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Vascular Endothelial Growth Factor Concentrations in Follicular Fluid Obtained from IVF-ET Patients: A Comparison of hMG, Clomiphene Citrate, and Natural Cycle

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***Purpose*:** Evaluation of vascular endothelial growth factor (VEGF) concentrations of follicular fluid (FF) in accordance with an ovarian stimulation protocol and ovarian response.
***Methods*:** The subjects of this study were 38 patients undergoing IVF-ET in our hospital. They were divided into three groups according to ovarian stimulation protocols; Group 1: hMG cycles ($n = 19$), Group 2: clomiphene citrate cycles ($n = 10$), Group 3: natural cycles ($n = 9$). They reclassified into three groups according to the number of oocytes harvested. VEGF concentration was measured in FF, employing ELISAs.
***Results*:** Group 1 shows lower VEGF concentrations in FF than Group 2 or Group 3. Excluding high responders from Group 1, no difference was found among these three groups. As for the reclassified groups, group of highest number of oocytes havested showed lowest VEGF concentrations in FF.
***Conclusions*:** VEGF concentrations in FF might negatively correlate with the number of follicles, irrespective of the ovulation induction protocol.

KEY WORDS: Follicular fluid; IVF-ET; ovarian stimulation; VEGF.

INTRODUCTION

Angiogenesis is a prominent histological component of the luteinization process (1) and angiogenic factors exist in the follicle fluid (FF) of human ovarian preovulatory follicles. Vascular endothelial growth factor (VEGF) is a powerful angiogenic factor and is produced by theca interna and granulosa cells in human ovary (2). Since VEGF increases vascular permeability, VEGF production is linked to the pathophysiology of ovarian hyperstimulation syndrome (OHSS) (3). OHSS is still one of the common complications in ovarian stimulation with gonadotrophin, and severe forms of it are sometimes life-threatening. In some reproduction centers, ovarian stimulation with gonadotrophin is avoided due to the possible risk of OHSS. Thus, clomiphene citrate or natural cycles are employed in IVF-ET protocol instead of human menopausal gonadotrophin (hMG). Since the mechanisms of ovulation induction are different among hMG cycles, clomiphen citrate cycles, and natural cycles, there arises a question whether or not this mechanical difference have any effect in the production of VEGF in a preovulatory follicle.

To address this question, we evaluated VEGF concentrations in FF in each of the ovarian stimulation protocols: hMG, clomiphene citrate, and natural cycles. We also examined the relationship of ovarian response and VEGF levels in FF.

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MATERIALS AND METHODS

Patients and Ovarian Stimulation Protocols

A total of 38 patients undergoing IVF-ET were entered in this study. Indications for IVF-ET were tubal occlusion in 12 patients, male infertility in 10, and unexplained in 9. Patients were divided into three groups according to the ovarian stimulation protocol.

Group 1 ($n = 19$; mean age: 31.7 ± 2.9) received controlled ovarian stimulation with hMG (humegone®: Organon Japan, Tokyo) under pituitary suppression with gonadotrophin-releasing hormone agonist (0.9 mg t.i.d., Suprecur®: Hoechst Japan, Tokyo). This nasal application was began in the mid luteal phase of the previous menstrual cycle and continued until the day of human chorionic gonadotrophin (hCG) administration. On Menstrual Day 5, hMG was started at a dose of 150 IU/day. The duration of hMG administration depended on the individual response. Follicle sizes were measured by transvaginal ultrasound scan. When a maximum follicle diameter of 18 mm was achieved, 10,000 IU of hCG was given i.m. in order to achieve follicle rupture; 36 h after the administration of hCG, oocyte retrieval (OR) was performed. The ovarian follicles are punctured with a 16-G needle (Cook, Australia), and follicular fluid was aspirated from the first punctured follicle only in order to avoid the contamination of flush fluid. Pure follicular fluid obtained from follicles with the diameter of over 18 mm was immediately centrifuged at $1800 \times g$ for 10 min at room temperature. The supernatant was collected and stored at $-80°C$ until the assay. On the day of hCG administration, serum was collected in order to measure the estradiol (E_2) level. According to E_2 levels, this group was subdivided into two groups: high responders ($n = 9$; mean age: 30.5 ± 3.0) and low responders ($n = 10$; mean age: 32.8 ± 2.7). The criteria for a high responder was that the E_2 level exceed 3000 pg/mL, and for the low responder it was that the E_2 level not exceed 2000 pg/mL.

In Group 2 ($n = 10$; mean age: 33.7 ± 4.8), clomiphene citrate was used for ovarian stimulation. On Menstrual Day 5, 50–100 mg/day of clomiphene citrate was started and continued until the Day 9. Monitoring of the follicle diameter was performed by transvaginal ultrasonography, and when the maximum diameter of the leading follicle reached 18 mm, 10,000 IU of hCG was administered.

In Group 3 ($n = 9$; mean age: 33.1 ± 4.14), no ovarian stimulation was employed (natural cycle). When the diameter of leading follicle reached 18 mm, 10,000 IU of hCG was administered.

In both Groups 2 and 3, OR and FF sampling and serum collection was done in the same fashion as in Group 1. Follicular fluid was obtained from the leading follicle only.

All patients included in this study were reclassified into three groups according to the number of oocytes harvested at OR, irrespective of the ovulation induction protocol. Group A ($n = 9$; mean age: 30.6 ± 3.0) over 10 oocytes were harvested, Group B ($n = 7$; mean age: 31.7 ± 2.4) 4–8 oocytes were harvested, and Group C ($n = 22$; mean age: 33.7 ± 4.1) under 3 oocytes. All members of Group 3 were included in Group C.

Measurements of VEGF and E_2

Immunoreactive VEGF was measured by a solid phase enzyme-linked immunosorbent assay (ELISA) according to the manufacture's instructions (R&D Systems INC, Minneapolis, MN). Intra- and interassay coefficients of variation (CV) were less than 10%. E_2 was measured by a commercially available radioimmunoassay method. Intra- and interassay coefficients of variation did not exceed 15%.

Statistical Analysis

To determine whether there were differences between the groups, an analysis of variance (ANOVA) test was performed for VEGF concentrations. The serum estradiol and VEGF relationship was analyzed employing regression analysis. All evaluations were done using *Statview* software. Values are indicated by means ± SD. $P < 0.05$ was considered significant.

RESULTS

There was no significant difference in mean age or other clinical backgrounds among these groups. Figure 1 demonstrates the comparison of the three groups on mean VEGF concentrations in FF. Group 2, clomiphene citrate cycle, shows the highest level (3151.0 ± 1597.9 pg/mL), and Group 1 shows lower level of VEGF concentrations in FF compared to the other two groups. Statistical significance was found between Groups 2 and 1 (2055.7 ± 1059.6 pg/mL) only ($P = 0.035$). In Group 2 versus 3 (3148.9 ± 1986.4 pg/mL), and Group 3 versus 1, no statistical

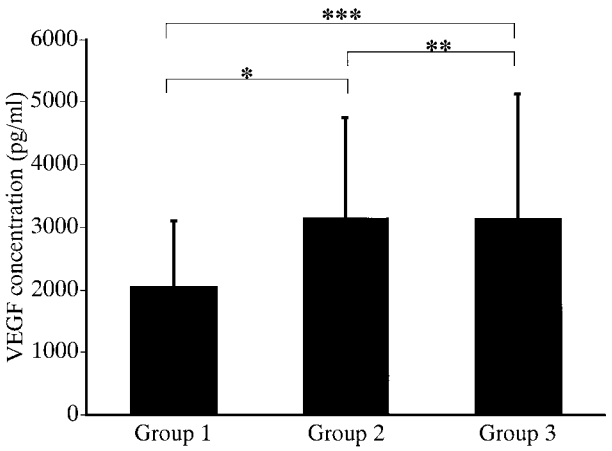

Fig. 1. This figure demonstrates the comparison of VEGF (pg/mL) levels in each group classified according to the ovarian stimulation protocol. Note that statistical significance is only found between Groups 1 and 3. $^{*}P = 0.035$, $^{**}P = 0.998$, $^{***}P = 0.067$.

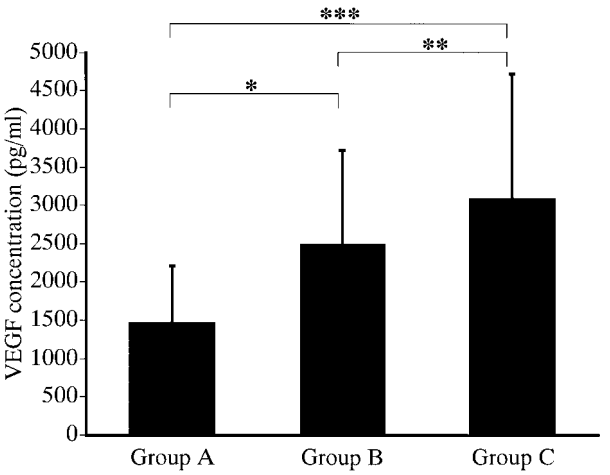

Fig. 3. The comparison of VEGF (pg/mL) concentrations in each group classified according to the number of oocytes harvested at OR. Statistical significance is found only between Group A and C. $^{*}P = 0.162$, $^{**}P = 0.329$, $^{***}P = 0.006$.

significances were found. Mean VEGF levels of high responders (1479.8 ± 732.1 pg/mL) were significantly lower than those of low responders (2574.0 ± 1067.3 pg/mL). Mean VEGF levels of high responders were significantly lower than the rest of the groups (Fig. 2). Low responders versus Group 2, and low responders and Group 3, showed no significant difference. The number of the oocytes harvested at OR was as follows; high responders: 18.4 ± 8.2, low responder: 4.9 ± 2.1, Group 2: 1.3 ± 0.5, and Group 3: 1.2 ± 0.4.

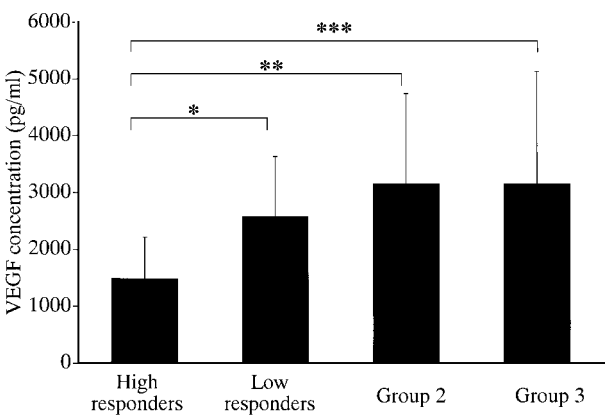

Fig. 2. Statistical significance is found between the high responders versus the low responders, high responders versus Group 2 and high responders versus Group 3. Comparison of high responders and other groups are indicated in this figure. $^{*}P = 0.020$, $^{**}P = 0.011$, $^{***}P = 0.031$.

To investigate the relationship between ovarian response and VEGF concentration in FF, serum concentrations of estradiol on the day of hCG administration were measured. Serum E_2 level negatively correlate with the VEGF concentration in FF (Correlation coefficient: $R = 0.440$, $P = 0.006$). In respect to the number of oocytes harvested at OR, Group A showed significantly lower levels of VEGF in FF than did Group C (Fig. 3).

DISCUSSION

Controlled ovarian hyperstimulation (COH) is a very important procedure in IVF-ET or other assisted reproductive technologies. COH can increase the number of mature follicles, but as number of mature follicles increases, the risk of OHSS also increases. The incidence of OHSS is reported to be between 0.2 and 1% of all assisted conception cycles (4).

Recently, in some institutions, ovarian stimulation with hMG has been avoided because of the known risk of OHSS, and so clomiphene citrate and natural cycles are employed in IVF-ET. Several reports on the relationship of OHSS and VEGF levels in serum or FF have been published. In some reports, expansion of vascular permeability in OHSS is attributed to locally produced VEGF (5–7). Other reports suggests that serum VEGF concentrations have a predictive value for OHSS (8,9), but other reports do not sustain this hypothesis (10,11). The exact role of VEGF in the pathogenesis of OHSS is still a focus of controversy.

In ovary, VEGF is mainly produced in granulosa cells, and the production of VEGF is augmented by the hCG stimulation (12). VEGF levels in preovulatory folicles are 10 times higher than those in serum. Anasti *et al*. (13) reported that the mean VEGF concentration in spontaneous ovulation (natural cycles) was 6900 ± 644 pg/mL. But to our knowledge, there is no available data in cases of clomiphen citrate cycle.

The purpose of our study was to investigate the differences of VEGF concentrations in FF according to the ovarian stimulation protocol; hMG, clomiphen citrate and natural cycles. Hormonal environment of the patients from each group was different due to the particular mechanism which stimulated the follicular growth in each of the protocols. In hMG cycles, extragenously administered gonadotrophin stimulated the follicles and E_2 levels much higher than in the other two groups. In clomiphene citrate cycles, endogenous gonadotrophin release was stimulated and the antiestragenic effects of clomiphene citrate in the peripheral tissue were characteristic to this protocol. So it is predictable that a difference of VEGF production levels might exist among these three groups.

In this study, VEGF concentrations in FF obtained from clomiphene citrate cycles were significantly higher than that from hMG cycles. Group 3, natural cycles, showed comparative level of VEGF concentrations in FF to Group 2. Although statistical significance was not found between Group 3 and Group 1, Group 1 showed lower level of VEGF concentrations in FF ($P = 0.067$). To investigate the reason of lower VEGF concentrations in FF obtained from hMG cycles, we divided Group 1 into two groups: high responders and low responders. High responders showed significantly lower levels of VEGF concentrations in FF than did the other three groups including low responders. On the other hand, VEGF concentrations in FF obtained from low responders did not show significant difference, when compared with Group 2 and Group 3. High responders largely contribute to the lower VEGF concentrations in FF of Group 1. In other words, when high responders were excluded from Group 1, Group 1 did not show significant difference. This observation implies that ovarian stimulation protocol does not exert effect on the production of VEGF in a follicle.

The observation that VEGF concentrations in FF from high responders showed lower value suggests the possibility of higher ovarian response having a negative effect on VEGF production in a follicle. So we examined the relationship between the number of oocytes harvested at OR and the VEGF concentration in FF. The finding that Group A shows significantly lower VEGF levels in FF than does Group C also sustained this notion. Increased numbers of matured follicles may suppress the production of VEGF in each one of the follicle at the time of ovulation. This notion is further strengthened by the observation that serum E_2 concentration is negatively correlated with the VEGF level in FF. But these findings do not deny the VEGF involvement in the pathophysiology of OHSS. Because, in high responders, increased number of matured follicle could overcome the suppressed production of VEGF in each one of the follicle. Although we do not have the available data, there is a possibility that total VEGF production in OHSS patients is higher than in other patients in whom OHSS did not develop.

In conclusion, the VEGF concentration in each of the follicle is negatively correlated to the number of matured follicles, irrespective of the ovarian stimulation protocol.

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