CLINICAL ASSISTED REPRODUCTION

The Potential Use of Maturation In Vitro of Human Oocytes in Low Responder Patients¹

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Purpose: To assess whether maturation in vitro of human oocytes (MIVHO) could be an alternative treatment in low responders to ovarian stimulation for in vitro fertilization (IVF).

Methods: Prospective case-control study. Spontaneously ovulatory women who volunteered were included in our program of MIVHO at the Instituto Valenciano de Infertilidad. Rates of oocyte retrieval, in vitro maturation, fertilization, and development up to the blastocyst stage were studied.

Results: A significantly increased rate of oocyte retrieval was found when the pickup was performed before follicular selection. No differences were found when MIVHO was used in a low responder patient with an ovarian content of early antral follicles > 5 as compared to normal responders.

Conclusions: MIVHO could be a successful choice in low responder patients with an acceptable number of early antral follicles. Oocyte retrieval should be performed before follicular selection in order to obtain more oocytes.

KEY WORDS: low responder; in vitro human oocyte maturation.

INTRODUCTION

Although there is no general agreement on the definition of a low responder (LR), it usually is accepted

that a LR is a patient who produces < 3–4 oocytes, has serum peak levels < 500 mg/ml after aggressive stimulation, and has undergone at least two previous unsuccessful attempts at ovarian stimulation (1). The incidence of this population varies among programs, ranging between 9 and 18% (1,2).

Once a LR has been identified during two stimulation cycles, the patient must be evaluated and the treatment of choice should be applied. Several strategies have been considered in order to improve the ovarian response of these women such as increasing the amount of gonadotropins injected (3) or trying to reduce or eliminate the administration of gonadotropin-releasing hormone-agonist (GnRH-a) (4). Some clinical trials have used growth hormone associated with gonadotrophins (5) or even used different stimulation protocols, such as clomiphene citrate (6). So far, the experience accumulated with the use of these protocols is controversial, leading in general to poor results. Although the use of in vitro fertilization (IVF) in a natural cycle is an old technique (7), there is a rekindling of interest in this method now (8,9). Based on the lack of response after ovarian stimulation with gonadotrophin, it has been proposed to apply this technique in LR patients. The disadvantage of this technique is that only one oocyte is recovered in each cycle.

Maturation in vitro of human oocytes (MIVHO) is a new technology for the treatment of human infertility. Immature oocytes have been obtained from women with polycystic ovaries and normovulatory patients (10,11). These oocytes may be potentially matured in vitro and transferred into the uterus without the use of ovarian stimulation.

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In this study we investigated whether MIVHO is an alternative treatment for LR patients in nonstimulated cycles. The hypothetical advantages are that it is not necessary to use gonadotropins; also, more oocytes will be obtained than in a natural cycle. Our approach was divided into two different steps. First, we studied which day of oocyte retrieval was the most favorable for MIVHO. When follicular selection takes place, there is a shift in the ratio of estrogen:androgen of the dominant follicle, increasing the amount of estrogen. The rest of the follicles will maintain high levels of androgens and, therefore, should become atretic. We considered a dominant follicle when follicle size was ≥ 10 mm by transvaginal ultrasound (12). A prospective study was designed comparing the results in terms of oocyte retrieval, in vitro maturation, and fertilization when ovum pickup was performed before or after follicle selection had been achieved.

Based on these results, we designed a second study in which normal and LR women to previous IVF cycles were compared using MIVHO. Developing follicles were punctured based on the result of the first study and the rate of oocytes recovered, matured in vitro, fertilization, and development up to the blastocyst stage were compared.

MATERIALS AND METHODS

Phase One

The study was approved by the Ethical Committee of the Instituto Valenciano de Infertilidad. All patients entering the study gave their informed consent to have their oocytes studied.

Oocytes were obtained from 20 patients (24 cycles) who volunteered to be included in an immature oocyte donation program between October 1, 1997 and April 30, 1998. The follow-up visits consisted of vaginal ultrasound scans performed from day 2–3 onward in which follicle number and diameter were evaluated. In the first visit a vaginal ultrasound was performed to identify any residual follicles from a previous cycle. Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) were evaluated on day 2–3 of the cycle and every other day. Cycles with any residual follicles or an abnormal baseline estradiol were canceled and rescheduled for the subsequent cycle.

The patients were randomly allocated in one of the two groups: group 1 ($n = 13$ cycles), in which ovum pickup was scheduled when all follicles were ≤ 10

mm; group 2 ($n = 11$ cycles) with at least one follicle > 10 mm in diameter when oocyte retrieval was planned. An aspiration pump was used with a pressure between 70 and 80 mm Hg, regular IVF needles (1.6×350 mm, 16g) (Swemed Laboratories, Billdal, Sweden) were used to collect the follicular aspirates in warm 10-ml plastic tubes (Falcon Plastic; Becton-Dickison) containing 2 ml of heparinized medium (Medicult, Copenhagen, Denmark).

Using hand-drawn pipettes to separate oocyte–cumulus masses from erythrocytes and follicular cells, oocytes were identified under the dissecting microscope. Oocyte–cumulus complexes were cultured in M-199 medium (Sigma, St. Louis, MO) at 37°C in an atmosphere of 5% CO_2 in air. Culture was supplemented with 0.075 IU/ml recombinant FSH (Gonal-F, Serono laboratories, Madrid, Spain), 2 ng/ml recombinant (EGF) (Sigma), 0.5 IU/ml human chorionic gonadotropin (hCG) (Profasi, Serono), 1 mg/ml E_2 (Sigma), 0.4% (HSA) (Sigma), and 0.33 mmole pyruvate. Each oocyte was placed individually in 20-ml drops under mineral oil. Oocytes were mechanically decumulated 24–36 hr after and the nuclear maturation was evaluated by the presence of the first polar body.

Hormonal levels were analyzed using a commercially available microparticle enzyme immunoassay kit (Abbott Laboratories, Abbott Park, IL). Intra- and interassay coefficients of variation were 4.1% and 8.5%, respectively, for FSH, 4.1% and 5.7% for LH, and 4.6% and 6.1% for E_2 .

Phase Two

Oocytes were obtained from 16 patients who volunteered to be included in MIVHO. In all cases they had been stimulated with gonadotropins in at least two previous cycles and were classified in two groups according to their ovarian response: those having < 4 oocytes collected in two different cycles of ovarian stimulation ($n = 6$) and those with a normal response ($n = 10$) in previous stimulated IVF cycles. A vaginal ultrasound on day 2–3 was performed in order to determine the number of early antral follicles (2–5 mm). In this phase, oocytes that reached metaphase II stage were donated to women included in our oocyte donation program. They were inseminated using intracytoplasmic sperm injection (ICSI) (13). Fertilization was evaluated after 18 hr of culture in standard conditions (37°C and 5% of CO_2) and further cocultured with human endometrial epithelial cells as described previously (14). Development of the embryos was followed up to the blastocyst stage, and then cryopre-

Table I. Patients Characteristics in the Two Groups Established According to Follicular Diameter on the Day of Ovum Pick-up^a

	Group 1	Group 2	P value
Cycles	13	11	
Age	24.8 ± 3.2	26.3 ± 3.1	NS
Follicular diameter (range)	7.3 ± 1.4 (6–9)	11.8 ± 0.7 (9–14)	< 0.05
Day of oocyte retrieval	7.3 ± 1.5	9.1 ± 0.8	NS
Retrieval rate	68.1% (79/116)	51.7% (60/116)	NS
E ₂ on retrieval date (pg/ml)	50.1 ± 4.2	89.5 ± 3.7	NS

^a Values are means ± SD; NS, not significant.

served for future genetic diagnosis before an eventual transfer.

All patients had spontaneous and regular menses, and none was under any medication that could interfere with the ovarian cycle.

Statistical Analysis

Comparative analysis of quantitative variables such as age, day of puncture, and serum hormone levels was made by ANOVA. Categorical and or dichotomized variables, i.e., follicular diameter, maturation, fertilization, and development to blastocyst stage, were analyzed by chi square test (χ^2) and Fisher's exact test. To compare the number of oocytes in each group, the binomial test was performed. A $P < 0.05$ was considered statistically significant. Statistical analysis was performed with use of Sigma Stat (Jandel Corporation, USA).

RESULTS

Phase One

Oocytes were identified in 59.9% of the cases (139 oocytes). Sixty-two (44.6%) of the recovered oocytes reached metaphase II stage. Table I shows the results in both groups according to the follicular diameter. Obviously, follicular size was bigger ($P < 0.05$) in group 2, in which ovum pickup was scheduled 2 days later than in group 1. The recovery rate was 68.1% in group 1 and 51.7% in group 2 ($P < 0.05$). The rate of oocyte that reached metaphase II stage was higher in the first group, although the difference did not reach statistical significance. Serum E₂ levels in the day of oocyte retrieval were significantly ($P < 0.05$) higher in group 2 as compared to group 1 (50.1 ± 4.2 vs. 89.5 ± 3.7).

Phase Two

As shown in Table II, age of the patients in both groups was comparable. No significant differences were found among the hormonal milieu on day 3 of the cycle (FSH: 6.3 ± 0.6 vs. 8.4 ± 0.9; LH: 3.9 ± 0.5 vs. 2.4 ± 0.9; E₂: 50.5 ± 3.9 vs. 39.6 ± 7.6). Parity was significantly higher in LR patients. (0.5 ± 0.2 vs. 2.4 ± 0.9, $P < 0.05$).

Table III shows a similar retrieval rate in both normal and LR (66.2% vs. 57.7% in LR group, $P = NS$). Maturation rate was 47.7% in normal responder versus 46.7% in LR patients, ($P = NS$). Fertilization rate was 68% in normal responder and 57.1% in LR patients, ($P = NS$). The rate of embryos that reached the blastocyst stage was 47.1% in normal responder versus 50% in LR patients, ($P = NS$).

In LR group, oocytes were obtained from four of six patients, while no oocytes were obtained in other two women. Two and three follicles, respectively, were punctured in these patients in whom no oocytes were retrieved. The number of early antral follicles (2–5 mm) observed in the basal vaginal ultrasound study were <5 in these two patients. However, early antral follicles in LR patients in whom oocytes were obtained ranged from 6 to 10 (8.0 ± 1.6). The control group had between 8 and 12 early antral follicles (8–12, mean 11 ± 2.1).

Table II. Endocrine Parameters in the Two Groups of Patients Included in Phase Two of the Study^a

	Control	Low responder	P value
No. of patients	10	6	
Age	23.7 ± 1.2	27.8 ± 1.8	NS
Parity	0.5 ± 0.2	2.4 ± 0.9	< 0.05
Day of oocyte retrieval	7.0 ± 1.4	7.4 ± 1.1	NS
FSH (mIU/ml)	6.3 ± 0.6	8.4 ± 0.9	NS
LH (mIU/ml)	3.9 ± 0.5	2.9 ± 0.9	NS
E ₂ (pg/ml)	50.5 ± 3.9	39.6 ± 7.6	NS

^a All values are means ± SD; NS, not significant.

Table III. Results of Phase Two Study

	Control	Low responder	P value
No. of patients	10	6	
Retrieval (%)	53/80 (66.2%)	15/26 (57.7%)	NS ^a
Maturation (%)	25 (47.7%)	7 (46.7%)	NS
Fertilization (%)	17 (68%)	4 (57.1%)	NS
Blastocyst formation (%)	8 (47.1%)	2 (50%)	NS

^a NS, Not significant.

DISCUSSION

Low responder patients are one of the most difficult and interesting groups in reproductive medicine. Several strategies have been considered in order to increase the ovarian response. So far, the experience accumulated with the use of different protocols is controversial, leading in general to poor results. Now, oocyte donation is the best alternative treatment for LR patients (15) and shows the best results in terms of live birth rates that are similar to other etiologies.

Currently there is an increase of interest in the use of IVF in natural cycles (8,9). This technique has been proposed even as a treatment for LR based on the lack of response to gonadotropins. Thornton *et al.* (16) applied the MIVHO technique in immature oocytes that were obtained in the course of follicle aspiration during IVF in unstimulated cycles in which a single dominant follicle had been stimulated with hCG 34–36 hr before. Using this method they obtained better results than fertilized the ovulatory oocyte. However, the pregnancy rate achieved is very low.

Our first study shows that the day of oocyte retrieval is a critical parameter in terms of oocyte pickup and in vitro maturation. The best results were found when oocytes were retrieved before follicle selection occurred. This finding confirms previous observations of our group in which we obtained better rates of maturation, fertilization, and embryo cleavage when oocytes obtained before follicular dominance were employed (17). This fact is in agreement with the idea that after the ovulatory follicular selection, the resting pool of secondary follicles will become atretic and only the dominant follicle will have the hormonal milieu necessary to achieve the oocyte maturation and ovulation. Based on these studies and on Gougeon's (18) design about the dynamics of the human follicular growth, we carried out follicle aspiration when one of the follicles reached a diameter close to 10 mm.

In our second study and based on these previous findings, we planned to assess whether MIVHO during

a natural cycle could be a successful technique for assisted reproduction in LR patients. We studied a group of women included in our protocol of in vitro maturation who had previously undergone an ovarian stimulation for ovum donation.

No differences were found between either group except for the parity that was unexpectedly higher in the LR group. This datum may be due to a lower mean age in the normal responder group and, therefore, they had not fulfilled their gestational desire yet. It must be emphasized that patients who had low response to gonadotropin stimulation presented at least one pregnancy in the last 2 previous years. We should not forget that women with a low response to gonadotropin stimulation may achieve pregnancy spontaneously.

FSH, LH, and E₂ levels assessed on the third day of the menstrual cycle also showed a normal range. Although the LR group showed higher FSH levels and lower E₂ levels, no statistically significant differences between the two groups were found. This may be consistent with the hypothesis of an early increase in FSH levels in patients with low response who will present higher levels in the future.

In the LR group, we differentiated two subgroups according to the number of early antral follicles identified by transvaginal ultrasound examination on day 3 of a natural cycle (<5 or ≥5 follicles). Transvaginal ultrasound has contributed remarkably to the success of IVF programs (19). With high-resolution vaginal probes, a cohort of small follicles measuring 2–5 mm in diameter can be detected already at the end of the previous cycle. The number of these early antral follicles may predict the number of oocytes to be collected in an IVF cycle. This group of follicles may represent the functional ovarian reserve and permits the identification of a subgroup of patients for whom ovarian stimulation will be ineffective (20,21). Chang *et al.* (22) found that the antral follicle count correlates with the number of oocytes retrieved and the number of embryos transferred in the same cycle. They did not find any pregnancies in the group with an antral follicle count of ≤ 3 follicles.

No statistically significant differences were found between the two groups regarding the retrieval, maturation, fertilization, and embryo development rates, although only 15 oocytes were retrieved out of 53 follicles punctured in the LR group. Interestingly, two patients in this particular subgroup had no oocytes inside their follicles. In those LR patients in whom we could obtain oocytes, the number of eggs collected was lower. We could hypothesize that together with the low response of their ovaries, a lower oocyte quality or

even a higher chance of an empty follicle syndrome may occur. Also, as the number of follicles in the baseline ultrasound decreases, the chances to obtain oocytes in the retrieval diminish. These data support our hypothesis about the usefulness of MIVHO in LR patients. Based on our data we may differentiate two groups in LR patients: those with a normal range of early antral follicles in whom oocytes were obtained and women with a lower number of follicles in whom oocytes were not retrieved. Therefore, we must focus our effort in those women who present a low response to aggressive stimulation with gonadotropins but whose ovaries show a normal range of antral follicles between 2 and 5 mm when a basal ultrasound study is performed.

Several hypotheses have been proposed that attempt to attribute the weak response to ovarian stimulation in the presence of a normal follicular pool to factors such as (a) lower ability of these ovaries to develop an appropriate vascular network responsible for the distribution of the circulating FSH (23), (b) a reduced capacity of these recruited follicles to amplify the action of FSH by secretion of insulin growth factor-I (IGF-I) by the theca compartment (24), or (c) the presence of a decreased number of FSH receptor available within the granulosa cells (23). In previously published data from our group (25), we suggested that women with poor response to gonadotropins in the presence of normal basal FSH concentrations had an abnormal follicular flow impedance that may be the consequence of an abnormal regulation of some of the factors implicated in the development of multifollicular growth to the preovulatory stage.

The purpose of our study was to investigate whether these follicles were accessible to puncture with a needle as with a control group and with similar results. When we compared the result between this subgroup with the control group, we did not find significant differences in the number of oocytes retrieval, maturation, and fertilization rates and blastocysts developed. Therefore, we think that this method may be considered as a therapeutic alternative in LR patients. In fact, the results achieved are at least comparable or even better than those obtained after aggressive stimulation with gonadotropin. Moreover the cost of this technique is lower, as these patients will not be treated with high doses of hormones.

In conclusion, although our results with MIVHO and fertilization need further improvement, we think that this technique could be a successful choice in patients who show a low ovarian response to gonadotropin stimulation but with a normal rate of early antral

follicles. In order to discriminate this group, an ultrasound scan in basal conditions could be an enormous help in order to determinate LR in those who are able to use this program. In these cases, an aspiration in a natural cycle may be performed and the oocyte obtained can be matured and fertilized in vitro. Nevertheless, this report refers to a limited number of cases, and therefore further studies should be carried out before this novel approach could be used.

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