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OHSS

Ovarian hyperstimulation syndrome inhibition by targeting VEGF, COX-2 and Calcium pathways: a preclinical randomized study

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Abstract

Objective: The efficacy of vascular endothelial growth factor (VEGF), COX-2, calcium and aromatase inhibitors in an ovarian hyperstimulation syndrome (OHSS) rat model was tested.
Methods: One hundred and eight female Wistar rats were randomly divided in nine groups. The control group received saline, while the OHSS group received rec-FSH for 4 consecutive days. The other seven groups received rec-FSH (4d) and Bevacizumab twice, Parecoxib daily, Verapamil daily, Parecoxib daily and Bevacizumab twice, Verapamil daily and Bevacizumab twice, Parecoxib and Verapamil daily, Letrozole and Meloxicam daily, respectively. All groups received also hCG at the 5th day.
Results: All intervention groups were characterized by reduced vascular permeability compared to the OHSS group, which in the groups of Verapamil (Calcium inhibition) and Parecoxib + Verapamil (COX-2 + Calcium inhibition) presented significant statistical difference. The Verapamil group showed the lowest corpus luteum formation, while the Parecoxib (COX-2 inhibition), the Parecoxib + Verapamil (COX-2 + Calcium inhibition), the Bevacizumab + Parecoxib (VEGF + COX-2 inhibition) and the Bevacizumab + Verapamil (VEGF + Calcium inhibition) groups were also characterized by lower corpus luteum numbers compared to the OHSS group. Furthermore, lower graafian follicle formation was observed in the above groups, while the ovarian weight and the hormonal profile were not significantly affected.
Conclusions: Studying the different check points of the VEGF pathway, we conclude that targeting calcium pathways could be beneficial for the vascular permeability control in an OHSS animal model.

Keywords

Calcium inhibition, Cox-2 inhibition, ovarian hyperstimulation, VEGF inhibition

History

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potential life threatening complication of controlled ovarian stimulation. Women with OHSS are characterized by increased ovarian angiogenesis, vascular permeability and circulatory dysfunction unrelated to hyperestrogenemia [1]. However, the main OHSS symptom is the increased vascular permeability combined with augmented albumin levels in the abdominal cavity, while in more severe syndrome forms the fluid accumulation may lead even to pleural effusion [2]. Current treatments such as avoidance of hCG triggering for oocyte maturation, embryo transfer postponing and patient intensive follow up are not sufficient for syndrome prevention.

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Vascular endothelial growth factor (VEGF) constitutes a physiological regulator of angiogenesis and a mediator of vascular endothelial cell permeability [3]. VEGF expression is critical for the ovarian angiogenesis, the normal follicular growth and the corpus luteum function [4]. Several studies in humans and rodents have associated the VEGF with the onset of OHSS. Specifically, VEGF has been proposed as the main capillary permeability agent in OHSS ascites fluid [5], while plasma VEGF levels have been correlated with the OHSS phenotype [6,7]. Furthermore, experiments in human and rat endothelial cells have shown an induction of VEGF expression after hCG administration that leads to increased vascular permeabilities [8,9].

Previous studies have used capergoline (Cb2), a dopamine agonist, that seems to prevent the incidence of severe OHSS in humans and Meloxicam, a cyclooxygenase-2 inhibitor, that attenuates the main symptoms of the syndrome in an OHSS rat model [10,11]. The current study is designed to inhibit VEGF various pathway check points, separately or in combination. We sought to investigate the impact of different medications/therapies on OHSS regarding abdominal vascular permeability, follicular

development, ovarian weight and hormonal profile. Highly potent and specific pathway inhibitors were tested: Bevacizumab (a humanized monoclonal antibody directed against VEGF that recognizes and neutralizes all isoforms of VEGF-A), Parecoxib (a selective COX-2 inhibitor) and Verapamil (a Calcium inhibitor). Furthermore, the combined effect of Meloxicam and Letrozole (an aromatase inhibitor) was tested. We also evaluated whether these treatments are effective when they are administered during the ovarian hyperstimulation and vascular permeability onset. The final aim of the study was to analyze effective treatment strategies from a physiology point of view and to understand how these clinically approved drugs may prevent iatrogenic ovarian hyperstimulation.

Materials and methods

Animal model

One hundred eight female immature Wistar rats, 22 days old weighing 45–50 g, were provided by the Breeding Laboratory Animal Institute and the experiments held out in the Research Laboratory of Reproductive Genetics, Department of Obstetrics and Gynecology, Ioannina University Medical School, Greece. They were kept under a controlled 12 h light–12 h dark life cycle and they had a standard diet and free access to water.

All research animals were treated in compliance with the European Union guidelines for the care and use of animals approved by our institution in accordance to principles of laboratory animal breeding and handling.

Interventions

To show a 66 % difference in vascular permeability test with a $p < 0.05$, a sample size of 12 animals in each group was required, namely 108 animals. The animals were divided in nine groups, after randomization using a computer-generated sequence. There was no difference in animal weight between these groups ($p = 0.15$). The interventions in each group are analyzed below:

- The 1st group (control group) received 0.2 mL of intraperitoneal saline for 5 consecutive days (days 22–26).
- The 2nd group (OHSS group) received 35 IU rec-FSH (Gonal–*f*, Merk Serono, UK) for 4 consecutive days (days 22–25) and 35 IU of hCG (Pregnyl, N.V. Organon, Schiphol-Rijk, The Netherlands) on the 5th day (day 26).

Groups 3–9 were treated similarly as the OHSS group. In groups 3–5, a single medication was added to gonadotropin treatment, while in groups 6–9 two medications in combination were administered.

- The 3rd group (OHSS + VEGF inhibition group) received 2 mg/kg Bevacizumab (Avastin, Roche, Los Angeles, CA) twice on the 3rd and the 5th day of stimulation.
- The 4th group (OHSS + COX-2 inhibition group) received 3 mg/kg Parecoxib (Dynastat, Pfizer, Kent, UK) daily.
- The 5th group (OHSS + Calcium inhibition group) received 5 mg/kg Verapamil (Isoptin, Lifepharma, Nicosia, Cyprus) daily.
- The 6th group (OHSS + VEGF/COX-2 inhibition group) received 25 mg/kg Bevacizumab twice, on the 3rd and the 5th day, and 35 mg/kg Parecoxib daily.
- The 7th group (OHSS + VEGF/Calcium inhibition group) received 25 mg/kg Bevacizumab twice, on the 3rd and the 5th day, and 55 mg/kg Verapamil daily.
- The 8th group (OHSS + COX-2/Calcium inhibition group) received 35 mg/kg Parecoxib and 55 mg/kg Verapamil daily.
- The 9th group (OHSS + Aromatase/COX-2 inhibition group) received 0.045 mg/kg Letrozole (Femara, Novartis, Berne, Switzerland) and 0.35 5 mg/kg Meloxicam (Movatec, Boehringer Ingelheim am Rhein, Germany) daily. Letrozole was diluted 10% w/v propylene glycol.

Medications were stored and used according to the manufacturers' instructions. All doses are recommended doses for efficacy in humans, but extrapolated to animal weight. Treatment was administered through injections in the abdominal region and parallel to gonadotropin administration with a minimal interval between injections. Higher doses of FSH (35 IU rec-FSH) were used to compare with previous studies in order to ensure ovarian hyperstimulation. All measurements were taken on the 7th day (48 after hCG administration).

Vascular permeability test

Alterations in vascular permeability, leakage and accumulation of albumin in the peritoneal cavity were measured by the Evans Blue dye method. Evans Blue dye combines with the albumin fraction and is thus distributed in the intravascular space [12]. The procedure was previously described [9]. Rats were anesthetized with ketamine and kept in thermal blanket to avoid hypothermia. Evans Blue dye was diluted in distilled water at a final concentration of 5 mM. A fixed volume of 0.2 ml was administered via the femoral vein in each rat. After 30 minutes the peritoneal cavity was infused with 5 ml of 0.9% saline (21 °C; pH 6) and massaged for 30 seconds. The accumulated fluid was extracted with a VasofixR BraunuleR (Bbraun) catheter to prevent tissue or vessel damage and it was collected in tubes containing 0.05 ml of 0.1 N NaOH to prevent any protein interference. Tubes were centrifuged at 900 *g* for 12 minutes at room temperature, and subsequently Evans dye concentrations were determined by measuring the dye absorption at 620 nm on a JENWAY 6305 UV/Vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). The level of the extravasated dye in the recovered fluid was expressed as μg/100 g of body weight.

Intra-abdominal albumin concentration

Another method for comparing vascular permeability alterations was the direct measurement of albumin in peritoneal fluid. Albumin concentration (mg/dl) was determined by immunoturbidimetry in an AU540 BECKMAN COULTER analyzer (Beckman Coulter International SA, Nyon, Switzerland) using the Medicon Microalbumin Reagent Kit (MEDICON HELLAS S.A., Athens, Greece).

Histology

The histological analysis of the ovaries was performed as previously described [13]. Briefly, ovaries from each experimental group were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 8 mm thickness and stained with hematoxylin for morphological observation. Three mice in each group have been randomly selected to be checked for ovarian follicles at different stages of development.

Hormone assay

Blood samples were collected by the jugular vein immediately after the recovery of the accumulated peritoneal fluid. Samples were kept for 10 minutes at room temperature and then centrifuged in 3000 rpm for 10 min. The serum was collected in 1.5 ml tubes and stored at −80 °C until use. Estradiol (E_2) and progesterone (PRG) levels were determined by the enhanced chemiluminescence assay (E_2 and PRG Reagent Packs, VITROS, Buckinghamshire, UK) on a Vitros 5600 Ea Analyzer (Johnson & Johnson, New Brunswick, NJy). The inter- and intra-assay coefficients of variation as indicated by manufactures were 13.8% and 8.5% for E_2 as well as 10.7% and 7.2% for PRG.

Statistical methods

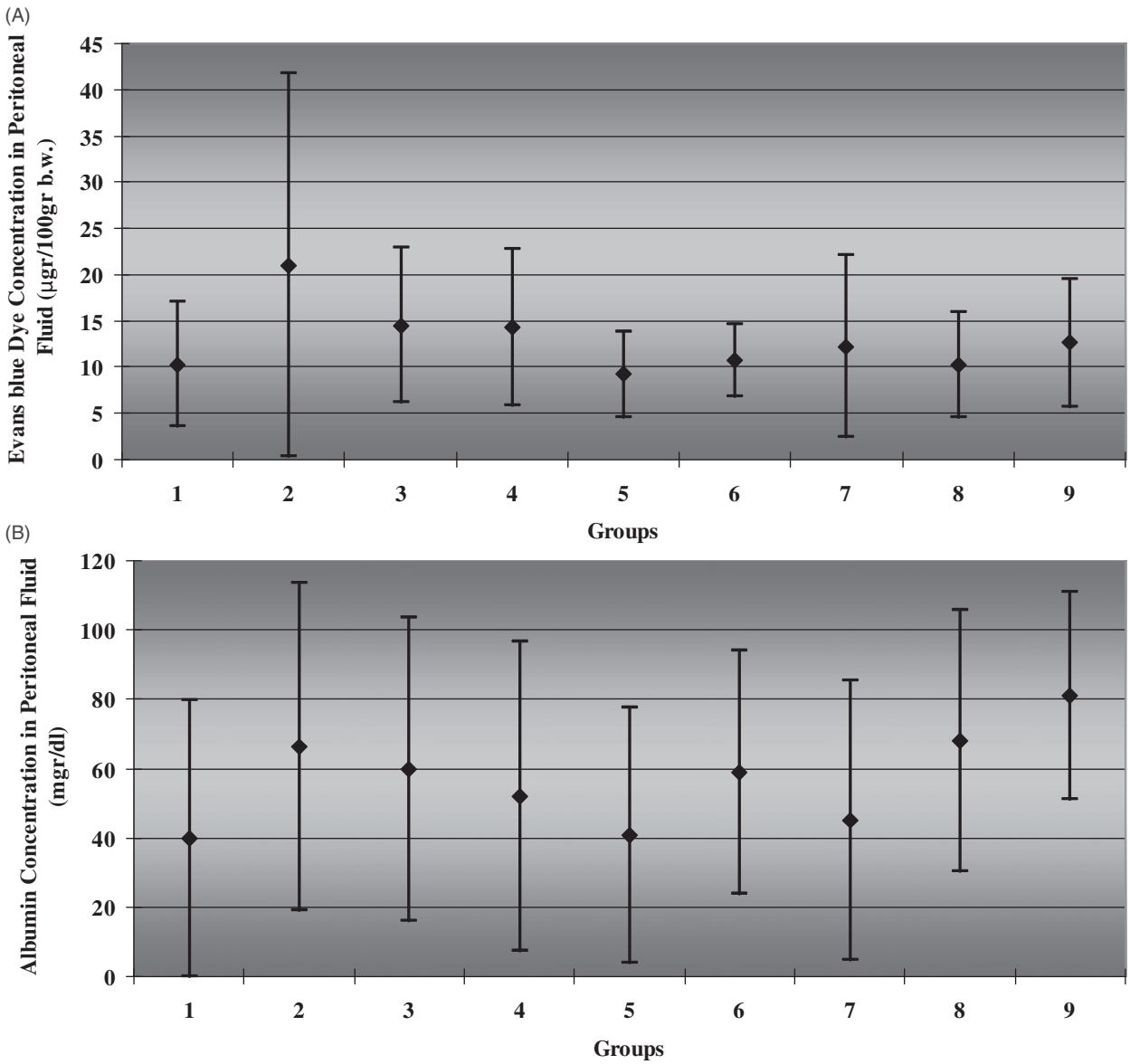
Ovarian weight, abdominal albumin accumulation in peritoneal fluid and hormonal levels were compared using one way ANOVA and Dunnett's method. p Value <0.05 was considered as statistically significant. Analyses were conducted with the Statistical Package for the Social Sciences Version 15.0 (SPSS, Inc., Chicago, IL).

Results

In the 108 female used in the current study, we evaluated the vascular permeability, the follicular development, the ovarian weight and the hormone levels to understand the efficacy of each medication on the OHSS.

Vascular permeability

All intervention groups showed lower Evans Blue Dye concentrations compared to the OHSS group (20.91 ± 20.83 μg/100 gr b.w.) (Figure 1). However, significant differences were observed only in the Verapamil group (Calcium inhibition, 9.2 ± 4.65 μg/100 gr b.w., $p = 0.023$), the Parecoxib + Verapamil group (COX-2 + Calcium inhibition, 10.17 ± 5.68 μg/100 gr b.w., $p = 0.046$) and as expected in the control group (10.25 ± 6.75 μg/100 gr b.w., $p = 0.048$) (Figure 1).



1	Control	**6**	Bevacizumab+Parecoxib (VEGF+COX-2 inhibition)
2	OHSS	**7**	Bevacizumab+Verapamil (VEGF+Calcium inhibition)
3	Bevacizumab (VEGF inhibition)	**8**	Parecoxib+Verapamil (COX-2+Calcium inhibition)
4	Parecoxib (COX-2 inhibition)	**9**	Letrozole+Meloxicam (Aromatase+COX-2 inhibition)
5	Verapamil (Calcium inhibition)		

Figure 1. Evans Blue Dye (A) and Albumin (B) concentrations on the 7th day (48 hours after hCG administration) in each group. Data shown as mean value ± standard deviation.

When we examined directly the albumin concentration in the peritoneal fluid, the Verapamil group (calcium inhibition, 40.63 ± 36.79 mg/dl, $p = 0.043$) and the control group (39.9 ± 39.87 mg/dl, $p = 0.039$) presented with significantly lower concentrations compared to the OHSS group (66.23 ± 47.09 mg/dl) (Figure 1).

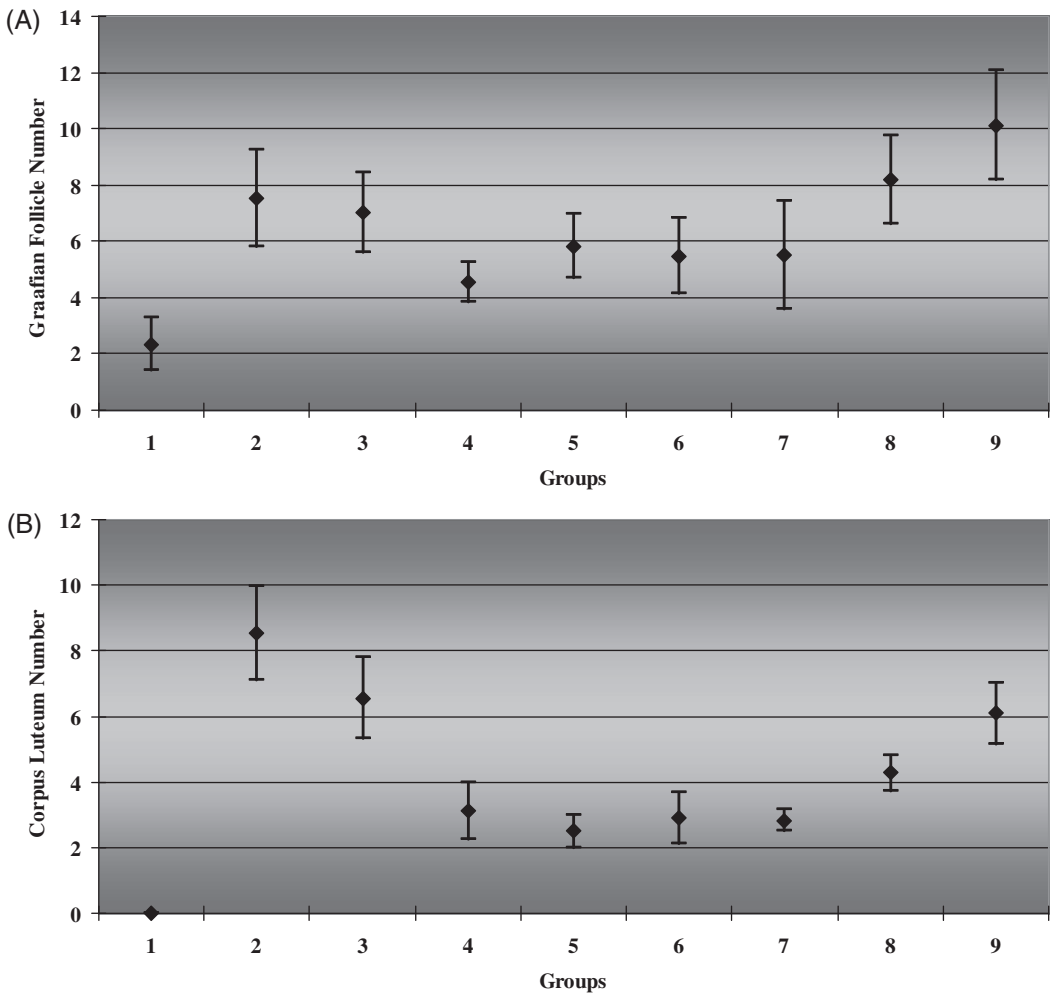
Consequently, the Verapamil group (calcium inhibition) had significantly lower vascular permeability compared to the OHSS group, according to both Evans Blue Dye and intrabdominal albumin measuring methods.

Follicular development

Graafian follicle and corpus luteum formation was not affected by any of the medication. However variations were observed between the groups. As expected, the control group presented with the lowest number of graafian follicles, while the Parecoxib (COX-2 inhibition), the Verapamil (Calcium inhibition), the Bevacizumab + Verapamil (VEGF + Calcium inhibition) and the Bevacizumab + Parecoxib (VEGF + COX-2 inhibition) groups were characterized by lower graafian follicle numbers compared to the OHSS groups (Figure 2). On the other hand, the highest number of corpus luteum was observed in the OHSS group (Figure 2). The Verapamil group (Calcium inhibition) showed the lowest corpus luteum formation, while the Parecoxib (COX-2 inhibition), the Parecoxib + Verapamil (COX-2 + Calcium inhibition), the Bevacizumab + Parecoxib (VEGF + COX-2 inhibition) and the Bevacizumab + Verapamil (VEGF + Calcium inhibition) groups were also characterized by lower corpus luteum numbers compared to the OHSS groups (Figure 2). The statistical analysis of the graafian follicles and the corpus luteum is only indicative due to the small number of samples analyzed in each group and therefore is not presented here.

Ovarian weight

The control group showed significantly lower ovarian weight as compared to the OHSS group (0.02 ± 0.01 g versus 0.08 ± 0.04 g, $p = 0.002$) and all intervention groups. Although the OHSS group showed the largest ovaries, the intervention groups showed comparable measurements (data not shown).



1	Control	**6**	Bevacizumab+Parecoxib (VEGF+COX-2 inhibition)
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3	Bevacizumab (VEGF inhibition)	**8**	Parecoxib+Verapamil (COX-2+Calcium inhibition)
4	Parecoxib (COX-2 inhibition)	**9**	Letrozole+Meloxicam (Aromatase+COX-2 inhibition)
5	Verapamil (Calcium inhibition)		

Figure 2. Graafian Follicle (A) and Corpus Luteum (B) numbers in each group. Data shown as mean value ± standard deviation.

Hormonal assay

No noteworthy differences were observed between the intervention groups and the OHSS group for estradiol (data not shown). Nevertheless, the Letrozole + Meloxicam (Aromatase + COX-2 inhibition) and the control groups showed significantly lower E_2 concentrations compared to the OHSS group (33.24 ± 12.86 pg/ml versus 43.18 ± 23.42 pg/ml, $p = 0.027$ and 33.68 ± 32.6 pg/ml versus 43.18 ± 23.42 pg/ml, $p = 0.028$, respectively).

On the other hand, PRG levels did not have significant differences between the intervention groups and the OHSS group (data not shown), while the control group had significant lower progesterone concentrations compared to the OHSS group (38.69 ± 10.16 nmol/l versus 50.84 ± 11.92 nmol/l, $p = 0.011$).

Discussion

The aim of this study was to test whether three different regimens, alone or in combination, administered during ovarian stimulation in a hyperstimulation rat model have significant effects on physiological parameters. The framework of the study was to inhibit pathways (VEGF, COX 2 and calcium pathways) with specific and efficient inhibitors and to associate the outcomes with certain pathway inhibition. The selected regimens target complementary mechanisms in the VEGF cascade and they are safe, stable, easily administered and already tested in humans for other indications. Bevacizumab, a VEGF-A neutralizer, Parecoxib, a selective COX-2 inhibitor, Verapamil, a calcium inhibitor, Meloxicam, a COX-1 over COX-2 inhibitor and Letrozole, an aromatase inhibitor, were tested separately or in combination during the ovarian stimulation in a hyperstimulation rat model. Their influence on vascular permeability, follicular development, ovarian weight and the levels of E_2 and PRG was evaluated.

The primary finding of the study was the reduced vascular permeability in the abdominal fluid in all intervention groups compared to the OHSS group. However, only in cases of Verapamil (calcium inhibition) and Parecoxib + Verapamil (COX-2 + Calcium inhibition) groups, the vascular permeability presented with significant reduction, reaching the levels of the unstimulated control group. According to our experiments, inhibiting VEGF seems to be an effective strategy for vascular permeability reduction, but calcium pathway blockage seems to offer greater reduction in albumin permeability.

The histological analysis showed, as expected, increased graafian follicle numbers and the highest corpus luteum formation in the OHSS group. The graafian follicle and corpus luteum formation was not affected by any of the medications tested. However, variations were observed between the groups. In the Parecoxib, Verapamil, Bevacizumab + Verapamil and Bevacizumab + Parecoxib groups decreased graafian follicle numbers were observed. Specifically, the Verapamil group showed the lowest corpus luteum formation, while the Parecoxib, the Parecoxib + Verapamil, the Bevacizumab + Parecoxib and the Bevacizumab + Verapamil groups were also characterized by lower corpus luteum numbers compared to the OHSS groups. Consequently the use of these medications and mainly of Verapamil does not disrupt the follicle formation process.

As regards the ovarian weight, the OHSS group was characterized by the heaviest ovaries while the unstimulated control group by the lightest ovaries. However, the intervention groups showed similar measurements with the OHSS group, indicating that the inhibition of the VEGF and its complementary mechanisms does not affect the ovarian weight increase during OHSS. Similar results have been observed concerning the levels of E_2 and PRG. Specifically, both hormones did not present significant differences between intervention groups and OHSS group. Only in the Letrozole + Meloxicam group, the estradiol levels were significantly lower compared to the OHSS group, reaching the respective levels of the control group. The estradiol rise was effectively inhibited in this group due to the inhibitory action of Letrozole against aromatase.

The potential effects of the medications used in the current study have been cited in the recent literature. Verapamil has been used during pregnancy [14], although it has been associated with possible problems in animal studies. Bevacizumab, although is not recommended for use in pregnant women, there are case reports supporting the opposite. Specifically, a pregnant woman has received intravitreal this treatment [15], while another woman accidentally received the same treatment on an unknown pregnancy [16]. In both cases, no short term adverse events have been reported on the mother and the fetus. Nevertheless, an early pregnancy loss after the same treatment [17] as well as hypertension of pregnancy due to anti-VEGF effects [18] has been reported. Furthermore, Parecoxib has been currently contraindicated during the third trimester of pregnancy although it could be used as a tocolytic agent [19]. On the other hand, Meloxicam has been already tested in ovarian hyperstimulation with conflicting results [11,20]. Finally, the follicular and luteal phase administration of Letrozole [21–23] has led to estradiol but not progesterone suppression. Although the safety precautions for Letrozole administration have not been specified yet, there are no studies associating Letrozole administration during follicular phase for ovulation induction with congenital malformations or teratogenicity [24,25].

This study sought to investigate the potential effect of different regimens that inhibit VEGF, COX-2 and calcium pathways on OHSS. It was also examined whether the administration of these medications during ovarian stimulation prevent vascular permeability. Indeed, all regimens demonstrated efficiency in reducing vascular permeability but only Verapamil showed the lowest albumin concentration intra-abdominally from all stimulated animals. Animals treated with Verapamil were also characterized by lower corpus luteum formation. Comparative studies with the administration of these medications after hCG (for oocyte maturation) should be performed in order to observe the magnitude of this effect on corpus luteum formation. The intention of this preclinical study is not to issue clinical practice guidelines, but to stimulate future clinical studies.

Declaration of interest

The authors report no declarations of interest.

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