Hormone-induced delayed ovulation affects early embryonic development

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Objective: To analyze the effects of delayed ovulation on embryonic development in mice, because intrafollicular oocyte development may be delayed during assisted reproductive technology (ART) treatment in humans.
Design: Experimental mouse study.
Setting: University hospital.
Animal(s): Female C57Bl/6 mice.
Intervention(s): Cetrorelix is used as a GnRH-antagonist in ART treatments. To assess the effect of delayed ovulation on embryonic development, cetrorelix was applied concomitantly with follicle stimulation by pregnant mare serum gonadotropin. Ovulation was induced by hCG. Controls were stimulated with pregnant mare serum gonadotropin without delaying ovulation. Suppression of ovulation was assessed from the number of tertiary follicles, ruptured follicles, and corpora lutea in mouse ovaries after cetrorelix treatment. Number and weight of embryos and placentas, as well as number of resorption sites and dead embryos, was determined on day 17.5 of pregnancy.
Main Outcome Measure(s): Inhibition of ovulation, embryonic development.
Result(s): Cetrorelix inhibited ovulation in mice, as shown by an increase in number of tertiary follicles concomitant with a significant inhibition of follicle rupture and corpora lutea formation. Delayed ovulation caused by Cetrorelix treatment led to a significant increase in resorption sites and a significant decrease in embryonic weight of offspring.
Conclusion(s): Preovulatory oocyte overripeness might have an effect on fertility and embryonic development during ART treatment. (Fertil Steril® 2011;95:2390–4. ©2011 by American Society for Reproductive Medicine.)

Key Words: GnRH-antagonist, cetrorelix, oocyte quality, oocyte maturation, embryo development

Preovulatory and postovulatory aging leading to oocyte overripeness (1) can occur in fertile females of any age as a result of delayed ovulation or delayed fertilization, respectively. Effects of postovulatory oocyte aging have been investigated extensively (2). However, prolonged preovulatory aging may also have an effect on oocyte developmental competence (3). Experiments in *Xenopus laevis* showed that preovulatory overripeness of oocytes leads to high embryo mortality and malformations (4), and studies in rats revealed chromosomal anomalies, changes in RNA and protein synthesis, and an increase in embryonic death and developmental defects in oocytes aged intrafollicularly (5–7). In humans, a prolonged follicular phase has been reported to increase the risk of congenital malformations (8). Because it is well established that oocyte quality determines the developmental potential of the embryo after fertilization (9), ART treatment leading to delayed ovulation may have an effect on oocyte developmental competence. These concerns are substantiated in current discussions of possible adverse health effects of assisted reproductive technologies, which include hormonal treatments, IVF, and embryo culture. Children conceived through these technologies are at increased risk of low birth weight, premature birth, malformations, and genomic imprinting disorders (10–15). Currently, little is known about the effects of preovulatory aging of oocytes, and the causal relationship between impaired oocyte quality because of intrafollicular overripeness and developmental defects remains elusive. To further analyze the effect of delayed ovulation on embryo development, we established a mouse model in which the LH surge was suppressed by cetrorelix. This GnRH antagonist binds competitively to the same receptor as GnRH, and equimolar amounts of cetrorelix and GnRH lead to a complete suppression of LH secretion (16). This compound is one of the most advanced GnRH antagonists available to date, and it is commonly used to suppress LH surges in women in assisted reproductive technology (ART) programs (17).

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MATERIALS AND METHODS

Animals

Adult mice (C57Bl/6) were maintained in 12-hour light and dark cycles with free access to food and water. Stage of ovarian cycle was determined by

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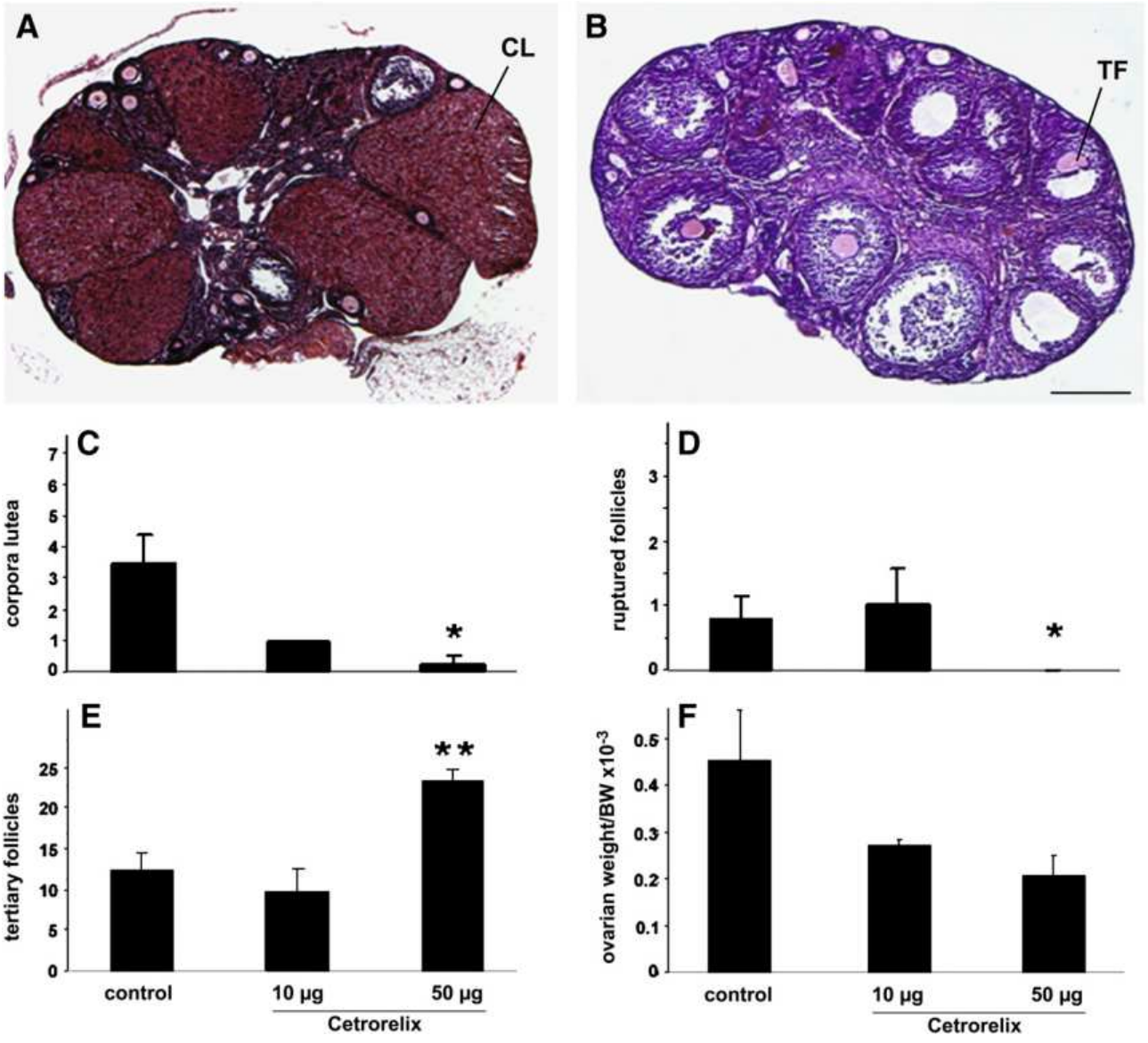
FIGURE 1

Ovaries of untreated (**A**) and cetrorelix-treated mice (**B**). Control ovaries show abundant corpora lutea (CL), whereas ovaries of cetrorelix-treated mice reveal high numbers of tertiary follicles (TFs). Scale bar = 200 μm. (**C**) Number of corpora lutea decreased dose dependently after cetrorelix-treatment (significant for 50 μg cetrorelix group). No ruptured follicles, as signs of ovulation, were observed in ovaries of mice treated with 50 μg cetrorelix (**D**), but the number of tertiary follicles increased significantly (**E**). After cetrorelix treatment, ovarian weight as percentage of body weight (BW) decreased slightly compared with controls (**F**). Controls, n = 9; cetrorelix-treated group, n = 4; *$P<0.05$ and **$P<0.005$ compared with controls.



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vaginal smear. All animal experiments were approved by the institutional animal care committee of the German government (LANUV G887/06).

Delayed Ovulation

Delayed ovulation was induced by SC injection of 10 and 50 μg cetrorelix per mouse per day (Merck Serono, Darmstadt, Germany) for 3 days starting on the second day of diestrus. Mice were killed by cervical dislocation 24 hours after the last cetrorelix injection, and ovaries were removed and weighted before paraffin embedding. Controls were investigated on the corresponding day of the ovarian cycle. Serial sections of complete ovaries of nine controls and four cetrorelix-treated mice were evaluated for total number of tertiary follicles, ruptured follicles, and corpora lutea.

Fertility and Embryonic Development

Embryonic development was assessed after delay of ovulation. Ovulation was suppressed with SC injection of 50 μg/day of cetrorelix starting on the first day of diestrus. Follicle growth was stimulated on days 1 and 4 of cetrorelix treatment by SC injection of 5 IU PMSG. Ovulation was induced on day 6 of treatment by SC injection of 7.5 IU hCG. Controls were stimulated with PMSG on the first day of diestrus, and ovulation

TABLE 1

Number of mice with resorption sites or intrauterine deceased embryos.

Treatment	No. of mice	No. of mice with normal embryos	No. of mice with resorption sites	No. of mice with deceased embryos
Controls	6	6	0	4
Cetrorelix	10	10	7	3

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was induced 48 hours later by hCG. Mice were mated the same evening after hCG injection with male C57Bl/6 mice overnight. The presence of a vaginal plug on the next morning was designated as 0.5 days post coitum (dpc). For each experimental group, 18 mice were mated, of which resulted 10 pregnancies in the cetrorelix-treated group and six pregnancies in the control group. Uteri were opened longitudinally on day 17.5 of pregnancy, and numbers of properly developed embryos, resorption sites, and intrauterine dead embryos (classified by no beating heart) were determined. Regularly developed embryos and placentas were removed and weighed.

FIGURE 2

Delayed ovulation induced by Cetrorelix reduced the number of regularly developed embryos per mouse at 17.5 dpc (**A**). Whereas no significant difference was observed in intrauterine deceased embryos (**B**), a significant increase in resorption sites was observed in the cetrorelix-treated group (**C**). (**D**) Resorption site (*arrow*) at dissection on 17.5 dpc in mice with cetrorelix-mediated delayed ovulation. Scale bar = 0.5 cm; Cetro = cetrorelix; controls, n = 6; cetrorelix treated group, n = 10; *$P<0.05$ compared with controls.

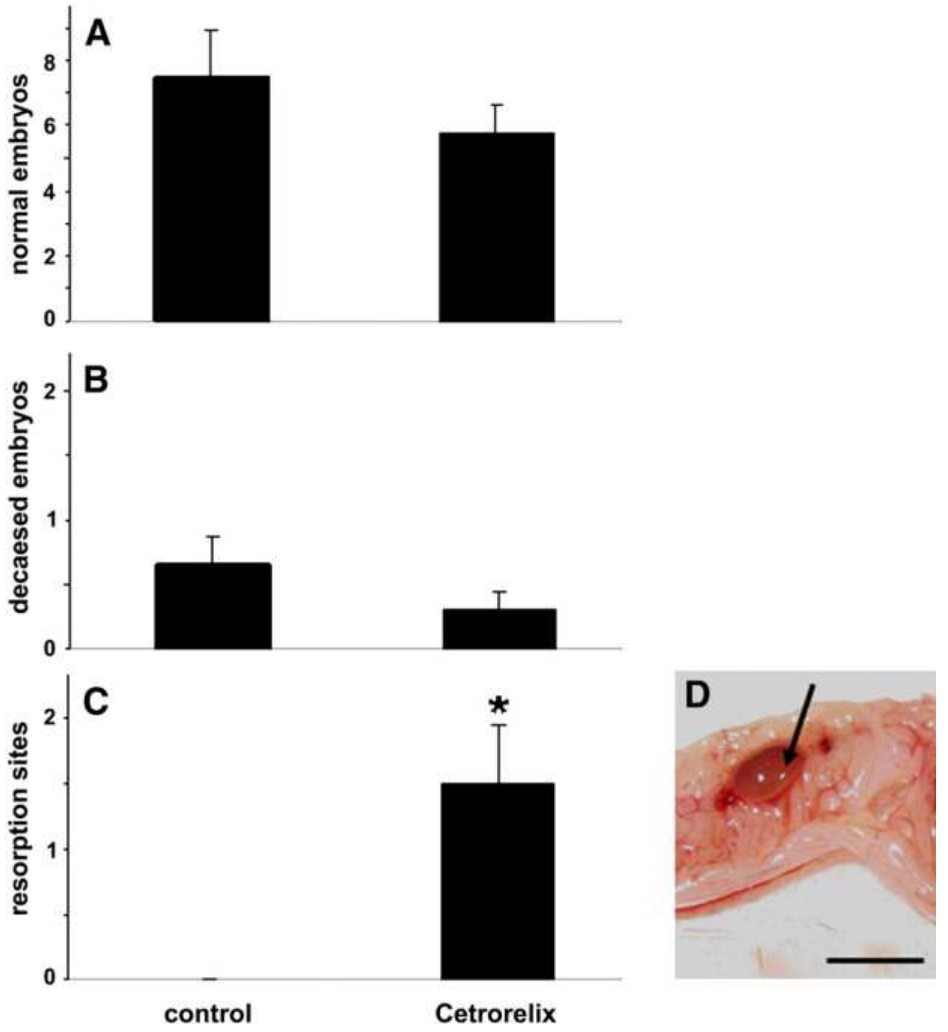

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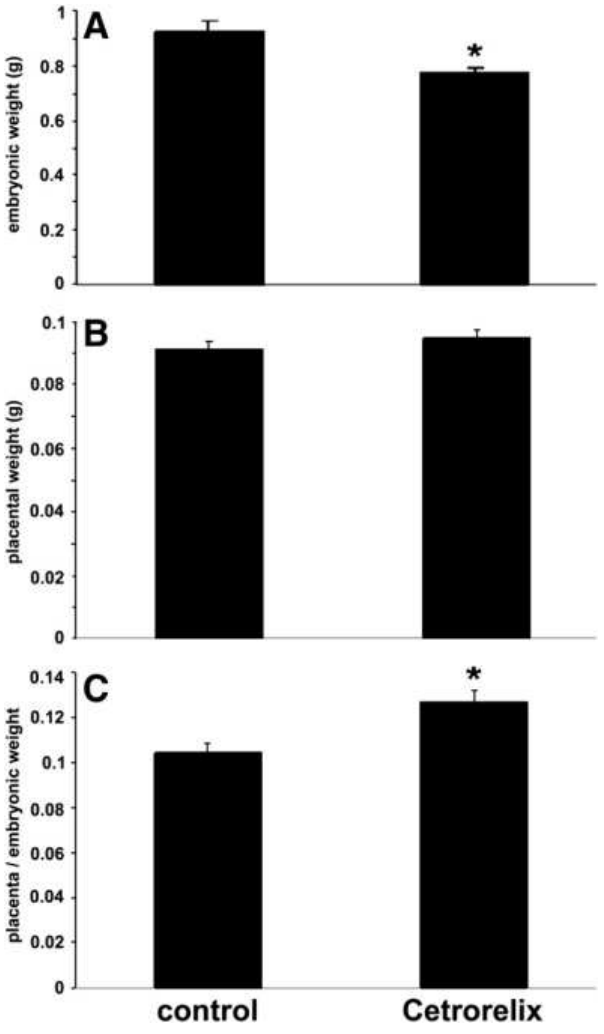
FIGURE 3

Weight of regularly developed embryos (**A**) and placentas (**B**) in cetrorelix-treated mice compared with controls at 17.5 dpc. A significant decrease in embryonic weight is observed (**A**), but placental weight was comparable to controls (**B**); this results in a significant increase in placental/embryonic weight ratio in the delayed ovulation group (**C**). Controls, n = 39; cetrorelix-treated group, n = 49; *$P<0.005$ compared with controls.



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Statistical Analysis

Exploratory data analysis and analyses of variances (Mann–Whitney test) were performed using the SPSS program for windows (SPSS, Inc., Chicago, IL). Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Suppression of Ovulation by Cetrorelix is Dose-Dependent

The GnRH antagonist cetrorelix inhibited ovulation in normal cycling mice for 3 days spanning the predicted time point of ovulation. Histologic examination revealed abundant corpora lutea in the ovaries of control mice (Fig. 1A), but hardly any corpora lutea in the ovaries of cetrorelix-treated mice, and instead high numbers of tertiary follicles (Fig. 1B). Counting all corpora lutea and tertiary follicles in serial sections of the ovaries demonstrated a dose-dependent effect of cetrorelix treatment. Injection of 50 μg cetrorelix significantly inhibited the formation of corpora lutea (Fig. 1C), whereas 10 μg was insufficient to reduce the number of corpora lutea significantly. The suppression of corpora lutea formation was paralleled by a significant increase in the amount of tertiary follicles (Fig. 1E) and the complete absence of ruptured follicles as histomorphologic signs of ovulation (Fig. 1D). Ovulation was not completely inhibited by 10 μg cetrorelix. Ovarian weight was reduced in all cetrorelix-treated mice; however, this reduction was not significant in any group (Fig. 1F). Based on these results, 50 μg cetrorelix was used to suppress ovulation in subsequent experiments.

Effect of Delayed Ovulation on Fertility and Development of Embryos and Placentas

Ovulation was suppressed by cetrorelix in mice during hormonal follicle stimulation for 5 days, and delayed ovulation was induced by hCG on day 6 of treatment. After successful mating, uteri were removed at 17.5 dpc and analyzed for numbers of normally developed embryos, deceased embryos and resorption sites. Mothers of both groups revealed no differences in pregnancy rate and showed normally developed embryos (Table 1). Dams with intrauterine deceased embryos, defined by smaller size and missing heartbeat, occurred in similar numbers in both experimental groups, whereas resorption sites occurred only in cetrorelix-treated mice (Table 1). The cetrorelix-mediated delayed ovulation led to a decrease in the mean number of normal embryos on 17.5 dpc (Fig. 2A), concomitant with a slight, but not significant, decrease in the number of deceased embryos (Fig. 2B). However, a significant increase in resorption sites was observed in mice that conceived after delayed ovulation (Fig. 2C). Intrauterine resorption sites were visible as balls of degenerating tissue (Fig. 2E), pointing to death in utero at approximately mid-gestation. Determination of the weight of healthy embryos and placentas at 17.5 dpc revealed a significant reduction of mean embryonic weight of the delayed ovulation group compared to controls (mean $\pm$ SEM, 0.92 $\pm$ 0.05 versus 0.77 $\pm$ 0.02 g; Fig. 3A), without any change in the placental weight (0.091 $\pm$ 0.0023 versus 0.095 $\pm$ 0.0026 g; Fig. 3B). As a consequence, the placental/embryonic weight ratio was significantly higher in the delayed ovulation group (Fig. 3C). Our data show that delayed ovulation induced by cetrorelix treatment resulted in an increase in resorption sites and a decrease in the weight of regularly developed embryos.

DISCUSSION

In this study, we analyzed the effects of preovulatory oocyte overripeness on embryonic and placental development after GnRH antagonist-induced delayed ovulation. In contrast to GnRH agonists, which suppress premature LH surges via pituitary desensitization, GnRH antagonists have the advantage of providing immediate and more complete suppression of gonadotropins by competitive occupancy of the GnRH receptor (18). The cetrorelix GnRH antagonist used in the present study is commonly used on women in ART programs (17). Besides reliably inhibiting premature LH peaks in women, cetrorelix also downregulates pituitary LHRH-receptors in female rats (19). Torres et al. (20) have compared the effects of treatment with GnRH agonists or antagonists in rats during normal cycle length. They found that cetrorelix more effectively suppressed ovulation and resulted in fewer implantation sites and developing

embryos compared to treatment with a GnRH agonist. In our mouse model, suppression of ovulation by cetrorelix was dose-dependent and led to an accumulation of tertiary follicles. These results show that the mouse responds similarly to cetrorelix and can be used as model for human response to treatment during ART.

Delayed ovulation resulted in fewer developed embryos, predominantly because of an increase in resorption sites. We observed a significant decrease in embryonic weight in the delayed ovulation group, whereas placental weight remained comparable to the control group. It has been shown previously that superovulation in mice results in smaller embryos by weight (21), and children resulting from ART are at increased risk for low birth weight (10). The ratio of placental weight to birth weight is known as a marker of fetal growth, and a significant increase in this ratio among IVF infants was described, which resulted from children being too small for their gestational age (22). This finding corroborates our findings that the increase in placental/embryonic weight ratio in the delayed ovulation group was due to a decrease in embryonic weight rather than an increase in placental weight. These results indicate that delaying ovulation hormonally in ART patients may have an effect on embryo development.

Previous studies demonstrated that hormonal hyperstimulation may have an effect on the sensitive developmental processes in mice and humans. Implantation rate and pregnancy outcome were reduced (21, 23), possibly caused in part by imprinting defects (24). Since oocytes undergo considerable molecular changes during intrafollicular growth and maturation, it is likely that delayed ovulation leading to oocyte overripeness has an additional impact on oocyte developmental competence. Delaying ovulation with pentobarbital sodium for 1–2 days increased the incidence of embryonic death and abnormal embryos in rats (5). In addition, Sommergruber et al. (25) obtained evidence for a reduced pregnancy rate after prolonged administration of cetrorelix in humans. The factors leading to impaired oocyte developmental competence remain ill defined, but are likely to include metabolic factors, cytoplasmic mRNA and protein stores, structural components, and epigenetic modifications of the maternal genome (26, 27) and a precise regulation of RNA polyadenylation (28, 29). These defects can impair the embryo proper and lead to placental dysmorphogenesis or dysfunction (30). It is imperative to evaluate whether the results reported here stem from impaired placental function or growth defects of the embryo proper. Because ART treatment has been shown to influence embryo development, including decreased embryonic weight (21) and increased congenital malformations (8), the possibility that delayed ovulation induced by Cetrorelix, or other GnRH antagonists, in patients undergoing ART could affect oocyte quality and subsequent embryo development must be addressed. Further evaluation of the mechanisms of these effects is necessary to minimize the risks of hormonal intervention in ART cycles.

REFERENCES

1. Jongbloet PH. Overripeness of the ovum. Observations on the etiology of Goldenhar's syndrome and related congenital dysplasias. Ned Tijdschr Geneeskd 1969;113:653–65.
2. Miao YL, Kikuchi K, Sun QY, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. Hum Reprod Update 2009;15:573–85.
3. Ottolenghi C, Uda M, Hamatani T, Crisponi L, Garcia JE, Ko M, et al. Aging of oocyte, ovary, and human reproduction. Ann N Y Acad Sci 2004;1034:117–31.
4. Mikamo K. Intrafollicular overripeness and teratologic development. Cytogenetics 1968;7:212–33.
5. Butcher RL, Blue JD, Fugo NW. Role of intrauterine environment on ova after normal and delayed ovulation. Biol Reprod 1969;1:149–51.
6. Butcher RL, Fugo NW. Overripeness and the mammalian ova. II. Delayed ovulation and chromosome anomalies. Fertil Steril 1967;18:297–302.
7. Peluso JJ, Butcher RL. RNA and protein synthesis in control and follicularly-aged rat oocytes. Proc Soc Exp Biol Med 1974;147:350–3.
8. Spira A, Spira N, Papiernik-Berkauer E, Schwarz D. Pattern of menstrual cycles and incidence of congenital malformations. Early Human Dev 1985;11:317–24.
9. Wang Q, Sun QY. Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reprod Fertil Dev 2007;19:1–12.
10. Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G, Wilcox LS. Low and very low birth weight in infants conceived with use of assisted reproductive technology. N Engl J Med 2002;346:731–7.
11. Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects–a systematic review. Hum Reprod 2005;20:328–38.
12. Shiota K, Yamada S. Intrauterine environment-genome interaction and children's development (3): Assisted reproductive technologies and developmental disorders. J Toxicol Sci 2009;34(Suppl 2):287–91.
13. Cox GF, Bürger J, Lip V, Mau UA, Sperling K, Wu BL, et al. Intracytoplasmic sperm injection may increase the risk of imprinting defects. Am J Hum Genet 2002;71:162–4.
14. Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. J Med Genet 2005;42:289–91.
15. Gosden R, Trasler J, Lucifero D, Faddy M. Rare congenital disorders, imprinted genes, and assisted reproductive technology. Lancet 2003;361:1975–7.
16. Weiss JM, König SJ, Polack S, Emons G, Schulz KD, Diedrich K, et al. Actions of gonadotropin-releasing hormone analogues in pituitary gonadotrophs and their modulation by ovarian steroids. J Steroid Biochem Mol Biol 2006;101:118–26.
17. Tur-Kaspa I, Ezcurra D. GnRH antagonist, cetrorelix, for pituitary suppression in modern, patient-friendly assisted reproductive technology. Expert Opin Drug Metab Toxicol 2009;5:1323–36.
18. Huirne JA, Homburg R, Lambalk CB. Are GnRH antagonists comparable to agonists for use in IVF? Hum Reprod 2007;22:2805–13.
19. Kovacs M, Schally AV, Csernus B, Rekasi Z. Luteinizing hormone-releasing hormone (LH-RH) antagonist Cetrorelix down-regulates the mRNA expression of pituitary receptors for LH-RH by counteracting the stimulatory effect of endogenous LH-RH. Proc Natl Acad Sci U S A 2001;98:1829–34.
20. Torres MM, Donadio N, Donadio NF, Brandão AC, Heck B. Comparison of embryo implantation in Wistar rats that underwent ovarian stimulation using exogenous gonadotropins associated with cetrorelix acetate or leuprolide acetate. Fertil Steril 2005;84:1235–40.
21. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Hum Reprod 2001;16:221–5.
22. Daniel Y, Schreiber L, Geva E, Amit A, Pausner D, Kupferminc MJ, et al. Do placentae of term singleton pregnancies obtained by assisted reproductive technologies differ from those of spontaneously conceived pregnancies? Hum Reprod 1999;14:1107–10.
23. Santos MA, Kuijk EW, Macklon NS. The impact of ovarian stimulation for IVF on the developing embryo. Reproduction 2010;139:23–34.
24. Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. Hum Reprod 2007;22:26–35.
25. Sommergruber M, Shebl O, Jesacher J, Gaiswinkler U, Krain V, Ebner T, et al. Antagonist und intrauterine Insemination—wann ist der geeignete Zeitpunkt der Ovulationsinduktion? J Reproduktionsmed Endokrinol 2006;4:260.
26. Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. Genomics 2002;79:530–8.
27. Krisher RL. The effect of oocyte quality on development. J Anim Sci 2004;82(ESuppl):E14–23.
28. De Leon V, Johnson A, Bachvarova R. Half-lives and relative amounts of stored and polysomal ribosomes and poly(A)+ RNA in mouse oocytes. Dev Biol 1983;98:400–8.
29. Kosubek A, Klein-Hitpass L, Rademacher K, Horsthemke B, Ryffel GU. Aging of Xenopus tropicalis Eggs Leads to Depolyadenylation of a Specific Set of Maternal mRNAs and Loss of Developmental Potential. PLoS 2010;5:e13532.
30. Fowden AL, Sibley C, Reik W, Constancia M. Imprinted genes, placental development and fetal growth. Horm Res 2006;65(Suppl 3):50–8.