**ABSTRACT**

**Aims:** Ginkgo biloba extract (EGb) has been widely applied in the treatment of cerebrovascular and neurological diseases. However, the effect of EGb761 on ovarian hyperstimulation syndrome (OHSS), a vascular disorder and life-threatening complication of in vitro fertilization and intracytoplasmic sperm injection therapy (IVF/ICSI), has not been evaluated.

**Materials and methods:** Forty female Wistar rats aged 22-days old (D22) were divided into eight groups: Control rats received intraperitoneal injection of saline for five consecutive days (D22–D26); OHSS model group received 10IU equine chorionic gonadotropin (eCG) for four consecutive days (D22–D25) and 30IU of human chorionic gonadotropin (hCG) on the 5th day (D26); Prophylactic treatment group received three doses of EGb761 (50, 100, and 200 mg/kg/day) 1 h before injection of eCG (hCG) for seven consecutive days; Therapeutic treatment group received three doses of EGb761 (50, 100, and 200 mg/kg/day) 48 h after injection of eCG (hCG) for seven consecutive days.

**Results:** All three doses of EGb761 therapeutic medication significantly reduced ovarian mass index of OHSS model rats (p < 0.01). Furthermore, therapeutic treatment group exhibited improved vascular permeability, decreased estradiol and progesterone levels, lower corpus luteum, and higher follicle numbers compared with the OHSS model. Elevated protein expression of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) in both ovary and kidney of the OHSS model was restrained by EGb761 therapeutic treatment.

**Conclusions:** EGb761 therapeutic medication decreases vascular permeability in OHSS rat model by inhibiting VEGF and VEGF expression, which may contribute to the treatment of OHSS.

**Introduction**

Ovarian hyperstimulation syndrome (OHSS) is a potentially life-threatening complication of in vitro fertilization and intracytoplasmic sperm injection (IVF/ICSI) therapy [1]. OHSS is essentially a vascular disorder with clinical symptoms associated with an overall increase in systemic vascular permeability. In cases of OHSS, fluid and serum proteins are constantly lost from the vasculature, leading to ascites and even progression to pleural effusion, thrombosis, and hypovolemic shock [2,3]. Studies in humans and rodents have investigated that vascular endothelial growth factor (VEGF) and its receptors VEGFR seem to play a key role in the pathogenesis of OHSS [4,5]. Moreover, these suggestions are reinforced by the findings that the occurrence of OHSS correlates with the serum concentration of VEGF, and the fact that VEGF content in the follicular fluid is frequently higher in patients with OHSS than that of individuals not affected by such complication [6]. Interestingly, both the clinical pregnancy rate and abortion rate in OHSS patients were significantly higher than those without the syndrome. These observations suggest that early pregnancy processes, such as implantation, trophoblast invasion, and placentation, may also be affected in patients with OHSS [7].

To date no specific therapy is available for OHSS, and current treatment principles include avoiding the use of human chorionic gonadotropin (hCG) to trigger oocyte maturation, postponement of embryo transfer, and intensive follow-up [3]. Obviously, these treatments are not effective in preventing development of the syndrome. The study of Zhang et al. has shown that Ginkgo biloba extract (EGb) 761 can inhibit the expression of VEGF protein and VEGF mRNA in rat aortic endothelial cells co-cultured with lysophosphatidylcholine [8]. EGb761 is standardized to contain 24% flavonoids, 6% terpenoids (ginkgolide 3.1%, bilobalide 2.9%), 5–10% organic acid, and other components. It has been extensively applied for neurological and vascular diseases, such as cerebral insufficiency, stroke, multi-infarct dementia, and myocardial ischemia [9]. However, whether EGb761 is effective in prevention or treatment of OHSS has not been studied.

The aim of the present study was to investigate the possible protective effect of EGb761 on the development of OHSS and explore the underlying mechanism.
Materials and methods

Animal models and interventions

Forty female immature Wistar rats aged 22 days old (D22) were provided by the Institute of Genome Engineered Animal Models for Human Disease of Dalian Medical University (Dalian, China). They were kept under a controlled 12-h light/12-h dark life cycle and were fed with a standard laboratory chow diet, with free access to food and water. The animals were randomly divided into eight groups (n = 5), and there was no difference in the initial body weight of each group (p = .382). The interventions of each group were conducted as below: (1) CON, control rats received intraperitoneal injection of saline (0.5 mL/100 g) for five consecutive days (D22–D26); (2) OHSS, OHSS model rats received 101 IU equine chorionic gonadotropin (eCG, Solarbio, China, 0.5 mL/100 g) for four consecutive days (D22–D25) and 301 IU of hCG (Ningbo Renjian Pharmaceutical Group Co. Ltd., China, 0.5 mL/100 g) on the 5th day (D26); (3) EGB761 + OHSS, prophylactic treatment group received (intraperitoneal injection) three doses of EGB761 (50 mg/kg/day, 100 mg/kg/day, and 200 mg/kg/day) 1 h before injection of eCG or hCG for seven consecutive days; (4) OHSS + EGB761, therapeutic treatment group received (intraperitoneal injection) three doses of EGB761 (50 mg/kg/day, 100 mg/kg/day, and 200 mg/kg/day) 48 h after injection of eCG or hCG for seven consecutive days. Medications were stored and used according to the manufacturers’ instructions. All doses are recommended to be efficacy in humans and extrapolated from animal’s weight. All animal experiments were approved by Animal Ethics Committee of Dalian Medical University (Permit Number: AEE18077).

Measurement of peritoneal and ovarian capillary permeability

Alterations in vascular permeability were measured by the Evans Blue (EB) dye method according to a protocol slightly modified from that described by Ujioka et al. [10]. Rats were inhalation anesthetized with diethyl ether and kept in thermal blanket to avoid hypothermia. EB dye powder (E8010, Solarbio, China) was diluted in distilled water at a final concentration of 5 mM. A fixed volume of 0.2 mL was administered via the caudal vein in each rat. Thirty minutes after the EB injection, the peritoneal cavity was irrigated with 5 mL of 0.9% normal saline (21 °C). The peritoneal irrigated fluid was collected into tubes and was centrifuged at 900 g for 12 min at room temperature. Immediately after the peritoneal irrigation, blood was obtained to allow the outflow of perfusate, and the left apex was slowly injected with 50 mL of saline for systemic circulation perfusion. After that, the ovaries were removed, weighed, and incubated in 65 °C N,N-dimethylformamide (D112007, Aladdin, China) for 24 h. EB concentrations were determined by measuring the dye absorption at 600 nm with a microplate reader (Spectra MR, DynexTechnologies, USA).

Hormone assay

The serum was collected from blood samples after centrifugation with 3000 rpm for 15 min. All samples were quick-frozen in liquid nitrogen and stored at −80 °C for further use. Estradiol (E2) and progesterone (PRG) levels were determined by the electrochemiluminescence assay (Roche, German) on an automatic electrochemiluminescence immunoassay system (Cobas e 601, Roche, Switzerland). The inter- and intra-assay coefficients of variation as indicated by manufactures were 13.8% and 8.5% for E2 respectively, 10.7 and 7.2% for PRG, respectively.

Histology analysis

Hematoxalin and eosin (H&E) staining: Ovaries and kidneys isolated from animals were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin as previously described [11]. Paraffin-embedded tissues were serially sectioned at 5 μm thickness and stained with H&E routinely for morphological observation. Three rats in each group have been randomly selected for detection of follicular development, and the images were obtained blind with the microscope (Olympus BX63, Japan) and Image Pro Plus software (Media Cybernetics, USA).

Immunohistochemical (IHC) analysis: Paraffin-embedded ovarian and renal tissues were used for the DAB Detection Kit (ZLI-9018, ZSGB-BIO, China) according to the manufacturer’s instructions. Sections were deparaffinized, hydrated, and rinsed before heat-mediated antigen retrieval in 0.01 M pH 6.0 sodium citrate buffer (C1010, Solarbio, China). After quenching of endogenous peroxidases with 3% H2O2, sections were incubated in nonspecific staining blockers for 15 min and with primary antibody against VEGF (WL00009b, Wanleibio, China) at a dilution of 1:100 at 4 °C overnight. Sections were then incubated in biotinylated secondary antibody for 15 min followed by avidin and biotinylated HRP (1:1) mixed solution incubations for 15 min. Finally, the sections were visualized with DAB and counterstained with hematoxalin, and antigen distribution was examined under light microscope.

Western blotting

Ovary and kidney samples were homogenized and lysed in lysis buffer (KGP2100, KeyGEN BioTECH, China). Protein extract was collected and subjected to BCA protein assays (Thermo, USA). Thirty micrograms of protein were separated by SDS-PAGE and subsequently transferred to PVDF membrane. Afterward, the membranes were blotted with 10% skim milk for 1 h at 37 °C and incubated with primary antibodies against VEGF (WL00009b, Wanleibio, China) and VEGFR1 (AF6204, Affinity Biosciences, USA) overnight at 4 °C. After incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG (ZB-2301, ZSGB-BIO, China) for 2 h at room temperature, the membranes were visualized with an enhanced chemiluminescence plus kit (P10100, NCM Biotech, China) and Bio-Rad ChemiDoc MP imaging system. Band density was quantified using ImageJ software.

Statistical analysis

The Shapiro–Wilk test was used to determine the distribution of normality for the continuous variables. Non-normally distributed data were expressed as the median and the interquartile ratio (IQR), and were analyzed by Kruskal–Wallis test followed by Dunn’s test. Those presented normal distribution were expressed as the means ± standard deviation (SD) and analyzed with ANOVA followed by least-significant difference (LSD) post hoc analysis. The data were analyzed by the SPSS 20.0 statistical software (SPSS Inc., USA), and p < .05 was considered statistically significant.
Results

Effect of EGb761 on ovarian and renal mass index of OHSS model

As shown in Figure 1 and Supplementary Figure S1, OHSS model rats exhibited significantly higher ovarian, renal, and liver mass indexes (organ mass/body weight) than that of the control group (all \( p < 0.01 \)), whereas pulmonary mass index was not affected (\( p = 0.089 \)). Compared with the model group, the ovarian mass index was significantly reduced by all three therapeutic treatment groups (all \( p < 0.01 \)), and renal mass index was significantly decreased by therapeutic EGb761 treatment at a dose of 100 and 200 mg/kg/day (both \( p < 0.01 \)). Whereas, all three prophylactic treatment groups had no effect on ovarian and renal mass indexes. Furthermore, only therapeutic EGb treatment at a dose of 200 mg/kg/day was able to reduce elevated liver mass index in OHSS rats (\( p < 0.05 \)). The aforementioned results demonstrated that therapeutic EGb761 treatment was effective in mitigating the edema of genitourinary organs in OHSS.

Effect of EGb761 on vascular permeability of OHSS model

EB concentration of all intervention groups was distinctly lower than that of the OHSS group in both peritoneal irrigated fluid and ovary. Furthermore, EB contents in the therapeutic treatment group were generally lower than those in the prophylactic treatment group, and dose-dependent manner was observed (Figure 2). Thus, both EGb761 prophylactic medication and therapeutic medication could alleviate the increase of vascular permeability in OHSS, and the therapeutic medication may have a more profound effect.

Effect of EGb761 on follicular development of OHSS model

As shown in the morphological analysis (Figure 4), the ovary of OHSS rats was characterized by the highest corpus luteum numbers and lowest graafian follicle numbers. Compared with the OHSS group, lower corpus luteum numbers and higher follicle numbers were observed in OHSS + EGb761 groups especially in the high-dose group (200 mg/kg/day). However, prophylactic medication of EGb761 appeared to have a limited effect on follicular development. Hence, therapeutic EGb761 agent was effective for the further development of follicles in OHSS and thus reduced the secretion of estrogen.

Effect of EGb761 on VEGF and VEGFR expression in ovary and kidney of OHSS model

By quantitative analysis of western blotting (WB) (Figure 5), we found that VEGF expression was obviously increased in ovary and kidney tissues in the OHSS group (both \( p \leq 0.01 \)), which was remarkably decreased in all three OHSS + EGb761 groups. Likewise, the expression of VEGFR in the ovary was significantly restrained by all three doses of EGb761 therapeutic agents, and the expression of VEGFR in the kidney was significantly suppressed by 100 and 200 mg/kg/day dose of EGb761 therapeutic agents. Correspondingly, protein localization analysis with IHC (Figure 6) also displayed a soared expression of VEGF in the kidney and ovary of the OHSS rats, which was distinctly damped after EGb761 treatment. In addition, there was no significant difference in pulmonary VEGF protein expression among the groups. However, consistent with the increased liver mass index in the OHSS group, VEGF protein was highly expressed in the liver of OHSS rats (\( p \leq 0.01 \)), which was observably decreased by
Figure 2. Effects of G. biloba extract (EGb761) on the EB dye content of peritoneal irrigated fluid (a) and ovary (b) in OHSS rats. EB was injected intravenously 30 min before execution. The level of EB dye in the peritoneal irrigated fluid was expressed as µg/100 g body weight (BW), and the ovarian EB content was expressed as µg/g tissue weight. The results are expressed as the median and IQR, and statistical analyses were conducted using Kruskal–Wallis test followed by Dunn’s test. For all analyses, values of $p < .05$ were considered statistically significant, $n = 3$ per group for (a) and $n = 4$ per group for (b). $**p \leq .01$ vs. CON group; $#p < .05$; $##p \leq .01$ vs. OHSS group.

Figure 3. Effects of G. biloba extract (EGb761) on serum concentrations of estradiol (a) and progesterone (b) in OHSS rats. The results of serum estradiol are presented as means ± SD, and statistical analyses were conducted using one-way ANOVA followed by least-significant difference (LSD) post hoc tests; data of serum progesterone are expressed as the median and IQR, and were analyzed by Kruskal–Wallis test followed by Dunn’s test. For all analyses, values of $p < .05$ were considered statistically significant, $n = 5$ per group. $**p \leq .01$ vs. CON group; $#p < .05$; $##p \leq .01$ vs. OHSS group.

Figure 4. Representative H&E staining of cross-sections of ovary isolated from rats of each group. Extensive corpus luteum (hollow star) numbers and minimum graafian follicle (black star) numbers were detected in the ovary of OHSS rats, whereas lower corpus luteum numbers and higher follicle numbers were observed in OHSS + EGb761 groups especially in the high-dose group (200 mg/kg/day). Images are shown at ×10 and ×20 magnification, Scale bars: 400 µm, $n = 3$ per group. EGb761, Ginkgo biloba extract; OHSS: ovarian hyperstimulation syndrome.
all three doses of therapeutic EGb761 medication (Supplementary Figures S1 and S2), indicating a certain contribution to reducing the peritoneal capillary permeability.

Discussion

In the present study, we explored the protective effects of EGb761 on OHSS by using a rat model, and the effects of prophylactic and therapeutic EGb761 medication were compared. The observed follicular development in ovarian morphology and increase in vascular permeability suggested that the OHSS rat model mirrored the human condition. In addition, ovarian and renal mass index, E2 and PRL hormonal level, corpus luteum numbers, and the protein expression of VEGF and VEGFR in the ovary and kidney were all augmented in OHSS model rats, whereas therapeutic EGb761 treatment
could effectively improve the aforementioned performance of OHSS.

VEGF, originally known as vascular permeability factor, is a signal protein that stimulates vasculogenesis and angiogenesis [12]. It is part of system that restores the oxygen supply to tissues when blood circulation is inadequate such as in hypoxic conditions. It has been suggested that the relative hypoxia in massively enlarged ovaries in OHSS rat model will require an increase in angiogenesis, as well as VEGF expression [13]. Moreover, inhibition of VEGF and inflammatory process was effective in the treatment of OHSS [14]. Thus, current studies support a crucial role of VEGF in the OHSS development, as reviewed by Soares et al. [15]. VEGF brings about increased vascular permeability by rearranging endothelial junction proteins, including cadherin and claudin-5. Using a rat model, Gómez et al. [16,17] found that both VEGF mRNA levels and vascular permeability increased in mesentery and ovary following gonadotropin stimulation. Consistently, the OHSS rat model in our study also showed a high protein expression of VEGF and VEGFR, as well as the aggravating vascular permeability in the peritoneum and ovary.

*G. biloba* extract, the dried leaf extract of ginkgo species with the main component flavonoids and terpene lactones, has a variety of health benefits. Extract of *G. biloba* is recognized as a strong antioxidant that can eliminate excess free radicals in the body and prevent lipid peroxidation. It shows a high efficacy without significant side effects in clinical application. In the early 1960s, extract of *G. biloba* has been applied in some countries for treatment of cerebrovascular diseases and neurological diseases. In addition, it has been considered to have immune-boosting, anti-aging effects, and beneficial for neurodegenerative disorders [18]. The study of Zhang et al. [8] has found that Egb can protect rat aortic endothelial cells from lysophosphatidylcholine by inhibiting the expression of VEGF protein and VEGF mRNA. However, the role of Egb in OHSS and the underlying mechanism has not been studied so far. Here, we suggested that Egb could decrease vascular permeability in OHSS rat model by inhibiting VEGF expression, which may contribute to the prevention and treatment of OHSS. Moreover, the therapeutic medication was superior to prophylactic medication, providing a theoretical basis for the clinical application of Egb in the treatment of OHSS. As a descriptive study, there are several limitations, especially lacking of evidence from animals with specific gene knockout or in vitro evidence to clarify whether changes in VEGF expression actually mediate the effect of Egb on OHSS or are concomitant, which will be verified next.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This study was supported by Dalian Medical Science Research Project [Grant Number: 18Z1001].

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