Embryonic early-cleavage rate is decreased with aging in GnRH agonist but not in antagonist protocols

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Abstract

Purpose The aim of this study was to evaluate the correlation between embryonic early-cleavage status and the age of patients receiving either a GnRH agonist long protocol or a GnRH antagonist protocol.

Methods This retrospective study included 534 patients undergoing a fresh cycle of oocyte retrieval and day-3 embryo transfer. Of the 534 patients treated, 331 received a GnRH agonist long stimulation protocol (GnRH agonist group) for ovarian stimulation and 203 patients received a GnRH antagonist protocol (GnRH antagonist group).

Results By logistic regression analysis, the rate of embryonic early-cleavage was significantly decreased with increasing age of women in the agonist ($P<0.001$) but not in antagonist groups ($P=0.61$). Based on the results of this study, maternal age is a critical factor for embryonic early-cleavage in agonist protocol but not in antagonist protocol. The results also showed that early-cleavage embryos were of better quality and resulted in

a higher pregnancy rate than late-cleavage embryos in the GnRH agonist group. However, embryo quality and pregnancy rate was not significantly different between early and late cleavage embryos in the GnRH antagonist group.

Conclusions We conclude that embryonic early-cleavage status is negatively correlated with aging in women receiving GnRH agonist long down-regulation but not in GnRH antagonist protocols. We also conclude that early cleavage of the zygote is not a reliable predictor for pregnancy potential using the GnRH antagonist protocol.

Keywords Aging · Embryonic early cleavage · GnRH agonist long protocol · GnRH antagonist protocol

Capsule Embryonic early-cleavage status is negatively correlated with aging in women receiving GnRH agonist long down-regulation but not in GnRH antagonist protocols. Early cleavage of the zygote is not a reliable predictor for pregnancy potential using the GnRH antagonist protocol.

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Introduction

In order to reduce multiple pregnancies and achieve a maximal rate of implantation, selection of the most viable embryo for transfer has become a high priority in assisted reproduction treatment. Traditionally, embryo selection is performed by using embryo morphology as the guideline. Currently, observation of embryonic first mitosis has been emphasized. Several studies have shown that embryonic early cleavage, which is the time period 25–27 h post insemination for in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI), can be an additional indicator of viable embryos [1–3].

In Bos-Mikich et al. (2001) study, there is a statistical analysis (Student's *t*-test) to support the finding that the group of patients that presented at least one early cleavage embryo was younger than the group of patients without any early cleavage embryo [4]. However, there has been no statistical analysis supporting this observation. Moreover, most of these previous studies were only using the gonadotrophin-releasing hormone (GnRH) agonist long protocol for pituitary suppression.

Recently, a GnRH antagonist protocol has become available in assisted reproduction therapy [5]. We wondered whether the effects of these two different protocols upon embryonic early cleavage were the same. Therefore, the aim of this study was to evaluate the correlation between embryonic early-cleavage status and the age of patients receiving either a GnRH agonist long protocol or a GnRH antagonist protocol. Furthermore, we compared the pregnancy rate between the early-cleavage and late cleavage subgroups in both GnRH agonist and antagonist protocols.

Materials and methods

Study subjects

This study was a retrospective study of IVF/ICSI outcome based on the medical records of patients in the Fertility Unit of MacKay Memorial Hospital from the time period, January 2006 to December 2008. The study protocol was approved by the Institutional Review Board of MacKay Memorial Hospital in Taipei, Taiwan.

To minimize confounding factors, patients diagnosed with polycystic ovaries, a severely poor response (fewer than two retrieved oocytes and an E₂ level ≤ 300 pg/ml on the day of human chorionic gonadotrophin (hCG) administration), and cycles for oocyte donation were excluded from the study. Only patients undergoing their first stimulated cycle were included and only one treatment cycle per patient was analyzed. A total of 534 embryo transfer cycles were included in this study. There were 331 cycles treated with an agonist protocol and the other 203 cycles were treated with an antagonist protocol. Patients with male factor infertility (which was defined by the presence of any of the following parameters: sperm concentration $<15 \times 10^6$ /ml, motility <40 %, and normal morphology <4 %) were treated with ICSI, while others without male factor infertility were treated with conventional insemination for IVF.

Ovarian stimulation protocols

Two stimulation protocols were used in this study; the GnRH agonist long protocol and the GnRH antagonist protocol. In the GnRH agonist group, leuprolide acetate (Takeda Pharma GmbH, Stolberg, Germany) was given at a daily dose of 1 mg, starting on day 21 of the previous cycle. Recombinant follicle-stimulating hormone (FSH; Gonal-F; Serono Laboratories, Aubonne, Switzerland) and human menopausal gonadotropin (hMG; Menopur; Ferring GmbH, Kiel, Germany) were given together from the second day of menstrual cycle until the day of hCG administration. The doses were adjusted according to the patient's ovarian response. In the GnRH antagonist group, recombinant FSH and hMG together were administered daily from the second day of the menstrual cycle. The doses were

also adjusted according to the patient's individual ovarian response. Once the dominant follicle reached 14 mm in mean diameter, cetrorelix (Cetrotide; Serono, Baxter Oncology GmbH, Halle, Germany) was administered subcutaneously at a dose of 0.25 mg daily until the day of hCG administration.

In both groups, 10,000 IU hCG (Profasi; Serono Laboratories, Aubonne, Switzerland) was administered when at least two follicles reached 18 mm in diameter, and oocyte retrieval was performed 34–36 h later. Upon completion of oocyte collection and IVF/ICSI, embryos were graded morphologically by the same embryologist. Embryo transfer was performed 72 h after the oocyte retrieval. Embryonic early cleavage, which occurs at 25–27 h post insemination for IVF/ICSI, defined as early cleavage [6, 7]. In each stimulation protocol, patients who had at least one early-cleavage embryo transferred were designated as the 'early-cleavage' subgroup. Patients who had no early-cleavage embryos transferred were designated as the 'late-cleavage' subgroup.

The luteal phase was supported by vaginal supplementation with 200 mg micronized natural progesterone (Progeffik; Effik, Paris, France) three times a day. To assess treatment outcome, serum hCG was measured 14 days after oocyte retrieval. An increase in serum hCG levels above 50 IU/l indicated pregnancy.

Assessment of fertilization, early cleavage and embryo quality

Normal fertilization was confirmed by the presence of two pronuclei and two polar bodies at 16–20 h (day 1) after insemination for IVF or ICSI. On the same day, early cleavage examination was performed at 25–27 h after insemination. Embryos displaying two cells at inspection were designated as 'early cleavage'. The embryos that had not yet cleaved to the 2-cell stage were designated as 'late cleavage'. Embryos were further examined for their quality at 44–46 h (day 2) and at 66–68 h (day 3). The day 2 and day 3 embryo scoring system (modified from Veeck, 1999) [8] was as follows: for day 2 embryos, namely: score 1: blastomeres of equal size and no cytoplasmic fragmentation; score 2: blastomeres of equal or unequal size and cytoplasmic fragmentation ≤ 10 %; score 3: blastomeres of equal or unequal size and 11–49 % overall cytoplasmic fragmentation; score 4: blastomeres of equal or unequal size and cytoplasmic fragmentation ≥ 50 %. For the day 3 embryos, namely: score 1: blastomeres of equal size with 6–8 blastomeres and no cytoplasmic fragmentation; score 2: blastomeres of equal or unequal size with 6–8 blastomeres and cytoplasmic fragmentation ≤ 20 %; score 3: blastomeres of equal or unequal size with 6–8 blastomeres and 21–49 % overall cytoplasmic fragmentation; score 4: blastomeres of equal or unequal size with 6–8 blastomeres and cytoplasmic fragmentation ≥ 50 % or fewer than 6 blastomeres. Ongoing pregnancy was defined when the pregnancy had completed ≥ 20 weeks of gestation.

Statistical analysis

Statistical analysis was performed using SPSS software version 12.0 for Windows (Statistical Package for Social Sciences, Inc., USA). The differences of means between two variables were calculated using the Mann–Whitney *U* test. The differences in binary variables were calculated using the chi-square test.

To evaluate the correlation between age and early-cleavage status of the embryo, logistic regression analysis was used. In order to analyze the correlation, graphs were drawn in a logistic regression model with the X-axis as patients' age and the Y-axis as the ratio of early-cleavage embryos. A *P* value of <0.05 was considered statistically significant.

Results

A total of 534 treatment cycles were analyzed. Of these, 331 cycles were from the agonist group while 203 cycles were from the antagonist group. Cycle characteristics of the agonist and antagonist groups are given in Table 1. The embryonic early-cleavage rate and the pregnancy rate were higher in the agonist group (45 and 56 %, respectively) than the antagonist (28 and 45 %, respectively). But the ongoing pregnancy rate between two groups was similar.

A total of 3425 embryos were analyzed; 2259 embryos were from the agonist group and 1166 embryos were from the antagonist group. In the agonist group, 926 (41 %) of 2259 embryos were early-cleavage embryos; the day 2 and day 3 mean embryo scores were 1.82 ± 0.03 and 1.93 ± 0.03 , respectively (Table 2); the day 2 and day 3 mean number of blastomeres were 4.01 ± 0.04 and 7.28 ± 0.06 , respectively (Table 2). The remaining 1333 (59 %) embryos were late-cleavage embryos; the day 2 and day 3 mean embryo scores for those were 2.11 ± 0.03 and 2.52 ± 0.03 , respectively. The

day 2 and day 3 mean number of blastomeres were 3.17 ± 0.04 and 6.16 ± 0.06 , respectively (Table 2). There were highly significant differences in day 2 and day 3 embryo scores between early and late-cleavage embryo subgroups (1.82 ± 0.03 vs. 2.11 ± 0.03 and 1.93 ± 0.03 vs. 2.52 ± 0.03 , $P < 0.001$) (Table 2). There were also dramatically significant differences in the day 2 and day 3 mean number of blastomeres between the early and late-cleavage embryo subgroups (4.01 ± 0.04 vs. 3.17 ± 0.04 and 7.28 ± 0.06 vs. 6.16 ± 0.06 , $P < 0.001$) (Table 2). In the agonist group, 216 cycles had at least one early-cleavage embryo for transfer while the remaining 115 cycles had all late-cleavage embryos for transfer. The pregnancy rate and ongoing pregnancy rate were significantly higher in the early-cleavage subgroup than late cleavage subgroup (64 % vs. 41 % and 47 % vs. 27 %, respectively, $P < 0.001$) (Table 2).

In the antagonist group, 323 (28 %) of 1166 embryos were early-cleavage embryos while the remaining 843 (72 %) embryos were late-cleavage embryos. There were no significant differences in day 2 and day 3 embryo scores or in day 3 number of blastomeres between early and late-cleavage embryo subgroups in the antagonist group (1.92 ± 0.04 vs. 2.11 ± 0.04 , 2.02 ± 0.06 vs. 2.10 ± 0.06 , and 7.29 ± 0.10 vs. 7.18 ± 0.10 , $P > 0.05$) (Table 3). However, there was a highly significant difference in day 2 number of blastomeres (4.11 ± 0.06 vs. 3.21 ± 0.06 , $P < 0.001$) (Table 3). In the antagonist group, 98 cycles had at least one early-cleavage embryo for transfer while the remaining 105 cycles had all late-cleavage embryos for transfer. However, there were no significant difference in pregnancy rate and ongoing pregnancy rate between the early-cleavage subgroup and late cleavage subgroup (46 % vs. 44 % and 40 % vs. 38 %, respectively, $P > 0.05$) (Table 3).

To examine the relationship between embryonic early-cleavage status and age of women, a logistic regression analysis was performed (Figs. 1 and 2). In the agonist group, there was a significant correlation between age and early-cleavage status ($P < 0.001$). A negative correlation was noted between

Table 1 Characteristics of the patients in the agonist and antagonist groups

	Agonist group	Antagonist group	<i>P</i> value
No. of cycles	331	203	
Age of patients	33.9 ± 0.2	34.7 ± 0.3	NS
Duration of stimulation (days)	10.3 ± 0.1	9.9 ± 0.1	NS
No. of ampoules used per cycle	35.4 ± 0.6	34.4 ± 0.3	NS
Peak E ₂ level	2347 ± 71	1731 ± 81	<0.05
No. of oocytes retrieved per cycle	10.0 ± 0.3	9.2 ± 0.4	NS
No. of mature oocytes per cycle	9.0 ± 0.3	8.2 ± 0.3	NS
No. of fertilized oocytes per cycle	6.8 ± 0.3	6.2 ± 0.3	NS
Total no. of embryos	2259	1166	
Embryonic early- cleavage rate	41 % (926/2259)	28 % (323/1166)	<0.001
Pregnancy rate	56 % (185/331)	45 % (91/203)	<0.05
Ongoing pregnancy rate	40 % (134/331)	39 % (79/203)	NS

Values are the mean $\pm$ SEM. NS no significance

Table 2 Embryonic score and mean number of blastomeres of the early-cleavage and late-cleavage subgroups in the agonist group

	Early-cleavage subgroup (<i>n</i> =216)	Late-cleavage subgroup (<i>n</i> =115)	<i>P</i> value
Day 2 embryonic score	1.82±0.03	2.11±0.03	<0.001
No. of day 2 blastomeres	4.01±0.04	3.17±0.04	<0.001
Day 3 embryonic score	1.93±0.03	2.52±0.03	<0.001
No. of day 3 blastomeres	7.28±0.06	6.16±0.06	<0.001
Pregnancy rate (%)	64 % (138/216)	41 % (47/115)	<0.001
Ongoing pregnancy rate	47 % (102/216)	27 % (32/115)	<0.001

Values are the mean ± SEM

embryonic early-cleavage status and the age of patients with coefficients (R^2) of 0.49 receiving a GnRH agonist protocol for controlled ovarian stimulation. In the antagonist group, there was no significant correlation ($P=0.61$) between embryonic early-cleavage status and the age of patients and showed low coefficients (R^2) of 0.01.

Discussion

In the present study, we demonstrated that embryonic early-cleavage rate was significantly negatively correlated with the age of women using GnRH agonist long protocol but not in antagonist protocol. Consistent with previous studies [1–3], we also observed that embryonic early-cleavage was a good predictor for early embryonic development and pregnancy outcome using a GnRH agonist long protocol (Table 2). However, we did not find the same relationship using a GnRH antagonist protocol (Table 3). Also, we found that embryonic early-cleavage rate was significantly lower using the GnRH antagonist protocol than the agonist protocol (Table 1). In our previous study [9], we observed that the early-cleavage

rate was significantly lower with the GnRH antagonists and was not a reliable predictor of pregnancy in women older than 35. In the present study, we also observed this relationship without any age limitation. It is possible that the first mitosis of zygote (early cleavage) could be retarded if insulin-like growth factor (IGF), epidermal growth factor (EGF) and the MAP kinase (MAPK) signal transduction pathway are inhibited by GnRH antagonists [9].

To achieve a singleton pregnancy without reducing the implantation rate should be the primary goal in assisted reproduction treatment. Up to now, embryonic morphology has been one of the most useful tools to achieve this goal. Currently, embryonic early-cleavage observed 25–27 h after insemination has been suggested to be another available parameter for embryo selection [1–3]. Although several studies have observed that embryonic early-cleavage rate seemed to occur more frequently in young women (<35 years) [1, 2, 4], there was no statistical correlation analysis to analyze the correlation between female age and embryonic early-cleavage rate. Moreover, all these previous studies used a GnRH agonist long protocol for pituitary suppression. In the present study, we found that embryonic early-cleavage rate was

Fig. 1 Correlation between age and early cleavage status in a logistic regression model when using the GnRH agonist long protocol (*n*=331). After logistic regression, $P<0.001$

Table 3 Embryonic score and mean number of blastomeres of the early-cleavage and late-cleavage subgroups in the antagonist group

	Early-cleavage subgroup (<i>n</i> =98)	Late-cleavage subgroup (<i>n</i> =105)	<i>P</i> value
Day 2 embryonic score	1.92±0.04	2.11±0.04	NS
No of day 2 blastomeres	4.11±0.06	3.21±0.06	<0.001
Day 3 embryonic score	2.02±0.06	2.10±0.06	NS
No. of day 3 blastomeres	7.29±0.10	7.18±0.10	NS
Pregnancy rate	46 % (45/98)	44 % (46/105)	NS
Ongoing pregnancy rate	40 % (39/98)	38 % (40/105)	NS

Values are the mean ± SEM. NS no significance

significantly negatively correlated with the age of women using either GnRH agonists but not using antagonists by logistic regression analysis.

As women age, the decline in fertility rate mostly depends upon decreasing oocyte quality [10]. Aging was shown to be associated with decreasing in the fraction of mitochondria in the ooplasm, and increased frequencies of dilated smooth endoplasmic reticulum and Golgi complexes in the oocytes of the primordial follicles [11]. Among these ultrastructural organelles, mitochondria play a vital role in the metabolism of energy-containing compounds in the ooplasm, providing adenosine triphosphate (ATP) for fertilization and preimplantation embryonic development [12]. Loss of mitochondrial activity in oocytes obtained from aging women in IVF treatment could lead to lower embryonic development and pregnancy rates [12, 13]. Furthermore, the age-associated reduction in embryonic developmental competence may be related to an inadequate capacity to generate ATP at levels sufficient to support normal chromosomal segregation [14] or normal mitotic, and other physiological activities within blastomeres, with resultant developmental abnormalities during

preimplantation embryogenesis [15]. Efficiency of the mitochondrial matrix in the conversion of pyruvate to ATP is necessary for diverse cellular processes in developing embryos, including cell division, DNA replication, and genomic activation [12]. Therefore, if mitochondrial dysfunction occurs with aging, the rate of first cell division of preimplantation embryos should be affected. In fact, in the present study we observed that the rate of early cleavage of zygotes was indeed decreased in aging women.

In the present study, the embryo quality and pregnancy rate showed no significant difference between early and late cleavage embryos in the GnRH antagonist group (Table 3). Previous studies have shown that continuous exposure to GnRH antagonists will induce down-regulation of the GnRH receptor and a decrease in GnRH receptor gene expression [16, 17]. With the discovery of extra-pituitary GnRH receptors in reproductive tissues, including the uterus, endometrium, oocyte-cumulus complex, pre-implantation embryo and placenta [18–20], it is possible that using GnRH antagonists may lead to down-regulation of these receptors. Some in-vitro studies using cancer cell lines to investigate the effect of GnRH

Fig. 2 Correlation between age and early cleavage status in a logistic regression model when using the GnRH antagonist protocol (*n*=203). After logistic regression, *P*=0.61

antagonists [21, 22] also showed GnRH antagonists may affect cell growth by decreasing the synthesis of insulin-like growth factor (IGF) and inositol 1, 4, 5-trisphosphate (IP₃). Another study showed that a decrease in epidermal growth factor (EGF) receptor mRNA and EGF peptide occurs in the presence of GnRH antagonists in vitro [23]. Both EGF and IGF receptors form the basis for the generation of an important mitogenic cascade, mediated by MAP kinase (MAPK) [24, 25]. Based on the results of the above studies, it is highly possible that first mitosis of the zygote (early cleavage) could be retarded if IGF, EGF and the MAPK signal transduction pathway are inhibited by GnRH antagonists.

In IVF cycles, administration of the GnRH antagonist is suspended once hCG is given, and the embryo is transferred between 5 and 7 days after the last dose of GnRH antagonist. The half-life of the GnRH antagonist stated by the manufacturer is 30 h and the time interval between the last dose of the GnRH antagonist and the early cleavage of zygotes is about 75 to 90 h. Consequently, the GnRH antagonist may still have some effect on zygotes to delay the first mitosis. Furthermore, the average embryo score on days 2 and 3 was also not significantly different between early-cleavage and late-cleavage embryos in the GnRH antagonist group (Table 3). Therefore, the effect of the GnRH antagonist on the pre-implantation embryo may only be limited to the first 2 days after insemination or ICSI. The potential detrimental effect of the GnRH antagonist upon the developing embryo may be significantly diminished after 2 days of insemination. This could be the reason for a lack of any significant negative effect of GnRH antagonists on pregnancy and live birth outcome [26–28]. Based on the results in Table 3 and previous reports, early cleavage of the zygote is not a reliable predictor for pregnancy potential using the GnRH antagonist protocol. During the embryonic culture period, decreased time to exposure the room air is important. If early embryonic cleavage is not a reliable predictor in GnRH antagonist protocol, we can neglect this procedure to reduce the embryonic exposure the room air.

In conclusion, the results of this study showed that early-cleavage rate was negatively correlated with aging in using GnRH agonist but not in using antagonist protocols. The results also showed that early cleavage of the zygote seems not to be a powerful predictor for embryonic viability using the GnRH antagonist protocol. Further studies are warranted to confirm these findings.

Conflict of interest All the authors declare that they have no conflict of interest.

Compliance with ethical requirements This article is a retrospective study. For this type of study formal consent is not required. This article does not contain any studies with animal subjects.

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