Optimized hormonal stimulation is critical for production of viable embryos and establishment of subsequent implantation

Tao Tao,¹ Alfred Robichaud,¹ Sylvie Nadeau,¹ Rejean Savoie,¹
Bernard Gallant,¹ and Rodney J. Ouellette^{1,2}

Submitted March 23, 2005; accepted October 27, 2005; published online January 4, 2006

Purpose: To investigate the effects of follicle number and size at the time of hCG administration, and ovarian stimulation length on the outcome of in vitro fertilization and pregnancy rate.

Methods: During the ovarian stimulation regimen, the follicular number and size were determined by transvaginal ultrasonographic examination. Ovulation was induced as early as three or more follicles were at least 16 mm in their greatest diameter.

Results: The fertilization rates were significantly increased with the longer length of stimulation (10–12 days: 75.4% and 13–16 days: 83.2%). However, no significant differences in the chemical pregnancy, clinical pregnancy, and implantation rates were found between 10–12 days (53.7%, 43.9%, and 21.8%) and 13–16 days (50.0%, 43.8%, and 23.4%) of stimulation. There were no significant differences in fertilization and chemical pregnancy rates between two groups with $\geq$ and <18 follicles in the ovaries on the day of hCG (human chorionic gonadotrophin) administration. However, the clinical pregnancy and implantation rates (47.2% and 26.0%) in the group with <18 follicles were significantly higher than those (33.3% and 15.5%) in the group with ≥ 18 follicles, respectively.

Conclusions: Excessive and rapid ovarian stimulation appears to decrease the survival of embryos at later stages after transfer. The advantage of prolonged stimulation may outweigh the potential adverse effects in some patients.

KEY WORDS: Embryo and pregnancy; follicle; oocyte; ovary.

INTRODUCTION

In the majority of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles, follicular size and estrogen level are commonly used as indicators of oocyte maturity (1,2). The ovarian stimulation with gonadotropin may be prolonged in some patients who have a suboptimal response until

an acceptable cohort of oocytes reaches a follicular size consistent with oocyte maturity. It is expected that the majority of developed follicles contain a competent and mature oocyte at the time of oocyte retrieval. However, different sizes of follicles are generally observed and most of the follicles need to be aspirated. The criteria used to determine adequate follicular development for oocyte retrieval differs from program to program. The duration of stimulation also varies among patients. Several studies have investigated the effect of the duration of gonadotropin exposure on oocyte quality, but results are controversial. It has been reported that delaying hCG administration decreases fertilization (3) and

¹ Conceptia Reproductive Clinic, Beausejour Medical Research Institute, Georges-L.-Dumont Hospital, Moncton Canada.

² To whom correspondence should be addressed at Institut de Recherche Médicale Beauséjour, Pavillon Hôtel Dieu, 35 Rue Providence St, Moncton, New Brunswick E1C 8X3, Canada; e-mail: rodneyo@health.nb.ca.

pregnancy rates (4,5). In contrast, authors found that 24–48 h delay of hCG administration does not impair oocyte recovery, fertilization, or pregnancy outcomes in a randomized study (6–8).

The purpose of this study was to investigate the effect of duration of ovarian stimulation with gonadotropin on the outcome of in vitro fertilization and pregnancy rate, and to examine the relationship of follicle number and size at the time of hCG administration to the subsequent development of the obtained oocytes.

MATERIALS AND METHODS

Patients and Stimulation

This was a prospective, controlled study, which was approved by the local ethics review committee. Data were obtained from 60 patients undergoing IVF (38 cases) and ICSI (22 cases) at the Conceptia Reproductive Clinic at Moncton, New Brunswick, Canada during a 2-year period from December 2000 to December 2002. The patients, ranged in age from 28 to 42 years, were referred to our clinic with a history of more than 2 years infertility. Patients had the following primary etiologies for their infertility: Male factor, tubal factor, unexplained, endometriosis, anovulatory and the polycystic ovary syndrome. Ovarian stimulation was performed with a GnRH analog (SUPREFACT, Berry Technologies Inc.) downregulation utilizing our long, short, or micro-flare protocols with recombinant FSH (Gonal F, Serono) and LH (Luvisis, Serono) or human menopausal gonadotrophin (Pergonal, Serono). The proportions of cycles that used long, short, and micro-flare protocol were around 70%, 15%, and 15%, respectively. Dose adjusted according to patient's age, body mass index, and previous response to ovarian stimulation (range between 100 and 500 IU). Stimulations were monitored by measurement of serum LH, FSH, hCG, Estradiol, and progesterone level. During the stimulation procedure, dose was also adjusted according to hormone level and follicular response.

Examination of Follicular Development and Ovulation Induction

During the ovarian stimulation regimen, the patients underwent transvaginal ultrasonographic evaluation of endometrial thickness and measurement of follicular number and size, starting on the morning of

day 6 of stimulation. Ultrasound measurements were performed by freezing the image when the follicle appeared maximal and two dimensions perpendicular to each other were measured, the average follicular diameter was then calculated. Ovulation was induced with recombinant hCG (Profesi, Serono) when three or more follicles were at least 16 mm in their greatest diameter, as long as serum progesterone remained in a follicular phase range. In this study, the proportion of different size of follicles, rate of fertilization, and rates of chemical and clinical pregnancy were compared between two groups with $\geq$ and <18 follicles in the ovaries on the day of hCG administration. The rates of oocyte maturation and fertilization, and rates of chemical and clinical pregnancy were compared between 10–12 days and 13–16 days of stimulation.

Oocyte Retrieval and Classification

All oocyte retrievals were done by transvaginal aspiration under ultrasound guidance 36–37 h after hCG administration. The medium used for flushing the follicles is modified human tubal fluid (Irvine Scientific, Irvine, CA) (mHTF). The retrieved oocytes were classified as follows into four categories. 1, Germinal vesicle (GV): this is the most immature stage. There is hardly any cumulus and the corona cells are tightly packed around the oocyte. A large clear nucleus is usually present and can only be seen after removal of the corona cells. 2, Metaphase I (MI): the oocyte is usually surrounded with a tight layer of corona cells and the corona in turn is enclosed tightly with a few cumulus cell layers. Denuding of the corona and cumulus reveals an oocyte with no GV or polar body. 3, Metaphase II (MII): this is the optimal level of maturity and most appropriate for fertilization. Corona cells are visible around the oocyte and these are enclosed by several layers of expanded fluffy cumulus cells. Denuding of corona and cumulus cells reveals a polar body in the perivitelline space. 4, postmature (Luteinized): very few corona cells are visible as the oocyte is enclosed with several layers of fully expanded fluffy cumulus. the oocyte is pale and usually surrounded by dark brown to black cells.

IVF and ICSI

After retrieval, oocytes were rapidly isolated from follicular fluid, rinsed, and placed in IVF-20 medium (Scandinavian IVF, Sweden). Oocytes were inseminated 3–5 h later either by classical IVF with a mean concentration of 200 000 motile spermatozoa

Table I. Proportion of Different Size of Follicles in the Ovaries with $\geq$ or <18 Follicles on the Day of hCG Administration

Group	Age	Number of follicles ($\pm$ SEM)			
		Total	Large (≥ 16 mm, %)	Middle (11–15 mm, %)	Small (≤ 10 mm, %)
≥ 18	32.0 ± 0.5	21.8 ± 0.7	10.8 ± 0.6 (50.2 ± 2.6) ^b	6.1 ± 0.6 (28.5 ± 2.7)	4.9 ± 0.8 (21.2 ± 3.2) ^a
<18	33.8 ± 0.5	11.7 ± 0.5	7.5 ± 0.4 (64.5 ± 2.8) ^a	3.1 ± 0.3 (25.6 ± 2.4)	1.1 ± 0.2 (9.9 ± 2.1) ^b

Note. Values with different letters (on the baseline) in the same column are significantly different ($p < 0.05$).

per mL or by ICSI. Spermatozoa for IVF and ICSI were prepared with the swim-up technique and density gradient centrifugation method according to our standard protocols, respectively. For ICSI cases, cumulus cells were first removed by hyaluronidase (Sigma) and mechanical treatments. ICSI was performed as described by Palermo *et al.* (9). Single motile spermatozoon of the best available morphologic appearance was injected into each mature oocyte. Care was taken to inject as little extra media volume (2 to 3 pL) as possible.

Embryo Culture and Classification

The occurrence of fertilization was determined 16–19 h after insemination. Oocytes with two pronuclei were individually placed in 20 μ L droplets of G1.2 medium (Scandinavian IVF, Sweden) under Ovoil for culture. Incubation was performed at 37°C, in a humidified atmosphere containing 5% CO₂ for 48 h. Transfer was performed approximately 72 h after oocyte retrieval. One to three of the best embryos were replaced at the 6–10 cell stage. The chemical pregnancies were defined as a positive pregnancy test result on luteal day 16 with a rising titer confirmed by a second hCG measurement. The clinical pregnancies were diagnosed by ultrasonographic evidence of embryonic heart activity.

Statistical Analysis

Statistical analysis was performed using *T*-Test for Equality of Means and Binomial Distribution Test when appropriate. Data are presented as means $\pm$ SEM.

RESULTS

General Results

In total of 60 cases (38 IVF; 22 ICSI), 678 oocytes were obtained from 942 follicles, including 528 large follicles (≥ 16 mm), 256 medium follicles (11–15 mm), and 158 small follicles (≤ 10 mm). The recovery rate was 72.0% (678/942).

Effect of Follicle Number and Size. The distribution of different sizes of follicles in two groups is shown in Table I. The proportion of large follicles in the group with <18 follicles in the ovaries on the day of hCG administration are significantly higher than that in the group with ≥ 18 follicles in the ovaries on the day of hCG administration, while the proportions of small follicles in the group with <18 follicles in the ovaries on the day of hCG administration are significantly lower than that in the group with ≥ 18 follicles in the ovaries on the day of hCG administration.

As shown in Table II, the fertilization rates were not significantly different between the two groups. Moreover, the chemical pregnancy rates after transfer were also not significantly different between the two groups. However, the clinical pregnancy and implantation rates (47.2% and 26.0%) in the group with <18 follicles in the ovaries on the day of hCG administration were significantly higher than those (33.3% and 15.5%) in the group with ≥ 18 follicles in the ovaries on the day of hCG administration, respectively.

Effect of Ovarian Stimulation Length. As indicated in Table III, there were no significant differences in the proportion of GV oocytes to total

Table II. Fertilization, Pregnancy, and Implantation Rates According to Follicular Number on the Day of hCG Administration

Group	No	Number of oocytes		Number of pregnancies (%)			
		Total	Fertilized (%)	Chem	Clin	Live birth	Implantation rate
≥ 18	24	15.2 ± 1.1	11.3 ± 0.7 (76.1)	12 (50.0)	8 (33.3) ^b	8 (33.3) ^b	15.5% (11/71) ^b
<18	36	8.7 ± 0.6	6.6 ± 0.5 (77.3)	20 (55.6)	17 (47.2) ^a	17 (47.2) ^a	26.0% (26/100) ^a

Note. Values with different letters (on the baseline) in the same column are significantly different ($p < 0.05$). Chem: Chemical; Clin: Clinical.

Table III. Distribution of Oocytes at Different Stages in Two Durations of Gonadotropin Exposure

Days of stimulation	No. of oocytes				
	Total	GV (%)	MI (%)	MII (%)	PM (%)
10–12	519	60 (11.6)	34 (6.6) a	423 (81.5) a	2 (0.3) b
13–16	143	14 (9.8)	2 (1.4) b	93 (65.0) b	34 (23.8) a

Note. Values with different letters (on the baseline) in the same column are significantly different ($p < 0.05$). GV: germinal vesicle; MI: metaphase I; MII: metaphase II; PM: postmature.

oocytes among the different durations of stimulation. However, the proportion of MI oocytes was significantly decreased with the longer length of stimulation (10–12 days: 6.6% and 13–16 days: 1.4%). The proportion of MII oocytes (the optimal level of maturity) in 10–12 days of stimulation (81.5%) was significantly greater than those in 13–16 days (65.0%) of stimulation, whereas the proportion of PM oocytes was significantly greater for the group of 13–16 day stimulation (23.8%).

Table IV shows that the fertilization rates were significantly increased with the longer length of stimulation (10–12 days: 75.4% and 13–16 days: 83.2%). However, there were no significant differences in chemical pregnancy, clinical pregnancy, and implantation rates between 10–12 and 13–16 days of stimulation.

DISCUSSION

Our results demonstrate that high numbers of follicles (≥ 18 follicles) in the ovaries on the day of hCG administration result in a lower clinical pregnancy rate and implantation rate, albeit no differences in fertilization rate and chemical pregnancy rate were observed in the study groups. It appears that excessive stimulation may affect the survival of embryos at later stages after transfer. Interestingly, our results also show that the proportions of small follicles in the group with ≥ 18 follicles in the ovaries on the day of hCG administration are significantly higher than that in the group with < 18 follicles in the ovaries on the

day of hCG administration. To our knowledge, this is the first time to describe the findings of proportion of large, medium, and small follicles in human stimulated ovaries. It is generally believed that larger follicles may result in higher oocyte recovery rates (10,11) and may yield more mature oocytes (12). In bovine, it was shown that oocytes derived from smaller follicles had increased fertilization abnormalities as a result of incomplete synthesis of maternal RNA transcripts (13,14). In mice, Eppig *et al.* (15) noted that even though oocytes from small follicles may be competent of completing nuclear maturation, they can still be deficient in maternal factors implicated in cytoplasmic maturation and essential for development of embryos. This could partially explain the reduction of clinical pregnancy in the group with ≥ 18 follicles in the ovaries on the day of hCG administration. Further study is required to compare the development of oocytes derived from different size of follicles. The viability of oocytes obtained from small follicles might be improved by performing in vitro maturation and postponing fertilization.

The scheduled administration of hCG in ovarian stimulation schemes used in assisted reproduction has made possible to control the ovulatory phenomenon. In the present study, examination of oocyte recovery, fertilization, pregnancy, and implantation rates according to the length of stimulation indicates that delaying hCG administration until an acceptable cohort of oocyte reaches a predetermined follicular size is beneficial. The criterion used for timing hCG administration in our clinic permits such delaying. Furthermore, no cycle

Table IV. Fertilization, Pregnancy, and Implantation Rates According to the Length of Stimulation

Days of stimulation	No	No. of oocytes fertilized (%)	No. of pregnancies (%)			
			Chem	Clin	Live birth	Implantation rate
10–12	41	9.4 $\pm$ 0.6 (75.4) b	22 (53.7)	18 (43.9)	18 (43.9)	21.8% (26/119)
13–16	16	7.3 $\pm$ 0.8 (83.2) a	8 (50.0)	7 (43.8)	7 (43.8)	23.4% (11/47)

Note. Values with different letters (on the baseline) in the same column are significantly different ($p < 0.05$). Chem: Chemical; Clin: Clinical.

was cancelled during this study. The stimulation length in our study is greater than that reported in most previous studies. Although some oocytes retrieved were postmature, there were still a sufficient number of optimal mature oocytes. It is known that prolonged stimulation correlates with poor ovarian response (5,16). The stimulations were prolonged to obtain a greater number of large follicles by the time hCG was administered. In our study, patients who received 13–16 days of gonadotropin stimulation had comparable clinical pregnancy and implantation rates. This indicates that in those patients, the advantage of prolonged stimulation, and thus the greater number of available oocytes may outweigh the potential adverse effects on the endometrium and on the quality of oocytes and embryos. This study has demonstrated that hCG may be administered over a range of several days (10–16 days) with acceptable clinical results. This flexibility can be quite useful for efficient patient scheduling. This result is contrary to some of the recent reports (4,16). This could be possibly due to the differences in the criteria used for inducing final oocyte maturation. In our study, hCG was administered as soon as at least three follicles were ≥ 16 mm on ultrasound, while in most other studies, triggering of final oocyte maturation is performed in the presence of at least three follicles with a maximum diameter of ≥ 17 mm (17–20) or ≥ 18 mm (21).

In our results, we also found that 8–9 days of stimulation resulted in high chemical pregnant rate (66.7%, 2/3), but no clinical pregnancy. This observation, albeit limited by the small number of patients, is consistent with previous reports that oocytes may undergo nuclear maturation and successful fertilization, however, yet not have completed cytoplasmic maturation, as indicated by the inability to progress beyond the blastocyst stage (15,22). This shows that although the occurrence of polar body extrusion, fertilization, and transferrable embryos are possible, they are not useful as indicators of developmental potential or maturity.

In conclusion, our findings show that excessive ovarian stimulation can decrease the survival of embryos at later stages after transfer. Techniques that avoid excessive follicular recruitment, such as using insulin sensitizing agent to reduce the basal follicular count in polycystic ovary in insulin-resistant patients, oral contraceptive for a few months prior to cycle initiation, or better gonadotrophin dose adjustment will have to be evaluated. Prolongation of ovarian stimulation does not affect the rates of pregnancy and im-

plantation in some patients. The broad range of stimulation time (10–16 days) will allow greater flexibility in the decision-making process for the timing of hCG administration in the ART cycles.

REFERENCES

1. Van Wissen B, Eisenberg C, Debey P, Pennehouat G, Auger J, Bomsel-Helmreich O: In vitro DNA fluorescence after in vitro fertilization (IVF) failure. *J Assist Reprod Genet* 1992;9:564–571
2. Chiu TTY, Tam PPL: A correlation of the outcome of clinical in vitro fertilization with the inositol content and embryotrophic properties of human serum. *J Assist Reprod Genet* 1992;9:524–530
3. Laufer N, DeCherney AH, Tarlatzis BC, Zuckerman AL, *et al.*: Delaying human chorionic gonadotropin administration in human menopausal gonadotropin-induced cycles decreases successful in vitro fertilization of human oocytes. *Fertil Steril* 1984;42:198–203
4. Clark L, Stanger J, Brinsmead M: Prolonged follicle stimulation decreases pregnancy rates after in vitro fertilization. *Fertil Steril* 1991;55:1192–1194
5. Catherino WH, Leondires M, McKeeby J, Cruess D, Segars JH, *et al.*: Prolonged ovarian stimulation in ART cycles does not improve clinical pregnancy rates in poor responders. *Fertil Steril* 2002;78:S49
6. Dimitry ES, Bates SA, Oskarsson T, Margara R, Winston RML: Programming in vitro fertilization for a 5- or 3-day week. *Fertil Steril* 1991;55:934–938
7. Dimitry ES, Oskarsson T, Conaghan J, Margara R, Winston RML: Beneficial effects of a 24-h delay in human chorionic gonadotropin administration during in vitro fertilization treatment cycles. *Hum Reprod* 1991;6:944–946
8. Tan SL, Balen A, Hussein E, Mills C, Campbell S, Yovich J, *et al.*: A prospective randomized study of the optimum timing of human chorionic gonadotropin administration after pituitary desensitization in in vitro fertilization. *Fertil Steril* 1992;57:1259–1264
9. Palermo G, Joris H, Devroey P, van Steirteghem AC: Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340:17–18
10. Ben-Rafael Z, Koof GS, Blasco L, Flickinger GL, *et al.*: Follicular maturation parameters associated with the failure of oocyte retrieval, fertilization, and cleavage in vitro. *Fertil Steril* 1986;45:51–57
11. Battaglia C, Regnani G, Petraglia F, Genazzani AR, Artini PG, Volpe A: The use of a starting dose of recombinant follicle stimulating hormone for controlled ovarian hyperstimulation: A randomized pilot study. *Gynecol Endocrinol* 2000;14:311–315
12. Jones HW Jr, Acosta AA, Andrews MC, Garcia JE, Jones GS, Mayer J, *et al.*: Three years of in vitro fertilization at Norfolk. *Fertil Steril* 1984;42:826–834
13. Crozet N, Kanka J, Motlik, *et al.*: Nucleolar fine structure and RNA synthesis in bovine oocytes from antral follicles. *Gamete Res* 1986;14:65–74
14. Sirard MA, Florman HM, Liebfried-Rutledge ML, *et al.*: Timing of nuclear progression and protein synthesis

- necessary for meiotic maturation of bovine oocytes. *Biol Reprod* 1989;40:1257–1263
15. Eppig JJ, Schultz RM, O'Brien M, *et al.*: Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Dev Biol* 1994;164:1–9
 16. Ivani KA, Weckstein LN, Walker DM: Effect of length of stimulation in ART cycles on pregnancy rate. *Fertil Steril* 2003;80:181–182
 17. Borm G, Mannaerts B: Treatment with the gonadotropin-releasing hormone antagonist ganirelix in women undergoing ovarian stimulation with recombinant follicle stimulating hormone is effective, safe, and convenient: Results of a controlled, randomized, multicentre trial. The European Orgalutran Study Group. *Hum Reprod* 2000;15:1490–1498
 18. Fluker M, Grifo J, Leader A, Levy M, Meldrum D, Muasher SJ, *et al.*: Efficacy and safety of ganirelix acetate versus leuprolide acetate in women undergoing controlled ovarian hyperstimulation. *Fertil Steril* 2001;75:38–45
 19. Kolibianakis E, Bourgain C, Albano C, Osmanagaoglu K, Smitz J, van Steirteghem A, *et al.*: Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin-releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. *Fertil Steril* 2002;78:1025–1029
 20. Kolibianakis EM, Albano C, Kahn J, Camus M, Tournaye H, van Steirteghem AC, *et al.*: Exposure to high levels of luteinizing hormone and estradiol in the early follicular phase of gonadotropin-releasing hormone antagonist cycles is associated with a reduced chance of pregnancy. *Fertil Steril* 2003;79:873–880
 21. Garcia-Velasco JA, Isaza V, Vidal C, Landazabal A, Remohi J, Simon C, *et al.*: Human ovarian steroid secretion in vivo: Effects of GnRH agonist versus antagonist (cetrorelix). *Hum Reprod* 2001;16:2533–2539
 22. Morgan PM, Warikoo PK, Bavister BD: In vitro maturation of ovarian oocytes from unstimulated rhesus monkeys: Assessment of cytoplasmic maturity by embryonic development after in vitro fertilization. *Biol Reprod* 1991;45:89–93