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Original article

Effects of human menopausal gonadotropin on zona pellucida and pregnancy outcomes of ovarian stimulation protocols

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Abstract

Background: Human menopausal gonadotropin (hMG) has contributed many improvements to human assisted reproduction. However, effects of hMG on oocyte development and clinical results remain controversial.

Objective: This study was conducted to investigate the effects of hMG on the zona pellucida of oocytes, as well as clinical results in superovulation treatment.

Materials and Methods: This retrospective study was performed with 150 cycles of long-protocol treatment using recombinant follicle-stimulating hormone (r-FSH) with or without hMG. The number of retrieved oocytes, fertilization rate, implantation rate, pregnancy rate, and birefringence and thickness of the zona pellucida of oocytes were investigated.

Results: No significant differences were existed in r-FSH +hMG, and r-FSH groups in the number of retrieved oocytes (11.99±0.75 vs. 13.9±0.73, p=0.06), maturation rate (84.76% vs. 83.32%, p=0.42), pregnancy rate (37.31% vs. 37.66%, p=0.96), and embryo implantation rate (28.97% vs. 23.26%, p=0.30). However, fertilization rate (82.95% vs. 78.75%; p=0.02) was different. Zona pellucida birefringence was lower in the r-FSH +hMG group than in the r-FSH group (6.70±0.50 vs. 7.04±0.31; p=0.53). Thickness values of the metaphase-II zona pellucida of the r-FSH +hMG group on the first (19.20±0.14 vs. 18.75±0.10; p=0.01) and second (18.69±0.12 vs. 18.17±0.14; p=0.00) days of insemination were both higher than those of the r-FSH group.

Conclusion: hMG positively influenced the improvement of oocyte fertilization, as well as the birefringence and thickness of zona pellucida.

Key words: *Humans, Birefringence, Oocyte, Zona pellucida, Pregnancy.*

Introduction

In many protocols used to administer gonadotropin, the bioactivity of luteinizing hormone (LH) has not been considered in terms of the effectiveness of follicle-stimulating hormone (FSH) alone in supporting follicular development. In clinical practice, researchers observed that many patients show hypo responsiveness to FSH, in which follicles develop slowly, and estradiol level is low; therefore, the quality and fertility of oocytes are decreased, posing a great risk of miscarriage. In early follicular stage, FSH is important in follicle recruitment; LH may also participate in folliculogenesis and induction of ovulation. Follicular stimulation protocols have been designed to induce FSH and LH to mimic the physiology of normal human folliculogenesis (1-3). Studies have developed protocols for controlled ovarian stimulation (COS) by using low-dose human menopausal gonadotropin (hMG) based on FSH in late follicular phase. These studies have shown that the ovarian follicle requires a minimal amount of LH activity for steroidogenesis; as a result, the sensitivity of ovaries to FSH is increased, the dosage of FSH, and the time of ovary stimulation is reduced (4-7).

Especially for patients with advanced reproductive age, low ovary reaction and required high gonadotropin dose, the supplement of LH activity are more necessary (8, 9). Other trials have shown that exogenous LH is also essential for patients with iatrogenic LH deficiency with GnRH against down regulation (7). Decreased LH concentrations have been associated with low pregnancy rate

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and increased miscarriage rates in GnRH antagonist protocol (10, 11). The duration of treatment and per cycle gonadotropin dose can be reduced by adding hMG stimulation (12, 13). Exogenesis LH can be supplied with urine hMG, low-dose human chorionic gonadotropin (hCG), and recombinant luteinizing hormone (rLH). Among these factors, rLH is limited to some extent despite its high effectiveness in treating endogenous LH deficiency because of high costs.

The activity of hCG is also six times that of LH. Therefore, low-dose hCG can stimulate and selectively modulate ovarian follicle function and growth. Studies have shown that hCG supplementation in the mid-follicular phase yields favorable pregnancy results in low responders (14). However, the clinical application of hCG is influenced in terms of its long half-life. hMG exhibits similar levels of FSH or LH activity. hMG can also stimulate follicular development to induce FSH activity; furthermore, this substance can be used as LH supplement to induce LH activity. In addition, hMG is cost effective; as such, doctors and patients prefer this supplement. In the present study, hMG was selected as a LH supplement.

The present study was designed to understand the effect of hMG on the total consumption of FSH, oocyte yield, fertilization rate, pregnancy, and implantation rate in GnRHa long-protocol treatment. The zona pellucida birefringence (ZPB) and zona pellucida thickness (ZPT) of fertilized oocytes were observed non-invasively to explore the effect of hMG on the development of zona pellucida.

Materials and methods

Oral consent of examine zona pellucida was obtained from participants who included all patients in our center from January 2007 to February 2009. The protocol and design of this study were approved by the Ethics Committee of the People's Hospital of Guangxi.

Protocol

In this retrospective study, 150 cycles were retrospectively analyzed from tubal infertile women who underwent IVF-ET treatment at the People's Hospital of Guangxi from January 2007 to February 2009. A long conventional protocol was used, in which a subcutaneous dose of 0.1 mg/d of GnRH-agonist (Diphereline; Ipsen; France) plus r-FSH (Merk Serono; Ferring, Germany) was administered with or without hMG (Humegon; 75 IU/mL; Organon Laboratories, Saint-Denis, France). Individual variation in the consumption of FSH was determined according to the basal reproductive hormone level and basal antral follicle count. Clinical monitoring was performed daily by transvaginal pelvic ultrasound and serum E2 assay. Approximately 5000/10000 IU hCG (based on the number of follicles with diameter ≥14 mm and E_2 level) was administered to trigger ovulation when more than two dominant follicles reached the diameter of 18 mm. The oocytes were retrieved 36 hr after HCG by transvaginal ultrasound-guided aspiration using a single lumen ovum aspiration needle (COOK, Australia).

In gamete buffer (GB medium; COOK, Australia), cumulus oocyte complexes were collected under a stereomicroscope (SMZ-Olympus, Japan). We removed granulosa cells and sperms by repeatedly aspirating these cells with a needle of 135 mm in diameter, 6 hr after fertilization. The ova were then visualized under an inverted microscope with Hoffman interference optics and objective to 20×, 30×, and 40×; the stage was heated to 37°C by using a temperature controlling system (Minitub HT300; Tiefenbach, Germany). Considering the presence of the first/second polar body and germinal vesicle (GV), we classified the oocytes into three stages: mature oocytes (metaphase II); immature oocytes (metaphase I); and GV.

Observation of ZPB and ZPT

ZPB (Figure 1) and ZPT (Figure 2) were detected using a previously described method (15). Denude oocytes were transferred separately into 3-5 µL pre-warmed droplets of GB medium (COOK) overlaid with equilibrated mineral oil (Sage, Trumbull, CT, USA) in glass-bottomed dishes. The ZP was imaged

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separately using an Olympus IX71 microscope with the OCTAX Polar-AIDE system (Octax) with an oocyte equator, in which the oolemmas were clear. Data were stored for later analysis but not used for embryo selection.

The obtained oocyte images were then examined to analyze the ZPT along the radius direction. These data, along with the ZPB score, were saved for the subsequent quantitative analysis of ZPB intensity and ZPT. Our study excluded MI and GV oocytes because hMG could affect oocyte maturation, which is closely related to ZPB and ZPT. Therefore, only MII oocytes (fertilized and unfertilized) with a polar body were observed 6 hr after insemination.

Evaluation of pronuclei and embryo quality

Pronuclei were evaluated using system in which the presence and number of pronuclei were obtained 18-20 hr after insemination. The embryos were scored according to the degree of cytoplasm fragmentation and the number of blastomeres. Grade 1 embryos were considered the most viable embryo exhibiting the following characteristics: 8-10 cells; symmetrical blastomeres; few fragments; absent multinuclei; and rough, clear cytoplasm. Grade 2 embryos contained more than seven cells with uniform cell division and maximum 20% of cytoplasm fragment. Grade 3 embryos contained more than four cells with uneven cell division and certain proportion of fragments (>20%, <50%). Grade 4 embryos contained poorly viable (morphologically lowest) embryos with uneven cell division and >50% of fragments.

Sperm preparation and fertilization

Sperm cells were harvested by sperm-grade double-density centrifugation (40% and 80%) technique (COOK, Australia) after these cells were incubated in IVF medium at 37°C with 6% CO_2 for 1-2 hr. Oocytes were then fertilized with a progressive motile sperm with a density of 100,000-200,000/mL.

Embryo transplantation

For transplantation, embryo was selected on the basis of traditional morphological assessment depending on the third day or the fifth day of embryo development. We transplanted two high-quality embryos to patients younger than 35 years old and three high-quality embryos to those in advanced age or subjected to repeated IVF cycles. Embryos were incubated in a Petri dish (Falcon 3037, France) containing blastocyst medium (BM, COOK, and Australia) for 0.5-2.0 hr; these embryos were then transplanted into the uterus using an IVF transfer catheter (COOK, Australia) by ultrasound. In the luteal phase, progesterone was administered on the first day of transplantation and continued until the day of the pregnancy test.

Pregnancy evaluation

Clinical pregnancy is determined on the basis of the presence of gestational sac, as confirmed by ultrasound on the fourth week after the embryo is transplanted. Only clinical pregnancies were included in this analysis.

Statistical analysis

Statistical analysis was performed by ANOVA and χ^2 tests using GraphPad Prism 4. $P<0.05$ was considered statistically different.

Results

Among the 150 cycles subjected to the long protocol with GnRH agonist, 81 received r-FSH treatment. A total of 69 cycles were subjected to superovulation by using r-FSH plus hMG as exogenous LH administered in the late follicular development. The average age of female patients in the two groups was comparable (31.25±0.41 vs. 31.33±0.42 years, respectively; p=0.99). No statistical difference was observed between the two groups in terms of the average number of retrieved oocytes and the maturation rate (13.9±0.73 vs. 11.99±0.75, p=0.06; 83.32% vs. 84.76%, p=0.42). The average amount of FSH in the r-FSH+ hMG group was similar to that in the r-FSH group (1583±60.47 vs. 1591±49.43, p=0.14; Table I).

Further studies were preformed regarding fertilization, cleavage, and embryo development after superovulation was performed with or without hMG. The results in

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table II suggested that the fertilization rate was higher in the hMG group (82.95% vs. 78.75%; p=0.02). However, no difference was observed between cleavage and excellent embryo rates (94.90% vs. 94.76%, p=0.99; 57.91% vs. 57.29%, p=0.85, respectively) between the two groups.

Clinical results were compared between different superovulation protocols. In table III, the endometrial thickness and average number of transplanted embryos were comparable between r-FSH group and r-FSH+ hMG group (11.72±0.31 vs. 11.18±0.26, p=0.19; 2.20±0.05 vs. 2.24±0.05, p=0.59; respectively). The results also indicated that pregnancy rate, implantation rate, and miscarriage rate did not differ between the two groups (37.66% vs. 37.31%, p=0.96; 23.26% vs. 28.97%, p=0.30; 17.24% vs. 8.00%, p=0.54; respectively).

ZPB was evaluated 6 hr after fertilization was performed using the long protocol cycles of IVF in the two groups (Table IV). No difference was found between r-FSH group (7.04±0.31) and r-FSH+ hMG group (6.70±0.50) (p=0.53). However, D0ZPT and D1ZPT of r-FSH group were respectively lower than those of r-FSH+ hMG group (18.75±0.10 vs. 19.20±0.14, p=0.01; 18.17±0.14 vs. 18.69±0.12, p=0.00) when the ZPT of the fertilized oocytes were further analyzed at 6 or 20 hr after insemination on D0 or D1.

Table I. Comparison of the number of retrieved oocytes and oocyte maturation rate between r-FSH and r-FSH+ hMG groups in IVF cycles

	r-FSH group	r-FSH+ hMG group	p-value
Number of cycles	81	69	-
Age (years)*	31.25 ± 0.41	31.33 ± 0.42	0.99
Primary infertility (%)	41.98 (34/81)	31.88 (22/69)	0.26
Duration of infertility (years)*	4.78 ± 0.32	4.19 ± 0.33	0.08
Oocytes retrieved number	13.9 ± 0.73	11.99 ± 0.75	0.06
Maturation rate (%)	83.32 (949/1139)	84.76 (701/827)	0.42
Dosage of FSH (IU)*	1591 ± 49.43	1583 ± 60.47	0.14

r-FSH: recombinant follicle-stimulating hormone, hMG: human menopausal gonadotropin
* Data are presented as the mean±SE

Table II. Comparison of fertilization between r-FSH and r-FSH+ hMG groups in IVF cycles

	rFSH group	rFSH+ hMG group	p-value
Fertilization rate (%)	78.75 (897/1139)	82.95 (686/827)	0.02
Cleavage rate (%)	94.76 (850/897)	94.90 (651/686)	0.99
Excellent embryo rate (%)	57.29 (487/850)	57.91 (377/651)	0.85

Table III. Comparison of clinical outcomes between r-FSH and r-FSH + hMG groups in IVF cycles

	rFSH group	rFSH+ hMG group	p-value
Endometrial thickness (µm) (mean±SE)	11.72 ± 0.31	11.18 ± 0.26	0.19
Transplanted embryo (mean±SE)	2.20 ± 0.05	2.24 ± 0.05	0.59
Pregnancy rate (%)	37.66 (29/77)	37.31 (25/67)	0.96
Implantation rate (%)	23.26 (40/172)	28.97 (42/145)	0.30
Miscarriage rate (%)	17.24 (5/29)	8.00 (2/25)	0.54

Table IV. Comparison of birefringence intensity and thickness of zona pellucida between r-FSH and r-FSH + hMG groups in IVF cycles

	r-FSH group	r-FSH+ hMG group	p-value
Zona pellucida birefringence (score)	7.04 ± 0.31	6.70 ± 0.50	0.53
D0 Zona pellucida thickness (µm)	18.75 ± 0.10	19.20 ± 0.14	0.01
D1 Zona pellucida thickness (µm)	18.17 ± 0.14	18.69 ± 0.12	<0.001

Data are presented as the mean ±SE

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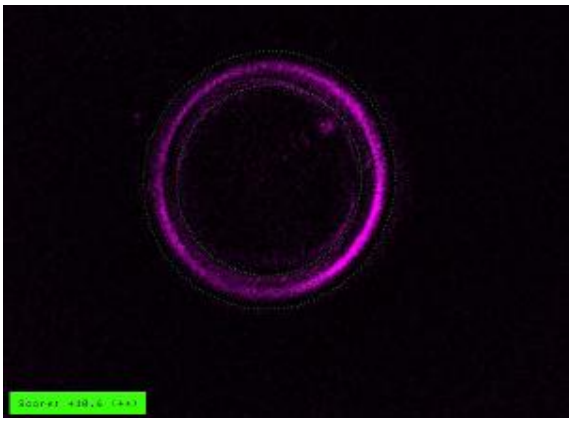
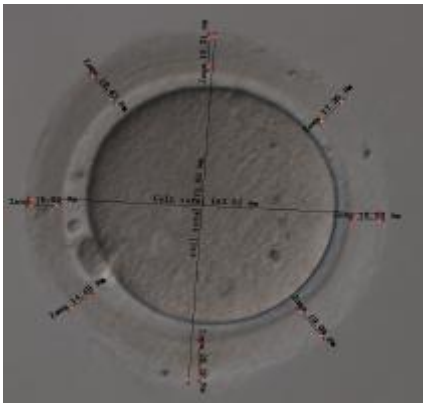


Figure 1. Examination of the zona pellucida birefringence scores of MII oocytes.



1	Cell total diameter	163.61 μ m
2	Cell total diameter	172.03 μ m
3	Zona pelucida thickness	18.63 μ m
4	Zona pelucida thickness	18.31 μ m
5	Zona pelucida thickness	17.35 μ m
6	Zona pelucida thickness	16.01 μ m
7	Zona pelucida thickness	19.08 μ m
8	Zona pelucida thickness	20.27 μ m
9	Zona pelucida thickness	14.45 μ m
10	Zona pelucida thickness	16.02 μ m

Figure 2. Examination of the zona pellucida thickness.

Discussion

All of the 150 tubal infertile patients undergoing IVF treatment received a standard long protocol in this research. The average amount of r-FSH, endometrial thickness, number of retrieved oocytes, and mature rate in the r-FSH+ hMG group were not different from those in the r-FSH group; this result is similar to those in the previous reports (5, 6). No evident differences were also found in cleavage, clinical pregnancy, and implantation rates between the two groups.

Patients who underwent pituitary down regulation and hMG injection, had improving fertilization rate. This finding may be attributed to the change in zona pellucida, although Gordon *et al* found that residual endogenous LH after down regulation is sufficient for normal ovarian response and estradiol synthesis and implantation is possibly higher when LH is co-administered (6). Our study demonstrated that the ZPB of oocytes was lower in the hMG+ r-FSH group than in the r-FSH group. This could be related to the higher LH level of patients treated with hMG supplement. An appropriate concentration of LH can possibly promote oocyte maturation and developmental potential (6).

In addition, a previous study regarding the zona pellucida showed that the ZPB of oocytes reduced gradually from a GV stage to a MII stage in the developmental process (16). Thus, administration of hMG could improve fertilization through maturity of oocytes zona pellucida. These results could be possibly attributed to the changes in paracrine in ovarian follicle caused by LH, such as cAMP, epidermal growth factor receptor (EGFR), and C-type natriuretic peptide (CNP)/ natriuretic peptide receptor 2 (NPR2) (17, 18). In meta-analysis, a high serum estradiol level was obtained when HCG was administered and a high number of metaphase II oocytes were retrieved in the hMG group when the gonadotropin releasing hormone antagonist (GnRH-A) protocol was used; however, no difference was observed in the rates of clinical

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pregnancy, implantation, and miscarriage (19). LH can also improve clinical pregnancy and implantation rates in low- and normal-responsive patients (6, 7, 20). LH is necessary to stimulate folliculogenesis and maturation or development potential of oocytes in clinical and experimental aspects (7, 21).

Loumaye *et al* reported that LH levels increase when exogenous LH is administered; this increase may produce medium-sized follicles to atresia, even if large amounts of FSH are used in superovulation; as a result, multiple follicle development was avoided (22). In the current study, a moderate reduction in the r-FSH+ hMG group was observed compared with the control group (11.99 0.75 vs. 13.9±0.73, respectively), which can be closely attributed to the efficacy of hMG in the controlled superovulation, although the overall number of retrieved oocytes represented no significant difference between the two groups (p=0.06). Fabregues *et al* showed a remarkable decrease in the number of non-dominant follicle with a diameter of 14-18 mm when hCG was administered (23).

The number of produced and fertilized oocytes significantly decreased in the FSH group rather than in the FSH +LH group. Previous studies revealed that exogenous LH activity (including hMG or r-LH) added in the mid-follicular phase of superovulation can optimize the controlled ovarian stimulation and ovulation outcome particularly in the subgroup of patients with advanced age and poor ovarian response (24, 25).

LH played an important role in promoting follicular development and oocyte quality. However, higher or lower than normal levels of LH negatively affect oocytes. The excessive secretion of LH can induce the release of androgen in theca cells, and this follicular microenvironment can induce not only the apoptosis of granulosa cells but also follicular atresia (26). A series of morphological changes in follicular granulosa cells and oocytes were observed during follicular atresia and degradation. Excessive LH can also negatively affect oocyte maturation; as a result, overly mature oocytes are produced and meiosis and mitosis are accelerated. In addition, granulose cells are luteinized and oocyte quality is reduced because of a premature and excessive secretion of LH before ovulation occurs; by contrast, low LH levels can produce E2 at concentrations less than normal values (7).

Westergaard *et al* and Weghofer *et al* reported that LH level <0.5 U/L in the mid-follicular phase likely increases the probability of abortion because of embryo polyploidy, although follicle growth, fertilization, and pregnancy remain unaffected (27, 28). This result showed that LH at an appropriate range could maintain normal levels of follicular androgen and improve follicular development and oocyte quality. Low “threshold” likely results in an insufficient synthesis of E_2, whereas LH that exceeds the “ceiling” level is possibly detrimental to follicular development (29).

Conclusion

In conclusion, hMG supplement did not elicit evident effects on normal-responsive patients except fertilization and ZPB and ZPT of oocytes. Further studies should be conducted to determine the function of LH on oocyte development.

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Conflict of interest

There is not any conflict of interest in this paper.

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