quercetin + PBS group, low-dose quercetin group, medium-dose quercetin group and high-dose quercetin group, with 13 mice in each group.

On days 3-8 of gestation, mice in the control and model groups were gavaged with PBS; mice in the quercetin + PBS group and the high-dose quercetin group were gavaged with high-dose quercetin (2.5 mg/ml); mice in the low-dose and medium-dose quercetin group were gavaged with low-dose (0.25 mg/ml) and medium-dose (1.25 mg/ml) quercetin, respectively. Gavage was performed twice at 09:00 and 15:00, with each time 0.2 ml and a total of 0.4 ml each day. On day 7 of gestation, mice in both the control group and quercetin + PBS group received with a 0.2 ml injection of PBS into the tail vein and mice in the model group, low-dose quercetin group, medium-dose quercetin group and high-dose quercetin group were injected with 0.2 ml of LPS (50 μg/ml). The specific procedure was shown in Fig. 1.

Humane endpoints were as follows: Inability to eat or drink without anesthesia or sedation or stand for up to 24 h; mice in the absence of anesthesia or sedation exhibits poor condition including hypothermia (body temperature <37˚C); mice show the appearance of central nervous system depression, tremor, paralysis, pain that does not respond to analgesics. 5 female mice were sacrificed prematurely according to the humane endpoints.

General observation. The body mass of each group of pregnant mice was measured in the morning at gestation day 1, 3, 7 and 9. After tail vein administration, the general state [including appearance, activity, autonomic activities, response to stimuli, degree of eye closure (27), and eye secretions; Table SI], diarrhea, vaginal bleeding and pregnancy discharge of pregnant mice in each group were observed and recorded in time. The body weight loss in D9 (compared with that in D7) was calculated as: Degree of weight loss (%)=[body weight (D9)-body weight (D7)]/body weight (D7) x100%.

Embryo loss rate and mean weight of surviving embryos. Mice were sacrificed by cervical dislocation under anesthesia on the morning of day 9 of gestation and complete uterine tissues were removed by dissecting the uterus. The death was confirmed by cessation of heartbeat, respiration, congestion and temperature of the skin. Embryos were observed and the gross weight and net weight of the uterus, the number of normal embryos and the number of dead embryos were recorded (or the number of implantation sites if the embryos were resorbed or expelled prior to the execution).

Criteria for determining dead embryos were uterus with 'bamboo-like' changes and embryos of different sizes with black or purplish-brown color. Following the intrauterine death, the size of the embryo does not continue to grow but shrinks, resulting in a bamboo-like appearance of the uterus, section by section without excessive expansion and with a knot between each section.

Embryo loss rate (%)=total number of dead embryos/(total number of dead embryos + total number of normal embryos) x100%.

Mean embryo weight=(gross uterine weight-net uterine weight)/number of surviving embryos.

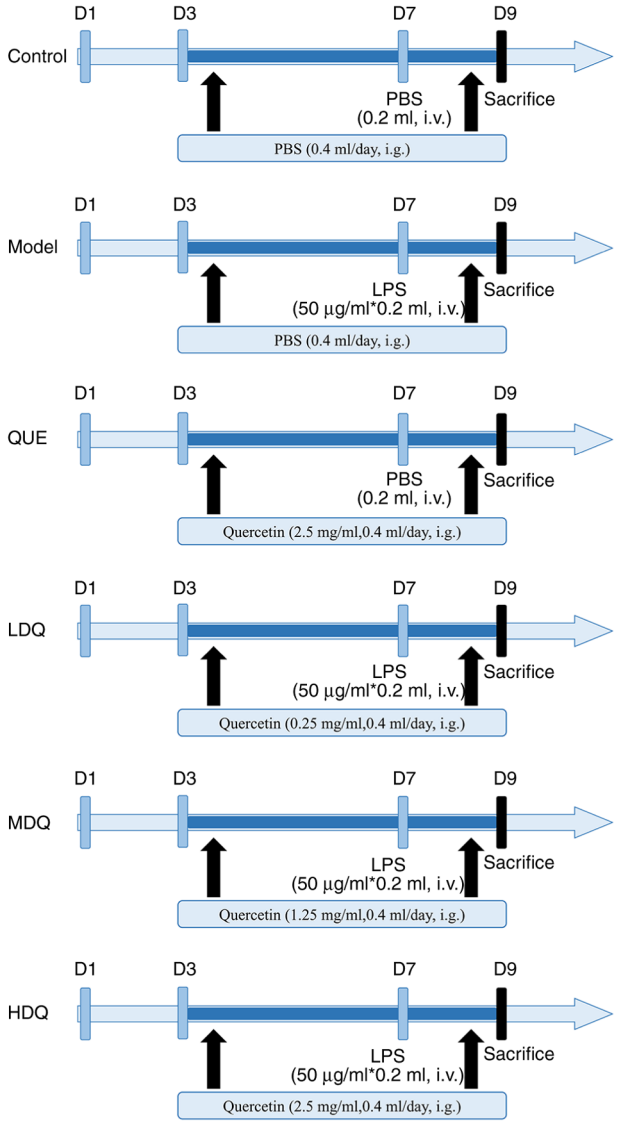

Figure 1. Diagram of the workflow of the animal research. MDQ, the medium-dose quercetin group; Control, the control group; Model, the model group; QUE, the quercetin + PBS group; LDQ, the low-dose quercetin group; MDQ, the medium-dose quercetin group; HDQ, the high-dose quercetin group; D1, the 1st day of pregnancy; PBS, phosphate buffered saline; LPS, lipopolysaccharide; i.v., intravenous injection; i.g., oral gavage.

Histological analysis. Implantation site tissue including embryo specimens from the control and model group were fixed with 4% paraformaldehyde at room temperature for 48 h. Specimens were embedded in paraffin, sectioned at 3 mm, and routinely stained at room temperature with hematoxylin (Solarbio, cat. no. G1150) for 3-8 min and eosin (Solarbio, cat. no. G1100) for 1-3 min. 100x and 200x light microscopes (Olympus, cat. no. BX53) were used to observe the pathological changes of the implantation sites.

Collection of drug targets. The 2D structure diagram of quercetin (SDF format) was achieved from PubChem (28) and then was uploaded to PharmMapper platform (29) to predict potential targets within *Homo sapiens*. Uniprot database (30) was applied to correct and normalize all the retrieved target names.

Collection of disease targets. SA-related genes were obtained from the databases of OMIM (31), GeneCards (32) and DisGeNet (33) using the keywords 'spontaneous abortion' and 'pregnancy loss' within 'Homo sapiens'. The SA-related genes from the searches were combined and duplicates were removed. Gene names were standardized by the Uniprot database (30).

Venn analysis. Venn online tool (34) was utilized to generate a Venn diagram and obtain mutual targets by intersecting the targets of quercetin with the targets of SA.

Protein-protein interaction (PPI) network construction and core target identification. The overlapping targets were submitted to the STRING database (35) to retrieve the PPI network within the context of 'Homo sapiens' and at a medium confidence level (0.04). The nodes that were discrete from the main network were hided. Next, the PPI network in TSV file format was imported into Cytoscape (v3.7.1) (36) for visualization. The cytoNCA plugin was employed to calculate the degree for each target and the core targets were identified by applying a degree value exceeding the average degree (37).

Enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The Metascape database (38) was used to analyze the enriched GO and KEGG pathways with the intersecting targets. $P<0.01$ was defined as the criteria for statistical significance. The top 20 KEGG pathways and the top 20 terms within the categories of molecular function (MF), biological process (BP) and cellular component (CC) were collected based on the smallest to largest P-value. The above results were then visualized using the Microbiome platform (http://www.bioinformatics.com.cn/).

RNA extraction and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). The gene expression levels were quantified using RT-qPCR. RNA extraction, cDNA synthesis, and qPCR were performed according to the manufacturer's protocols. Total RNA was isolated from uterus tissues with TRIzol Plus RNA Purification Kit (Thermo Fisher Scientific, cat. no. 12183-555). Ultraviolet spectrophotometer and electrophoresis were used to test RNA purity and quantification. The RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, cat. no. 11752-050). RT-qPCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, cat. no. 4367659) with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad), the reaction volume of which was 20 μl. PCR cycling conditions (denaturation, annealing and extension, times and temperatures) are as follows:

For initial denaturation: 95˚C for 60 sec, followed by 40 cycles: 95˚C for 15 sec for denaturation; 63˚C for 25 sec for annealing and extension. GAPDH) was served as the internal reference, and the $2^{-\Delta\Delta Cq}$ (39) method was applied to calculate the relative expression. The experiments were performed in biological triplicate for each group.

All primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Primer sequences were as follows: GAPDH were F: 5'-GAAGGTCGGTGTGAACGGATTTG-3'; R: 5'-CATGTAGACCATGTAGTTGAGGTCA-3'. Primer

sequences for Akt1 were F: 5'-GAAGGTCGGTGTGAACGGATTTG-3'; R: 5'-GCATGAGGTTCTCCAGCTTCA-3'. Primer sequences for ESR1 were F: 5'-CAGGCTTTGGGGACTTGAATC-3'; R: 5'-CCAGACGAGACCAATCATCAGA-3'. Primer sequences for JAK2 were F: 5'-GCTACCAGATGGAAACTGTGCG-3'; R: 5'-GCCTCTGTAATGTTGGTGAGATC-3'. Primer sequences for MAPK1 were F: 5'-TCAAGCCTTCCAACCTCCTGCT-3'; R: 5'-AGCTCTGTACCAACGTGTGGCT-3'. Primer sequences for MAPK3 were F: 5'-CAACACCACCTGCGACCTT-3'; R: 5'-CCACATACTCCGTCAGAAAGC-3'. Primer sequences for PGR were F: 5'-GTCCGAGTTATGAGAACCCTTGA-3'; R: 5'-GATTTGGTGAAAAGGTGATTCTCTGG-3'. Primer sequences for PI3K were F: 5'-GATGTGGCTGACGCAGAAAG-3'; R: 5'-GGTTGCTGCTCCCGACATT-3'. Primers for SGK1 were F: 5'-GCTCGATTCTACGCAGCTGAA-3'; R: 5'-CCCTGGGAGTCTAGGAGAA-3'.

Statistical analysis. Microsoft Office Excel 2021 (Microsoft Corporation) was used to create database and SPSS 26.0 statistical analysis software (IBM Corp.) was used for data processing and analysis. Quantitative data that conformed to normal or near-normal distribution were described by mean ± standard deviation. Then t-test or one-way ANOVA was conducted and Tukey's or Scheffe's post hoc comparison was used to evaluate differences between groups. In case the data deviated from a normal distribution, they were represented by the median (upper quartile, lower quartile), namely *M (P25, P75)*, analyzed by Kruskal-Wallis H test and multiple comparisons were performed by Kruskal-Wallis one-way ANOVA. Count data were described as percentages, compared using the χ^2 test or Fisher's exact probability method. Multiple comparisons were conducted using the Bonferroni method with a corrected test level of $\alpha'=0.008$. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of general information and weight between different groups. The pregnant mice in the control group and the quercetin + PBS group were in a good health, had shiny fur and were responsive, whereas the pregnant mice in the model group and medium-dose quercetin group had obviously rough and dull fur and were unresponsive, accompanied by diarrhea. About half of them had blood stains and pregnancy residues on their vaginas. In the high- and low-dose quercetin group, the pregnant mice showed an improved state than those in the model group and medium-dose quercetin group, but some of them had rough fur, diarrhea and were less responsive, accompanied by blood stains and pregnancy residues on their vaginas. After autopsy, it was found that 45.92% of the embryos from abortion mice in the model group had been absorbed or discharged. 53.57% of aborted embryos in the low-dose quercetin group, 84.78% in the medium-dose quercetin group and 55.88% in the high-dose quercetin group had been absorbed or discharged.

As shown in the Fig. 2B, the body weights of pregnant mice in the control group and the model group exhibited a steady increase from day 3 to day 5 of gestation. Conversely, pregnant mice in the medium- and high-dose quercetin group displayed a decline pattern in their body weights during this period. Subsequent to the tail vein injection, the body weights of mice in the control group and quercetin + PBS group continued to show consistent growth. In contrast, the other four groups exhibited varying degrees of weight decline after the injection.

Following the tail vein injection on gestation day 7, the body weights of pregnant mice in low-, medium-, high-dose quercetin and the model groups descended markedly ($P<0.0001$). Notably, the model group exhibited the most substantial decrease in body weight with the rate of 10.94±3.13%. Among three quercetin groups, the medium-dose quercetin group experienced the greatest reduction in body weight while the low-dose one showed the lowest weight loss rate of 5.92±2.47%. However, the body weight growth rate of the control group and the quercetin + PBS group remained similar, with slight growth rates of 0.50±4.14% and 1.03±1.90%, respectively ($P=0.998$; Table I).

Effect of quercetin on the number of surviving and dead embryos and the mean weight of surviving embryos. In the control group and the quercetin + PBS group, the implantation sites were evenly distributed, with normal embryonic development. The uterus was in red and shaped like a bead, with a small amount of bruising in the uterine cavity. In the model group, the development of embryos was not synchronized with each other and the size of the implantation sites was reduced significantly. Some disappeared or were necrotic, leaving dark brown or dark red bamboo-like appearances of the uteri, section by section with a knot between each section, and obvious bruising was seen in the uterine cavity. The low- and medium-dose groups showed shrinkage in size and a whitening color of the implantation sites, but no bleeding spot or bruising was seen in the uterine cavity. While the high-dose quercetin group displayed a superior embryo status, there was a small amount of bleeding spot or bruising in the uterine cavity (Fig. 2A).

Compared with the model group, the number of embryo deaths decreased in both the control group and quercetin + PBS group, while the number of surviving embryos increased in the quercetin + PBS group ($P=0.028$, $P=0.028$ and $P=0.034$, respectively). Meanwhile, the medium-dose quercetin group exhibited a markedly higher number of embryo deaths in comparison to the control group ($P=0.035$). No significant difference was observed in the numbers of surviving and dead embryos between the control group and quercetin + PBS group ($P=1.000$ and $P=1.000$, respectively; Table II).

Regarding the mean weight of surviving embryos, both the control group and quercetin + PBS group exhibited significantly higher values than the model group ($P<0.0001$), which were 0.0267±0.0019, 0.0258±0.0025 g and 0.0174±0.0008 g, respectively. The high-, medium- and low-dose quercetin groups all demonstrated higher mean weight than the model group, with the high-dose quercetin group displaying the highest mean weight of 0.0218±0.0014 g among three quercetin groups ($P=0.049$, $P=0.376$ and $P=0.457$, respectively). Moreover, the mean weight in the high-, medium- and low-dose quercetin groups were all lower than that in the quercetin + PBS group ($P=0.0096$, $P=0.0011$ and $P<0.0001$, respectively). The results are shown in Fig. 2C and Table SII.

Table I. Decrease in body weight following the tail vein injection.

Group	n	Body weight (D7)/g	Body weight (D9)/g	*t*	P-value	Degree of weight loss/%
Control	13	30.67±1.91	30.78±1.66	-0.336	0.742	-0.50±4.14
Model	13	29.88±1.42	26.63±1.86	12.857	<0.0001	10.94±3.13[a]
QUE	13	31.75±1.33	32.07±1.12	-1.905	0.081	-1.03±1.90[b]
LDQ	13	31.75±2.17	29.88±2.36	8.733	<0.0001	5.92±2.47[a,c,e]
MDQ	13	29.78±1.51	27.67±2.00	5.900	<0.0001	7.09±4.29[a,d,e]
HDQ	13	30.43±1.86	28.60±2.01	6.550	<0.0001	6.01±3.18[a,c,e]

F-value of degree of weight loss was 25.785 (P<0.0001). [a]P<0.0001 vs. the Control; [b]P<0.0001, [c]P<0.01 and [d]P<0.05 vs. the Model group; [e]P<0.0001 vs. the QUE group. Control, the control group; Model, the model group; QUE, the quercetin + PBS group; LDQ, the low-dose quercetin group; MDQ, the medium-dose quercetin group; HDQ, the high-dose quercetin group.

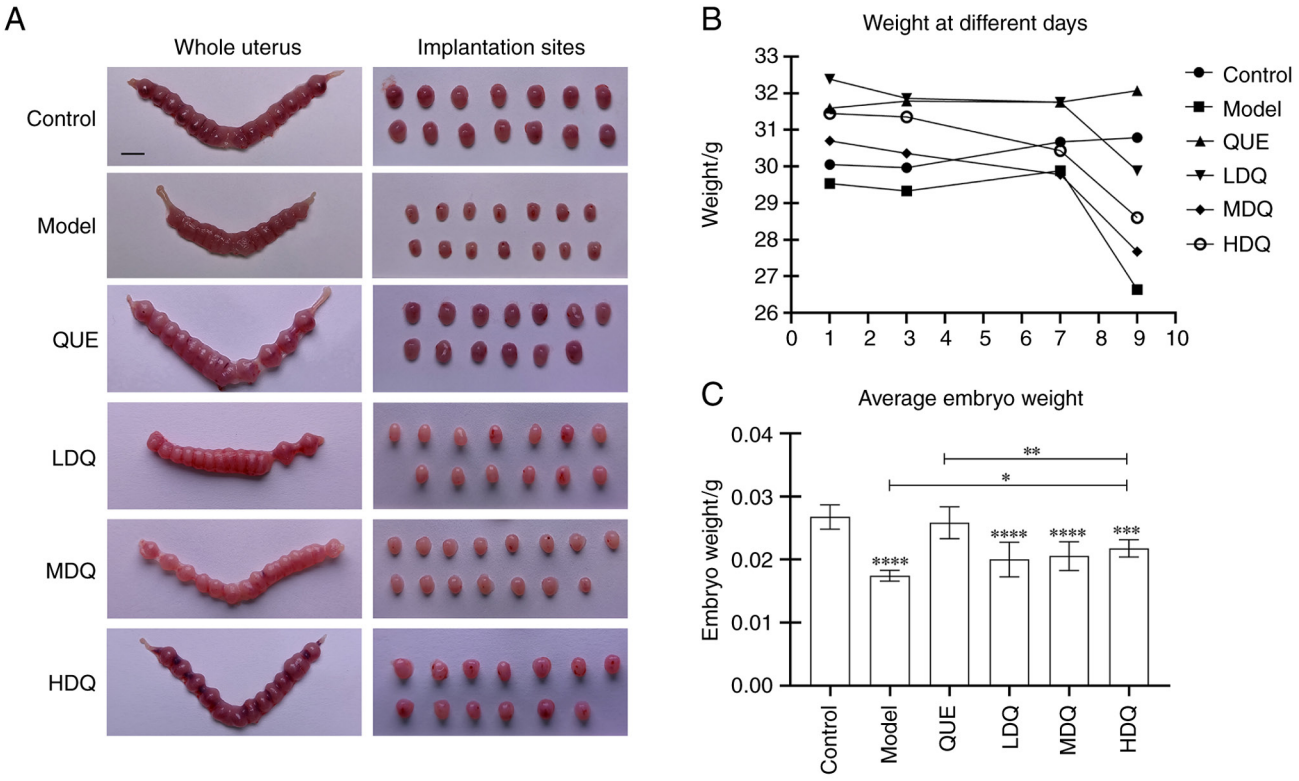

Figure 2. Animal research of quercetin in treating spontaneous abortion. (A) Diagram of uterus and implantation sites of mouse. Scale bar, 5 mm. (B) Line chart of the mouse weight of different pregnant days in the six groups. (C) Bar chart of the average weight of surviving embryos in the six groups. ****P<0.0001, ***P<0.001 vs. the Control group, **P<0.01, *P<0.05. Control, the control group; HDQ, the high-dose quercetin group; LDQ, the low-dose quercetin group; MDQ, the medium-dose quercetin group; Model, the model group; QUE, the quercetin + PBS group.

Effect of quercetin on the rate of embryo loss in SA mice. The embryo loss rate of the model group was 59.39%, while there was no embryo loss in the control group and the quercetin + PBS group, showing a statistically significant difference (P<0.0001). As for the three quercetin groups, the medium-dose quercetin group shared the highest rate of embryo loss (51.69%), accompanied by the rates of 15.73 and 38.64% in the low- and high-dose quercetin groups. Statistically speaking, the high- and medium-dose quercetin groups displayed an increased number of dead embryos and a higher rate of embryo loss in comparison to the low-dose quercetin group (P<0.0001 and P<0.0001, respectively). The low-dose quercetin group exhibited a reduction in both the quantity of dead embryos and the rate of embryo loss. Moreover, the high-dose quercetin group showed a significantly elevated number of dead embryos and a higher rate of embryo loss when compared with the high-dose quercetin + PBS group (P<0.0001). Interestingly, no significant difference was found in the rate of embryo loss between the medium-dose quercetin group and the model group (P=0.159; Table III).

Effect of quercetin on litter size and birthweight of the pups. To confirm that quercetin does prevent SA, the results of another set of experiments showed that all pregnant mice in each group

Table II. Total, surviving and dead embryos.

Group	n	Total embryos/n	Surviving embryos/n *M* (upper, lower quartile)	Dead embryos/n *M (P25, P75)*
Control	13	13.54±1.45	14.00 (13.00,14.50)	0.00 (0.00,0.00)[b]
Model	13	12.69±1.32	0.00 (0.00,13.00)	11.00 (0.00,13.00)
QUE	13	13.77±1.30	14.00 (12.50,15.00)[b]	0.00 (0.00,0.00)[b]
LDQ	13	13.69±1.23	13.00 (12.50,14.00)	0.00 (0.00,0.00)
MDQ	13	13.69±1.25	0.00 (0.00,14.50)	12.00 (0.00,13.50)[a,c]
HDQ	13	13.54±1.27	12.00 (0.00,14.00)	0.00 (0.00,13.00)

The F-value of total embryos was 1.259 (P=0.291); the H-values of surviving and dead embryos were 14.117 (P=0.015) and 21.545 (P=0.001), respectively. [a]P<0.05 vs. the Control group; [b]P<0.05 vs. the Model group; [c]P<0.05 vs. the QUE group. Control, the control group; Model, the model group; QUE, the quercetin + PBS group; LDQ, the low-dose quercetin group; MDQ, the medium-dose quercetin group; HDQ, the high-dose quercetin group.

could produce offspring, and there was no significant difference in litter size and birthweight of the pups (Table SIII and Fig. S1).

Effect of LPS on the morphology of implantation site tissue. Fig. S2 shows the representative hematoxylin and eosin staining for the implantation site tissue sections of the control and model group, including the embryo. The findings showed that the embryos of normal mice were structurally intact with regularly and tightly arranged cells, while those of aborted mice were characterized by irregularly and loosely arranged cells with the significant infiltration of inflammatory cells and red blood cells, the structures of which were not as intact as the former.

Screening potential targets of quercetin and SA. A total of 291 candidate quercetin targets were obtained using PharmMapper platform and 53, 1,498 and 197 SA-related targets were estimated in OMIM, GeneCards and DisGeNet databases respectively by using the keywords 'spontaneous abortion' and 'pregnancy loss'. Finally, a total of 1,622 disease-associated targets were determined after removing 126 duplicates.

The Venn online tool was applied to visualize the Venn diagram. A total of 80 targets of quercetin intersection with SA were obtained.

PPI network construction and core target screening. The PPI network of the 80 intersecting targets was generated by the STRING database (Fig. 3A). After removing the nodes that were discrete from the main network, 79 targets and 579 edges were finally presented. The TSV format result was downloaded and input into Cytoscape (3.7.1) for visualization. The degree value of each target was obtained using the cytoNCA plug-in and the average degree value of 14.658 was calculated using Excel software. Consequently, 34 core targets that had higher degree value than the average value were screened out (Fig. 3B and Table SIV). Among them, the top three targets in terms of degree value were AKT1, albumin (ALB) and caspase-3 (CASP3).

Enrichment analysis of GO and KEGG pathway. GO and KEGG enrichment analysis were performed by importing the 80 intersecting targets into the Metascape database.

The GO enrichment analysis of quercetin treating SA yielded a total of 1,062 terms (P<0.01), including 923 entries for BP, 44 entries for CC and 95 entries for MF. BP was mainly involved in 'hormone responses', 'regulation of defense responses', 'inflammatory responses', 'cell migration' and 'phosphatidylinositol 3-kinase signaling'. CC was mainly involved in 'outer plasma membrane', 'extracellular matrix', 'serine peptidase complex' and 'serine endopeptidase complex'. MF was mainly comprised of 'endopeptidase activity', 'serine hydrolase activity', 'serine peptidase activity', 'serine endopeptidase activity', 'peptidase activity', 'organic acid binding', 'nuclear receptor activity' and 'carboxylic acid binding'. The Fig. 3C shows the top six entries in BP, CC and MF sorted from the smallest to largest P-value.

A total of 130 KEGG pathways were responsible for in the treatment of SA (P<0.01). To unravel the basic molecular mechanisms leading to quercetin in the treatment of SA, the top 20 pathways were selected in order of P-value from smallest to largest. As shown in Fig. 3D, quercetin was not only related to cancer and diabetes, but also associated with signaling pathways of PI3K-Akt, Ras, Rap1, MAPK, TNF, VEGF and C-type lectin receptor.

Effect of quercetin on the relative mRNA expression of AKT1, ESR1, JAK2, MAPK1, MAPK3, PGR, PI3K and SGK1. The relative mRNA expressions of AKT1, MAPK1, PGR and SGK1 in the model group were lower than those in the control group (P<0.0001, P=0.012, P=0.0002 and P=0.001, respectively), and the expressions of the same targets in the quercetin groups were higher than those in the model group. But the expression changes of ESR1 and MAPK3 showed a reverse trend: the expressions in the model group were higher than those in the control group (P=0.013 and P=0.0004, respectively), while the ones of quercetin groups were lower than that in the model group. There was no statistical difference between the expressions of JAK2 in the model and the control group, as well as PI3K. LD-QUE group shared the highest expression of JAK2 among the six groups. Data and pictures are shown in Table SV and Fig. S3.

Table III. Effect of quercetin on the rate of embryo loss in SA mice.

Group	n	Total embryos/n	Surviving embryos/n	Dead embryos/n	Embryo loss rate /%
Control	13	176	176	0	0.00
Model	13	165	67	98	59.39
QUE	13	179	179	0	0.00
LDQ	13	178	150	28	15.73[a]
MDQ	13	178	86	92	51.69[c,d]
HDQ	13	176	108	68	38.64[b,d]

The chi-square value between the six groups was 296.438 (P<0.0001). [a]P<0.0001, [b]P<0.001 and [c]P>0.008 vs. model group; [d]P<0.0001 vs. the LDQ group. QUE, the quercetin + PBS group; LDQ, the low-dose quercetin group; MDQ, the medium-dose quercetin group; HDQ, the high-dose quercetin group.

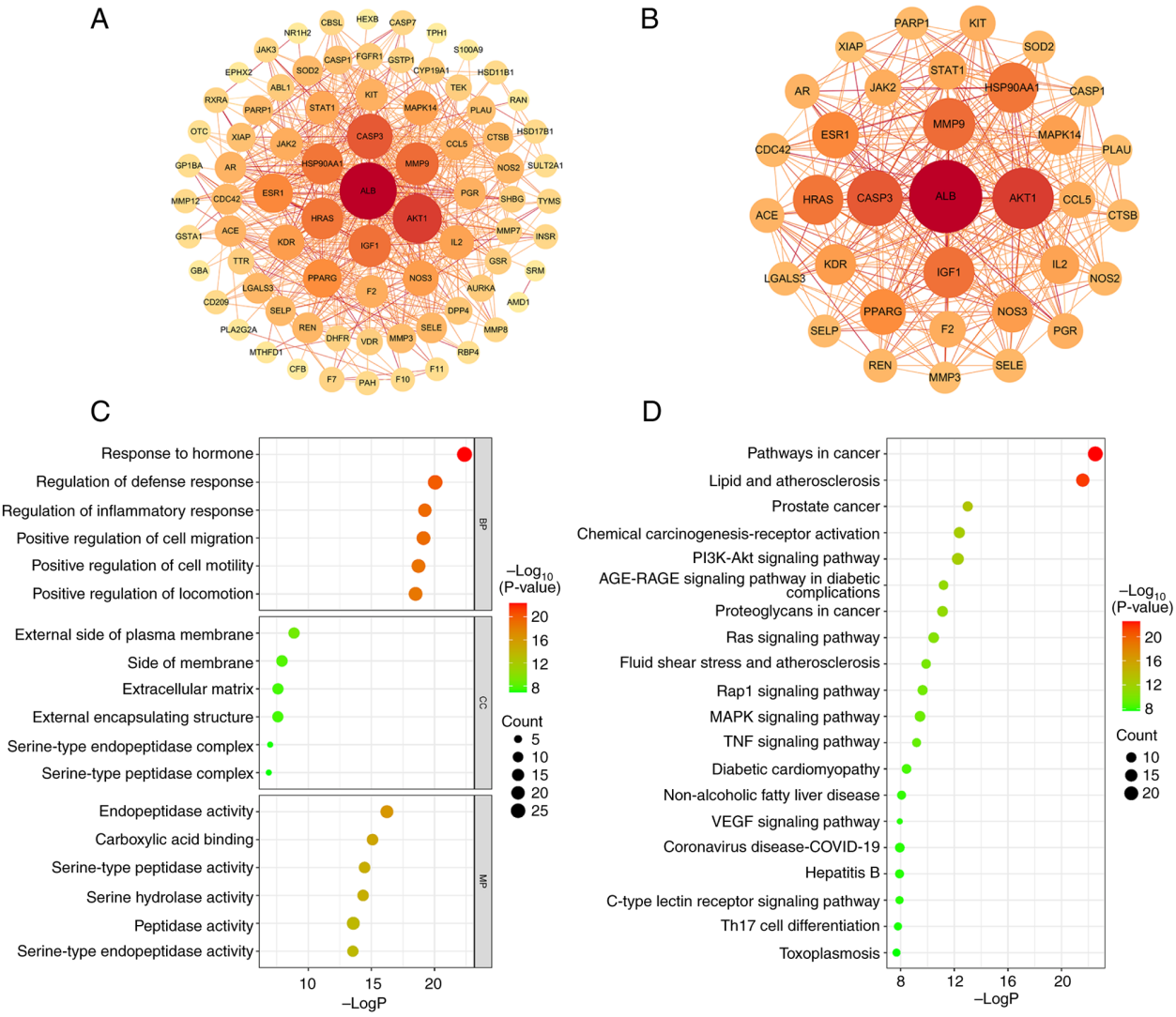

Figure 3. Network pharmacology analysis of quercetin and spontaneous abortion. (A) PPI network. (B) Core network: the larger the node, the darker the color and the more important is the target. (C) Top GO enriched terms of quercetin in treating spontaneous abortion. (D) Top KEGG enriched pathways of quercetin in treating spontaneous abortion. PPI, protein-protein interaction. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Discussion

As the most common complication of early pregnancy, the etiology of SA is complex, with maternal immunologic abnormalities being one of the important etiologic factors (1). A normal pregnancy shares similarities with a successful allogeneic hemizygous transplantation, hinging on the intricate equilibrium between innate and adaptive immune responses at

the maternal-fetal interface, that is, the balance of maternal-fetal immune tolerance. Disruption in the functioning of cellular components within the maternal-fetal interface, coupled with the excessive production of inflammatory cytokines, can lead to maternal-fetal rejection, thereby giving rise to miscarriage and other unfavorable pregnancy outcomes (40). Pregnancy can be divided into three major stages: Implantation, development and growth and delivery (41). Inflammation is present during implantation, but excessive inflammation leads to an imbalance in maternal-fetal immune tolerance, so a successful pregnancy depends on the timely elimination of inflammation and the establishment of a balance of maternal-fetal immune tolerance (42).

LPS is derived from gram-negative bacteria and is a potent initiator of inflammation. The model used in the current study was the LPS-induced SA mouse model. LPS induces macrophage polarization towards M1 type (43), increasing the secretion of inflammatory cytokines such as IL-1β, IL-6 and TNF-α (44), which leads to an imbalance in the local immune microenvironment in the uterus, presenting a pro-inflammatory microenvironment and leading to embryo loss. The results of the present study showed that following LPS injection through the tail vein on day 7 of gestation, all groups of pregnant mice displayed varying degrees of poor condition including unresponsiveness and rough hair, diarrhea and weight loss, with or without vaginal bleeding and discharge of gestational material. The embryo loss rate in the model group was 59.39%, markedly higher than that of the control group ($P<0.001$), validating the effectiveness of the LPS-induced SA mouse modeling.

The present study showed that quercetin could lessen the rate of embryo loss and elevate the average weight of embryos to a certain extent. Quercetin may play a role in promoting the expulsion or resorption of dead embryos after miscarriage and promote the uterus to return to the pre-pregnancy state. Quercetin is a natural flavonoid and is the main active ingredient in the herbs that in traditional Chinese medicine (TCM) are used to 'tonify' kidneys and 'stabilize' the fetus, such as *Cuscuta* and *Herba Taxilli* (45-47) that are the main components of the basic formula for the Shoutai Pill (48). *Cuscuta* and *Herba Taxilli* are commonly used in TCM in treating and preventing miscarriage (48). Studies have revealed that Shoutai Pill regulates the expression levels of cytokines such as IL-2, IL-4, IL-6, IL-10 and interferon-γ (IFN-γ), maintains the balance of Th1/Th2 cytokines and improves endometrial tolerance to promote embryo implantation (49-51). It also reduces the expression of Th17 in the decidual tissue (52), thus maintaining normal pregnancy. It was previously found that *Cuscuta* and *Herba Taxilli* serve as what TCM terms sovereign drug and assistant drug, respectively, in SA treatment (53,54). Relevant studies have confirmed that quercetin decreases expression of IL-1β (55), IL-6 (56) and TNF-α (57) to maintain the balance of local immune microenvironment and therefore to contribute a favorable environment for embryo growth and development. Liu *et al* (58) investigated the mechanism of action of quercetin by establishing a PM2.5-exposed pregnant mouse model and found that quercetin could prevent miscarriage by inhibiting the expression of cytokines such as IL-6 and IL-8 and upregulating the level of heme oxygenase-1 in peripheral blood. Quercetin has a strong antioxidant capacity and some animal experimental findings have illustrated that quercetin can hinder the expression of TNF-α and IL-6 in the placenta of LPS-treated mice and rescue the abnormal oxidative stress, inflammatory response and angiogenic factor imbalance at the placental interface (26). Moreover, Cao *et al* (59) found that quercetin counteracted hyperglycemia-induced nitrosative stress and oxidative stress, decreased the levels of oxidative stress markers and increased the expression of superoxide dismutase 1 and 2 and ultimately reduced the rate of embryonic malformations in diabetic pregnant mice. In addition, *in vitro* experiments have demonstrated that quercetin at appropriate concentrations can improve biological function, mitochondrial membrane potential and morphology of trophoblast cells under hypoxic conditions (60). Decidual natural killer (NK) cells are the primary immune cell population at the maternal-fetal interface, with low cytotoxicity (61), which enhance decidual cell invasion as well as spiral artery remodeling through the secretion of matrix metalloproteinases, vascular endothelial growth factor and interleukins, regulating maternal-fetal immune tolerance and placental developmental capacity (62-65). Evidence has shown that cytokines such as IL-15, IL-18 and TGF-β can convert highly cytotoxic peripheral blood NK cells into low cytotoxic decidual NK cells (66,67). TGF-β, among the above three cytokines, is abundant in decidual microenvironment, which is the key to drive the transformation of placental extravillous trophoblast cells into decidual extravillous trophoblast cells according to bioinformatic analysis (68). Hu *et al* (69) found that quercetin is capable to up-regulate TGF-β expression, as well as to promote the polarization of M2 macrophages, which is beneficial to the maintenance of normal pregnancy.

Network pharmacological results uncovered that the effects of quercetin on SA were mainly related to hormone response and the modulation of defense response, inflammatory response, cell migration and motility, involving signaling pathways such as VEGF, MAPK and PI3K-Akt. Among them, the PI3K-Akt signaling pathway is importantly involved in cell proliferation, apoptosis and autophagy (70-72). It has also been suggested that the establishment and retention of a normal pregnancy tightly associate with PI3K-Akt pathway (73). Song *et al* (74) discovered that quercetin directly bonds to PI3K, which led to the conclusion that PI3K was a molecular target of quercetin. VEGF is a key angiogenic factor in the process of metamorphosis (75) and the lack of bone morphogenetic protein receptor type 2 in mouse uterine decidualization during the early stage of placental development inhibits VEGF signaling, which affects the process of decidualization and leads to the abnormal development of embryonic blood vessels (76). Relevant animal experiments have demonstrated that a high quercetin diet increases VEGF-A and VEGF-R2 levels in mice, enhances angiogenesis and contributes to the reduction of systemic inflammation and insulin resistance (77). The MAPK signaling pathway is a classical signaling pathway in trophoblast cells and abnormalities in this pathway impact the physiological functions of trophoblast cells, leading to adverse pregnancy outcomes (78). Quercetin disrupts skeletal fibrillar actin of macrophages and inhibits LPS-induced macrophage adhesion and migration by downregulating focal adhesion kinase–paxillin and modulating the MAPK pathway (79). In addition, it has been proposed that the process of embryonic implantation is similar to tumor invasion (80). For instance, the maternal-fetal interface microenvironment in the first trimester of pregnancy is similar to

the tumor microenvironment in hypoxic, acidic and immune features (81). While several relevant signaling pathways identified in the present study have been demonstrated to modulate tumorigenesis and tumor development (82-87).

In summary, quercetin decreased the embryo loss rate, increases the mean weight of surviving embryos and promotes the resorption or expulsion of dead embryos in a mouse model of SA. Network pharmacological studies revealed that quercetin can treat SA by regulating multiple signaling pathways such as PI3K-Akt, VEGF, MAPK and core targets such as AKT1, ALB and CASP3. RT-qPCR showed that quercetin could upregulate AKT1, MAPK1, PGR and SGK1, and downregulate ESR1 and MAPK3, some of which are closely related to SA. Currently, the studies of quercetin on reproductive medicine are scattered. Although the present study has elucidated the influence of quercetin on embryo loss rate and mean weight of embryos and explored its potential molecular mechanisms based on network pharmacology, the further validation is needed. Our group intends to select Clark classical recurrent abortion model mice to explore and validate the specific mechanisms of quercetin in SA treatment from the cellular and molecular perspective, which can provide solid evidence to sustain the application of quercetin in the field of reproductive medicine.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YL, SW and YT conceived and designed the study. SW, QZ, YT and LM conducted the animal experiments. ZF, HL and XZ performed the network pharmacology analysis. SW analyzed the experimental data. SW drafted the manuscript, which was reviewed and edited by YL. YL and SW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (ethics approval no. IACUC-20220919-25) by the Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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