GnRH Antagonist Improved Blastocyst Quality and Pregnancy Outcome After Multiple Failures of IVF/ICSI-ET with a GnRH Agonist Protocol

Katsuhiko Takahashi,^{1,4} Tetsunori Mukaida,¹ Tatsuhiko Tomiyama,² Tetsuya Goto,³ and Chikahiro Oka³

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Background: To determine the efficacy of a gonadotrophin-releasing hormone (GnRH) antagonist, cetrorelix, in improving the quality of embryos and pregnancy outcome, we performed a study in patients with a history of multiple failures of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycles with a GnRH agonist (GnRHa) long protocol.

Methods: Forty women with no live births after conventional IVF or ICSI embryo transfer (ET) and subsequent blastocyst transfer (BT) with a GnRHa long protocol entered this study. The treatment protocol consisted of a daily dose of clomiphene citrate 100 mg for 5 days and gonadotrophin injections daily from cycle day 4 onward. Cetrorelix, 0.25 mg/day, was started when the leading follicle reached 14 mm. Induction of ovulation was triggered with human chorionic gonadotrophin (HCG) ($N = 36$) or GnRHa ($N = 4$). It was possible to perform BT in 38 patients.

Results: Comparison of the results with the results for BT with the previous GnRHa protocol showed no significant differences in number of oocytes retrieved or the zygote- and blastocyst-development rate. With the cetrorelix protocol, however, number of patients whose embryos had developed to at least one expanded blastocyst on day 5 was significantly higher than with the GnRHa protocol (25 vs. 9) ($p < 0.001$), and 16 of the women became pregnant (42.1%), with 7 delivering 9 infants, 4 ending in abortion (25%), and 5 in progressing.

Conclusions: The use of a GnRH antagonist in controlled ovarian hyperstimulation improves the outcome of pregnancy of patients with a history of multiple failure of IVF/ICSI-ET in a GnRHa protocol, most likely due to improvement of the quality of the blastocysts generated.

KEY WORDS: Blastocyst; GnRH agonist; GnRH antagonist; multiple ET failures.

INTRODUCTION

Multiple implantation failure after transfer of embryos is one of the important problems in assisted reproductive technology (ART) that need to be

solved. Since the major cause of implantation failure is believed to be poor quality of the embryos transferred, many researchers have devised a variety of methods to obtain better quality human embryos and increase the pregnancy rate after in vitro fertilization-embryo transfer (IVF-ET), e.g., by coculture of embryos with fallopian tube cells (1), assisted hatching of embryos before transfer (2), changing the constituents of the culture medium, and transferring embryos at the blastocyst stage (3).

Blastocyst transfer (BT) has been found to result in a higher pregnancy rate and fewer multiple gestation deliveries in selected patients (3,4), nonselected

¹ Hiroshima HART Clinic, 5-7-10 Ohtemachi, Naka-ku, Hiroshima 730-0051, Japan.

² Osaka HART Clinic, 2-6-20 Umeda, Kita-ku, Osaka 530-0001, Japan.

³ Tokyo HART Clinic, 1-22-2 Higashi, Shibuya-ku, Tokyo 150-0011, Japan.

⁴ To whom correspondence should be addressed; e-mail: hart@enjoy.ne.jp.

patients (5,6), and patients with a history of multiple IVF–ET failure (7). Racowsky *et al.* (8) found no benefit of BT when fewer than three zygotes had developed to the eight-cell stage on day 3 after insemination. Nevertheless, all investigators agreed that BT is useful in selecting good-quality embryos for transfer and improving the prognosis. The pregnancy rate after BT in patients with a history of multiple conception failure after IVF is as high as in patients who had never been treated by IVF, and factors affecting pregnancy outcome after BT have been the number of embryos transferred and blastocyst quality, but not maternal age or history of infertility (9).

Most of the above studies were carried out using gonadotrophin-releasing hormone agonists (GnRHa) in a so-called “long protocol,” because the long protocol had been shown to be much more effective than stimulation without administration of a GnRHa (10). However, the long protocol has some disadvantages, such as the flare-up effect, a lower response in some patients because of prolonged pituitary suppression, a need for larger doses of gonadotrophin, and a high incidence of ovarian hyperstimulation syndrome (OHSS). Since the GnRH (gonadotrophin-releasing hormone) receptor mRNA is expressed in human granulosa cells and the ovary, prolonged use of GnRHa may exert a direct action on the ovary (11).

Recently GnRH antagonists have been introduced into the ovarian stimulation protocol to overcome some of these disadvantages, and lower numbers of oocytes retrieved and lower serum estradiol (E2) levels have been found in the patients treated with a GnRH antagonist than those treated by the GnRHa long protocol (12–14). Nevertheless, the results of all of the studies showed higher fertilization rates, mean numbers of good-quality embryos, and pregnancy rates, as well as a low incidence of OHSS, indicating the usefulness of GnRH antagonists in ovarian stimulation protocol. Minaretzis *et al.* (15) observed better embryo quality in patients treated with GnRH antagonist than in those treated with the GnRHa protocol, and Craft *et al.* (16) reported a favorable clinical outcome of IVF when a GnRH antagonist was used in patients who had a poor response to GnRHa.

We therefore designed this study to investigate the efficacy of a GnRH antagonist protocol on blastocyst quality and on the clinical outcome of women who had a history of multiple failure of conventional IVF/ICSI–ET and subsequent BT with a GnRHa protocol.

MATERIALS AND METHODS

Patients

The entry criteria for the women in this study were conception failure or spontaneous abortion 3 or more times (3–14 times) after conventional IVF–(ICSI) ET and subsequent BT with a GnRHa long protocol. BTs were carried out between September 2000 and April 2001.

Forty of the women who met these criteria agreed to receive the GnRH antagonist cetrorelix (Cetrotide, Asta Medica, Frankfurt, Germany) for the BT cycle to be repeated. All women were informed of the possibility of side effects of cetrorelix and that cetrorelix is not yet licensed in Japan. The mean age of the patients was 37.1 years, and the mean number of conventional day 2 or 3 ET cycles they had previously received was 6.1 (Table I). The indications for ART treatment were infertility due to tubal factors in 7 patients, due to endometriosis in 3, polycystic ovaries (PCO) in 6, uterine factors, such as a uterine anomaly or small uterus, in 4, male factors in 8, and of unexplained etiology in 12 (Table I). IVF was performed in 10 patients, and ICSI was performed in 8 for male factor and 22 for low fertilization rate in previous IVF. Two patients whose embryos failed to develop to blastocysts were excluded. BTs in the GnRH antagonist study were carried out between January and December 2001.

Ovarium Stimulation Protocol

In the GnRHa long protocol, all women received a GnRHa, nafarelin (Nasanyl, Yamanouchi, Tokyo, Japan) nasal spray, 200 μ g twice a day, starting on the day 7 before the expected date of the start of the next menstrual period, and received human menopausal gonadotrophin (HMG), 225–300 IU daily, starting on day 3 of the menstrual cycle. Follicle measurements

Table I. Clinical Data of the Patients

No. of patients	40
Mean age (year)	37.1 $\pm$ 3.6 (26–42)
Mean no. of previous ET cycles	6.1 $\pm$ 3.7 (3–14)
Initial indications for ART	
Tubal factors	7
Endometriosis	3
PCO	6
Uterine factors	4
Male factors	8
Unexplained factors	12

and serum E2 level determinations were started on day 7 or 8 of the cycle. When the leading follicle reached 18 mm in diameter, nafarelin and HMG were discontinued and human chorionic gonadotrophin (hCG), 10,000 IU, was administered.

In the cetrorelix protocol, clomiphene citrate, 100 mg a day, was given to the patients for 5 days starting on day 3 of the menstrual cycle and HMG, 225–300 IU a day, was started on day 4 of the cycle. Daily measurements of follicles by vaginal ultrasound and of serum E2 concentration were started on day 7 or 8, and cetrorelix, 0.25 mg a day by daily subcutaneous injection, was started on the day when the diameter of the leading follicle reached 14 mm or more (usually day 8 or 9). HMG and cetrorelix were discontinued when the diameter of the leading follicle reached 20 mm, and hCG (10,000 IU) was administered to all of the patients except four to induce ovulation. The four exceptions were patients with PCO who had experienced mild to moderate OHSS in a previous long protocol and had more than 20 follicles in this cycle, and nafarelin nasal spray, 400 μ g, was used instead of hCG to prevent OHSS.

Oocyte Fertilization, Embryo Culture, and Transfer

Oocytes were retrieved about 36 h after hCG or nafarelin administration. The methods of IVF, ICSI, blastocyst culture, and cryopreservation with vitrification, and BT have been described previously (17,18). Up to three blastocysts were transferred to each patient 5 or 6 days after oocyte retrieval, and supernumerary blastocysts were cryopreserved. A daily dose of 50-mg progesterone was given for

luteal support till 16 days after oocyte retrieval, when serum hCG was measured.

Pregnancy was determined on the basis of the presence of a gestational sac. Statistical analysis was performed with Student's *t* test.

RESULTS

Of the 40 patients in this study, 10 underwent IVF-BT and 30 underwent ICSI-BT in both the GnRHa long protocol and cetrorelix protocol. In the cetrorelix protocol, oocyte retrieval was successful in all patients, including the four patients given nafarelin instead of hCG to induce ovulation. However, BT was cancelled in two cycles of ICSI because no blastocysts had developed.

The outcome of IVF-BT with the GnRHa long protocol and with the cetrorelix protocol is shown in Table II. The mean total dose of HMG administered was 2197.5 ± 988.4 IU in the GnRHa protocol and 1745.0 ± 443.0 IU in the cetrorelix protocol. The mean number of oocytes retrieved was 18.5 ± 14.8 in the GnRHa protocol and 15.8 ± 7.1 in the cetrorelix protocol. The mean serum E2 concentration on the day of oocyte retrieval was 3192.1 ± 2303.0 pg/mL in the GnRHa protocol and 2671.5 ± 1292.1 pg/mL in the cetrorelix protocol. Since nearly half of the patients lived far away, and local nurses administered their daily dose of HMG and hCG, it was only possible to measure the serum E2 concentration of all patients on the day of oocyte retrieval. The mean number of normal zygotes (2PN) was 13.3 ± 11.7 (fertilization rate: 71.9%) in the GnRHa protocol and 11.7 ± 4.4 (74.1%) in the cetrorelix protocol. The

Table II. Outcome of IVF-BT Obtained Previously with a GnRHa Long Protocol and Obtained in the Same Patients with a Cetrorelix Protocol

	GnRHa long protocol	Cetrorelix protocol
No. of patients	10	10
Total dose of HMG (IU)	2197.5 ± 988.4	1745.0 ± 443.0
No. of oocytes retrieved	18.5 ± 14.8	15.8 ± 7.1
Serum E2 on the day of oocyte retrieval	3192.1 ± 2303.0	2671.5 ± 1292.1
No. of 2PN (%) ^a	13.3 ± 11.7 (71.9) ^a	11.7 ± 4.4 (74.1) ^a
No. of blastocysts (%)	4.5 ± 3.2 (33.8)	5.5 ± 3.9 (47.0)
No. of patients whose embryos developed to expanded blastocysts	2	8***
No. of blastocysts transferred	2.2 ± 1.2	2.3 ± 1.0
No. of BT cycles	10	10
No. of pregnancies (%)	3 (30)	4 (40)
No. of abortions (%)	3 (100)	0**

^aper MII oocyte.

p* < 0.01; *p* < 0.001.

number of blastocysts that developed was 4.5 ± 3.2 (blastocyst development rate: 33.8%) in the GnRHa protocol and 5.5 ± 3.9 (47.0%) in the cetorelix protocol. The mean number of blastocysts transferred was 2.2 ± 1.2 in the GnRHa protocol and 2.3 ± 1.0 in the cetorelix protocol. There were three pregnancies (pregnancy rates: 30%) in the GnRHa protocol and four (40%) in the cetorelix protocol. There were no statistically significant differences between any of the above values obtained in the GnRHa protocol and in the cetorelix protocol. However, the number of patients whose embryos had developed to at least one expanded blastocyst on day 5 was 2 (20%) in the GnRHa protocol and significantly lower than 8 (80%) in the cetorelix protocol ($p < 0.001$), and no abortions occurred in the cetorelix protocol, while all three pregnancies ended in abortion in the GnRHa protocol ($p < 0.01$). One of the four patients who achieved pregnancy with the cetorelix protocol has delivered a healthy infant, and the other three patients are in ongoing pregnancy.

The outcome of ICSI-BT in the GnRHa long protocol and in the cetorelix protocol is shown in Table III. There were no statistically significant differences between the two protocols in the total doses of HMG administered, the serum E2 concentrations on the day of oocyte retrieval, the numbers of oocytes retrieved, the numbers of normal zygotes obtained, the numbers of blastocysts that developed, and the numbers of blastocysts transferred, the same as the outcome of IVF-BT (cf. Table II). In the cetorelix protocol, however, the number of patients whose embryos had developed to at least one expanded blastocyst on day 5 was 17 and was significantly higher than 7 in the GnRHa protocol ($p < 0.001$), and the outcome

with the cetorelix protocol was significantly superior to the outcome with the GnRHa protocol in terms of the numbers of pregnancies (12 vs. 2, $p < 0.001$) and abortion rate (33.3% vs. 100%, $p < 0.005$).

With the cetorelix protocol, six patients delivered eight healthy babies, and two PCO patients who received nafarelin instead of hCG for ovulation induction are in ongoing pregnancy.

DISCUSSION

GnRHa have long been used in controlled ovarian hyperstimulation (COH) regimens for IVF-ET to prevent a premature luteinizing hormone (LH) surge, thereby enabling retrieval of an increased number of oocytes (19,20), a higher fertilization rate, transfer of more embryos (19), a lower cancellation rate (10), and widening of the implantation windows (21). This resulted in a higher pregnancy rate than in COH without using GnRHa. However, use of GnRHa is associated with need for a higher dose of HMG, a higher incidence of OHSS, further pituitary suppression in poor responders, the necessity of full luteal support, and the possibility of development of an ovarian cyst due to the flare-up effect. It is also possible that since GnRH receptors are present in the ovary, prolonged use of GnRHa has an adverse effect on folliculogenesis.

The recent development of GnRH antagonists, such as cetorelix, has allowed us to exclude the GnRHa from the COH protocol. Moreover, the use of GnRH antagonists has been shown to be capable of improving pregnancy outcome in "difficult responders" to GnRHa protocols, and a better outcome of IVF with the cetorelix protocol, which included

Table III. Outcome of ICSI-BT Obtained Previously with a GnRHa Long Protocol and Obtained in the Same Patients with a Cetorelix Protocol

	GnRHa long protocol	Cetorelix protocol
No. of patients	30	30
Total dose of HMG (IU)	2087.0 ± 606.0	1974.0 ± 538.3
No. of oocytes retrieved	11.9 ± 7.1	10.3 ± 4.0
Serum E2 on the day of oocyte retrieval	1613.1 ± 1388.6	1318.9 ± 756.7
No. of 2PN (%) ^a	6.4 ± 4.5 (64.1) ^a	5.7 ± 2.4 (70.7) ^a
No. of blastocysts (%)	2.8 ± 1.9 (43.8)	2.5 ± 1.5 (43.3)
No. of patients whose embryos developed to expanded blastocysts	7	17**
No. of blastocysts transferred	2.0 ± 0.9	2.0 ± 0.8
No. of BT cycles	30	28
No. of pregnancies (%)	2 (6.7)	12 (42.9)***
No. of abortions (%)	2 (100)	4 (33.3)**

^aper MII oocyte.

** $p < 0.005$; *** $p < 0.001$.

clomiphene citrate and HMG, was obtained not only in poor responders to previous GnRHa protocols but also in patients with PCO (16). These results led us to suspect that prolonged use of GnRHa might adversely affect oocyte quality in some patients who had multiple failures of conception after IVF or ICSI-ET.

The results of this study showed that a clomiphene–gonadotrophin–cetorelix protocol that did not include a GnRHa improved pregnancy outcome in patients with a history of multiple conception failures with a GnRHa protocol. Our COH protocol including a GnRH antagonist was associated with retrieval of a smaller number of oocytes and a lower serum E2 concentration than the COH protocol using a GnRHa, although the differences were not statistically significant, and the fertilization rate and blastocyst development rate with the two protocols were comparable. However, a significant difference was observed in the number of patients whose embryos developed to the expanded blastocyst stage or beyond it, with 80% of the IVF patients and 60.7% of the ICSI patients having at least one expanded blastocyst with the cetorelix protocol, as opposed to only 20 and 23.3%, respectively, with the GnRHa protocol. Although the pregnancy rates of the IVF patients were similar with both protocols, the pregnancy rate of the ICSI patients was significantly higher with the cetorelix protocol than the GnRHa protocol, and the abortion rate was significantly lower with the cetorelix protocol than the GnRHa protocol. These results suggest that the higher pregnancy rate and lower abortion rate with the cetorelix protocol are most likely due to improvement of the quality of blastocysts generated. The transfer of higher quality of embryos clearly improved the pregnancy outcome with conventional ET, and even more clearly with BT. Using the scoring system, transfer of at least one expanded blastocyst has been demonstrated to result in a significant increase in the implantation and ongoing pregnancy rates (22). All of the ongoing pregnancies achieved in the present study were in women who had received at least one expanded blastocyst, and these results supported the findings by Minaretzis *et al.* (15) and Craft *et al.* (16). As to the quality of the embryos that developed during COH, Garcia-Velasco *et al.* (14) found that the follicular fluid E2 concentration was significantly lower in patients that received a GnRH antagonist than in those that received a GnRHa, but no significant differences were found between the two groups in follicular fluid progesterone concentration or testosterone concentration, and they suggested that the sig-

nificant effect of GnRH antagonist on ovarian follicular steroidogenesis might favor the development of good-quality embryos. Minaretzis *et al.* (15) also reported that patients treated with a GnRH antagonist yielded more mature oocytes and embryos of better quality than those treated with GnRHa.

The majority of the 30 patients who underwent ICSI (22/30, 73.3%) were not those with infertility due to male factors but due to PCO, endometriosis, or of unknown etiology with a history of low fertilization rate. Low fertilization rates by IVF have been reported in patients with endometriosis (23) and PCO (24), and they are thought to be due to oocyte dysfunction. It is possible that the oocytes of these patients are more adversely affected by prolonged use of the GnRHa and that GnRH antagonist rescues this condition.

None of the four patients who received nafarelin by nasal spray instead of hCG to induce ovulation developed any signs or symptoms of OHSS despite the number of oocytes retrieved (range, 22–33) being similar to that with the previous GnRHa long protocol. This is the first report of successful pregnancy after ovarian stimulation with a GnRH antagonist followed by GnRHa administered by nasal spray, not by injection. Itskovitz-Eldor *et al.* (26) reported that GnRHa (0.2-mg triptorelin) injection after the GnRH antagonist ganirelix led to the achievement of four pregnancies in eight women thought to be at high risk for OHSS, and that none developed any signs or symptoms of OHSS. Although it is difficult to compare the biological activity of two different types of GnRH antagonists, cetorelix, and ganirelix, they are useful in preventing OHSS without worsening the clinical outcome.

In conclusion, the clomiphene citrate–gonadotrophin–cetorelix protocol is very useful for improving the quality of embryos and achieving pregnancy and healthy delivery in patients with a history of multiple failure of conception or delivery after embryo transfer or subsequent blastocyst transfer with the GnRHa long protocol. This protocol is also useful for reducing OHSS in PCO patients by using GnRHa for induction of ovulation.

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