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Investigation of short- and long-term effects of ovarian hyperstimulation syndrome on ovarian reserve: an experimental study

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Summary

Purpose: To investigate the short and long term effects of ovarian hyperstimulation syndrome (OHSS) on serum levels of vascular endothelial growth factor (VEGF) and endothelin-1 and ovarian follicular reserve (OFR). *Materials and Methods:* An experimental case-control study was conducted on a university animal laboratory with 20 immature (22-day-old) virgin female Wistar Albino rats. Firstly, rats were divided into two groups. Group 1 (n = 10): control and Group 2 (n = 10): experimental OHSS induced rats. Secondly, Group 2 was randomly divided into two groups on the day of OHSS development (27th day) as follows: Group 3 (n = 5): 27-day-old OHSS induced rats and Group 4 (n = 5): 27-day-old OHSS induced rats supervised for seven days. Group 1 was divided into two groups to constitute age-matched controls as follows: Group 5 (n = 5): 27-day-old rats, Group 6 (n = 5): 35-day-old rats. The comparisons of Group 3 vs Group 5 and Group 4 vs Group 6 were performed. Main outcome measures were OFR, serum levels of VEGF, and endothelin-1. *Results:* While the OFR and primordial follicle number (PFN) of Group 3 were significantly lower than those of Group 5 ($p < 0.05$); VEGF and endothelin-1 levels and atretic follicle number (AFN) were significantly higher in Group 3 compared to Group 5 ($p < 0.05$). In Group 4, PFN was significantly lower ($p < 0.05$) and AFN was significantly ($p < 0.05$) higher than Group 6. However, there were no statistically significant difference between Group 4 and Group 6 regarding the parameters of OFR, serum levels of VEGF, and endothelin-1. *Conclusion:* This experimental OHSS model revealed increased serum VEGF and endothelin-1 levels and decreased OFR during short-term of OHSS. OHSS showed detrimental effect on PFN of rats during long-term.

Key words: Endothelin-1; Ovarian follicle reserve; Ovarian hyperstimulation syndrome; VEGF.

Introduction

Ovarian hyperstimulation syndrome (OHSS) is the most serious complication of ovulation induction with gonadotropin and human chorionic gonadotrophin (hCG). This iatrogenic condition is potentially lethal and occurs in 0.3 to five percent of stimulated ovarian cycles [1]. Some forms of OHSS may arise from the following conditions: [1] pregnant women with polycystic ovary syndrome that respond excessively to endogen gonadotropin; [2] abnormally high serum hCG levels in molar pregnancies; [3] women with primary hypothyroidism; [4] gonadotroph adenoma inducing co-secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), causing a rise in estradiol (E2) levels with ovarian enlargement without ascites [2, 3]. Clinical manifestations of OHSS are massive extravascular fluid accumulation and hemoconcentration similar to that in syndromes due to capillary leakage. The patients may be complicated by renal failure, hypovolemic shock, thromboembolic episodes, and adult respiratory distress syndrome. The pathophysiology of this syndrome has not been completely evaluated, the increased capillary permeability triggered by the release of vasoactive substances secreted by the ovaries under hCG stimulation plays a key role in this syndrome [4].

The angiogenic molecule, vascular endothelial growth factor (VEGF) is the most important mediator of hCG-dependent ovarian angiogenesis. It is known that VEGF is expressed in human ovaries [5] and that VEGF mRNA levels increase after hCG administration in granulosa cells [6, 7]. A circulatory dysfunction has been described in every woman treated with gonadotropins for in vitro fertilization. It is not known, whether the gonadotropins up-regulate VEGF receptor-2 (VEGFR-2) expression and whether increased vascular permeability is also found with mild stimulation [8]. High concentrations of VEGF have been demonstrated in ascitic fluid from patients with OHSS [6].

Endothelin-1, an endothelial derived peptide, is a potent vasoconstrictor that increases capillary permeability in several tissues [9]. High levels of endothelin-1 were found in follicular fluid in patients undergoing ovulation induction

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[10]. Endothelin-1 concentration was found to be 100–300 fold higher in the follicular fluid than in the plasma. Moreover, a positive correlation between endothelin-1 and FSH concentration in the follicular fluid were found, suggesting that endothelin-1 may play a role in ovarian function as well as in OHSS [11]. In this study the authors aimed to investigate the short and long term effects of OHSS on ovarian histology and serum levels of VEGF and endothelin-1 in a rat model.

Materials and Methods

Experimental design

This study was approved by Firat University Animal Use Committee and conducted at Firat University Animal Laboratory (FUTDAM). Twenty immature (22-day-old), weighing 41- 49 grams, female Wistar Albino rats were used for all experiments. They were housed individually as quinary groups in plastic cages with chip bedding, and ad libitum access to rat chow (pellet) and water. They were maintained on a 12:12 light:dark cycle (lights on at 07:00 AM) at room temperature.

Twenty immature (22-day-old) female rats were randomly divided into two groups. Group 1 (n = 10): 22-day- old rats. Group 2 (n = 10): experimental OHSS induced rats. These rats were randomly divided into two groups on the day of OHSS development (27^{th} day); Group 3 (n = 5): 27-day-old experimental OHSS induced rats euthanised and then ovarian tissue and serum samples were collected. Group 4 (n = 5): 27-day-old experimental OHSS induced rats supervised spontaneously for seven days. Group 1 divided into two groups to constitute age-matched controls; Group 5 (n = 5): 27-day-old normal rats group, Group 6 (n = 5): 35-day-old normal rats group. The comparisons of Group 3 vs Group 5 and Group 4 vs Group 6 were performed. The rats were divided into four age-matched groups. Ovarian histopathologic evaluation and serum level analysis of VEGF and endothelin-1 of all rats were performed.

OHSS induction and ELISA assays

To prepare the OHSS model, immature female Wistar rats were stimulated with 10 IU of FSH for four consecutive days followed by 30 IU of hCG on the 26^{th} day of life. The manifestation of the OHSS was demonstrated with daily weight gain and hematocrite elevation as illustrated by Ohba *et al.* [12]. All rats were euthanised with decapitation. Approximately three cc blood were collected from all rats and centrifuged at 2,500 rpms for four minutes to obtain serum samples. The serum samples were stored at -20°C until the analysis of VEGF and endothelin-1. The extracted serum samples were assayed by an enzyme linked immunosorbent assay (ELISA) using commercially available kit for VEGF and endothelin-1, according to the manufacturer's instructions.

Ovarian morphology

After laparatomy, ovaries were removed and cleaned of adhering tissue in culture medium, weighed, and used for subsequent assays. Ovarian tissue was fixed with ten percent formaldehyde and then paraffin-embedded tissue samples were cut into four μm sections for estimation of mean ovarian follicle count. The sections were stained with masson trichrome to determine ovarian follicle reserve under light microscope. The four μm step sections were mounted at 50 μm intervals onto microscope slides to prevent counting the same structure twice, according to the aforementioned method described [13]. Follicles were classified as primordial, primary, secondary, and tertiary follicles. An atretic follicle was defined as the follicle that presented more than ten pycnotic nuclei per follicle; in the smallest follicles, the criterion for atresia was a degenerate oocyte, precocious antrum formation, or both [14].

Main outcome measures were as follows: age of rat (days), weight of rat (gr), hematocrit of rat (%), weight of ovary (mgr), serum levels of VEGF (pg/ml) and endothelin-1 (ng/ml), total follicle count with determination of primordial, primary, secondary, and tertiary follicle numbers [15]. Atretic follicle, corpus luteum (CL), and corpus albicans were also determined. CL was investigated for regression of angiogenesis and ovarian stromal fibrosis and these findings were scaled as 0 = absence, 1 = moderate presence, and 2 = high presence. Ovarian follicle cysts were counted macroscopically and scaled as 0 = absence and 1 = presence [16].

Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences (SPSS) version 12.0. Results were presented as mean and standard deviation and number and percentage where applicable. Age-matched comparison of Group 3 vs Group 5 and Group 4 vs Group 6 were performed. Differences between groups for ordinal variables were analyzed using Mann-Whitney *U* test and differences in the categorical variables of groups was assessed using Chi-squared test. *P* values < 0.05 were considered as statistically significant.

Results

All of the experiments were completed successfully in all of the groups. Main outcome measures of the groups were weight, hematocrit and ovarian weight of rats and are presented in Table-1; ovarian morphology (Figure 1) and follicle counts are presented in Table 2, and serum levels of VEGF and endothelin-1 are presented in Table 3.

The comparison of Group 3 vs Group 5 showed significantly low ovarian follicle reserve and primordial follicle count in Group 3 ($p < 0.05$) and significantly high atretic follicle count and serum VEGF and endothelin-1 levels in Group 3 ($p < 0.05$). The weights and hematocrits of rats on days four and six were significantly high in Group3 and total ovarian weight on day six was significantly high in Group 3, too. Primary and secondary follicle counts of Group 3 were lower than of Group 5, but the difference was not significant.

The comparison of Group 4 vs Group 6 showed significantly high total ovarian weight, hematocrit, and weights of rats on days four, six, and 13 in Group 4 ($p < 0.05$). While primordial follicle count was significantly low, AFN was significantly high in Group 4 ($p < 0.05$). In Group 4, serum VEGF and endothelin-1 levels were higher than Group 6 and ovarian follicle reserve was lower than Group 6, but the differences were not significant.

Discussion

The present study is the first one to evaluate the short- and long-term effects of OHSS on ovarian reserve in an experimental model. It was shown that OHSS increased the serum levels of VEGF and endothelin-1 and had detrimen-

Table 1. — *The weight, hematocrit % and ovarian weight of all rats in the study*

Parameters	Short-term			Long-term		
	G3 (n=5, OHSS)	G5 (n=5, control)	*p* value	G4 (n = 5, OHSS)	G6 (n = 5, control)	*p* value
Weight on day 0 (gr)	44.4 ± 3	45 ± 2.3	NS	45.6 ± 2.8	47 ± 1.6	NS
Weight on day 4 (gr)	63.4 ± 3	54 ± 1.4	< 0.05[a]	58.6 ± 2.6	53.4 ± 1.1	< 0.05[a]
Weight on day 6 (gr)	69 ± 4.1	60 ± 2.1	< 0.05[a]	71.8 ± 2.1	61.4 ± 2.3	< 0.05[a]
Weight on day 13 (gr)	decapitation	decapitation	-	105.6±3.6	83.6 ± 1.1	< 0.05[a]
Htc on day 0 (%)	37.6 ± 1	37 ± 1.3	NS	37.2 ±1.4	37.6 ± 1.5	NS
Htc on day 4 (%)	40.6 ± 1	37 ± 0.6	< 0.05[b]	40.6 ± 1.3	37.4 ± 0.9	< 0.05[b]
Htc on day 6 (%)	42 ± 1.2	37 ± 0.8	< 0.05[b]	42.2 ± 0.8	37,5 ± 1.8	< 0.05[b]
Htc on day 13 (%)	decapitation	decapitation	-	42.2 ± 0.8	38.2 ± 1.3	< 0.05[b]
Ovarian weight (mgr)	69.4 ± 7	53 ± 7	< 0.05[a]	123±18.2	60.6 ± 5.8	< 0.05[a]

Note: The values are presented as mean ± SD and %; [a] = MWU test; [b] = Chi-square test; NS = Non-significant; Htc = hematocrit

Table 2. — *Ovarian histopathology and follicle counts of all rats in the study*

Parameters	Short-term			Long-term		
	G3 (n=5, OHSS)	G5 (n=5, control)	*p* value	G4 (n = 5, OHSS)	G6 (n = 5, control)	*p* value
Primordial follicle count	11 ± 10.2	25±12	<0.05[a]	8.4 ± 5.0	17.8±13.8	< 0.05[a]
Primary follicle count	16.4 ± 2.8	19 ± 4	NS	20 ± 7.5	20.6 ± 4.2	NS
Secondary follicle count	10.8 ± 3.1	12 ± 2.8	NS	14.4 ±.3	9.6 ± 5.8	NS
Tertiary follicle count	4.4 ± 1.1	2.8 ± 3.1	NS	2.2 ± 0.8	1.4 ± 1.1	NS
Ovarian follicle reserve	42.6 ± 10	60 ± 1.5	< 0.05[a]	45± 11.1	49.4 ± 7.6	NS
CL count	0 ± 0	0 ± 0	NS	1.8 ± 1.8	0 ± 0	< 0.05[a]
Corpus albicans count	0 ± 0	0 ± 0	NS	0±0	0 ± 0	NS
Total corpus count	0 ± 0	0 ± 0	NS	1.8 ± 1.8	0 ± 0	< 0.05[a]
Follicle cyst count	0 ± 0	0 ± 0	NS	0 ± 0	0 ± 0	NS
Angiogenesis in CL	0 ± 0	0 ± 0	NS	0.4 ± 0.5	0 ± 0	< 0.05[a]
Fibrosis	0 ± 0	0 ± 0	NS	0.4 ± 0.5	0 ± 0	< 0.05[a]
Atretic follicle count	3.4 ± 1.9	0.6 ± 0.9	< 0.05[a]	2.6 ± 2	0.4 ± 0.5	< 0.05[a]

Note: Values are presented as mean±SD; [a] = MVU test; NS = Non significant; CL = Corpus luteum.

Table 3. — *Serum VEGF and endothelin-1 levels of all rats in the study*

Parameters	Short-term			Long-term		
	G3 (n = 5, OHSS)	G5 (n = 5, control)	*p* value	G4 (n = 5, OHSS)	G6 (n = 5, control)	*p* value
VEGF(pg/ml)	2717± 324	1,596 ± 1113	< 0.05[a]	2,250 ± 952	1,777 ± 1016	NS
Endotelin-1 (ng/ml)	0.8 ± 01	0.55 ± 0.1	< 0.05[a]	0.9 ±0.1	0.8 ± 0.1	NS

Note: Values are presented as mean±SD; [a] = MWU test; NS = Non significant

tal effect on ovarian follicle reserve during short-term. These effects proceeded during long-term with insignificance. In this study the authors compared the groups according to age matching to eradicate the age bias [17].

It is reported that VEGF and IL-8 are the key mediators in pathogenesis of OHSS [18]. VEGF induces neoangiogenesis and permeability via VEGF-2 receptors on the surface of endothelial cells [19]. These changes are reversed 98% by anti-VEGF antibody [20]. In the pathogenesis of OHSS, the main stimulator of vascular hyperpermeability is the hypoxia-induced VEGF production in developing multiple corpus luteums [21- 24]. Busso *et al.* reported that gonadotrophin induced OHSS could be prevented by VEGF antagonization [25].

Investigators reported that follicular fluid VEGF levels of women either undergoing in vitro fertilization (IVF) or non-stimulated were in correlation with degree of follicular luteinization [26, 27]. Other researchers demonstrated that during ovulation induction, follicular fluid VEGF concentrations were higher in advanced reproductive age women compared with younger women or it could be said that there was an association, positive correlation, between follicular fluid VEGF levels and patient's age [28-30]. In the present study, the authors observed

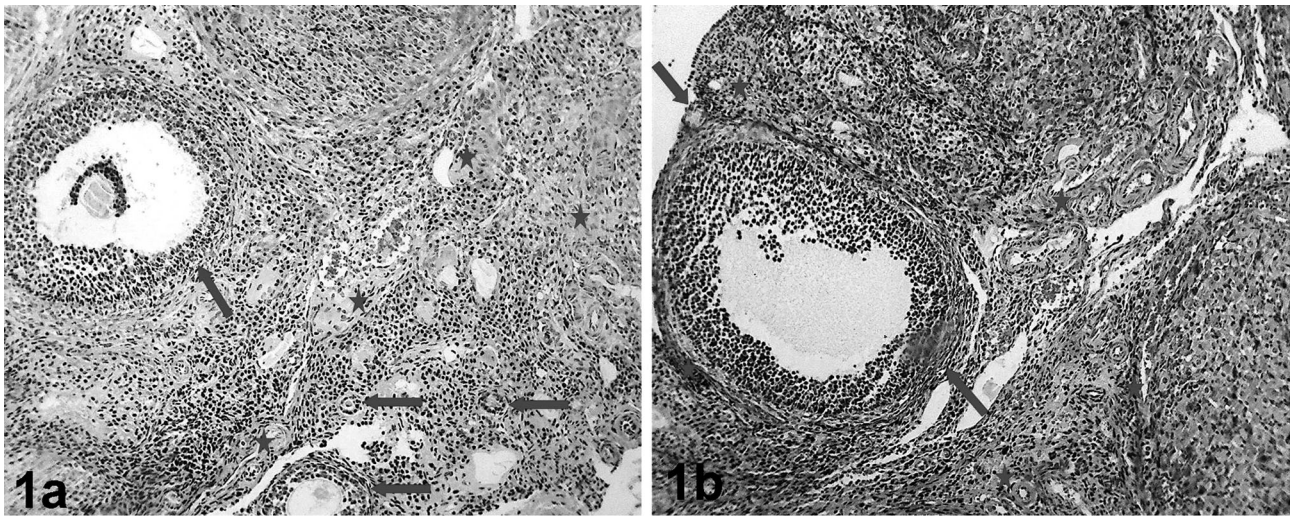

Figure 1. — Masson trichrome staining images of rat ovary under light microscope. 1a: ovarian histology of control rat; 1b: decreased ovarian follicle reserve and increased stromal fibrosis of hyperstimulated rat ovary. Arrow = different types of follicles; star = stromal fibrosis areas.

significantly increased serum VEGF and endothelin-1 levels especially during short-term of OHSS. This increment might be due to relative hypoxia of stromal tissue of hyperstimulated ovary [24, 31]. The comparison of serum VEGF and endothelin-1 levels between the non-stimulated rats showed increment with age.

The preovulatory follicle provides a unique physiological example of rapid growth accompanied by neovascularization: two processes that are generally characteristic of pathologies such as wound repair or malignancy. During the hours preceding ovulation, follicular growth is accompanied by elevated levels of messenger RNA for VEGF. Following ovulation, rapid infiltration of capillaries through the follicular wall is essential for the formation of the CL [24]. Growth and regression of CL are accompanied by growth and regression of the luteal vascular bed. VEGF is the main regulator of angiogenesis, inducing endothelial cell proliferation, migration, vascular permeability, and vessel lumen formation [32]. VEGF-dependent angiogenesis is crucial for follicular growth, and corpus luteum formation and function [33]. In the ovary VEGF can be hormonally regulated, but in other systems, the main regulator of VEGF expression is hypoxia [34-36]. The mediator of this process is hypoxia-inducible factor-1alpha (HIF1A) [34]. Avascularization and decrement of local oxygen concentrations of granulosa cells are related to ovulation [24, 36].

VEGF inhibition in the mid- or the late luteal phase induces functional luteolysis due to premature and selective death of endothelial cells [37]. Most of the studies reported that disruption of ovarian blood supply resulted with ovarian follicular reserve decrement [38-40]. Atilgan *et al.* reported that unilateral total salpingectomy induced atretic follicles, stromal fibrosis, and macroscopic follicular cystic formation on the same side ovary [40]. In another study, the researchers reported that bilateral tubal ligation performed with uni/bipolar cautery increased the numbers of CL, but decreased the regression level of angiogenesis in CL only during short-term of surgery [16]. Both of two experimental study showed the effect of hypoxia on ovarian follicular reserve and development. In the present study, angiogenesis in CL was significantly higher in rats with OHSS than normal age-matched rats during long-term. Hypoxic conditions increased the vascularisation of CL and decreased ovarian follicular reserve. These results indicate the relationship between hypoxia-induced angiogenesis and VEGF. The present study observed significantly decreased ovarian follicular reserve especially during short-term of experimental OHSS.

In the present study, endothelin-1 levels were high in rats with OHSS. It is reported that in addition to VEGF, hCG may trigger activation of the renin-angiotensin system and kinin-kallikrein system together with releasing of endothelin-1, that also increases vascular permeability [41].

In conclusion, according to the similarity of OHSS between human beings and rats, it can be said that VEGF and endothelin-1 might have a trigger function on the onset of OHSS. Multiple follicular development in OHSS may bring out relative hypoxia and induce the expression of angiogenic substances. These substances induce the manifestation of OHSS while leading to damage on ovarian follicular reserve.

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