Effect of Angelica Sinensis extract on the angiogenesis of preovulatory follicles (F1−F3) in late-phase laying hens

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ABSTRACT In order to form follicles and ovulate normally, there must be abundant blood vessels. *Angelica sinensis* (Oliv.) Diels (AS), as a traditional Chinese medicinal herb, has the effects of tonifying the blood and activating the blood circulation. However, the effect of AS on angiogenesis in hen-follicles remains to be discovered. In this study, we identified vascular richness, granulosa layer thickness, expression of platelet endothelial cell adhesion molecule-1 (**CD31**) and the content of vascular endothelial growth factor A (**VEGFA**) in granulosa layers to elucidate the effect of AS extract on angiogenesis in preovulatory follicles (F1−F3) of late-phase laying hens (75 wk). Based on network pharmacology, we predicted beta-sitosterol, ferulic acid, and caffeic acid as the main active components of AS, and hypoxia-inducible factor-1α (**HIF1α**), vascular endothelial growth factor receptor 2 (**VEGFR2**) as hub targets of AS in angiogenesis. The intersection targets were enriched by Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways, and the hub targets were verified by immunofluorescence and western blot. Molecular docking of active components with hub targets was performed and verified in vitro. The results showed that AS extract promoted angiogenesis in preovulatory follicles and increased granulosa cell layer thickness, CD31 expression and content of VEGFA. Experiments in vitro and in vivo demonstrated that AS extract promoted the expression of HIF1α and VEGFA, up-regulated the phosphorylation levels of VEGFR2. These results further demonstrated the reliability of molecular docking and network pharmacology findings. In summary, AS extract can promote angiogenesis in the preovulatory follicles in late-phase laying hens.

Key words: Angelica Sinensis extract, preovulatory follicle, angiogenesis, granulosa cell, follicle microvascular endothelial cell

2023 Poultry Science 102:102415
https://doi.org/10.1016/j.psj.2022.102415

INTRODUCTION

Egg production performance in hens mainly depends on the vascular development of follicles. In hens, oocytes do not endogenously synthesize vitellin, which is mostly transported into follicles via the vascular system in other organogenesis (Wei et al., 2016; Lin et al., 2019). Similarly, the supply of macronutrients and hormones is necessary for follicle development (Robinson et al., 2009). Most of the nutrients in follicles are obtained from the stroma through an abundant vascular network, so a separate capillary network is formed on each follicle. It has been reported that selective development and sustained growth of follicles depends on the development of the vascular network in follicles (Zeleznik et al., 1981; Guzmán et al., 2021). This process of rapid accumulation of nutrients in preovulatory follicles within a few days urgently requires the generation of large numbers of blood vessels (Kim et al., 2016). Thus, angiogenesis is a critical regulator of preovulatory follicle development (Devesa and Caicedo, 2019).

AS, a traditional Chinese medicinal herb, has the effect of tonifying blood and activating blood circulation. Due to the properties and efficacy of AS, it has been widely used as a functional food or dietary supplement (Wei et al., 2016). The effect of AS in promoting angiogenesis has been demonstrated in vitro and in vivo. AS extract has been reported to enhance proliferation,

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Received October 24, 2022.

Accepted December 5, 2022.

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migration and lumen formation of follicular microvascular endothelial-like cells (**FMEC**) (Chen et al., 2022). In addition, AS extract has been shown to be able to promote the development of subintestinal capillaries in the zebrafish model by elevating expression of VEGFA (Lam et al., 2008). Modern medical studies have shown that multiple bioactive components in AS extracts such as polysaccharides, volatile oils and organic acids have become the main research objects (Chen et al., 2021). Angelica polysaccharide has strong hematopoietic biological activity, and can enhance immunity, anti-oxidation, anti-tumor and protect the liver (Cao et al., 2018; He et al., 2022). Studies have shown that angelica polysaccharide can up-regulate expression of eNOS, NO, VEGF, Akt and down-regulate expression of LDH at the gene level (Zhang et al., 2021). Ferulic acid has been shown to attenuate oxidative stress by up-regulating GSH, NADPH, Nrf2, NQO1, HO-1 and reducing ROS (Ma et al., 2010). In addition, ferulic acid can improve endothelial cell proliferation and enhance angiogenesis (Lin et al., 2010). Caffeic acid restores antioxidant levels and promotes hematopoiesis (Chen et al., 2017), which inhibits monocyte adhesion by up-regulating the Nrf2/EpRE pathway (Zhang, Liu, Duan, Li, Peng and Wu, 2021). Beta-sitosterol promotes angiogenesis in ischemic/reperfusion injured gerbil brain by regulating expression of vWF, VEGFA, and Flk-1 (Choi et al., 2002).

Oocytes in the center of preovulatory follicles in hens are surrounded by the theca cell in the outer layer and the granulosa cell in the inner layer. The vascularized theca layer is separated from the granulosa cells of the inner layer by the basal lamina (Zhu et al., 2019). VEGFA is the most typical proangiogenic factor, which induces endothelial cell survival, proliferation, migration, invasion, lumen formation, and vascular maturation (Cébe-Suarez et al., 2006). VEGFA, both paracrine and autocrine, is crucial for the follicular development (Trau et al., 2016). VEGFA levels in follicle granulosa cells (**GC**s) are significantly increased, enhancing angiogenesis within the theca layer and promoting rapid growth of preovulatory follicles (Kim et al., 2016). VEGFA is mainly located in the avascular granulosa layers (Hunter et al., 2004; Kim et al., 2017), that stimulates the growth of blood vessels into the basal lamina and supplies GCs with oxygen, nutrients, and hormones may exist (Fraser and Duncan, 2005). VEGFR2 is the most important receptor involved in VEGF-induced angiogenesis, which regulates migration, proliferation, differentiation, and survival of endothelial cells as well as alters permeability and expansion of vascular. VEGFA-mediated proliferation of endothelial cell depends on activation of multiple pathways downstream of VEGFR2 (Cébe-Suarez et al., 2006). VEGFR2 is mainly expressed in the vascular endothelium of the theca layer in follicles of hens. Expression of VEGFR2 was increased in healthy follicles compared to atretic follicles (Kim, Lee and Johnson, 2016). HIF1 is a major driver of angiogenesis, including the oxygen-regulated α subunit and the ubiquitously expressed β subunit. HIF1α accumulates and translocates to the nucleus where it forms dimers with HIF1β and binds to the VEGF promoter to induce angiogenesis when hydroxylation of HIF1 is prevented under chronic hypoxia (Jiang et al., 1997; Wiesener et al., 1998; Ma et al., 2018). HIF1α has been demonstrated to have a crucial role in the formation of follicles and ovulation, as well as it is a key regulator of ovarian gene expression (Alam et al., 2009; Rico et al., 2014).

In this study, we investigated the effect of AS extract on angiogenesis in preovulatory follicles (F1−F3) of late-phase laying hens and explored the targets related to angiogenesis in AS extract based on a network pharmacology approach. Then we validated the hub targets by immunofluorescence (**IF**) and Western blot techniques. Finally, molecular docking and in vitro cell experiments verified the hub targets of AS extract in promoting angiogenesis. This study provides a theoretical basis for the development of feed supplements in hens from Angelica sinensis extract. This study provides a theoretical basis for AS extract as feed supplements in late-phase laying hens.

MATERIALS AND METHODS

Ethics Statement

Animal experiments in this study was performed according to the Regulations on Administration of Animal Experiments (Ministry of Science and Technology of China, Approval No.2006-398) and approved by the Animal Care and Use Committee of Northeast Agricultural University (Harbin, Heilongjiang, China).

Preparation of AS Extract

In this experiment, AS was purchased from Minxian Hetai Chinese Medicinal Materials Company (Gansu, China), and identified by professors at the Institute of Traditional Chinese Veterinary Medicine, Northeast Agricultural University. AS was stored at 4°C. Dry AS was given a 2-hour soak in water that was ten times its weight, which was heated to boiling with high fire and maintained for 15 min, and then changed to low fire heating for 45 min. After filtration and centrifugation, the extract was concentrated to a crude drug concentration of 1 g/mL and stored at 4°C for in vivo experiments. For cell experiments, AS extract was lyophilized at −50°C with an extraction yield of 22.3% and stored in an −80°C freezer.

Laboratory Animal Feeding and Experimental Design

A total of 60 healthy 70-wk-old Hy-line brown laying hens and 15 healthy 30-wk-old Hy-line brown laying hens were purchased from Sunshine Chicken Farm (Harbin, China); These hens were maintained in individual cages (400 mm × 350 mm × 320 mm). Cages were

randomly placed in a ventilated room with a temperature of 25 ± 2°C, a humidity of 65 ± 10% and 16 h illumination. Diets and water were offered daily for ad libitum intake. After the hens were adapted to the environment for one week, the 71 wk laying hens were randomly divided into 4 groups: control group, low-dose of AS extract treatment group, medium-dose of AS extract treatment group and high-dose of AS extract treatment group. The 31 wk laying hens were taken as the positive control group. All groups were provided with a normal diet daily during a consecutive 4 weeks experimental period. Meanwhile, low-dose of AS extract treatment group was given AS extract at a dose of 1 g/chicken, medium-dose of AS extract treatment group was given AS extract at a dose of 2 g/chicken and high-dose of AS extract treatment group was given AS extract at a dose of 4 g/chicken. At 35 and 75 wk of age, all hens were euthanized at 3 h after laying. Following that, the theca layers and granulosa layers of each group's F1, F2, and F3 were collected and stored at −80°C.

Hematoxylin-Eosin Staining

Preovulatory follicles F1, F2, and F3 were collected, and then fixed with 4% paraformaldehyde. These follicles were dehydrated, embedded in paraffin, sectioned, and stained after deparaffinization. Measure the thickness of granulosa layer and theca layer, and observe the integrity of granulosa layer.

Determination of VEGFA by Enzyme-Linked ImmunoSorbent Assay (ELISA)

Granulosa layers of F1, F2, and F3 from each group were weighed, and were added with 9 times the mass of phosphate buffered saline (PBS, PH 7.2−7.4) for adequate homogenization. Centrifuged at 3,000 rpm/min for 20 min at 4°C and carefully collected the supernatant for testing. The ELISA kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing Jiancheng, China). VEGFA concentrations were detected using the method that complies with the manufacturer's instructions.

Network Pharmacology

Collection and Screening of AS Compounds and Prediction of Compound-Related Targets Major active chemical ingredients of AS were obtained from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php), TCMID database (http://bidd.group/TCMID/) and previous studies (Chen et al., 2022). The screening criteria of the TCMSP database were set to oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥0.18. We obtained the selected components' structural information through PubChem (https://pubchem.ncbi.nlm.nih.gov/). In addition, the targets of active compounds were predicted by SwissTargetPrediction (http://swisstargetprediction.ch/) and TCMID. A drug-component-target database was established and a network diagram was constructed by Cytoscape 3.9.1 software.

Data mining and screening of angiogenesis targets Disease or functional gene targets were queried through the GeneCards database (https://www.genecards.org/) with the keyword "Angiogenesis" (Wu et al., 2021). A total of 5038 related gene targets were obtained, and 631 related gene targets were selected using the correlation coefficient "Relevance score ≥ 1.810106397".

GO enrichment analysis and KEGG pathway enrichment analysis of intersection targets The intersection of the predicted target and the obtained angiogenesis-related gene target was taken to obtain the intersection gene target. Through the David database (https://david.ncifcrf.gov/), GO functional (Cell Component, Molecular Function and Biological Process) enrichment analysis and KEGG pathway enrichment analysis were performed on the collected intersection targets ($P < 0.05$ indicated significant differences).

Hub Targets Screening by Topology Analysis of PPI Networks We entered the intersection target into the STRING database (https://cn.string-db.org/), after which protein-protein interactions (**PPI**s) were predicted (confidence scores > 0.9). After the above results were imported into Cytoscape 3.9.1 software for visualization, the Centiscape plug-in was used to screen the important hub target network. The hub target was selected by combining the hub target network with the angiogenesis correlation coefficient "Relevance score" in GeneCards.

Molecular Docking

The hub target proteins of angiogenesis in the PPI network were selected for molecular docking with the active components of AS. Appropriate target proteins were selected in the Uniprot database (https://www.uniprot.org/) and 3D structure files were downloaded in PDB format by linking to the RCSB PDB database (https://www.rcsb.org/). 3D structure files in SDF format for drug ingredients were downloaded from PubChem database and converted to PDB format files by Open Babel 3.1.1. Simulated molecular docking of the hub target protein to the active component of AS was performed using the software AutoDockTools-1.5.6 and AutoDock Vina (Trott and Olson, 2010). Finally, 3D structures were presented by PyMoL 2.3.0.

Cell Culture

According to the previous method, follicle granulosa cells (**GC**s) and follicular microvascular endothelial-like cells (**FMEC**s) were isolated from preovulatory follicles of 30-wk-old hens (Chen et al., 2022). Hens were euthanized and preovulatory follicles (F1−F3) were removed. The avascular area on the back of the yolk was quickly cut using a scalpel after fixation of the follicle pedicle with hemostatic forceps. Gravity

expelled the granulosa layer and yolk together, and then washed the granulosa and theca layers with PBS. Granulosa layers were digested in collagenase II (Solarbio, Beijing, China; C8150) for 8 min at 37°C in a water bath and digestion was stopped with an equal amount of M199 medium (Hyclone, Beijing, China; SH30253.01B) containing 10% FBS. Primary GCs were obtained by filtration with 70 μm nylon mesh and cultured in M199 medium containing 10% FBS and 1% penicillin streptomycin. Theca layers were digested in a water bath for 60 min at 37°C in collagenase I (Solarbio, Beijing, China; C8140), and digestion was stopped using DMEM medium (Hyclone, Beijing, China; SH30022.01B) containing 10% FBS. Primary FMECs were obtained by filtration with 70 μm nylon mesh and centrifuged. DMEM medium was used to suspend the cell pellet and attached to the centrifuged Percoll (precooled 35% Percoll was centrifuged at 4°C for 15 min at 30,000 g 4°C in advance). After centrifugation at 400 g for 15 min, the solution was divided into three layers and the middle layer was the desired FMECs pellet. Pellets were resuspended in DMEM medium with 10% FBS, 50 μg/mL ECGs (Sigma-Aldrich, St. Louis) and 1% penicillin streptomycin, and were cultured at 37°C, 5% CO2. Primary GCs were cultured for 3 days before drug-addition. Cells of 3 to 6 passages from the primary FMEC were used for subsequent experiments. GCs and FMECs were treated with 100 and 200 μg/L AS extracts for 48 h.

Immunofluorescence

The paraffin sections of preovulatory follicles tissue were dewaxed, and the slides were put into the Improved Citrate Antigen Retrieval Solution (Beyotime, Shanghai, China; P0083). The slides were boiled in a microwave oven at high heat and then turned off for 8 min, and heated at low heat for 7 min. GC and FMEC were fixed with 4% paraformaldehyde for subsequent operation. These tissue sections and cells were permeabilized with 0.3% triton X-100 (Beyotime, Shanghai, China; P0096). Following blocking with homologous serum, tissue sections were incubated with primary antibodies against CD31 (1:1000, ABclonal, Wuhan, China; A0378), VEGFA (1:200, ABclonal, Wuhan, China; A12303), VEGFR2 (1:500, ABclonal, Wuhan, China; A11127), P-VEGFR2 (1:500, ABclonal, Wuhan, China; AP0382), HIF1α (1:1000, ABclonal, Wuhan, China; A0378). The epitope region used for creating the antibodies used are 90% homologous between rabbit and chicken according to protein blast. It was then coupled with FITC (green) (1:200, Bioss, Beijing, China; bs-0295G-FITC), Alexa Fluor594 (red) (1:200, Bioss, Beijing, China; bs-0295G-AF594) secondary antibodies, nuclear staining with DAPI (blue) (Biosharp, Hefei, China; BL105A), and mounting with Antifade Mounting Medium (Biosharp, Hefei, China; BL739A). Images were obtained using a fluorescence microscope (Nikon, Tokyo, Japan).

Western Blot

Total protein was extracted from theca layer and granulosa layer using RIPA Lysis Buffer (Beyotime, Shanghai, China; P0013B) and Protease Inhibitor Cocktail (AbMole, Shanghai, China; M5293), Phosphatase Inhibitor Cocktail (AbMole, Shanghai, China; M7528). Total protein content was determined using a BCA kit (Meilunbio, Dalian, China; MA0082). Proteins were transferred to Polyvinylidene fluoride membranes following migration through SDS-PAGE gels of gradient concentrations. Following blocking with skimmed milk powder, membranes were incubated with primary antibodies to protein to detect CD31 (1:1000, ABclonal, Wuhan, China; A0378), VEGFA (1:200, ABclonal, Wuhan, China; A12303), VEGFR (1:500, ABclonal, Wuhan, China; A11127), P-VEGFR (1:500, ABclonal, Wuhan, China; AP0382), HIF1α (1:1000, ABclonal, Wuhan, China; A0378), β-actin (1:2000, ABclonal, Wuhan, China; AC038). Secondary antibodies were combined with primary antibodies and protein bands were visualized with a chemiluminescence system (Tanon, Shanghai, China) and quantified with Image J software.

Statistical Analysis

Results are presented as the mean ± S.E.M and all statistical analyses were performed using GraphPad-Prism 8.0. Statistical significance of data between groups was assessed by one-way ANOVA multiple comparisons and Tukey test. P values < 0.05 were considered statistically significant. All experiments were repeated at least three times.

RESULTS

Effect of AS Extract on Angiogenesis of Preovulatory Follicles (F1−F3) in Late-Phase Laying Hens

In order to investigate the effect of AS extract on angiogenesis of preovulatory follicles in late-phase laying hens in vivo, we observed the apparent vascular status of follicles in each group. The number of vessels on the surface of preovulatory follicles was lower in the 75 wk group compared with the 35 wk group. After treatment with AS extract, the number of vessels on the surface of preovulatory follicles was significantly increased in the 75 wk + L, 75 wk + M, and 75 wk + H groups compared with the 75 wk group (Figure 1A). We found that the number of follicles larger than 2 mm in diameter was higher in the 75 wk + L, 75 wk + M, and 75 wk + H groups than in the 75 wk group (Figure 1B). Therefore, we used Hematoxylin-Eosin Staining to observe the morphological changes in the granulosa layer of preovulatory follicles (F1−F3). Compared with the 35 wk group, the granulosa layer of preovulatory follicles (F1−F3) was thinner ($P < 0.001$) and loosely arranged in the 75 wk group. However, the granulosa layer (F1−F3) in the

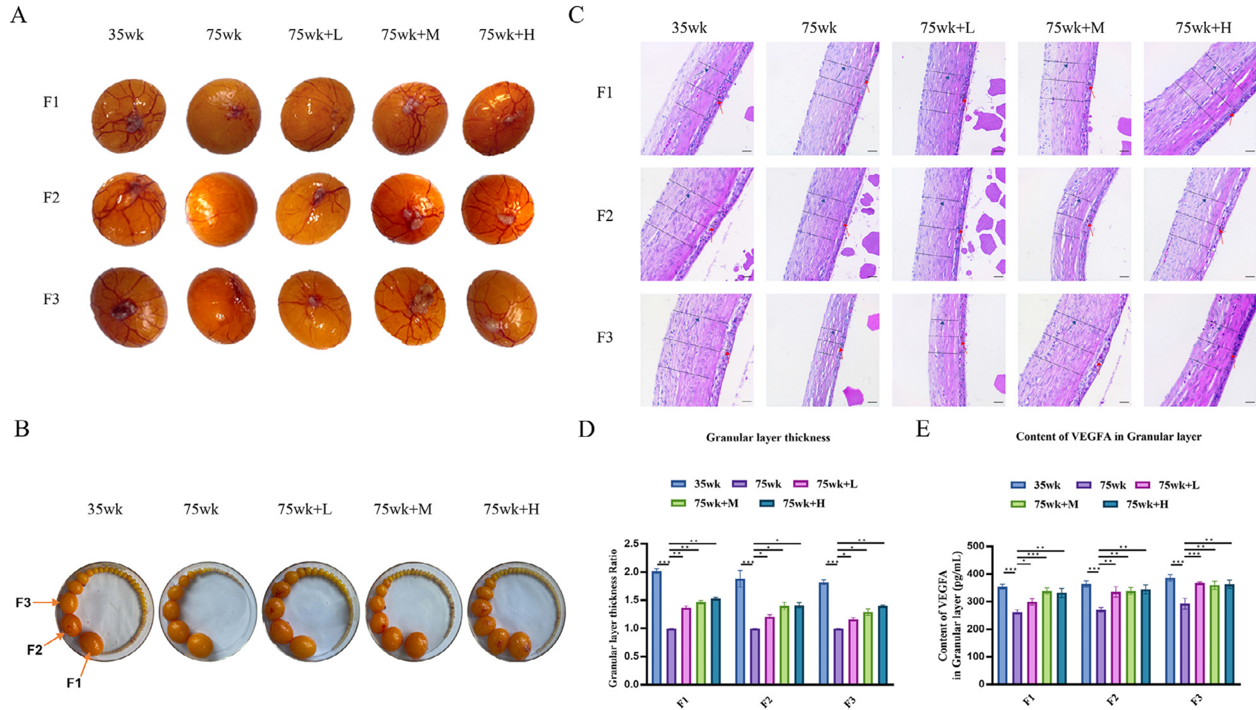

Figure 1. Effects of AS extract on preovulatory follicles appearance, granulosa layer thickness and VEGFA content. (A) Apparent vessels of preovulatory follicles (F1−F3) in each group. (B) The number of follicles with a diameter greater than 2 mm. The 3 preovulatory follicles marked by arrows were F1, F2, and F3 from large to small. (C) Granulosa layer thickness was measured by HE staining of follicles in each group. The red arrows indicated granulosa layer and the black arrows indicated theca layer. The 3 black lines indicated the thickness of the granulosa layer that was measured randomly. Scale bar: 50 μm. (D) Measurement of granulosa layer thickness. (E) VEGFA content in granulosa layer of each group. All experiments were performed in triplicate, and the data are the mean ± S.E.M ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

AS extract treatment group was thickened to varying degrees more than that in the 75 wk group ($P < 0.05$, $P < 0.01$) (Figure 1C, D).

Effect of AS Extract on VEGFA Expression in Granulosa Layer of Preovulatory Follicles (F1−F3)

GCs are critical cells for follicle development and can secrete VEGFA to promote angiogenesis in the theca layer. VEGFA content in the granulosa layer of follicles was detected by ELISA. As shown (Figure 1E), VEGFA content in preovulatory follicles (F1−F3) of the 75 wk group was lower than that in the 35 wk group ($P < 0.01$, $P < 0.001$). VEGFA was significantly increased in the AS extract-treated group compared with the 75-wk group. ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

Effect of AS Extract on CD31 Expression in Theca Layer of Follicles F1 to F3

CD31 is a vascular endothelial marker used to assess angiogenesis. IF showed that CD31 expression in the theca layer of preovulatory follicles (F1−F3) was lower in the 75-wk group than in the 35-wk group ($P < 0.01$, $P < 0.001$). CD31 expression levels increased after AS extract treatment ($P < 0.05$, $P < 0.01$ and $P < 0.001$) (Figure 2A-F). Western blot revealed that CD31 protein expression levels were significantly lower in the 75 wk group than in the 35 wk group ($P < 0.001$). CD31 expression levels increased after AS extract treatment in a dose-dependent manner. ($P < 0.05$, $P < 0.01$ and $P < 0.001$) (Figure 2G−L). This corresponds to the VEGFA results described above.

Exploring the Potential Mechanism of AS Extract Promoting Angiogenesis based on Network Pharmacology

In order to explore the mechanism of AS extract promoting angiogenesis in preovulatory follicles (F1−F3), we used the method of network pharmacology. First, beta-sitosterol, ferulic acid and caffeic acid were the main active components of AS obtained by database and literature mining. The reliability of the compounds was verified by querying the literature. The corresponding 216 targets were predicted in the Swiss Target Prediction database (Figure 3A). Six hundred thirty-one related proteins were queried and filtered in GeneCards, resulting in 54 targets common to predicted targets (Figure 3B, E). We performed enrichment analysis of these targets. KEGG enrichment analysis showed that these targets were associated with VEGF signaling pathway, HIF1 signaling pathway and endocrine resistance pathway (Figure 3C). GO enrichment analysis showed that these targets were mainly present in the plasma membrane, macromolecular complex, nucleoplasm and other regions of the cell and were involved in biological

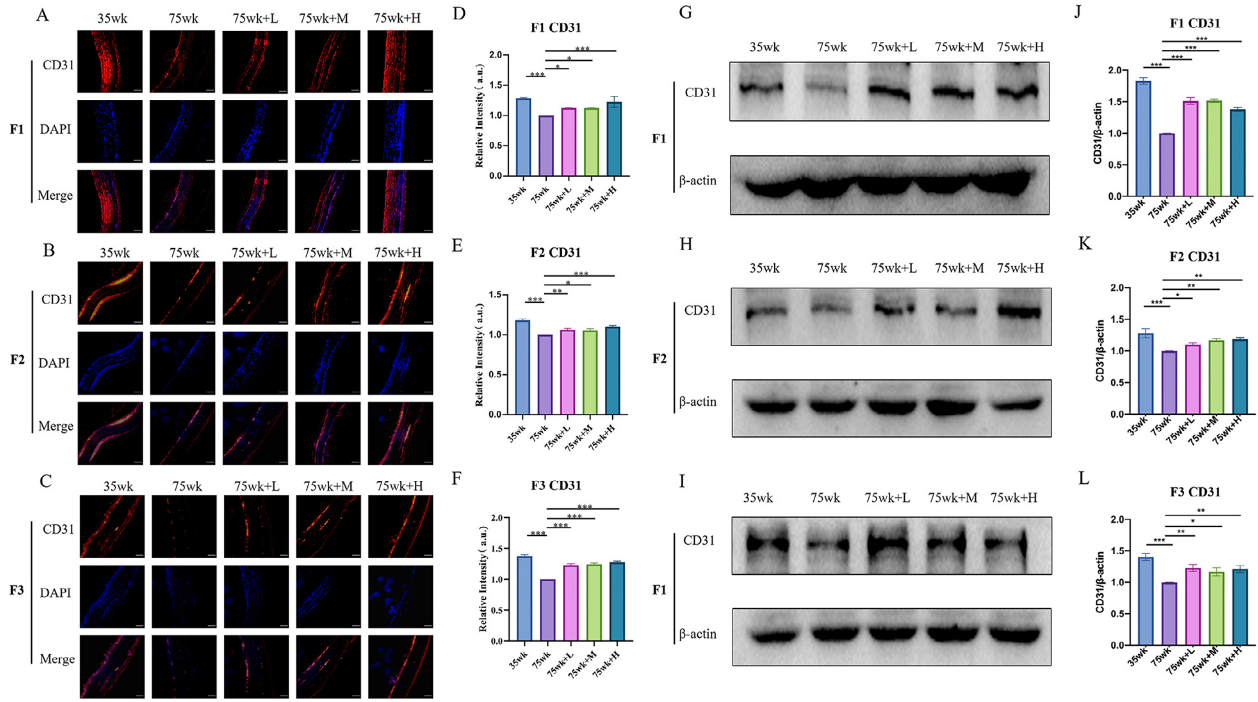

Figure 2. Effect of AS extract on the expression of CD31 in the theca layers. (A−C) Immunofluorescence (IF) showed the expression of CD31 in preovulatory follicles (F1−F3). Scale bar: 100 μm. (D−F) The relative fluorescence intensity of CD31 in preovulatory follicles (F1−F3) was quantified. (G−L) Western blot detected the expression of CD31 in preovulatory follicles (F1−F3). All experiments were performed in triplicate, and the data are the mean ± S.E.M ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

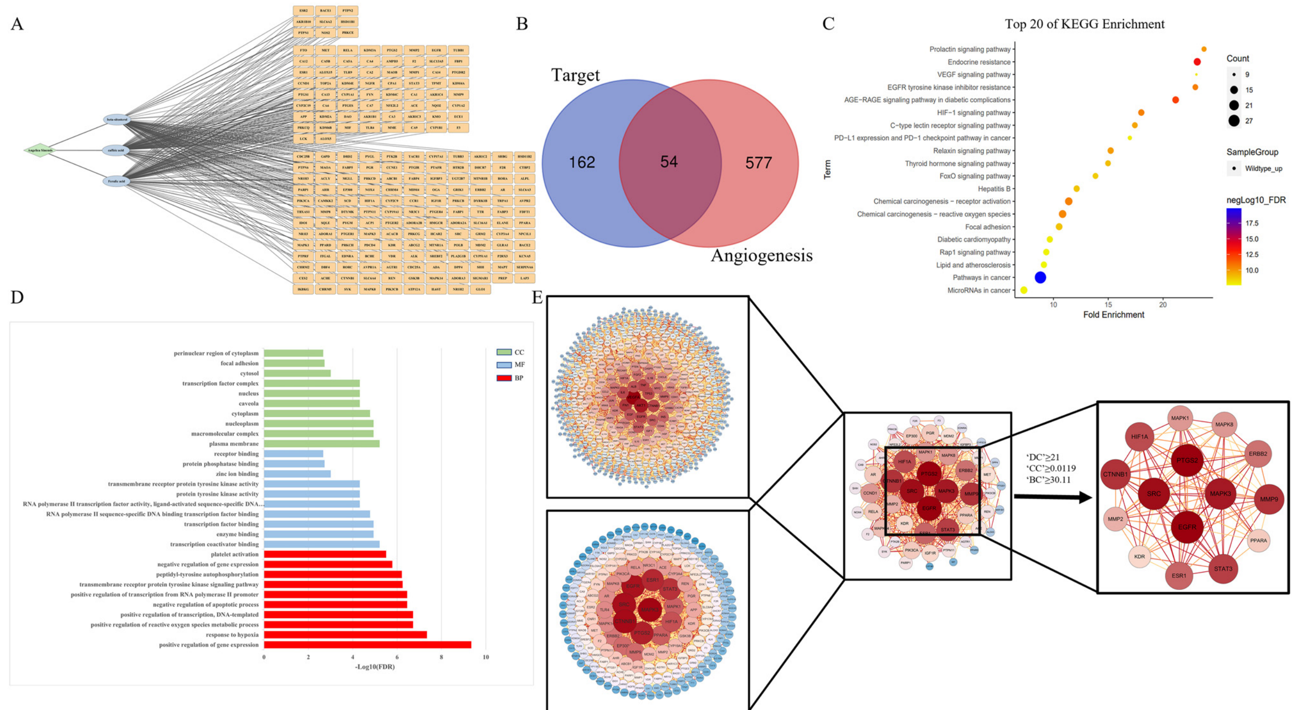

Figure 3. Network pharmacology to explore the target of AS in angiogenesis. (A) AS-component-target map. (B) 54 intersection genes of angelica target and angiogenesis. (C) KEGG pathway enrichment analysis of 54 intersection gene targets. (D) GO enrichment analysis of 54 intersection gene targets. (E) Topology of the PPI network showed the core objectives of this study.

processes such as regulation of gene expression, response to hypoxia, positive regulation of reactive oxygen species metabolic process, positive regulation of transcription, transmembrane receptor protein tyrosine kinase signaling pathway and platelet activation through molecular functions such as transcription factor binding, protein tyrosine kinase activity, transmembrane receptor protein tyrosine kinase activity, protein phosphatase binding, receptor binding. In conclusion, the effect of AS extract on angiogenesis seems to be closely related to hypoxia response, transmembrane receptor protein tyrosine kinase activity, gene expression and

phosphorylation, especially in the plasma membrane and nucleus (Figure 3D). To further screen hub targets, 54 intersection targets networks were analyzed by using cytoscape and centiscape. The screening condition was 'DC $\geq$ 21 AND CC $\geq$ 0.0119 AND BC $\geq$ 30.11' to derive the hub targets network (Figure 3E). Combined with the hub targets network, KEGG enrichment analysis, and angiogenesis correlation coefficients (Supplementary Figure 1), we identified HIF1α and VEGFR2 as hub targets for further validation.

Effect of AS Extract on VEGFA and HIF1α Expression in Granulosa Layer of Preovulatory Follicles (F1–F3)

VEGFA plays an important role in angiogenesis. HIF1α acts as a hypoxia-inducible factor and is involved in the regulation of VEGFA. The results of IF showed that VEGFA and HIF1α were mainly expressed in the granulosa layer of follicles. Levels of VEGFA and HIF1α in the granulosa layer of preovulatory follicles (F1–F3) were higher in the 35 wk group than in the 75 wk group ($P < 0.01$ and $P < 0.001$). After treatment with AS extract, the expression levels of VEGFA and HIF1α were higher than those in the 75 wk group ($P < 0.05$, $P < 0.01$ and $P < 0.001$) (Figure 4).

Western blot was used to detect the expression levels of VEGFA and HIF1α in the granulosa layer of preovulatory follicles (F1–F3). VEGFA and HIF1α expression levels in the granulosa layer of preovulatory follicles (F1–F3) were lower in the 75 wk group than in the 35 wk group ($P < 0.001$). Compared with the 75 wk group, VEGFA and HIF1α expression levels were significantly increased in the granulosa layer of preovulatory follicles (F1–F3) in the AS extract treatment group (Figure 5) ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

Effect of AS Extract on P-VEGFR2 Expression in Theca Layer of Preovulatory Follicles (F1–F3)

VEGFR2 plays a major role in mediating VEGFA-induced angiogenesis in vivo. The results of IF showed that P-VEGFR2 and VEGFR2 were mainly expressed in the theca layers (Figure 6). Levels of VEGFR2 expression did not change significantly in each group (Figure 6G–L). Whereas levels of P-VEGFR2 in the theca layer of preovulatory follicles (F1–F3) were lower in the 75 wk group than in the 35 wk group ($P < 0.001$), AS extract treatment significantly increased levels of P-VEGFR2 expression in the theca layer of preovulatory follicles (F1–F3) compared with 75 wk (Figure 6A–F) ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

Levels of VEGFR2 expression in theca layer of preovulatory follicles (F1–F3) were detected by Western blot. The levels of P-VEGFR2 expression in the theca layer of preovulatory follicles (F1–F3) in the 75 wk group was lower than that in the 35 wk group ($P < 0.001$). The levels of P-VEGFR2 expression were significantly increased in the 75 wk + L, 75 wk + M, and 75 wk + H groups compared with the 75 wk group (Figure 7) ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

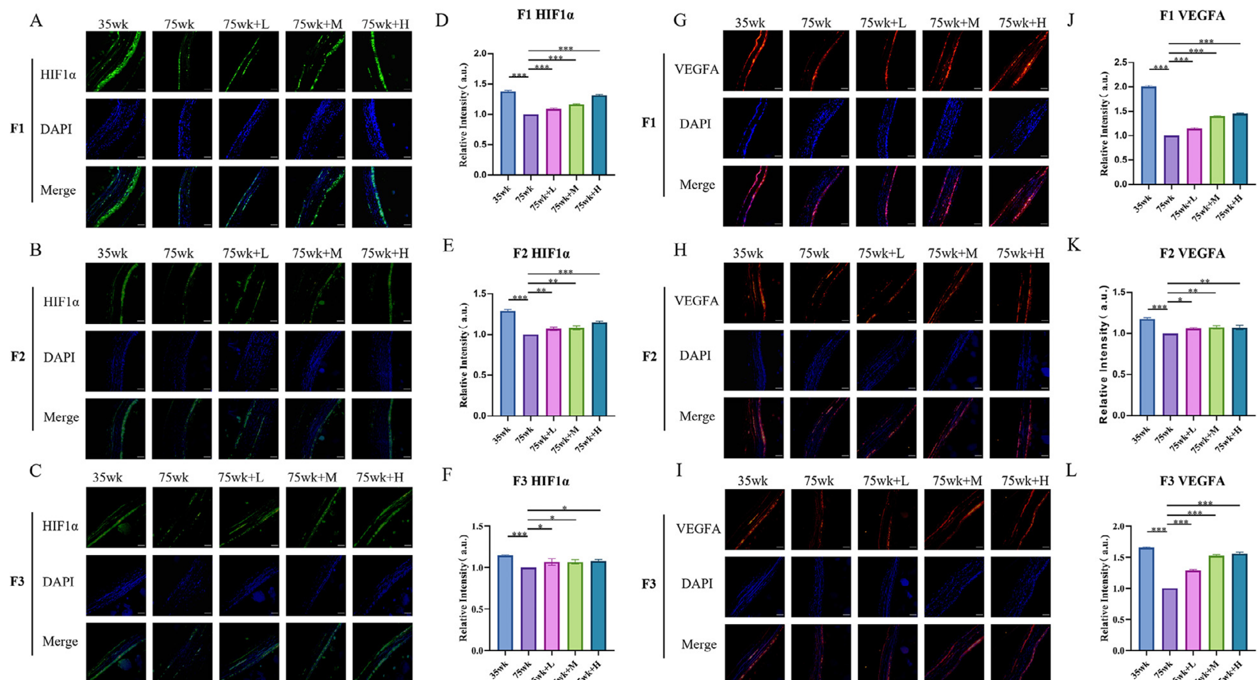

Figure 4. IF examined the effect of AS extract on HIF1α and VEGFA expression in the granulosa layer of preovulatory follicles (F1–F3). (A–C) IF detected the expression of HIF1α in preovulatory follicles (F1–F3). (D–F) The relative fluorescence intensity of HIF1α in preovulatory follicles (F1–F3) was quantified. (G–I) IF detected the expression of VEGFA in preovulatory follicles (F1–F3). (J–L) The relative fluorescence intensity of VEGFA in preovulatory follicles (F1–F3) was quantified. Scale bar: 100 μm. All experiments were performed in triplicate, and the data are the mean $\pm$ S.E.M (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

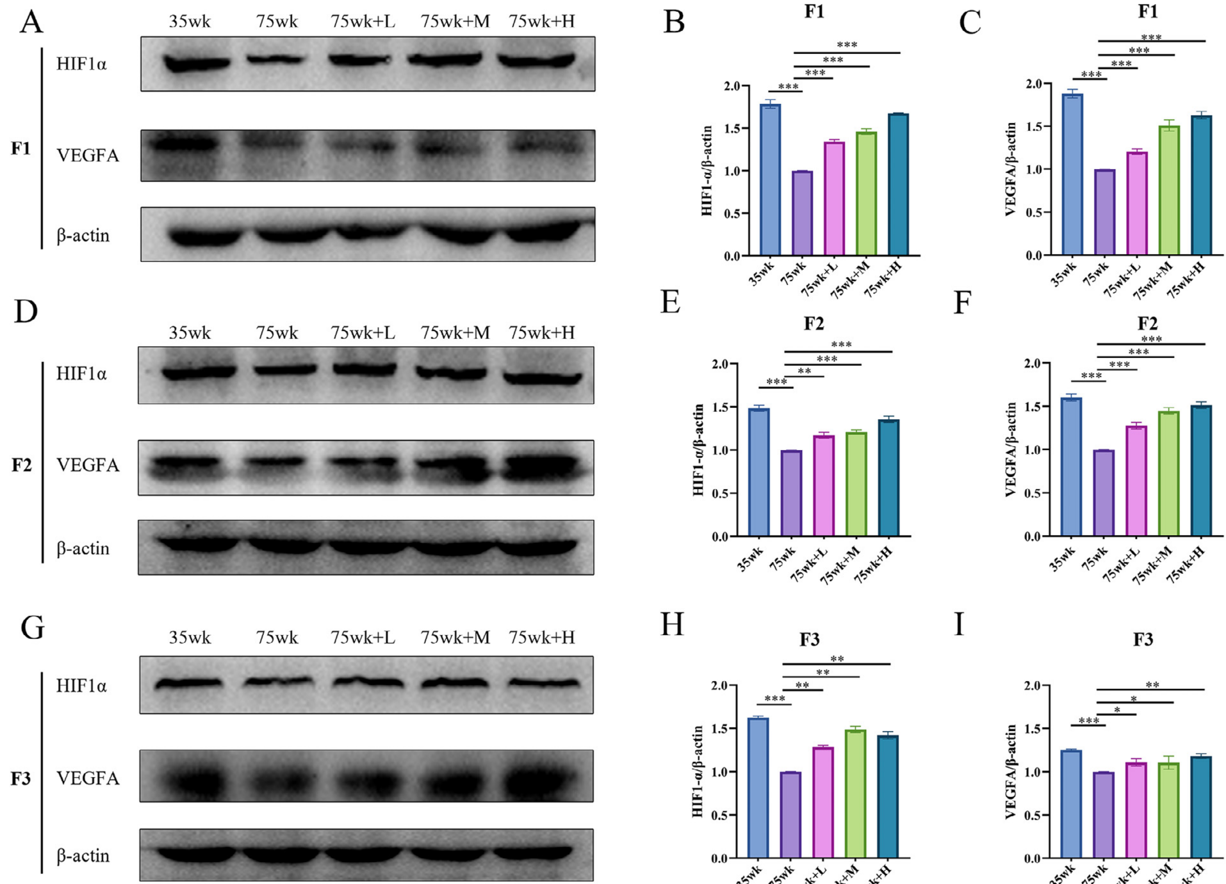

Figure 5. Western blot detected the effect of AS extract on HIF1α and VEGFA expression in the granulosa layer of preovulatory follicles (F1−F3). (A−C) Expression of HIF1α and VEGFA in the granulosa layer of preovulatory follicle F1. (D−F) Expression of HIF1α and VEGFA in the granulosa layer of preovulatory follicle F2. (G−I) Expression of HIF1α and VEGFA in the granulosa layer of preovulatory follicle F3. All experiments were performed in triplicate, and the data are the mean ± S.E.M ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

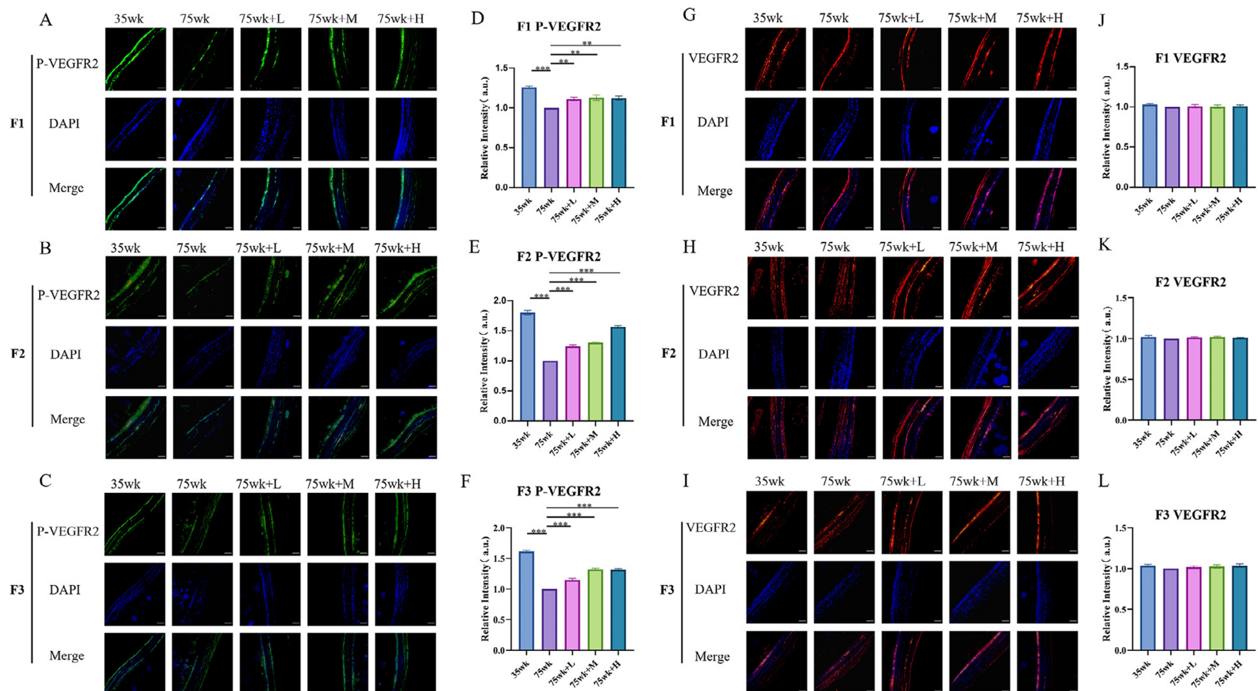

Figure 6. IF examined the effect of AS extract on VEGFR2 and P-VEGFR2 expression in the granulosa layer of preovulatory follicles (F1−F3). (A−C) IF detected the expression of P-VEGFR2 in preovulatory follicles (F1−F3). (D−F) The relative fluorescence intensity of P-VEGFR2 in preovulatory follicles (F1−F3) was quantified. (G−I) IF detected the expression of VEGFR2 in preovulatory follicles (F1−F3). (J−L) The relative fluorescence intensity of VEGFR2 in preovulatory follicles (F1−F3) was quantified. Scale bar: 100 μm. All experiments were performed in triplicate, and the data are the mean ± S.E.M ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

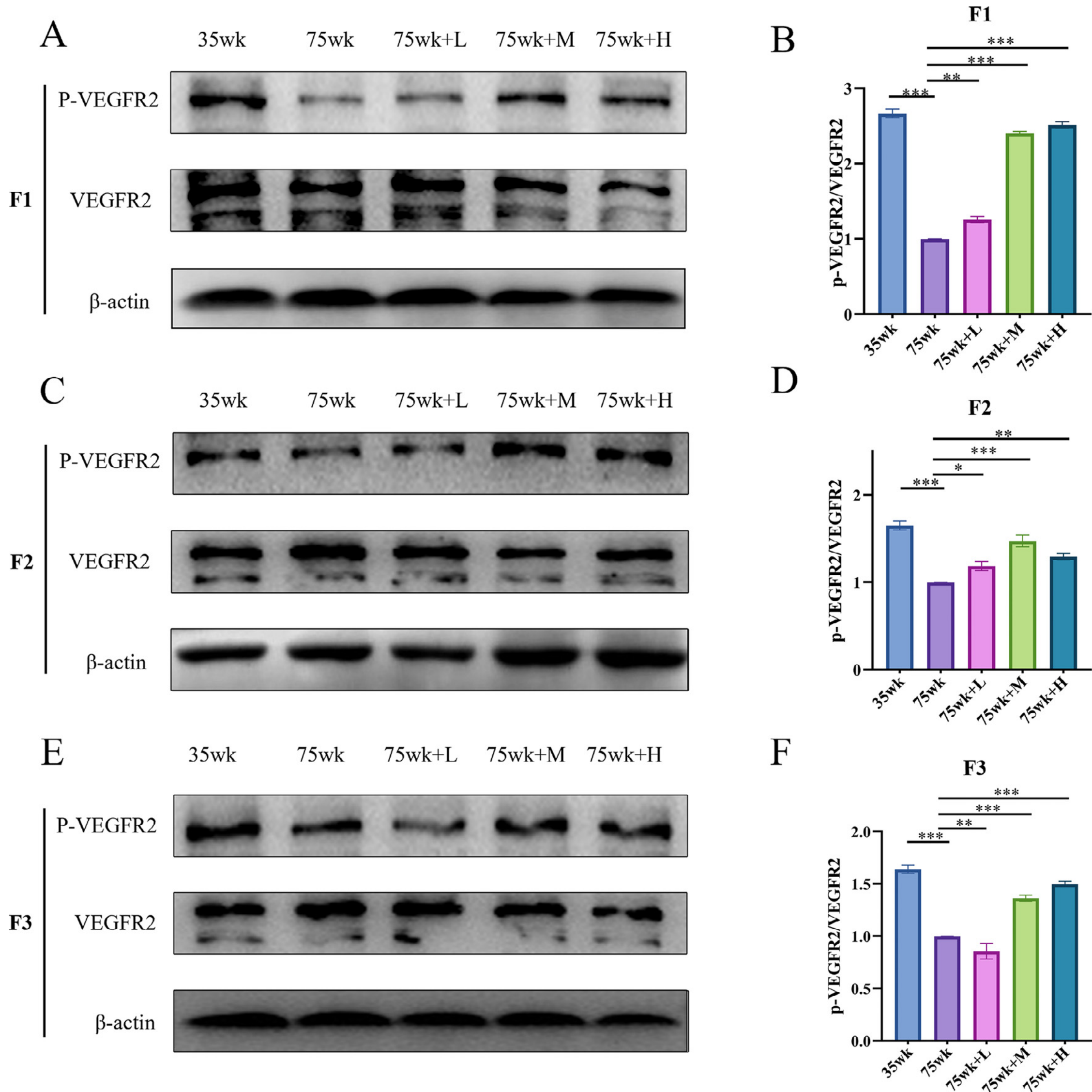

Figure 7. Western blot detected the effect of AS extract on P-VEGFR2/ VEGFR2 expression in the theca layer of preovulatory follicles (F1−F3). (A−B) Expression of P-VEGFR2/VEGFR2 in the theca layer of preovulatory follicle F1. (C−D) Expression of P-VEGFR2/VEGFR2 in the theca layer of preovulatory follicle F2. (E−F) Expression of P-VEGFR2/VEGFR2 in the theca layer of preovulatory follicle F3. All experiments were performed in triplicate, and the data are the mean ± S.E.M ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Simulation Molecular Docking of AS Components and Key Proteins

Molecular docking was used to simulate the binding of beta-sitosterol, ferulic acid, and caffeic acid to protein molecules VEGFR-2 and HIF1α. Molecular docking mimics the binding of beta-sitosterol, ferulic acid, and caffeic acid to protein molecules VEGFR-2 and HIF1α. In the docking pocket, they obtained the best 3D molecular docking structure. In this structure, the lowest binding energies of VEGFR2 protein to Beta-sitosterol, VEGFR2 protein to Ferulic acid, and VEGFR2 protein to Caffeic acid were −8.4 kcal/mol, −7.3 kcal/mol, and −7.6 kcal/mol, respectively (Figure 8A−C). The lowest binding energies of HIF1α protein to Beta-sitosterol, HIF1α protein to Ferulic acid, and HIF1α protein to Caffeic acid were −6.4 kcal/mol, −5.2 kcal/mol, and −5.5 kcal/mol, respectively (Figure 8D−F). Beta-sitosterol can form hydrogen bonds with amino acid residues ILE-1044 on VEGFR2 protein and LYS-401 on HIF1α protein (Figure 8A, D). Ferulic acid can form hydrogen bonds with amino acid residues CYS-919 and ASP-1046 on VEGFR2 protein and HIS-362 on HIF1α protein (Figure 8B, E). Caffeic acid can form hydrogen bonds with amino acid residues CYS-919 and ASP-1046 on VEGFR2 protein and GLY-26, LEU-27, and GLN-355 on HIF1α protein (Figure 8C, F). These hydrogen bonds may allow the two to form a tight and stable complex.

Effect of AS Extract on Key Proteins in GCs and FMECs of Follicle In Vitro

The IF results showed that the levels of HIF1α and VEGFA expression in GC significantly increased after 48 h of AS extract treatment ($P < 0.01$, $P < 0.001$) (Figure 9A−D). AS extract treatment significantly

A

VEGFR2-beta-sitosterol
Affinity=-8.4 (kcal/mol)

D

HIF1α-beta-sitosterol
Affinity=-6.4 (kcal/mol)

B

VEGFR2-Ferulic acid
Affinity=-7.3 (kcal/mol)

E

HIF1α-Ferulic acid
Affinity=-5.2 (kcal/mol)

C

VEGFR2-Caffeic acid
Affinity=-7.6 (kcal/mol)

F

HIF1α-Caffeic acid
Affinity=-5.5 (kcal/mol)

Figure 8. Molecular docking pattern diagram. (A−C) VEGFR2 docked into the docking pocket with active molecules beta-sitosterol, Ferulic acid, and Caffeic acid. The lowest binding energies were −8.4, −7.3, and −7.6 (kcal/mol), respectively. (D−F) HIF1α docked into the docking pocket with active molecules beta-sitosterol, Ferulic acid, and Caffeic acid. The lowest binding energies were −6.4, −5.2, and −5.5 (kcal/mol), respectively. Green was the protein structure; Pink was the binding molecule; Blue was the bound amino acid; Yellow dashed lines indicated hydrogen bond interaction forces that facilitate binding of the molecule to the active site.

Figure 9. Effect of AS extract on HIF1α, VEGFA and P-VEGFR2 in GCs and FMECs. (A−B) Expression level of HIF1α in GCs. (C−D) Expression level of VEGFA in GCs. (E−F) Expression level of P-VEGFR2 in FMECs. Scale bar: 50 μm. All experiments were performed in triplicate, and the data are the mean ± S.E.M ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

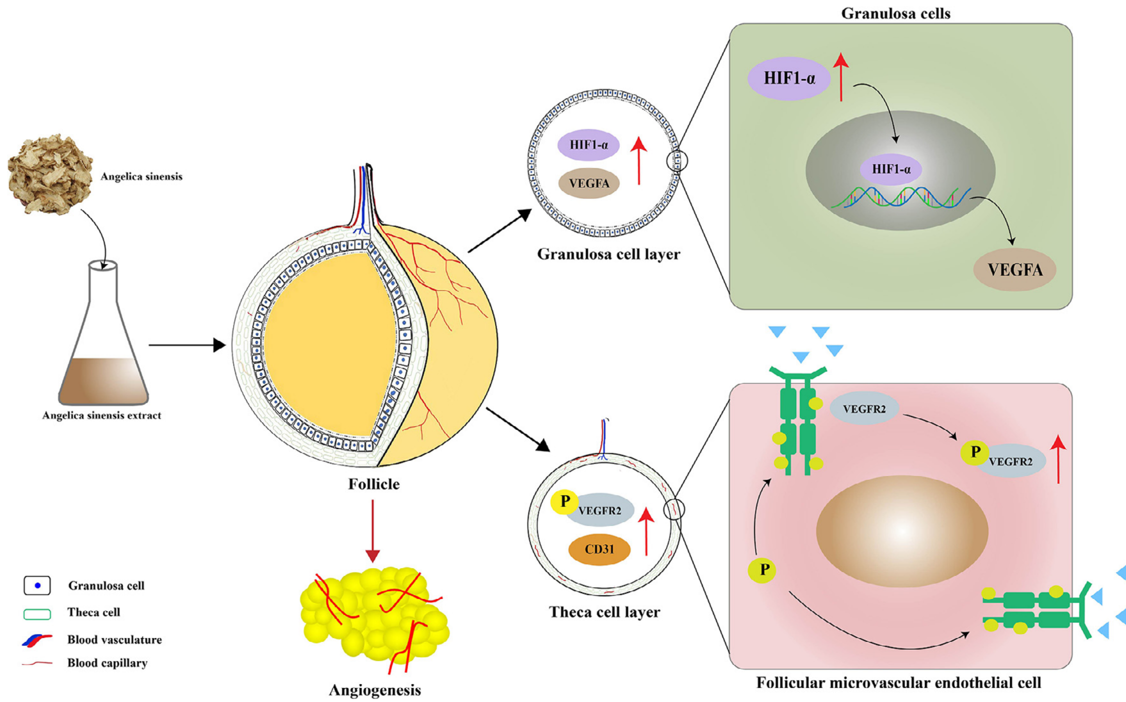

Figure 10. Mechanism of AS extract on angiogenesis in preovulatory follicles.

increased the phosphorylation levels of VEGFR2 protein in FMEC (Figure 9E−F).

DISCUSSION

AS, a traditional medicinal and edible plant, has long been used for tonifying, tonifying blood, promoting blood circulation and removing blood stasis, and also as a dietary supplement (Zhao et al., 2003). Available studies suggest that renal protection in AS may be associated with VEGF upregulation. Upregulation of VEGF reduces capillary loss and improves microstructural dysfunction (Song et al., 2009). AS extract can promote angiogenesis by regulating p38 and P-JNK 1/2 (Lam et al., 2008; Zhang et al., 2017). AS promotes angiogenesis and anti-apoptosis in rats with cerebral ischemia-reperfusion injury by regulating the p38MAPK/HIF-1α/VEGF-A/vWF signaling pathway (Cheng et al., 2017). In the ovary, cyclic development of follicles is accompanied by cyclic angiogenesis. Normal development of blood vessels is a prerequisite for normal ovulation (Guzmán et al., 2021). Previous studies have shown that the formation of a single capillary network around each follicle is required for follicle growth (Robinson et al., 2009). Densely distributed capillaries outside the theca of laying hens play a pivotal role in follicle growth because these capillaries provide nutrients, hormones and cytokines for development and maturation of follicle (Ma et al., 2020). Abundant capillaries transport yolk precursors from the liver into developing follicles, eventually forming preovulatory follicles F1 over 30 mm (Schneider, 2016). It has been demonstrated by immunofluorescence that vascular endothelial cells are mainly present in the theca layer (Trau et al., 2015). In this study, we examined apparent vessels and CD31 (CD31 used to localize endothelial cells in developing tissues) of preovulatory follicles (F1−F3) (Duffy et al., 2019; Wang et al., 2019). The results showed that the 75 wk group was less vascularized than the 35 wk group. The increase of CD31 of preovulatory follicles (F1−F3) in late-phase laying hens suggested that AS extract promoted angiogenesis of follicles.

The development of follicles is regulated by several follicle-derived factors. VEGFA and its receptor which play a crucial role in follicle angiogenesis (Tao et al., 2021b). GCs of follicles are the main site of VEGFA production (Trau et al., 2016; Kim et al., 2017), so the development of GCs is important for follicle angiogenesis (Kim et al., 2016). Studies on mammalian ovaries have shown that VEGFA, an angiogenic factor secreted by GC, plays a critical role in follicle growth and survival through the formation of new blood vessels within the theca layer. In laying hens, VEGFA is also essential for enhancing angiogenesis within the theca layer of preovulatory follicles (Kim et al., 2016). The results of this study showed that the thickness of the granulosa layers at 75 wk was significantly lower than that at 35 wk, which is consistent with previous findings (Hao et al., 2021). Thinning of the granulosa layer was one of the reasons for the low number of blood vessels in preovulatory follicles (F1−F3) in late-phase laying hens. The intervention of AS extract increased the thickness of the granulosa layer and improved the structure of the granulosa layer in preovulatory follicles (F1−F3) of late-phase laying hens. Meanwhile, VEGFA content in the granulosa layer of preovulatory follicles (F1−F3) was significantly increased after AS extract treatment.

Network pharmacology (Li, 2021) was used to explore the mechanism of angiogenesis promoted by AS. Through the analysis of AS-compound-angiogenesis target network, we found that the main active components related to angiogenesis were beta-sitosterol, ferulic acid, caffeic acid. Hub targets of AS affecting angiogenesis were focused on HIF1α and VEGFR2 by vascular correlation coefficient analysis of hub targets network. HIF1α is an important angiogenesis transcription factor, which accumulates stably under hypoxia and degrades rapidly after synthesis in normoxia (Yang et al., 2021). Activation of the transcription factor HIF1α adapts to the hypoxic environment via activating transactivation of downstream target genes. VEGFA is a well-established target of HIF1α promoting angiogenesis. The biological function of VEGFA is mainly mediated through binding to VEGFR2, which leads to tyrosine autophosphorylation of VEGFR2 and subsequent recruitment and activation of several key adaptor proteins (Shc, Grb2, Gab1, SHP1, and SHP2) to induce the transcription of angiogenesis gene (Sun et al., 2018). GO enrichment analysis showed that biological processes related to angiogenesis, such as hypoxia response (Choudhry and Harris, 2018; de Heer et al., 2020) and platelet activation (Tao et al., 2021a). These biological processes were mainly involved through molecular functions such as transcriptional coactivator binding, enzyme binding, transcription factor binding and transmembrane protein tyrosine kinase activity. KEGG analysis yielded major pathways related to angiogenesis, including the cancer pathway, VEGF signaling pathway, and HIF1 signaling pathway. The cancer pathway is closely related to VEGF signaling and HIF1 signaling pathway. The development of tumors is related to excessive cell proliferation, which requires a lot of oxygen consumption. Many genes responding to stress under hypoxia are regulated by the HIF1α signaling pathway and VEGFA plays a key role in tumor angiogenesis (Zhang et al., 2019).

HIF1α is a transcription factor that primarily regulates gene transcription in response to hypoxic conditions and is essential for both angiogenesis and inflammation (Ahmadian et al., 2013). It has been shown that VEGFA is an important regulator of follicle development and survival, while VEGFA expression in GCs is regulated by signals emitted by LH and FSH -mediated HIF1α (Rico et al., 2014). VEGFA expression is predominantly in the granulosa layer and spreads toward the theca layer (Hunter et al., 2004; Fraser and Duncan, 2005; Kim et al., 2017; Gao et al., 2020). VEGFR2 is expressed and acts in the main theca layer (Stassi et al., 2019). It has been shown that HIF1α accumulates in cells and binds to HIF1β to form dimers, which translocate into the nucleus to activate the target gene VEGF for transcription and promote VEGFA expression (DAMERT et al., 1997; Semenza, 2003; Ahluwalia and Tarnawski, 2012). VEGFA binds to its receptor VEGFR2 in the theca layer and induces dimerization and phosphorylation of VEGFR2, which activates multiple signaling cascades and affects a variety of processes, including endothelial cell proliferation, actin remodeling, cell migration, and vascular permeability (Herbert and Stainier, 2011). Eventually angiogenesis is promoted. This study found that changes in HIF1α and VEGFA expression occurred mainly in the granulosa layer of follicles, and VEGFR2 was mainly expressed in the theca layer, which is consistent with other studies (Stassi et al., 2019; Gao et al., 2020). AS extract promoted the expression of HIF1α protein and VEGFA protein in granulosa layer of preovulatory follicles (F1−F3) in vivo. Meanwhile, AS extract promoted phosphorylation of VEGFR2 in the theca layer. Phosphorylation of VEGFR2 has been shown to activate multiple downstream pathway expression and promote proliferation, migration, as well as angiogenesis in endothelial cells (Sakurai et al., 2005; Miller and Sewell-Loftin, 2021).

Beta-sitosterol, ferulic acid, and caffeic acid could bind to VEGFR2 and HIF1α proteins to form stable conformations in docking pockets when simulating molecular docking. These results preliminarily verified the possibility that AS extract affected cellular proteins. AS extract increased HIF1α and VEGFA expression in GCs and promoted VEGFR2 phosphorylation in FMECs. This provided a theoretical basis for molecular docking results.

CONCLUSION

The main active components and hub targets of AS extracts were analyzed by network pharmacology. In vitro and in vivo experiments verified and revealed that AS extract promoted angiogenesis of preovulatory follicles (F1−F3) in late-phase laying hens by promoting the expression of granulosa layer proteins HIF1α and VEGFA and increasing the phosphorylation level of theca layer protein VEGFR2 (Figure 10). Our study provides a theoretical basis for AS extract as feed supplements in late-phase laying hens.

ACKNOWLEDGMENTS

This work was supported by the national key research and development plan of China under grant number 2017YFD0502200.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: The work was mainly conceived and designed by Zhenlei Ping, Xin Chen, Hao Chen and Wenhui Yu; Zhenlei Ping, Xin Chen, Hao Chen, Lixue Fang, Kai Wu and Chang Liu performed the experiments; Zhenlei Ping, Xin Chen collected and analyzed experimental data; Xiaowen Jiang and Jun Ma managed the project; The manuscript was written by Zhenlei Ping, Xin Chen. All the authors read and approved the final manuscript.

Consent for publication: Written informed consent for publication was obtained from all participants.

DISCLOSURES

The authors have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102415.

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