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ORIGINAL ARTICLE

Comparison of follicular fluid amphiregulin and EGF concentrations in patients undergoing IVF with different stimulation protocols

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Abstract Epidermal growth factor (EGF)-like growth factors, such as amphiregulin (AR) and EGF, have emerged as mediators to propagate Luteinizing hormone (LH) stimulus for the oocyte maturation throughout the pre-ovulatory follicle, because cumulus cells and oocytes express few or no LH receptors. This study was to compare AR and EGF concentrations in follicular fluid (FF) among four controlled ovary stimulation (COS) protocols and to investigate the relationship between FF EGF-like growth factors and COS outcomes. Ninety-five patients who underwent in vitro fertilization–embryo transfer (IVF–ET) were treated by four different COS protocols, including gonadotropin-releasing hormone agonist (GnRH-a) long protocol, GnRH-a ultra-long protocol, GnRH-a short protocol, and GnRH antagonist protocol. FF was taken on oocyte retrieval day. FF AR and EGF concentrations were measured and their correlations with COS outcomes were analyzed. FF AR concentration was significantly different from each other among four COS protocol groups (GnRH-a ultra-long protocol group, 186.12 ng/ml; GnRH-a long protocol group, 128.35 ng/ml; GnRH antagonist protocol group, 108.23 ng/ml; GnRH-a short protocol group, 77.13 ng/ml, $p < 0.05$). FF AR concentrations were higher in GnRH-a ultra-long and long protocol groups, while number of oocytes retrieval, available embryos, and good quality embryos in these two groups were also significantly higher than GnRH-a short protocol group and GnRH antagonist protocol group. FF AR concentration was positively correlated with available embryos, but negatively correlated with serum LH level on hCG day. FF EGF concentration had no relationship with COS parameters. Different COS protocols might have variable effects on AR synthesis. FF AR might be a good indicator to predict the number of oocytes and embryos. FF AR elevation may result in increasing the number of oocyte retrieval and embryo generation, consequently increased cumulative pregnancy rate.

Keywords Amphiregulin · EGF · Human follicular fluid · GnRH analog · COS · IVF

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Introduction

Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) control the follicle development and ovulation [1]. The mid-cycle LH surge plays a pivotal role in the

oocyte maturation and ovulatory process, which includes a cascade of events such as resumption of meiosis, expansion and mucification of cumulus cells (CC), termination of granulosa cells division, and proteolysis of the follicular wall [1, 2]. However, the precise physiologic signaling network is complicated by which gonadotrophins successfully release a fertilizable oocyte at ovulation. LH can directly stimulate theca and mural granulosa cells, these cells express LH receptor (LHR). But CCs and oocytes express few or no LHRs and they are insensitive to direct LH stimulation [3]. From this point, there need to have some autocrine and paracrine signals to propagate LH stimulus throughout the preovulatory follicle [4].

In recent years, EGF-like growth factor family members have emerged as likely mediators of LH action in the follicle. EGF-like growth factor family includes EGF, AR, epiregulin, betacellulin, epigen, and neuregulins. These growth factors have an EGF-like motif organization synthesized as integral membrane precursors. They bind homo- and heterodimers of the EGF receptor (EGFR) to trigger a signaling network that mediates cell–cell interactions in cell development and oncogenesis [5]. Recently, more and more studies confirmed that EGF-like growth factors act to mediate the LH stimulation of oocyte maturation, ovulatory enzyme expression, and ovulation [6]. When either mouse preovulatory follicle cultures or isolated mouse germinal vesicle stage (GV) cumulus–oocyte-complexes (COCs) were incubated with AR, they can recapitulate the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation [2]. LH stimulation led to rapid transactivation of the EGF network in mice preovulatory follicles to amplify the initial LH signal for oocyte maturation [7]. Addition of AR resulted in complete stimulation of the resumption of meiosis in rat oocytes [8]. As for human beings, isolated human granulosa cells were found to show a dramatic up-regulation of AR in both the mRNA and protein levels with LH stimulation [9–11]. After human chorionic gonadotropin (hCG) injection, bioactive AREG accumulation was induced in human FF and incubation of mouse COCs with human FF-induced CC expansions and oocyte maturation [12].

There is considerable controversy as to whether the presence of EGF-like growth factors in FF can be used as a predictor of follicle maturity and oocyte quality. Ozornek et al. [13] reported an inverse correlation between FF EGF level and IVF outcome. Inoue et al. [14] also found that FF AR was inversely related to oocyte quality, fertility rate, and pregnancy outcome. However, human GV stage oocytes exposed to EGF can significantly increase maturation rate as compared to controls [15]. And supplementation of the maturation medium with AR improved GV oocytes the maturation in vitro at human [16]. Humaidan et al. [17] had compared FF AR levels from the GnRH-a versus the hCG during triggering of final oocyte maturation in IVF cycle, the authors considered different oocyte maturation rate might be achieved through the design of alternative protocol for triggering ovulation.

GnRH-a and GnRH antagonists have been used widely to prevent premature LH surges during COS for IVF–ET. Now the popular COS protocols include: GnRH-a long protocol, GnRH-a ultra-long protocol, GnRH-a short protocol, and GnRH antagonist protocol. Different COS protocols may impact growth factor synthesis in FF, and produce different quality oocytes in IVF. The aims of this study were to (1) compare AR and EGF concentrations in FF among four different COS protocols; (2) investigate the relationship between EGF-like growth factors and IVF outcomes.

Materials and methods

Subjects

Ninety-nine women who underwent IVF/intracytoplasmic sperm injection (ICSI) were prospectively recruited at Peking University Third Hospital Reproductive Centre from January 2011 to August 2011. This study was approved by the institutional ethics committee of Peking University. All patients were obtained informed consent for this study. All involved patients had regular menstrual cycles. Their fertile causes were tubal and/or male factors. Patients with uterine fibroids, endometriosis, endocrinopathies, such as polycystic ovary syndrome, premature ovary failure were excluded. Patients with history of poor ovarian response to COS or total IVF/ICSI times $\geq$3 were also excluded. The age of subjects was between 20 and 39 years old. Body mass index (BMI) was calculated as body weight (kg) divided by body height squared (m^2). BMI of subjects was between 18 and 25 kg/m^2. Day 3 serum FSH level was $\leq$10 IU/l.

Controlled ovarian stimulation protocols and oocytes retrieval

At last, we enrolled 95 subjects for the following reasons: three patients cancelled embryo transfer because of ovarian hyperstimulation syndrome (OHSS) or prevention of OHSS occurrence and one patient cancelled ET for serum progesterone (P) was more than 3 nmol/l on hCG day. 95 subjects were treated by four different COS protocols for IVF/ICSI which were GnRH-a long protocol (28 subjects), GnRH-a ultra-long protocol (20 subjects), GnRH-a short protocol (27 subjects), and GnRH antagonist protocol (20 subjects).

GnRH-a long protocol: 1.8 mg GnRH-a (triptorelin acetate) was administrated at the mid-luteal phase of the previous cycle. When patients came back 14 days later, ovarian stimulation was started if pituitary down-regulation was established (serum estradiol (E_2) <280 pmol/l, P <1 nmol/l, FSH <5 IU/l, and LH <5 IU/l).

GnRH-a ultra-long protocol: 3.75 mg GnRH-a (triptorelin acetate) was given on cycle day 1. Patients came back 28–32 days later and ovarian stimulation was started whose requirement was the same as long protocol.

GnRH-a short protocol: GnRH-a (triptorelin acetate, 0.1 mg/day) was administered from cycle day 2 until hCG injection day. Ovarian stimulation was started on day two or three if patients met the following conditions: serum E_2 <280 pmol/l, P <1 nmol/l, and FSH <10 IU/l.

GnRH antagonist protocol: ovarian stimulation was started on cycle day 2 if patients met the following conditions: serum E_2 <280 pmol/l, P <1 nmol/l, and FSH <10 IU/l. If one follicle ≥14 mm was observed, GnRH antagonist (ganirelix, 0.25 mg/day) was administered daily until hCG injection.

In these four protocols, ovarian stimulation was started with a starting dose varying from 150 to 300 IU/day recombinant FSH (r-FSH) according to patients' age and ovarian reserve. Dose of r-FSH can be adjusted and individualized per subject based on the follicular growth. When there were three or three more largest follicle reached 18 mm in diameter, hCG 10000 IU was administered to induce final oocyte maturation. Oocyte retrieval and fertilization were performed according to standard procedures, as described previously [18]. If the female age <35 years old, two embryos were transferred on day 3 and if female age ≥35 years old or the husband was diagnosed as severe oligozoospermia three embryos were transferred. Luteal phase supplementation was given on the evening of oocyte pick-up day until 14 days later (progesterone, 180 mg/day, intravaginally or 60 mg/d, i.m.). Embryo morphology was assessed according to the Society for Assisted Reproductive Technology (SART) embryo grading system [19]. Biochemical pregnancy was defined as a positive serum hCG 14 days after embryo transfer. Clinical pregnancy was confirmed as gestational sac observation under the transvaginal ultrasonographic at 7 weeks'.

Sample collection

During oocyte retrieval, FF from the leading follicle was aspirated from each ovary and collected. Any FF contaminated with blood was discarded. FF samples were centrifuged for 10 min at 500×g to separate red blood cells, leukocytes, and granulosa cells. The samples were frozen without preservatives and stored at −80 °C until assayed. We obtained 95 FF from each subjects and only a single FF sample from each patient was used for analysis. The volume of each FF was between 1.3 and 4.0 ml.

Fasting blood samples from all subjects were collected on day 2–5 of a natural cycle for basal FSH, LH, T, E_2 assay, on hCG injection day for LH, E_2, P assay and on oocyte retrieval day of COS cycle for AR, EGF assay. Serum was separated and frozen in aliquots at −80 °C for subsequent analysis.

Biochemical assay

FF EGF and AR concentrations were determined using commercially available sandwich ELISA kits (DuoSet Economy Pack; R&D Systems Inc.) in accordance with the manufacturer's instructions. The coefficients of variability (CV) for EGF and AR were functional sensitivity 0.1 pg/ml, intra-assay CV 4 %, inter-assay CV 8 %. Serum LH, FSH, E, P, T levels, and FF hCG level were determined by chemiluminescence immunoassay.

Statistical analysis

Kolmogorov–Smirnov test was used to analyze whether clinical parameters were normal distribution. Mean differences among groups were compared by one-way analysis of variance (ANOVA). Chi-square test was used to analyze the associations between categorical variables. Spear-man's test was used to determine the coefficient of correlation (r) of FF AR, EGF levels with other parameters. All analyses were performed by SPSS software version 18.0 (SPSS Inc. Chicago, IL, USA). Tests of statistical significance were two sided and taken as significant when $p < 0.05$.

Results

Patient characteristics and COS parameters

There were no significant differences among four COS protocol groups in terms of the following clinical characteristics: age, BMI, day 3 serum FSH, LH, E, T levels or follicle number per ovary (Table 1).

The duration of COS was significantly longer in GnRH-a ultra-long protocol and GnRH-a long protocol groups than GnRH-a short protocol and GnRH antagonist protocol groups (12.88 days, 12.78 days vs 10.00 days, 10.10 days, $p < 0.05$). The most of the total dose of gonadotrophins was used in GnRH-a ultra-long protocol group (3,151 IU), and the second in GnRH-a long protocol group (2,606 IU). Both the groups were administered significantly with total dose of gonadotrophins as compared to GnRH-a short protocol and GnRH antagonist protocol groups (1,916 and 2,166 IU, $p < 0.05$) (Table 2).

Table 1 Subjects characteristics in four COS groups

	GnRH-a long protocol ($n = 28$)	GnRH-a ultra-long protocol ($n = 20$)	GnRH-a short protocol ($n = 27$)	GnRH antagonist protocol ($n = 20$)	p value
Age (years)	30.55 ± 3.28	30.90 ± 3.32	31.35 ± 4.46	32.00 ± 3.55	NS
BMI	21.63 ± 2.31	23.14 ± 3.45	22.28 ± 3.12	23.70 ± 4.41	NS
Basal FSH (mIU/ml)	7.01 ± 1.64	6.51 ± 1.29	6.38 ± 1.68	7.41 ± 2.32	NS
Basal LH (mIU/ml)	5.25 ± 4.47	4.11 ± 2.37	3.80 ± 1.32	3.69 ± 1.40	NS
Basal E_2 (pmol/l)	163.74 ± 81.80	153.25 ± 49.53	137.15 ± 63.06	137.25 ± 58.85	NS
T (nmol/l)	0.87 ± 0.25	0.79 ± 0.26	0.79 ± 0.19	0.88 ± 0.53	NS
Follicle number per ovary (n)	5.24 ± 0.43	5.17 ± 0.33	5.21 ± 0.43	5.27 ± 0.89	NS

Continuous variables are expressed as mean ± SD

NS not significant

Table 2 Subjects characteristics in four COS groups

	GnRH-a long protocol ($n = 28$)	GnRH-a ultra-long protocol ($n = 20$)	GnRH-a short protocol ($n = 27$)	GnRH antagonist protocol ($n = 20$)
Duration of COS cycle (days)	12.78 ± 1.15[c]	12.88 ± 0.97[c]	10.00 ± 1.54[f]	10.10 ± 0.85[f]
Dose of total gonadotropins consumption (IU)	2606.01 ± 835.76[d]	3151.25 ± 943.02[e]	1916.96 ± 579.14[f]	2166.25 ± 668.34[f]
Serum E_2 on hCG day (pmol/l)	15202.22 ± 7507.78	15026.63 ± 8557.02	12803.60 ± 5559.57	11994.80 ± 4838.81
Serum LH on hCG day (IU/l)	0.83 ± 0.35[d]	0.44 ± 0.21[e]	2.76 ± 1.31[f]	2.75 ± 2.29[f]
Serum P on hCG day (nmol/l)	3.11 ± 1.62	3.57 ± 1.54	3.72 ± 1.39	3.53 ± 1.52
No. of oocytes retrieved	17.52 ± 7.63[c]	17.90 ± 8.51[c]	13.71 ± 5.66[f]	11.90 ± 1.92[f]
Fertilized rate	69.09 %	69.59 %	62.54 %	66.23 %
2PN rate	62.23 %	63.45 %	57.67 %	59.58 %
No. of total available embryo[a]	9.37 ± 4.93[c]	9.12 ± 4.12[c]	5.68 ± 3.23[f]	5.56 ± 2.04[f]
Total available embryo rate	78.89 %	76.54 %	72.20 %	73.04 %
No. of good quality embryo[b]	6.15 ± 3.96[c]	5.82 ± 3.08[c]	3.92 ± 2.47[f]	4.10 ± 2.95[f]
Good quality embryo rate	51.84 %	48.07 %	49.54 %	52.76 %
Clinical pregnancy rate	42.90 %	40.00 %	33.33 %	40.00 %

Continuous variables are expressed as mean ± SD

[a] No. of available embryo means that the sum of number of transferred embryos and number of cryopreserved embryos from each subjects

[b] Embryos considered good quality were morphologic grade I/V or II/V and at least seven blastomeres on day 3 after fertilization

[c] Significantly different from GnRH-a short protocol group and GnRH antagonist protocol group, $p < 0.05$

[d] Significantly different from GnRH-a ultra-long protocol group, GnRH-a short protocol group, and GnRH antagonist protocol group, $p < 0.05$

[e] Significantly different from GnRH-a long protocol group, GnRH-a short protocol group, and GnRH antagonist protocol group, $p < 0.05$

[f] Significantly different from GnRH-a long protocol group and GnRH-a ultra-long protocol group, $p < 0.05$

Serum LH level on hCG day in GnRH-a ultra-long protocol group was significantly lower than that in GnRH-a long protocol group (0.44 vs 0.83 IU/l, $p < 0.05$); Serum LH level on hCG day was similar in GnRH-a short protocol and GnRH antagonist protocol groups (2.76 vs 2.75 IU/l, $p > 0.05$), but both groups were significantly higher than GnRH-a long protocol group ($p < 0.01$). Serum E_2 and P level on hCG day did not differ among four COS group (Table 2).

In GnRH-a long protocol and GnRH-a ultra-long protocol groups, the number of oocytes retrieved (17.52 and 17.90 vs 13.71 and 11.90, $p < 0.05$), total available embryos (9.37 and 9.12 vs 5.68 and 5.56, $p < 0.05$), the number of high-quality embryos (6.15 and 5.82 vs 3.92 and 4.10) were significantly higher than that in GnRH-a short protocol and GnRH antagonist protocol groups. However, other parameters such as fertilization rate, two-pronuclear zygotes (2PN) rate, high-quality embryos rate, and total available embryos rate were not significantly different among four COS groups. Clinical pregnancy rate per transfer was similar among GnRH-a long protocol, GnRH-a ultra-long protocol, and GnRH antagonist protocol groups. However, clinical pregnancy rate was lowest in GnRH-a short protocol group, but it was not significantly

different from other three COS groups (42.9, 40.0, 40.0 and 33.3 %, Table 2).

FF and serum AR and EGF concentrations

FF AR concentration was significantly different among four COS groups, in a descending order as following: GnRH-a ultra-long protocol, GnRH-a long protocol, GnRH antagonist protocol, and GnRH-a short protocol groups (186.12, 128.35, 108.23, and 77.13 ng/ml, $p < 0.05$). No significant differences were found regarding FF EGF, FF hCG, serum AR, and serum EGF levels among four COS groups (Table 3).

AR level in FF was 2,000 times as high as that in serum. EGF level in FF was similar to that in serum. In FF, AR levels were about 13,600 times as high as EGF levels (Table 3).

Correlations of FF AR, EGF concentration with COS outcomes

FF AR concentration correlated positively with number of available embryos and correlated negatively with serum LH level on hCG day (Fig. 1). There was no significant correlation between FF AR concentrations and FF hCG levels. In addition, there were no significant relationships between FF EGF concentrations and FF hCG levels, the number of oocytes retrieved, available oocytes or good quality embryos.

Discussion

To our knowledge, this is the first study to compare FF AR and EGF concentrations with COS cycles and to analyze correlation between FF AR concentration and IVF outcomes. From this study, we identified that FF AR concentration was significantly different among four COS protocol groups. FF AR concentration was positively correlated with the number of available embryos and negatively correlated with serum LH level on hCG day. FF AR level was about 2,000 times as high as serum AR level on hCG day and 13,600 times as high as FF EGF level. FF EGF concentration had no correlation with COS outcomes.

Recently, a number of studies had confirmed EGF-like growth factors, such as AR or EGF could mimic LH function to stimulate the oocyte maturation of preovulatory follicle, FF AR was associated with IVF outcomes [2, 4, 12, 14]. For the first time, we compared FF AR concentration with four COS protocol groups and found it was significantly different from each other. In a descending order, FF AR concentration was highest in GnRH-a ultra-long protocol group, the second in GnRH-a long protocol group, the third in GnRH antagonist group, and the last in GnRH short protocol. We thought there were two aspects linked with this phenomenon. First, it was different that the type and dose of the GnRH analogs were used to prevent premature LH surge in four COS protocols. GnRH receptor was detected in preovulatory follicles and localized predominantly to the granulosa cell layer [20]. With follicle growing, GnRH receptor expression became more and it went to the highest level in granulosa cells of Graafian follicle. GnRH analogs (both agonist and antagonist), by binding these GnRH receptors, might influence synthesis of locally produced growth factors during folliculogenesis and then affect the secretion of hormones and quality of oocytes [21]. Singh et al. [22] recently reported when mice were treated with different doses of GnRH agonist, high dose treatment showed significantly increased expression of LHR protein in the ovaries. LHR is critical for its activation can increase MMP-mediated cleavage of membrane-bound EGF-like growth factors molecules [12, 17,

Table 3 Concentrations of AR, EGF, and hCG in FF and serum

	GnRH-a long protocol ($n = 28$)	GnRH-a ultra-long protocol ($n = 20$)	GnRH-a short protocol ($n = 27$)	GnRH antagonist protocol ($n = 20$)	p value
FF AR (ng/ml)	128.35 ± 55.17[a]	186.12 ± 99.23[b]	77.13 ± 31.94[c]	108.23 ± 30.18[d]	0.001
FF EGF (pg/ml)	8.57 ± 3.96	8.33 ± 4.62	10.14 ± 3.88	8.55 ± 4.35	NS
Serum AR on hCG day (pg/ml)	58.51 ± 8.03	56.04 ± 6.69	58.05 ± 10.11	61.53 ± 10.41	NS
Serum EGF of hCG day (pg/ml)	6.07 ± 6.52	5.57 ± 1.21	6.01 ± 3.14	6.54 ± 1.57	NS
FF hCG (mIU/ml)	145.66 ± 50.56	138.36 ± 58.94	130.74 ± 46.76	169.00 ± 38.38	NS

Continuous variables are expressed as mean ± SD

NS not significant

[a] Significantly different from GnRH-a ultra-long protocol group, GnRH-a short protocol group, and GnRH antagonist protocol group, $p < 0.05$

[b] Significantly different from GnRH-a long protocol group, GnRH-a short protocol group, and GnRH antagonist protocol group, $p < 0.05$

[c] Significantly different from GnRH-a ultra-long protocol group, GnRH-a long protocol group, and GnRH antagonist protocol group, $p < 0.05$

[d] Significantly different from GnRH-a ultra-long protocol group, GnRH-a short protocol group, and GnRH long protocol group, $p < 0.05$

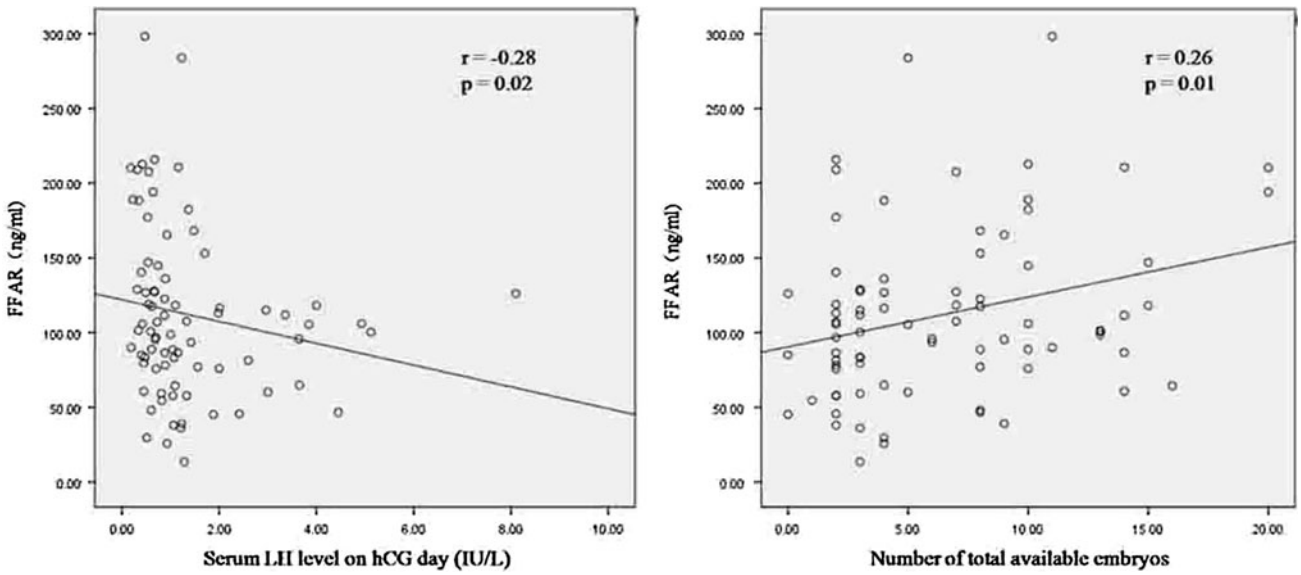

Fig. 1 Correlations of FF AR with number of available embryos and the serum on hCG day

23]. From this aspect, 3.75 and 1.8 mg GnRH-a in GnRH-a ultra-long and long protocol groups might stimulate mural granulosa cell to express more LHR than other protocols. After hCG injection, more AR was produced in these two groups. Second, we also found that in GnRH-a protocol and GnRH-a ultra-long groups, total gonadotropins consumption were significantly more than other two groups. Chen et al. [24] reported that gonadotropins (FSH and LH) could endogenously stimulate AR expression by PKC signal pathway and AR functionally mimicked FSH action on porcine oocyte meiotic resumption. Freimann et al. [9] studied when human granulosa cells were cultured with FSH, AR mRNA and protein increased markedly. Thus, more gonadotropins might induce more AR synthesis, as in our study higher FF AR concentration in GnRH-a long and ultra-long protocol groups follows higher total dose of gonadotropins.

The surge of gonadotrophins from the pituitary, including FSH and LH triggers a cascade of events to drive ovulation. AR has been considered as a favorable mediator to conduct FSH and LH signaling for oocyte nuclear maturation and cumulus expansion [4]. We found in GnRH-a long and ultra-long protocol groups, accompanied by high FF AR concentration, the number of oocytes retrieved, available embryos, and good quality embryos were also significantly higher than those in other groups. When using correlation analysis, FF AR concentration was positively correlated with the number of available embryos. Zamah et al. [12] also found human FF AR accumulation was useful to gonadotrophin stimulation and oocyte competence. Feuerstein et al. [25] found that elevated AR mRNA levels in CCs were positively associated with oocyte nuclear maturation in IVF patients. Conversely, Inoue et al. [14] reported high levels of AR appeared to be inversely correlated with the fertilization rate and embryo quality. FF AR activated the EGF receptor (EGFR) which was a tyrosine kinase on the CC. Sustained EGFR activity was necessary for the chronic phosphorylation of the ERK1/2 signaling molecules [26]. The latter induced the change in gene expression of CCs like Ptgs2, Has2, and Tnfaip6 which were critical for remodeling of the extracellular matrix to switch on COC expansion and ovulation [4]. Norris et al. [27] reported EGFR kinase signaling pathway was required for COC gap junction closure and an essential component to decrease oocyte cGMP level and then restarted the meiotic cell cycle in preovulatory follicle. From this point, high FF AR concentrations contributed to produce more good quality oocytes and available embryos. Increased number of embryos available for improving embryo selection and cryopreservation may consequently result in increased cumulative pregnancy rates [28]. Thus, GnRH-a long protocol using more doses of GnRH-a and gonadotropins via producing more AR can result in retrieving more oocytes and generating more available embryos to potentially increase cumulative pregnancy rates, which may be helpful for poor responders or old patients.

We also found FF AR concentration was negatively correlated with serum LH level on hCG day. In COS process, serum LH level usually was suppressed at a low range to prevent premature LH surge. We used hCG injection to mimic LH surge to trigger final oocyte maturation, and FF AR production was not the direct event of serum LH during COS [29, 30]. However, we speculated that serum LH level might have effects on LHR sensitivity on mural granulosa cell and further affects LH downstream

signaling pathway like MAPK system, which was critical for inducing AR synthesis [31]. Our hypothesis was that LHR sensitivity might increase and combine hCG more effectively under lower serum LH level environment. These changes may transfer downstream signaling and then result in increased AR production. This phenomenon needs further researches to confirm its mechanisms.

Some studies found AR was most abundant EGF receptor ligand in FF from infertile patients undergoing IVF [12, 14, 16, 17]. In our study, FF AR concentration of all subjects ranged from 40 to 300 ng/ml. Its mean level was about 120 ng/ml, which was far higher than FF EGF concentration and serum AR level (8 and 60 pg/ml). This result suggested AR was synthesized specially in follicles. Our findings were consistent with Inoue' result in which FF AR concentration was 108.4 ng/ml [14]. In other two studies FF AR concentrations were also high, 40,000–80,000 pg/ml and 50–70 ng/ml, respectively [12, 16]. FF AR concentration in our study was little higher than that in other studies. We thought this difference might be associated with different human races and ethnicities. Another possible reason was that different COS protocols were used in different studies.

We failed to find the correlation between FF AR concentration and FF hCG level as Zamah's [12] reported a positive association of AREG and hCG in FF. The differences may be from the following facts. First, AR mRNAs increased 3 h and remained elevated at 24 h post-hCG in granulosa cells obtained from rhesus macaques undergoing COS protocols [32]. But FF sample would not be obtained so early for oocytes retrieval started at 36 h after hCG injection with human being. Second, Jamnongjit and Conti [4, 23] described a model for gonadotropin-induced oocyte maturation that LH binded to LHRs on theca cells or mural granulosa cells and resulted in MMP-mediated cleavage of membrane-bound EGF molecules. It seemed LH or hCG in serum but not in FF was a critical factor for producing FF AR. Third, in Zamah's studies, he did not described the cause of infertile patients and the number of FF samples for hCG detection in detail. In our studies, all patients were infertile for tubal obstruction or male factor. Different reasons like endometriosis or endocrine diseases may have unequal effects on AR production.

FF EGF concentration was similar to serum EGF level on hCG day (8 and 6 pg/ml) and both of them were very low in our study. It indicated that the presence of EGF in FFwas derived from serum and was not produced locally. In Reeka' study, FF EGF concentration was very low too, < 10 pg/ml [33]. Westergaard et al. [34] reported FF EGF levels declined with increasing diameter of the follicle and the diameter of preovulatory follicle reached peak and FF EGF level went down. We did not find any difference about FF EGF in four COS groups. There were no correlation between FF EGF concentration and COS parameters, like oocytes number and FF hCG concentration. This finding was in accordance with other studies conclusions that paracrine actions of EGF-like growth factors, rather than EGF, mediate LH effects during the periovulatory period [2, 4, 8]. Gomez et al. [35] added EGF to culture media before insemination, but found no positive effects on either the fertilization rate or human blastomere development. Therefore, our results demonstrated that FF EGF had little effects on COS outcomes.

Different pituitary down regulation drugs were used in four COS protocols and the mechanisms of preventing premature LH surge were different. In GnRH-a ultra-long and long protocol groups, the days of COS cycle and total dose of gonadotropins consumption were significantly more than the other two groups. Accordingly, the number of oocyte retrieval, available embryos, and good quality embryos were significantly more in these two GnRH-a protocol groups. These results shed the same characters as many other studies. In Maheshwari et al. [36] report, there was an increased number of oocytes and an increase in the requirement for gonadotrophins when a long protocol was used as compared to a short protocol. Meanwhile, the fertilization rate, good quality embryo rate, and pregnancy rate were not significantly different among four groups in this study. This result was consistent with other studies in which a similar clinical efficacy was achieved when using GnRH antagonist compared with GnRH agonist [37, 38].

Based on this study, we had some limitations, such as (1) we could not get the FF from each separate follicle to compare every corresponding oocyte quality, (2) we could not obtain FF samples after hCG injection at every time course so we did not get the real information of AR metabolism characters in FF, and (3) the mean number of oocytes was 15 which was a little more than other studies usually reported. Main infertile reasons of patients in this study were tubal obstruction and male factors. The ovarian response to gonadotropin was more sensitive than other infertile patients.

In conclusion, FF AR concentrations varied from different stimulation protocols, which might impact on AR synthesis. FF AR might be a good indicator to predict the number of oocytes and embryos. The elevation of FF AR may result in increased the number of oocyte retrieval and embryos generation, consequently increased cumulative pregnancy rate.

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Conflict of interest None.

References

1. A. Ulloa-Aguirre, T. Zariñán, A.M. Pasapera, P. Casas-González, J.A. Dias, Multiple facets of follicle-stimulating hormone receptor function. Endocrine **32**, 251–263 (2007)
2. H. Ashkenazi, X. Cao, S. Motola, M. Popliker, M. Conti, A. Tsafriri, EGF-like growth factors as mediators of LH action in the ovulatory follicle. Endocrinology **146**, 77–78 (2005)
3. X.R. Peng, A.J. Hsueh, P.S. LaPolt, L. Bjersing, T. Ny, Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. Endocrinology **129**, 3200–3207 (1991)
4. M. Conti, M. Hsieh, J.Y. Park, Y.Q. Su, Role of the epidermal growth factor network in ovarian follicles. Mol. Endocrinol. **20**, 715–723 (2006)
5. Y. Yarden, M.X. Sliwkowski, Untangling the ErbB signalling network. Nat. Rev. Mol. Cell Biol. **2**, 127–137 (2001)
6. S. Friedmann, A. Dantes, A. Amsterdam, Ovarian transcriptomes as a tool for a global approach of genes modulated by gonadotropic hormones in human ovarian granulosa cells. Endocrine **26**, 259–265 (2005)
7. S. Panigone, M. Hsieh, M. Fu, L. Persani, M. Conti, Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol. Endocrinol. **22**, 924–936 (2008)
8. H. Ashkenazi, X. Cao, S. Motola, M. Popliker, M. Conti, A. Tsafriri, Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol. Endocrinol. **146**, 77–84 (2005)
9. S. Freimann, I. Ben-Ami, A. Dantes, R. Ron-El, A. Amsterdam, EGF-like factor epiregulin and amphiregulin expression is regulated by gonadotropins/cAMP in human ovarian follicular cells. Biochem. Biophys. Res. Commun. **324**, 829–834 (2004)
10. I. Ben-Ami, S. Freimann, L. Armon, A. Dantes, D. Strassburger, S. Friedler, A. Raziel, R. Seger, R. Ron-El, PGE2 up-regulates EGF-like growth factor biosynthesis in human granulosa cells: new insights into the coordination between PGE2 and LH in ovulation. Mol. Hum. Reprod. **12**, 593–599 (2006)
11. E. Rimon, R. Sasson, A. Dantes, A. Land-Bracha, A. Amsterdam, Gonadotropin-induced gene regulation in human granulosa cells obtained from IVF patients: modulation of genes coding for growth factors and their receptors and genes involved in cancer and other diseases. Int. J. Oncol. **24**, 1325–1338 (2004)
12. A.M. Zamah, M. Hsieh, J. Chen, J.L. Vigne, M.P. Rosen, M.I. Cedars, M. Conti, Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin. Hum. Reprod. **25**, 2569–2578 (2010)
13. M.H. Ozörnek, P. Bielfeld, J.S. Krüssel, J. Hirchenhain, R.S. Jeyendran, U. Koldovsky, Epidermal growth factor and leukemia inhibitory factor levels in follicular fluid: association with in vitro fertilization outcome. J. Reprod. Med. **44**, 367–369 (1999)
14. Y. Inoue, S. Miyamoto, T. Fukami, K. Shirota, F. Yotsumoto, T. Kawarabayashi, Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization–embryo transfer. Fertil. Steril. **91**, 1035–1041 (2009)
15. P.T. Goud, A.P. Goud, C. Qian, H. Laverge, J. Van der Elst, P. De Sutter, M. Dhont, In vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum. Reprod. **13**, 1638–1644 (1998)
16. I. Ben-Ami, A. Komsky, O. Bern, E. Kasterstein, D. Komarovsky, R. Ron-El, In vitro maturation of human germinal vesicle-stage oocytes: role of epidermal growth factor-like growth factors in the culture medium. Hum. Reprod. **26**, 76–81 (2011)
17. P. Humaidan, L.G. Westergaard, A.L. Mikkelsen, M. Fukuda, C. Yding Andersen, Levels of the epidermal growth factor-like peptide amphiregulin in follicular fluid reflect the mode of triggering ovulation: a comparison between gonadotrophin-releasing hormone agonist and urinary human chorionic gonadotrophin. Fertil. Steril. **95**, 2034–2038 (2011)
18. P.M. Kastrop, S.M. Weima, R.J. Van Kooij, E.R. Te Velde, Comparison between intracytoplasmic sperm injection and in vitro fertilization (IVF) with high insemination concentration after total fertilization failure in a previous IVF attempt. Hum. Reprod. **14**, 65–69 (1999)
19. C. Racowsky, M. Vernon, J. Mayer, G.D. Ball, B. Behr, K.O. Pomeroy, D. Wininger, W. Gibbons, J. Conaghan, J.E. Stern, Standardization of grading embryo morphology. J. Assist. Reprod. Genet. **27**, 437–439 (2010)
20. J.H. Choi, C.B. Gilks, N. Auersperg, P.C. Leung, Immunolocalization of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and type I GnRH receptor during follicular development in the human ovary. J. Clin. Endocrinol. Metab. **91**, 4562–4570 (2006)
21. R.M.J. Janssens, L. Brus, D.J. Cahill, J.A.F. Huirne, J. Schoemaker, C.B. Lambalk, Direct ovarian effects and safety aspects of GnRH agonists and antagonists. Hum. Reprod. Update **6**, 505–518 (2000)
22. P. Singh, A. Krishna, Effects of GnRH agonist treatment on steroidogenesis and folliculogenesis in the ovary of cyclic mice. J. Ovarian Res. **18**, 26 (2010)
23. M. Jamnongjit, A. Gill, S.R. Hammes, Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation. Proc. Natl. Acad. Sci. USA **102**, 16257–16262 (2005)
24. X. Chen, B. Zhou, J. Yan, B. Xu, P. Tai, J. Li, S. Peng, M. Zhang, G. Xia, Epidermal growth factor receptor activation by protein kinase C is necessary for FSH-induced meiotic resumption in porcine cumulus-oocyte complexes. J. Endocrinol. **197**, 409–419 (2008)
25. P. Feuerstein, V. Cadoret, R. Dalbies-Tran, F. Guerif, R. Bidault, D. Royere, Gene expression in human cumulus cells: one approach to oocyte competence. Hum. Reprod. **22**, 3069–3077 (2007)
26. Y. Reizel, J. Elbaz, N. Dekel, Sustained activity of the EGF receptor is an absolute requisite for LH-induced oocyte maturation and cumulus expansion. Mol. Endocrinol. **24**, 402–411 (2010)
27. R.P. Norris, M. Freudzon, V.O. Nikolaev, L.A. Jaffe, Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction **140**, 655–662 (2010)
28. A. Templeton, J.K. Morris, Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. N. Engl. J. Med. **339**, 573–577 (1998)
29. J.N. Hugues, E. Massé-Laroche, J. Reboul-Marty, O. Boîko, C. Meynant, I. Cédrin-Durnerin, Impact of endogenous luteinizing hormone serum levels on progesterone elevation on the day of human chorionic gonadotropin administration. Fertil. Steril. **96**, 600–604 (2011)
30. D. de Ziegler, Hormonal control of endometrial receptivity. Hum. Reprod. **10**, 4–7 (1995)

31. M. Shimada, I. Hernandez-Gonzalez, I. Gonzalez-Robayna, J.S. Richards, Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. Mol. Endocrinol. **20**, 1352–1365 (2006)
32. K.N. Fru, M. Cherian-Shaw, M. Puttabyatappa, C.A. VandeVoort, C.L. Chaffin, Regulation of granulosa cell proliferation and EGF-like ligands during the periovulatory interval in monkey. Hum. Reprod. **22**, 1247–1252 (2007)
33. N. Reeka, F. Berg, C. Brucker, Presence of transforming growth factor alpha and epidermal growth factor in human ovarian tissue and follicular fluid. Hum. Reprod. **13**, 2199–2205 (1998)
34. L.G. Westergaard, C.Y. Andersen, Epidermal growth factor (EGF) in human preovulatory follicles. Hum. Reprod. **4**, 257–260 (1989)
35. E. Gomez, M.J. de los Santos, A. Ruiz, J.J. Tarin, J. Remohi, A. Pellicer, Effects of epidermal growth factor in the final stages of nuclear and cytoplasmic oocyte maturation in humans. Hum. Reprod. **8**, 691–694 (1993)
36. A. Maheshwari, A. Gibreel, C.S. Siristatidis, S. Bhattacharya, Gonadotrophin-releasing hormone agonist protocols for pituitary suppression in assisted reproduction. Cochrane Database Syst. Rev. **10**(8), CD006919 (2011)
37. European and Middle East Orgalutran Study Group, Comparable clinical outcome using the GnRH antagonist ganirelix or a long protocol of the GnRH agonist triptorelin for the prevention of premature LH surges in women undergoing ovarian stimulation. Hum. Reprod. **16**, 644–651 (2001)
38. H.G. Al-Inany, M.A. Youssef, M. Aboulghar, F. Broekmans, M. Sterrenburg, J. Smit, A.M. Abou-Setta, GnRH antagonists are safer than agonists: an update of a Cochrane review. Hum. Reprod. Update **17**, 435 (2011)