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Establishing a combined stimulation protocol hFSH followed by rFSH might represent a breakthrough in the IVF practice

S. GERLI, G.C. DI RENZO

Department of Obstetrics and Gynecology, Centre for Perinatal and Reproductive Medicine, University of Perugia, Perugia, Italy

Abstract. – INTRODUCTION: Controlled ovarian stimulation directly influences assisted reproductive technology (ART) outcomes. Indeed, several studies have shown that the total IU of gonadotropins used for ovarian stimulation inversely correlates with pregnancy rate. Nowadays, two main gonadotropins are used in ART protocols, human-derived and recombinant follicle-stimulating hormone (FSH). The difference between these two hormones is dramatic. Indeed, the human-derived FSH is an acidic isoform of the hormone while the recombinant is a less acid one. In particular, during a physiological menstrual cycle the acid isoform is produced during the follicular phase (probably it is more effective in recruiting follicles) while less acidic isoform is produced during the mid follicular phase (preovulatory).

In the present study, we aim to evaluate the efficacy of a protocol that mimics the physiological shift form an acidic to a less acid FSH isoform during oocyte maturation.

PATIENTS AND METHODS: A total of 308 infertile couples undergoing their first Intracytoplasmic Sperm Injection (ICSI) treatment were enrolled. All patients underwent a standard down-regulation protocol with GnRH analogue hormone. Patients were randomized in two groups: group 1, patients that received 225 IU of human-derived FSH (hFSH Fostimon, IBSA, Lodi, Italy) for 6 days from the second day of the cycle and then 225 IU of recombinant FSH (rFSH Gonal-F; Serono, Rome, Italy) from the 7th day of stimulation until hCG administration, and group 2, control group, patients that received 225 IU recombinant FSH alone from the second day of the cycle until hCG administration.

RESULTS: The combined protocol (hFSH + rFSH) resulted in significantly less IU of FSH necessary for ovarian stimulation together with the stimulation days. Furthermore, oocyte and embryo quality was higher in the group of patients treated with the combined protocol. Noteworthy, a significantly higher implantation rate and pregnancy rate were observed in favour of group 1 compared to group 2.

CONCLUSIONS: We demonstrated that establishing a stimulation protocol able to mimic the physiological differences in FSH isoforms, hFSH combined with rFSH positively impact on ART outcome.

Key Words:
IVF, Gonadotropin, Follicle-stimulating hormone.

Introduction

Controlled ovarian hyperstimulation is a crucial part of the assisted reproductive technology. This is achieved by the administration of exogenous gonadotropins to increase follicular recruitment and oocyte yield. For this purpose, FSH (follicle-stimolating hormone) preparations from different origin have been implemented in a variety of ovarian stimulation regimens with variable clinical outcomes. Until recently, gonadotropins used for ovarian stimulation have been extracted from the urine of postmenopausal women. Later on, two pure FSH preparations have become available thanks to the recombinant DNA technology: follitropin-alpha and follitropin-beta, which lack LH activity or other human proteins[1,2].

Clinical trials have shown that recombinant FSH (rFSH) is effective in terms of number of oocytes retrieved, number of embryos obtained, and total gonadotropin dose needed, without increasing the risk for the ovarian hyperstimulation syndrome (OHSS)[3-5]. In addition, rFSH has been shown to be as effective as as human-derived FSH (hFSH) or hMG with or without GnRH agonists[6-10]. Recently, the efficacy of rFSH compared to hFSH in terms of oocyte and embryo quality has been evaluated, and the results reported are highly in favour of hFSH. Authors speculated that the presence of LH activity in the hFSH preparation has a positive effect on oocyte maturation and embryo quality[11-13]. On the other hand several study have hypothesized that the main difference in efficacy between rFSH and hFSH may reside in the nature of FSH isoform activities[14,15]. FSH isoforms influence a variety of biological activities, cellular growth and development, steroidogenesis and protein synthesis. Human-derived FSH contains both acidic and mid-acidic iso-

Corresponding Author: Gian Carlo Di Renzo, MD; e-mail: direnzo@unipg.it

forms whereas recombinant FSH contains a higher proportion of less acidic isoforms. Less acidic FSH isoforms exhibit a high *in vitro* bioactivity, but they have a faster clearance and thus a shorter half-life compared to the acidic FSH isoforms[15,16]. Another study has shown that the slow clearance of the acidic isoforms results in more estrogenic follicles and follicular maturation and estradiol secretion[14]. Furthermore, several studies have reported significant changes in FSH heterogeneity during certain physiological conditions including puberty and the menstrual cycle[17-21]. Acidic FSH isoforms are routinely produced during follicular and luteal phases (i.e. hFSH) when the E_2 level is low whereas less acidic FSH isoforms are produced during mid-cycle (i.e. rFSH) when the E_2 level is high. This shift towards the production and secretion of less acidic/sialylated FSH molecules in the mid-cycle and preovulatory phases of the cycle may be an important mechanism to regulate the intensity of the FSH stimulus during the final steps of follicular maturation[22]. Taking into account literature date, in the present paper we reported the clinical outcome of a stimulation protocol that aim to mimic the physiological cycle during ovarian stimulation by using a combined protocol of both hFSH and rFSH, starting with the hFSH preparation during the follicular phase and rFSH preparation during the mid-follicular phase until hCG administration. We evaluated the efficiency of this combined stimulation protocol on oocyte and embryo quality, as well as on pregnancy and implantation rates.

Patients and Methods

Patient Selection

A total of 308 infertile couples undergoing their first intracytoplasmic sperm injection (ICSI) treatment were enrolled from June 2009 to March 2011. Inclusion criteria were: women of 27-38 years old; infertility due to tubal abnormalities, male factor or idiopathic infertility; serum hormonal profile (FSH and LH < 12 mIU/ml, E_2 < 50 pg/ml and prolactin < 30 ng/ml) within the normal range; regular ovulatory menstrual cycles; presence of normal uterine cavity; body mass index (BMI) ≥ 20-≤ 26 kg/m^2 and first IVF treatment. Exclusion criteria for the female partner were: poor response; polycystic ovarian syndrome (PCOS); endometriosis. For the male partner were: azoospermia; clinical signs of infection detected in semen analysis within 12 months before treatment.

Randomization was computer based and took place after the confirmation of down-regulation and immediately before gonadotropin administration in order to minimize post-randomization withdrawals. All patients were counselled about the nature of the study and gave their written informed consent for their participation to the randomization procedure. Participating patients were registered in our local Ethical Committee register that approved the study.

The primary end point was clinical pregnancy. The secondary endpoints were: implantation rate (clinical pregnancy on number of embryo transferred), total dose of FSH administered, total number of days of stimulation, serum estradiol levels and endometrial thickness on the day of hCG administration, number of mature oocytes retrieved, embryo quality, fertilization rate, embryo cleavage rate, pregnancy rate, live birth and miscarriage rates, cancellation rate, and incidence of moderate or severe OHSS.

Stimulation Protocol

All patients underwent a standard down-regulation protocol with GnRH analogue hormone (triptroline, Decapeptyl 0.1 mg/day, Ipsen, Milan, Italy). The patients were randomized in two groups: group 1 (n = 150), patients that received 225 IU of human-derived FSH (hFSH Fostimon, IBSA, Italy) for 6 days from the second day of the cycle and then 225 IU of rFSH (Gonal-F; Serono, Rome, Italy) from the 7th day of stimulation until hCG administration, and group 2, control group, (n = 158), patients that received 225 IU recombinant FSH alone from the second day of the cycle until hCG administration. The patients with a poor response to gonadotropin treatment were withdrawn from the study. Patients with excessive response to gonadotropins were counselled about the risk for OHSS and were advised to interrupt the stimulation cycle or to undergo oocyte retrieval with cryopreservation of any resultant embryos for replacement in the subsequent cycle. Oocyte maturation was triggered by the administration of 10,000 IU of human chorionic gonadotropin (hCG) (Gonasi HP, IBSA, Italy), indeed, hCG injection was performed when the dominant follicle was 18-19 mm and there were at least two follicles of 16-17 mm were identified by ultrasound. Oocyte retrieval was performed 36 h after hCG administration and the harvested oocytes were denuded from their cumulus cell and were assessed for their maturity. Mature oocytes underwent ICSI, and the resultant embryos were scored according to established crite-

ria[23,24]. In particular, oocyte quality was assessed by taking into account nuclear maturity cytoplasm appearance. Indeed, it is generally accepted that good-quality human MII (matured metaphase) oocytes should have a clear, moderately granular cytoplasm that does not contain inclusions, a small perivitelline space (PVS) containing a single unfragmented polar body and a round, clear, colourless zona pellucida (ZP).

Ultrasound guided embryo transfer took place 48 h following insemination. The luteal phase was supported with the administration of 50 mg/day of progesterone.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (2236 Avenida de la Playa La Jolla, CA, USA). The parameters were compared using the two tailed Student's *t*-test for independent data and χ^2-test, setting the significance level at $p \leq 0.05$.

Results

During the study, three cycles were cancelled due to the increased risk for OHSS; one in group 1 and two group 2. This difference was not statistically significant. Of the 308 studied patients, 305 underwent oocyte retrieval, 149 patients in group 1 and 156 in group 2.

The two groups were comparable regarding demographic data, infertility factor distribution, duration of stimulation, estradiol level and endometrial thickness on the day of hCG administration (Table I). Furthermore, the mean number of oocyte retrieved did not differ between two groups (Table II)

Analysing the effects of the two different stimulation protocols, it was possible to notice that the combined protocol (hFSH + rFSH) resulted in significantly less IU of FSH necessary for ovarian stimulation together with the stimulation days (Table II).

Furthermore, percentage of MII oocyte was higher when the stimulation was performed with hFSH followed by rFSH (Table II).

A significant difference was also identified in favour of group 1 versus group 2 in terms of grade 1 embryos (Table II). Noteworthy, a significantly higher implantation rate (28.6% vs. 13.4%) and pregnancy rate (47% vs. 33%) were observed in favour of group 1 compared to group 2 (Table II).

It is very likely that the increased oocyte and embryo quality is positively affecting the pregnancy rate, indeed, it was possible to observe an increased pregnancy rate in group 1 (Table II).

Discussion

Recombinant FSH has introduced an alternative to urine-derived FSH for ovarian stimulation regimens. Several comparison studies have shown that recombinant FSH is more effective than hFSH (HMG or highly purified FSH) and the absence of LH activity in rFSH does not affect follicular growth[3-5]. However, recent reports demonstrate that human-derived FSH is considerably better than recombinant FSH in terms of oocyte and embryo quality and pregnancy and implantation rates, although the number of retrieved oocytes is higher in favour of rFSH[11-13]. Among the factors that affect oocyte quality during stimulation protocols there are woman age, basal hormonal profile, profound suppression of LH during down regulation and estradiol concentration per growing follicle.

Indeed, there is evidence that estradiol have a crucial role in oocyte maturation[25-27]. Tesarik and Mendoza[28,29] have reported that estradiol exerts a beneficial effect on cytoplasmic maturation via a non-genomic calcium-mediated mechanism, which contributes to oocyte capacitation for fertilization and early post-fertilization development. Significantly higher pregnancy rates have been re-

Table I.

	hFSH + rFSH (grp 1, n = 150)	rFSH (grp 2, n = 158)	*p*
Age (years)	30 ± 4	30 ± 5	NS
Infertility duration (years)	6.9 ± 3.9	7.2 ± 4.8	NS
D3 FSH (IU/l)	6.6 ± 2.1	7.0 ± 2.7	NS
BMI (kg/m^2)	24.6 ± 4.7	24.9 ± 4.6	NS
Infertility diagnosis, n (%)			NS
Tubal factor	30 (20)	29 (18)	NS
Male factor	75 (50)	79 (50)	NS
Unexplained	45 (30)	50 (32)	NS

Table II.

	hFSH plus rFSH (grp 1, n = 149)	rFSH (grp 2, n = 156)	p
Total Gn used (IU)	2106 ± 719	2536 ± 1099	< .0001
Stimulation days	12.3 ± 1.0[a]	14.1 ± 1.2[b]	< .0001
Endometrium on hCG day (mm)	11.5 ± 2.1	11.6 ± 2.3	NS
Serum E2 on hCG day (pg/ml)	3261 ± 1055	3009 ± 1096	NS
Oocyte retrieved	10.6 ± 5.8 1	10.0 ± 6.8 2	NS
MII oocytes, %	70.2	55.5	< 0.001
E2/oocyte ratio (pg/ml)	219 ± 83	180 ± 62	NS
Grade I embryos/all embryos at day 3, %	59.8	42.5	< 0.001
Embryos transferred, n	2.9±0.9	2.5 ± 1.2	NS
Implantation rate, %	28.6	13.4	< 0.01
Pregnancy rate, %	47	33	< 0.01
Abortion rate, %	9	15	NS

ported in women with an intermediate estradiol/oocyte ratio between 70 and 140 pg/ml[30].

Additionally, profound suppression of LH during the down-regulation protocols affects oocyte quality and clinical outcome. It has been reported that suppression of LH below the level < 0.5 IU/l is associated with a reduced cohort of embryos and a reduced estradiol/oocyte ratio[31,32]. On the other hand, other studies have shown that a low concentration of endogenous LH (< 3 mIU/ml) in the late follicular phase is associated with lower fertilization rates and higher biochemical pregnancy rates. It has been suggested that when using recombinant FSH only, it may be of clinical benefit to add LH in the late follicular phase or to reduce further the GnRH analogue dosage[31-34]. Conversely, it has been reported that patients showing an high degree of LH suppression respond similarly to those moderately suppressed, and only 6% of patients would benefit from exogenous LH administration[30]. Recombinant FSH lacks any LH activity by definition; nevertheless, it remains highly effective in stimulating follicle growth and maturation. Another factor that could affect oocyte maturity and development may be the nature of FSH isoforms used for ovarian stimulation. It has been shown that different FSH isoforms influence different of biological function, cellular growth and development, steroidogenesis and protein synthesis[35-37]. Because of their structural differences, FSH isoforms differ in their ability to bind to target cell receptors in their half-life and in their ability to induce a biological response *in vivo* and *in vitro*[22,38-41]. Several differences have been identified between recombinant and human-derived FSH, in particular, rFSH contains a higher proportion of less acidic isoforms, whereas human-derived FSH contains a higher proportion of acidic forms. This difference impact on their biological activity, rate of clearance and biological function. It has been shown that the less acidic isoforms have a faster circulatory clearance and, thus, a shorter circulatory half-life[15] compared to the acidic isoforms[42,43]. However, recent work has shown that the slow clearance of the acidic isoform results in better follicular maturation and estradiol secretion than the less acidic isoform[14]. In our paper the estradiol level per oocyte at hCG day was slightly higher in the combined protocol hFSH+rFSH compared to rFSH group. Although the number of retrieved oocytes does not significantly differ between the two groups, significant differences were observed in favour of the combined protocol compared to the rFSH group in terms of the proportion of mature and immature oocytes. Also statistically higher grade I embryos was found in group 1 compared to group 2. This likely explains the differences found between the two groups in terms of implantation and pregnancy rates. We can speculate that embryos derived from the combined protocol group have a high proportion of good quality embryos that in turn improve IVF outcome. Our findings might also be explained by the fact that the combined protocol mimic the physiological cycle, where more acidic FSH isoforms are produced during the follicular phase of the menstrual cycle while less acidic FSH isoforms are produced from mid cycle onward i.e. preovulatory phase and luteal phase. Several evidence reported significant differences regarding the *in-vitro* biological potency among the various FSH isoforms; in particular, it has been suggest that the shifts towards the production and secretion of more basic or acidic FSH molecules is related to specific physiological conditions (e.g. puberty, menstrual cycle and menopause). This might represent an important

mechanism through which the anterior pituitary regulates gonadal function[20,21]. Other studies have previously shown that almost all stored FSH isoforms may be released from the pituitary gland with few or no modifications in their number and pH values and that the charge distribution of the circulating isoforms changes according to the phase of the menstrual cycle[19,44,45]. The shift towards the production and secretion of less acidic/sialylated FSH molecules during a specific cycle phase may be an important mechanism to regulate the intensity of the FSH stimulus during the final steps of follicular maturation.

Conclusions

We can conclude that establishing a new protocol that mimic the physiological condition (i.e. follicular phase supported with an acidic FSH; luteal phase supported with a less acidic FSH), might be a real breakthrough in the IVF clinical practice.

Conflict of Interest

None to declare.

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