Impaired fertility in T-stock female mice after superovulation

Dagmar Zudova^{1,*}, Andrew J. Wyrobek¹, Jack Bishop² and Francesco Marchetti¹

¹Biology and Biotechnology Research Program, L-448, Lawrence Livermore National Laboratory, 7000 East Avenue, Livermore, CA 94550, USA and ²Developmental Genetic Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

Correspondence should be addressed to Francesco Marchetti; Email: marchetti2@llnl.gov

*Dagmar Zudova is currently at Genetic Laboratory, Sanatorium Pronatal, 140 00 Prague, Czech Republic

Abstract

Superovulation of female mice with exogenous gonadotrophins is routinely used for increasing the number of eggs ovulated by each female in reproductive and developmental studies. We report an unusual effect of superovulation on fertilization in mice. *In vivo* matings of superovulated T-stock females with B6C3F1 males resulted in a two-fold reduction ($P < 0.001$) in the frequencies of fertilized eggs compared with control B6C3F1 matings. In addition, approximately 22 h after mating, only 15% of fertilized eggs recovered in T-stock females had reached the metaphase stage of the first cleavage division versus 87% in B6C3F1 females ($P < 0.0001$). Matings with T-stock males did not improve the reproductive performance of T-stock females. To investigate the possible cause(s) for the impaired fertilization and zygotic development, the experiments were repeated using *in vitro* fertilization. Under these conditions, the frequencies of fertilized eggs were not different in superovulated T-stock and B6C3F1 females (51.7 ± 6.0 and $64.5 \pm 3.8\%$, $P = 0.10$). There was a seven-fold increase in the frequencies of fertilized eggs that completed the first cell cycle of development after *in vitro* versus *in vivo* fertilization in T-stock females. These results rule out an intrinsic deficiency of the T-stock oocyte as the main reason for the impaired fertility after *in vivo* matings, and suggest that superovulation of T-stock females may induce a hostile oviductal and uterine environment with dramatic effects on fertilization and zygotic development.

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Introduction

Exogenous gonadotropins are commonly used for superovulation in humans and animals to increase the number of oocytes for use in many fields of biology and assisted reproductive technology. New hormonal stimulation protocols are being introduced continuously into clinical practice to improve the quality of ovulated eggs, the chance of successful fertilization and pregnancy outcome (Licciardi *et al.* 1999). However, despite significant progress, fertilization and implantation failures remain high (Dubey *et al.* 1997, 1998, Maman *et al.* 1998) and it is still unclear how hormonal stimulation impacts oocyte quality and oviduct and uterine environments.

The mouse is an acknowledged animal model in reproductive medicine, genetics and toxicology. The protocol for superovulation of female mice is well established and many factors that can influence the outcome are known (Hogan *et al.* 1994, Ozgunen *et al.* 2001, Tarin *et al.* 2002). Among the most important factors are the age and strain of females. Generally, 3–6-week-old females ovulate the maximum number of eggs obtainable from a given strain; however, mice at this precise age are usually not available from commercial suppliers and fully mature

females are normally used for cytogenetic studies. Mouse strains fall into two categories: high responders, which ovulate 30–50 eggs per mouse, and low responders, which ovulate 15 or fewer eggs. C57BL/6J, BALB/cByJ and SJL/J strains are among the high ovulators, while A/J, C57/L and 129/J are among low ovulators (Hogan *et al.* 1994).

Adverse effects of superovulation on reproductive outcomes have been reported in rodents. In mice, delayed embryonic development, increased abnormal blastocyst formation, fetal growth retardation and increased numbers of resorption sites were observed in superovulated females with respect to naturally ovulating females (Allen & McLaren 1971, Beaumont & Smith 1975, Ertzeid & Storeng 1992, 2001, Ertzeid *et al.* 1993, Van der Auwera & D'Hooghe 2001). It has been suggested that superovulation may impair oocyte quality by recruiting immature oocytes that have not experienced a normal period of follicular maturation (Takagi & Sasaki 1976, Maudlin & Fraser 1977, Elbling & Colot 1985). However, other studies have suggested that abnormal embryonic development after superovulation with gonadotrophins is predominantly induced by effects of the hormone treatment on the

maternal oviductal and uterine environment (Elmazar *et al.* 1989, Van der Auwera *et al.* 1999). No adverse effects of superovulation have been reported on fertilization or cleavage during the early phases of mouse preimplantation development. In rats, fertilization and implantation failure are common consequences of superovulation with pregnant mare's serum (PMSG; Miller & Armstrong 1981a,b, Walton & Armstrong 1983, Walton *et al.* 1983). In addition, superovulation in rats often results in ovulation occurring 24 h before the administration of human chorionic gonadotropin (hCG; Miller & Armstrong 1982, Walton *et al.* 1983). Thus, ovarian stimulation may alter oocyte/embryo quality as well as the uterine milieu, but the underlying mechanisms remain poorly understood.

The T-stock is a random-bred stock of mice carrying seven recessive mutations (*a*, non-agouti; *b*, brown; *c^{ch}*, chinchilla; *p*, pink-eyed dilution; *d*, dilute; *se*, short ear; *s*, piebald spotting) that has been used extensively in the specific locus and dominant lethal tests (Russell & Russell 1992). This multiple recessive tester stock was formed at Oak Ridge National Laboratory (ORNL) from a cross between the NB inbred strain homozygous for six recessive mutations (*a*, *b*, *c^{ch}*, *p*, *d* and *se*) and a non-inbred stock homozygous for three of the same recessive mutations plus an additional one (*s*; Russell 1951). T-stock females showed consistently higher levels of dominant lethality after mating with mutagen-treated males when compared with other mouse strains (Generoso *et al.* 1979), suggesting that T-stock eggs may have a reduced capacity for repairing the DNA damage carried by the sperm.

We recently showed that chromosomal aberrations in first-cleavage (1-Cl) zygote metaphases are predictive of abnormal embryonic outcomes (Marchetti *et al.* 2004); therefore, we began a study of the induction of chromosomal aberrations in 1-Cl zygotes after mating T-stock females with mutagen-treated males. However, during the course of the study we discovered an unusual effect of superovulation on T-stock females. We report here that superovulation in T-stock females greatly reduces the number of eggs that are fertilized and the number of fertilized eggs that reach the metaphase stage of the first cleavage division. Both these effects were significantly reduced after *in vitro* fertilization (IVF) suggesting that the major determinants of the impaired fertility occur in the female reproductive tract.

Materials and Methods

Animals

B6C3F1 mice were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). T-stock mice were produced at Taconic (Germantown, NY, USA) using three sublines provided by ORNL. The animals were housed under 14-h dark/10-h light cycle. Mice were fed a standard pellet diet *ad libitum*, and had free access to drinking water. The use of vertebrate animals in these experiments was conducted in accordance with the principles and procedures outlined

in the NIH Guide for the Care and Use of Laboratory Animals and was approved by the LLNL Institutional Animal Care and Use Committee.

Superovulation, timing of ovulation and doses of hormones

T-stock and B6C3F1 females, 10–14 weeks old, received an i.p. injection of 7.5 IU PMSG (Sigma Chemical Co., St Louis, MO, USA) to augment the number of maturing ovarian follicles, followed 48 h later by an i.p. injection of 5.0 IU hCG to induce ovulation. With this superovulation protocol, ovulation is expected to occur between 11 and 14 h after administration of hCG (Edwards & Gates 1959, Hogan *et al.* 1994, Marchetti & Mailhes 1995). Both groups of females were injected with hormonal aliquots prepared from the same lot of gonadotrophins.

Another group of T-stock females was superovulated as described above and euthanized at 0, 4, 8, 12 or 16 h after the administration of hCG to determine the timing of ovulation. Oviducts were isolated and analyzed for the presence of eggs.

Collection of zygotes for cytogenetic analysis

Immediately after hCG injection, females were mated with untreated B6C3F1 or T-stock males. For matings using B6C3F1 males, at least three repetitions, each using a group of 12 superovulated females, were performed, while a group of 12 T-stock males was mated once with both B6C3F1 and T-stock females. Females were removed from males after 8 h and checked for the presence of vaginal plugs. This was done to assure that sperm were already present in the female reproductive tract at the time of ovulation to increase the synchronicity of post-fertilization development among the eggs collected from different females. Twenty four hours after hCG injection, mated females received an i.p. injection of 0.08 mg colchicine in 0.2 ml distilled water to arrest development of the zygotes at the first mitotic division and were euthanized 6 h later by CO₂ inhalation. Eggs from all mated females within a repetition were flushed from the oviducts, pooled and processed according to the mass harvest procedure (Mailhes & Yuan 1987). Prepared slides were stained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed under a fluorescent microscope. Each egg or zygote recovered on the slide was classified into one of the following five groups according to its appearance (Marchetti & Wyrobek 2003): unfertilized oocytes, oocytes with meiotic chromosomes (Fig. 1A) or degenerating chromatin without a sperm head or tail (Fig. 1B); developmentally arrested zygotes, zygotes showing female meiotic chromosomes and a sperm head or tail (Fig. 1C), or occasionally male meiotic chromosomes; degenerated zygotes, zygotes with degenerating chromatin and a sperm head or tail, or fragmented pronuclei (Fig. 1D); pronuclei, zygotes with two well-defined pronuclei showing the difference in size between paternal (larger) and

Figure 1 Photomicrographs of the various types of unfertilized and fertilized eggs recovered after *in vivo* mating of superovulated T-stock females with B6C3F1. (A) Unfertilized egg with meiotic chromosomes and polar body. (B) Unfertilized egg with degenerating chromatin. (C) Developmentally arrested zygote with maternal meiotic chromosomes and a sperm head (Sp) in the early stage of decondensation. (D) Zygote with pronuclear fragmentation. (E) Zygote with maternal (smaller) and paternal (larger) pronuclei. (F) Zygote at the metaphase stage of the first mitotic division.

maternal (smaller) pronuclei (Fig. 1E); and zygotes, zygotes with mitotic chromosomes (Fig. 1F).

Collection and capacitation of spermatozoa for IVF

Epididymal spermatozoa were obtained from the cauda epididymis of B6C3F1 males as described by Lowe *et al.* (1996). After 20 min at 37°C in 250 µl M16 medium (Sigma), 25 µl sperm suspension was diluted in 475 µl water and 10 µl of this suspension was used to estimate sperm concentration with the aid of a hemocytometer chamber. Fertilization drops of 0.5 ml were prepared by adding an aliquot of undiluted sperm suspension in M16 medium supplemented with 15 mg/ml bovine sperm albumin (Sigma) under mineral oil (Sigma) to give a final concentration of 1×10^6 spermatozoa/ml. The fertilization

drops were kept at 37°C in 5% CO₂ for 100 min to capacitate spermatozoa (Sakkas *et al.* 1995).

Collection of oocytes for IVF and cultivation of zygotes

Oocytes for IVF studies were collected from females euthanized 15 h after hCG. Ovaries along with the oviduct and part of uterus were isolated and placed into warm Hank's balanced salt solution (Sigma). The cumulus–oocyte complexes were collected by tearing the ampullary region of the oviduct and washed twice in fertilizing medium. Approximately 20 cumulus–oocyte complexes were transferred into each fertilization drop and kept at 37°C in 5% CO₂ for 5 h. Oocytes were then washed three times in cultivation medium (M16 medium supplemented with

4 mg/ml BSA; Hogan *et al.* 1994) and transferred into 100 µl cultivation medium under mineral oil (approximately 10 oocytes per drop). They were cultured at 37 °C in 5% CO₂ for 21 h.

The morphological appearance of each egg was evaluated at the end of the cultivation period under a stereomicroscope (Leica Wild MZ 8). Eggs were assumed to be fertilized if they had two, or occasionally three, well-defined pronuclei or were at the two-cell embryo stage showing two blastomeres of similar size. Eggs with three pronuclei were assumed to have originated by polyspermic fertilization. Cells exhibiting nuclear fragmentation and cellular debris enclosed by the zona pellucida were classified as degenerated oocytes/embryos (Tarin *et al.* 2002). These eggs may represent either unfertilized or fertilized eggs that had degenerated during the culturing time. Single cells, without visible pronuclei and exhibiting normal morphology, were categorized as unfertilized eggs. The fertilization rate was calculated by dividing the number of pronuclear zygotes and two-cell embryos by the total number of eggs.

Statistical evaluation

A chi-square test with adjustment for overdispersion (Collett 1991) was used for the analysis of the data because the observations within the T-stock, both *in vivo* and *in vitro*, did not follow a Poisson distribution.

Results

In vivo matings

The same B6C3F1 males were used to breed with T-stock and B6C3F1 females. Both superovulated T-stock and B6C3F1 females bred and ovulated normally (Table 1). About 64% of T-stock females had vaginal plugs (compared to 82% in B6C3F1; *P* = 0.26), and ovulated an average of 34.6 eggs per female compared to 33.3 eggs per female in B6C3F1. However, there was a two-fold reduction (*P* < 0.001) in the frequencies of eggs that were fertilized (Table 1). Interestingly, at the time of zygote collection the oviducts isolated from mated T-stock females still had swollen ampullas containing eggs heavily surrounded by cumulus cells, while swollen ampullas were

no longer observable in mated B6C3F1 females and the eggs were almost completely without cumulus cells. In addition, only 15% of the fertilized eggs in T-stock females reached the metaphase stage of the first cleavage division at 30 h after hCG (compared to 87% in B6C3F1; *P* < 0.001). Overall, 1-Cl metaphases were found in 69% of the eggs recovered from B6C3F1 females but only in 6% of the eggs from T-stock females.

Major differences were found in the percentages of the various types of unfertilized and fertilized eggs recovered from T-stock and B6C3F1 females (Table 2). There were two types of unfertilized egg: oocytes with meiotic chromosomes at the metaphase stage of the second meiotic division (Fig. 1A) and oocytes with degenerating chromatin and no recognizable chromosomes (Fig. 1B). Oocytes with meiotic chromosomes represented approximately 40% of the unfertilized eggs in T-stock females, while they represented only 8% of the unfertilized eggs in B6C3F1 females. There were also major differences among the fertilized eggs in T-stock versus B6C3F1 females. Fertilized eggs with maternal meiotic chromosomes associated with sperm heads in various stages of decondensation (Fig. 1C), or sperm tail only, represented approximately 13% of fertilized eggs in T-stock females but were not seen in B6C3F1 females (the historic frequency of these eggs in B6C3F1 females is 0.5%). Degenerated fertilized eggs with no recognizable chromosomes, or fragmented or damaged pronuclei (Fig. 1D) represented more than 65% of the fertilized eggs in T-stock females compared to 12% in B6C3F1 females. Zygotes with two well-defined pronuclei showing the difference in size between paternal (larger) and maternal (smaller) pronuclei (Fig. 1E) were also significantly higher in T-stock females (*P* = 0.02). It is possible that these zygotes would have reached the metaphase stage with a later harvest time. However, even assuming that pronuclear zygotes are normal zygotes, these data show that only approximately 21% of fertilized eggs in T-stock females were able to form pronuclei, undergo DNA synthesis and reach the metaphase stage of the first cleavage division (Fig. 1F). This is far below the approximately 87% seen in B6C3F1 females.

To rule out the possibility of physiological incompatibility between T-stock eggs and B6C3F1 sperm, 12 T-stock

Table 1 Fertilization rate and development in matings using B6C3F1 males. Data shown as percentages ± SE; the percentage range is shown in parentheses. % Zygotic development indicates the number of 1-Cl metaphases/fertilized eggs. % 1-Cl metaphase indicates the number of 1-Cl metaphases/total number of eggs. Statistical comparisons were made with the chi-square test.

	B6C3F1 females	T-stock females
% Matings	81.7 ± 5.4 (54.4–100)	63.6 ± 4.8 (50.0–72.7)
No. of mated females	67	34
No. of eggs per female	33.3 ± 1.6 (29.5–41.6)	34.6 ± 3.7 (30.6–52.6)
Total eggs analysed	1126	618
% Fertilized eggs	80.1 ± 2.4 (74.8–90.7)	40.3 ± 6.4* (30.5–59.8)
% Zygotic development	86.6 ± 2.8 (77.3–93.9)	14.9 ± 8.2* (0–32.8)
% 1-Cl metaphase	69.4 ± 3.0 (58.0–85.2)	6.0 ± 3.8* (0–13.5)

* *P* < 0.001.

Table 2 Types of unfertilized and fertilized eggs in matings using B6C3F1 males. Data shown as percentages $\pm$ SE; the percentage range is shown in parentheses. Percentages in subcategories under the headings Unfertilized eggs and Fertilized eggs are based on data within those categories only. Statistical comparisons were made with the chi-square test.

	B6C3F1 females	T-stock females
Total eggs	1126	618
Unfertilized eggs (%)	19.9 $\pm$ 2.4 (9.3–25.2)	59.7 $\pm$ 6.4* (40.2–68.5)
Meiotic chromosomes (%)	7.6 $\pm$ 5.0 (0–36.4)	38.5 $\pm$ 14.4* (4.7–74.6)
Degenerated (%)	92.4 $\pm$ 5.0 (63.6–100)	61.5 $\pm$ 14.4 (25.4–95.3)
Fertilized eggs (%)	80.1 $\pm$ 2.4 (74.8–90.7)	40.3 $\pm$ 6.4* (30.5–59.8)
Meiotic chromosomes (%)	0	12.9 $\pm$ 6.3* (0–30.4)
Degenerated (%)	12.0 $\pm$ 2.6 (2.0–19.5)	65.9 $\pm$ 11.0* (50.0–98.2)
Pronuclei (%)	1.5 $\pm$ 0.5 (0–3.3)	6.4 $\pm$ 3.2† (1.8–15.1)
1-Cl metaphases (%)	86.6 $\pm$ 2.8 (77.3–93.9)	14.9 $\pm$ 8.2* (0–32.8)

* $P < 0.001$.

† $P = 0.02$.

males were used to breed with both T-stock and B6C3F1 females. The results of this single mating showed that even when T-stock males were used to mate with superovulated T-stock females, the frequencies of fertilized eggs and of 1-Cl metaphases did not improve, while the results with B6C3F1 females were in line with the historical control data for B6C3F1 male and female breedings (Table 3). The fertilization rate in matings of T-stock males and females was half that of matings using B6C3F1 males and T-stock females (Table 2). However, among the four matings that were performed using B6C3F1 males and T-stock females, two produced fertilization rates below 35% with the lowest being 30%. More importantly, two out of four of the matings using B6C3F1 males and T-stock females did not produce a single zygote at the metaphase stage as it was observed with T-stock males and females. These findings rule out a male effect and interstrain incompatibility and suggest an abnormal response of T-stock females to superovulation.

Timing of ovulation in T-stock females

The fact that oocytes with meiotic chromosomes were common in T-stock females may indicate that ovulation in T-stock females occurs later than in B6C3F1 females, or that T-stock oocytes have a slower rate of degeneration

after ovulation. To estimate the timing of ovulation in T-stock females, oocytes were collected from the ampullas at 0, 4, 8, 12 and 16 h after administration of hCG. No eggs were found up to 12 h after hCG, while at 16 h after hCG an average of more than 30 eggs per female was collected. Also, cytogenetic analysis of 129 oocytes collected 16 h after hCG showed that 94% were at the metaphase of the second meiotic division (MII), 2% were at the metaphase of the first meiotic division and 4% showed no chromosomes or degenerating chromatin. All MII metaphases analyzed had a normal haploid count of chromosomes, except one that had 19 dyads. These results show that the timing of ovulation after administration of exogenous hormones is normal in T-stock females, and that ovulated oocytes are at the normal stage of meiotic maturation and have a normal haploid number of chromosomes.

Effects of hormonal doses

To determine whether dosage of exogenous hormones had an adverse effect on hormonal regulation of ovulation and function of reproductive organs in T-stock females, the doses of PMSG and hCG were reduced to 2.5 I.U. Reducing the hormone amount affected the oestrus induction, as indicated by the fact that only 42% (15 out of 36) of females mated, and decreased the number of eggs that were ovulated (average, 11.5 eggs per female). However, there was no improvement in fertilization rate (33.9 ± 2.9 compared to $40.3 \pm 6.3\%$ after regular hormonal dosage) and in the frequency of 1-Cl zygotes (9.3 ± 4.0 compared to $6.0 \pm 3.8\%$ after regular hormonal dosage).

IVF

To investigate the role of the oviductal and uterine environment on the impaired fertility in T-stock females, the experiments were repeated using IVF (Table 4). T-stock and B6C3F1 females had similar frequencies of unfertilized oocytes (26.9 compared to 27.5%), whereas they differed in the incidence of degenerated oocytes/embryos (21.8 compared to 8.0%, respectively). There was also a statistical significant difference between T-stock and

Table 3 Types of unfertilized and fertilized eggs in matings using T-stock males. Percentages in subcategories under the headings Unfertilized eggs and Fertilized eggs are based on data within those categories only. Statistical comparisons were made with the chi-square test.

	B6C3F1 females	T-stock females
Total eggs	86	83
Unfertilized eggs (%)	30.2	79.5*
Meiotic chromosomes (%)	0.0	74.2*
Degenerated (%)	100.0	25.8*
Fertilized eggs (%)	69.8	20.5*
Meiotic chromosomes (%)	0.0	29.4*
Degenerated (%)	20.0	70.6*
Pronuclei (%)	8.3	0.0*
1-Cl metaphases (%)	71.7	0.0*

* $P < 0.001$.

Table 4 Fertilization rate and development after IVF using B6C3F1 sperm. Data shown as percentages ± SE; the percentage range is shown in parentheses. Statistical comparisons were made with the chi-square test.

	Source of oocytes	
	B6C3F1 females	T-stock females
Total eggs	375	317
Unfertilized (%)	27.5 ± 2.7 (15.6–34.3)	26.5 ± 2.9 (15.8–38.1)
Degenerated (%)	8.0 ± 2.1 (1.6–14.3)	21.8 ± 4.9* (11.4–47.9)
Pronuclei (%)	1.9 ± 1.3 (0–9.1)	9.1 ± 3.5* (0–27.3)
Two-cell embryos (%)	62.7 ± 4.6 (45.5–82.8)	42.6 ± 5.8* (21.9–68.4)
Two-cell embryos and pronuclei (%)	64.5 ± 3.8 (52.2–82.8)	51.7 ± 6.0† (23.3–69.0)

* $P < 0.05$.

† $P = 0.10$.

B6C3F1 females in the frequencies of two-cell embryos (42.6 compared to 62.7%, respectively; $P < 0.05$). Nevertheless, IVF resulted in a seven-fold increase ($P < 0.001$) in the frequencies of T-stock eggs that completed the first cell cycle of development and produced two-cell embryos with respect to *in vivo* matings (Figure 2). In addition, as also observed after *in vivo* fertilization (Table 2), there were significantly more eggs at the pronuclear stage ($P < 0.05$) in T-stock females than in B6C3F1 females. Three of the 29 eggs at the pronuclear stage in T-stock females had three pronuclei, suggesting polyspermic fertilization, while all seven pronuclear eggs in B6C3F1 had two pronuclei ($P = 0.3$). When the frequencies of two-cell embryos and pronuclear eggs were combined to generate the fertilization rate, there was no significant difference between T-stock and B6C3F1 females (51.7 compared to 64.7%; $P = 0.10$). These results show that under *in vitro* conditions, superovulated T-stock eggs are as competent as B6C3F1 eggs to undergo fertilization and complete the first cell cycle of development.

Discussion

We report an unusual effect of superovulation on fertility in T-stock female mice. *In vivo* matings following induced ovulation with exogenous hormones yielded a significant reduction in the frequencies of eggs that were fertilized and in the frequencies of fertilized eggs that reached the metaphase stage of the first cleavage division. IVF of superovulated eggs improved to near-normal levels both the frequencies of fertilized eggs (approximately 1.5-fold) and the frequencies of fertilized eggs that completed the first cell cycle of development and formed two-cell embryos (seven-fold). These findings suggest that superovulated T-stock females have a hostile oviductal and uterine environment that is detrimental to sperm function and embryo development. To our knowledge this is the first report of such a dramatic effect of superovulation on fertilization and the early phases of embryonic development in mice.

The *in vivo* experiments indicate that two aspects of normal reproduction were affected by superovulation in T-stock females: (1) the ability of the sperm to fertilize the egg, as indicated by the reduced frequency of fertilized eggs, and (2) the ability of the fertilized egg to undergo activation and initiate development, as indicated by the increased frequencies of fertilized eggs that had failed to resume meiosis and that were unable to form pronuclei. Successful fertilization requires the binding of the sperm to the zona pellucida and fusion with the egg membrane (Ducibella 1998). This triggers a cascade of events, including a calcium-dependent release of cortical granules to block polyspermy, that results in the activation of the egg and initiation of mammalian embryonic development (Ducibella 1998, Abbott *et al.* 1999). The ability of the oocyte to respond to the fertilizing sperm is acquired gradually before ovulation when the oocyte undergoes both nuclear and cytoplasmic maturation (Ducibella & Buetow 1994, Eppig *et al.* 1994, Ducibella 1996). Nuclear maturation refers to the processes associated with the resumption of meiotic maturation and progression to the metaphase stage of the second meiotic division, while cytoplasmic maturation refers to the acquisition of the egg's ability to release and respond to intracellular

Figure 2 Comparison of embryonic development after *in vivo* matings and IVF with B6C3F1 sperm for T-stock and B6C3F1 females. For *in vivo* matings, a total of 1126 eggs from seven repetitions and 618 eggs from four repetitions were analyzed for B6C3F1 and T-stock females, respectively. For IVF, a total of 357 eggs from seven repetitions and 317 eggs from seven repetitions were analyzed for B6C3F1 and T-stock females, respectively. Bars represent SEM.

calcium. Because the two phenomena may be independent, oocytes that have completed nuclear maturation can still be deficient in cytoplasmic maturation and vice versa (Eppig *et al.* 1994). Thus, although nuclear maturation was not affected in T-stock females, as indicated by the fact that over 94% of the oocytes recovered 16 h after hCG were at MII, it cannot be excluded that superovulation may have resulted in improper cytoplasmic maturation. This may have affected the ability of T-stock oocytes to be fertilized and to respond properly to the fertilizing sperm. However, the majority of our findings point to an abnormal response of the female reproductive tract as the major determinant of the impaired fertilization.

First, the ovulatory response of T-stock females to the administration of exogenous hormones was normal. In fact, ovulation took place between 12 and 16 h after hCG, over 34 eggs per females were ovulated and their chromosomal constitution was normal. This compares well with the timing of ovulation in B6C3F1 females, which is known to occur between 11 and 14 h (Tiveron *et al.* 1992). Also, data from unrelated experiments that were taking place in our laboratory at the same time as the experiments with T-stock females indicated that by 16 h after hCG an average of 36 eggs were collected from superovulated B6C3F1 females (results not shown). Secondly, the observation that 30 h after hCG, eggs were still surrounded by cumulus cells in mated T-stock females suggests that the reduced fertilization rate observed in our study after *in vivo* matings may be due to an abnormal rate of sperm transportation within the female reproductive tract that significantly reduced the number of sperm reaching the site of fertilization in the oviduct. Sperm progression and function within the uterus and the fallopian tubes is strongly regulated by the oviductal epithelium (Smith 1998) and ovarian endocrine activity (Hunter 1994), and superovulation may have affected this process. Thirdly, and more importantly, IVF significantly increased the frequencies of superovulated eggs that were fertilized and able to complete the first cell cycle of development. If the effects observed after *in vivo* matings were the result of intrinsic deficiencies of the oocyte, IVF should not have improved the reproductive performance of T-stock oocytes. We therefore propose that the main reason for the reduced fertilization rate and zygotic development in superovulated T-stock females is a hostile uterine environment that is detrimental to sperm function and embryo development.

Exogenous administration of gonadotrophins results in higher concentrations of circulating steroids due to excessive estrogenic secretion after ovulation (Walton & Armstrong 1981, Miller & Armstrong 1982, Ertzeid & Storeng 1992). Elevated blood levels of steroids result in alterations of the uterine milieu that can produce an environment unsuitable to sustain embryonic development (Elmazar *et al.* 1989, Ertzeid *et al.* 1993, Van der Auwera *et al.* 1999, Ertzeid & Storeng 2001). Administration of exogenous hormones in T-stock females may create

disturbances in hormonal balances altering the oviductal and uterine milieu (i.e. changes in pH, Ca^{2+} ion concentration, epithelial secretions, etc.) and trigger a hostile environment in the female reproductive tract which negatively affects sperm progression, capacitation and/or fertilization processes (Hunter 1994, Hunter *et al.* 1999). It is worth noting that because of the strategy utilized in the present study to synchronize post-fertilization development among the eggs collected from different females, sperm were exposed to the reproductive tract environment for a number of hours prior to fertilization, which may, in part, account for the adverse impact of superovulation on fertilization in T-stock females. Following fertilization, the unsuitable oviductal environment manifested a negative influence on zygote development, arresting development of the new embryos around the time of pronuclear formation. In support of this hypothesis, during the necropsy, we observed morphological changes in the reproductive tracts of superovulated T-stock females. The uterine horns were approximately 1.5 times thicker than in superovulated B6C3F1 females and in non-superovulated T-stock females, while the ovaries were larger and exhibited prominent luteinized follicles. The hypertrophic uterus may be the morphological manifestation of an altered uterine environment. These findings are similar to the observations in immature rats where uterus enlargement was reported in stimulated females 3 days after PMSG and was associated with excessive serum estradiol levels (Miller & Armstrong 1981b).

It is interesting to note that T-stock females tend to have smaller litter sizes than other strains of mice after natural matings and that this is due to a considerably higher incidence of spontaneous post-implantation mortality (Larsen & Generoso 1984). It is possible that hormonal regulation of reproductive function is already defective in T-stock females under normal conditions and that superovulation causes an even greater alteration of circulating steroid levels that has a negative effect on the uterine ability to sustain pregnancy.

Differences between T-stock and B6C3F1 females were still observed after IVF. There were significantly more degenerated eggs in T-stock females. It is possible that these eggs represent immature eggs that degenerated during the culturing time or, alternatively, because oocytes would still be exposed to the hostile reproductive tract for a few hours before being collected for IVF, they represent another manifestation of the adverse effects of superovulation on oocyte competency. Also, the frequencies of two-cell embryos were lower in T-stock females with respect to B6C3F1 females suggesting a difference in the timing of the first cell cycle between the two strains of females. It is known that the length of the first cell cycle is different in various mouse strains and that both paternal and maternal genotypes have an effect (Niwa *et al.* 1980, Shire & Whitten 1980a,b). In our study, higher frequencies of eggs at the pronuclear stage in T-stock females were observed both *in vivo* and *in vitro* (Tables 2 and 4). This is in

agreement with the findings of Larsen & Generoso (1984), who reported slower rates of development for T-stock embryos. Therefore, it is possible that the pronuclear eggs observed after IVF would have reached the two-cell stage at later times. When the frequencies of pronuclear eggs and two-cell embryos are combined to estimate the frequencies of eggs that were fertilized *in vitro*, no difference between T-stock and B6C3F1 females was observed ($P = 0.1$).

In conclusion, we found that T-stock females have an abnormal response to superovulation. Although an intrinsic deficiency of T-stock oocytes could not be excluded completely, the results of IVF experiments suggest that superovulation in T-stock females results in a hostile uterine environment with dramatic effects on sperm function and embryo development. Additional research is needed to uncover the reason(s) for these findings. It also remains to be determined whether some of the seven recessive loci are responsible for impaired fertilization and zygotic development. Finally, these results suggest that T-stock females can be a useful animal model for investigating the impact of ovarian stimulation with gonadotropins on fertilization and early embryonic development.

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References

- Abbott AL, Fissore RA & Ducibella T 1999 Incompetence of preovulatory mouse oocytes to undergo cortical granule exocytosis following induced calcium oscillations. *Developmental Biology* **207** 38–48.
- Allen J & McLaren A 1971 Cleavage rate of mouse eggs from induced and spontaneous ovulation. *Journal of Reproduction and Fertility* **27** 137–140.
- Beaumont HM & Smith AF 1975 Embryonic mortality during the pre- and post-implantation periods of pregnancy in mature mice after superovulation. *Journal of Reproduction and Fertility* **45** 437–448.
- Collett D 1991 *Modeling Binary Data*. London: Chapman and Hall.
- Dubey AK, Penzias AS, Emmi AE, Layman LC, Reindollar RH & Ducibella T 1997 Failed fertilization after intracytoplasmic sperm injection: the extent of paternal and maternal chromatin decondensation. *Fertility and Sterility* **68** 714–717.
- Dubey AK, Penzias AS, Reindollar RH & Ducibella T 1998 Technical and physiological aspects associated with the lower fertilization following intra cytoplasmic sperm injection (ICSI) in human. *Theriogenology* **49** 33–41.
- Ducibella T 1996 The cortical reaction and development of activation competence in mammalian oocytes. *Human Reproduction Update* **2** 29–42.
- Ducibella T 1998 Biochemical and cellular insights into the temporal window of normal fertilization. *Theriogenology* **49** 53–65.
- Ducibella T & Buetow J 1994 Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Developmental Biology* **165** 95–104.
- Edwards R & Gates A 1959 Timing of the stages of the maturation divisions, ovulation, fertilization and the first cleavage of eggs of adult mice treated with gonadotropins. *Journal of Endocrinology* **18** 292–304.
- Elbling L & Colot M 1985 Abnormal development and transport and increased sister-chromatid exchange in preimplantation embryos following superovulation in mice. *Mutation Research* **147** 189–195.
- Elmazar MM, Vogel R & Spielmann H 1989 Maternal factors influencing development of embryos from mice superovulated with gonadotropins. *Reproductive Toxicology* **3** 135–138.
- Eppig JJ, Schultz RM, O'Brien M & Chesnel F 1994 Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Developmental Biology* **164** 1–9.
- Ertzeid G & Storeng R 1992 Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *Journal of Reproduction and Fertility* **96** 649–655.
- Ertzeid G & Storeng R 2001 The impact of ovarian stimulation on implantation and fetal development in mice. *Human Reproduction* **16** 221–225.
- Ertzeid G, Storeng R & Lyberg T 1993 Treatment with gonadotropins impaired implantation and fetal development in mice. *Journal of Assisted Reproduction and Genetics* **10** 286–291.
- Generoso WM, Cain KT, Krishna M & Huff SW 1979 Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. *PNAS* **76** 435–437.
- Hogan B, Beddington R, Constantini F & Lacy E 1994 *Manipulation of the Mouse Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Hunter RH 1994 Ovarian regulation of sperm progression in the fallopian tubes. *Zygote* **2** 363–366.
- Hunter RH, Petersen HH & Greve T 1999 Ovarian follicular fluid, progesterone and Ca²⁺ ion influences on sperm release from the fallopian tube reservoir. *Molecular Reproduction and Development* **54** 283–291.
- Larsen MM & Generoso WM 1984 Analysis of spontaneous early embryonic lethality in mice. *Mutation Research* **128** 65–72.
- Licciardi FL, Kwiatkowski A, Noyes NL, Berkeley AS, Krey LL & Grifo JA 1999 Oral versus intramuscular progesterone for in vitro fertilization: a prospective randomized study. *Fertility and Sterility* **71** 614–618.
- Lowe X, O'Hogan S, Moore D, Bishop J & Wyrobek A 1996 Aneuploid epididymal sperm detected in chromosomally normal and Robertsonian translocation-bearing mice using a new three-chromosome FISH method. *Chromosoma* **105** 204–210.
- Mailhes JB & Yuan ZP 1987 Cytogenetic technique for mouse metaphase II oocytes. *Gamete Research* **18** 77–83.
- Maman E, Lunenfeld E, Levy A, Vardi H & Potashnik G 1998 Obstetric outcome of singleton pregnancies conceived by in vitro fertilization and ovulation induction compared with those conceived spontaneously. *Fertility and Sterility* **70** 240–245.
- Marchetti F & Mailhes JB 1995 Variation of mouse oocyte sensitivity to griseofulvin-induced aneuploidy during the second meiotic division. *Mutagenesis* **10** 113–121.
- Marchetti F & Wyrobek AJ 2003 PAINT/DAPI analysis of mouse zygotes to detect paternally transmitted chromosomal aberrations. *Advances in Experimental Medicine and Biology* **518** 131–145.
- Marchetti F, Bishop JB, Cosentino L, Moore D II & Wyrobek AJ 2004 Paternally transmitted chromosomal aberrations in mouse zygotes determine their embryonic fate. *Biology of Reproduction* **70** 616–624.
- Maudlin I & Fraser LR 1977 The effect of PMSG dose on the incidence of chromosomal anomalies in mouse embryos fertilized in vitro. *Journal of Reproduction and Fertility* **50** 275–280.
- Miller BG & Armstrong DT 1981a Superovulatory doses of pregnant mare serum gonadotropin cause delayed implantation and infertility in immature rats. *Biology of Reproduction* **25** 253–260.

- Miller BG & Armstrong DT** 1981b Effects of a superovulatory dose of pregnant mare serum gonadotropin on ovarian function, serum estradiol, and progesterone levels and early embryo development in immature rats. *Biology of Reproduction* **25** 261–271.
- Miller BG & Armstrong DT** 1982 Infertility in superovulated immature rats: role of ovarian steroid hypersecretion. *Biology of Reproduction* **26** 861–868.
- Niwa K, Araki M & Iritani A** 1980 Fertilization in vitro of eggs and first cleavage of embryos in different strains of mice. *Biology of Reproduction* **22** 1155–1159.
- Ozgunen KT, Erdogan S, Mazmanoglu N, Pamuk I, Logoglu G & Ozgunen T** 2001 Effect of gonadotrophin dose on oocyte retrieval in superovulated BALB/c mice. *Theriogenology* **56** 435–445.
- Russell WL** 1951 X-ray-induced mutations in mice. *Cold Spring Harbor Symposium on Quantitative Biology* **16** 327–336.
- Russell LB & Russell WL** 1992 Frequency and nature of specific-locus mutations induced in female mice by radiations and chemicals: a review. *Mutation Research* **296** 107–127.
- Sakkas D, Manicardi G, Bianchi PG, Bizzaro D & Bianchi U** 1995 Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biology of Reproduction* **52** 1149–1155.
- Shire JG & Whitten WK** 1980a Genetic variation in the timing of first cleavage in mice: effect of paternal genotype. *Biology of Reproduction* **23** 363–368.
- Shire JG & Whitten WK** 1980b Genetic variation in the timing of first cleavage in mice: effect of maternal genotype. *Biology of Reproduction* **23** 369–376.
- Smith TT** 1998 The modulation of sperm function by the oviductal epithelium. *Biology of Reproduction* **58** 1102–1104.
- Takagi N & Sasaki M** 1976 Digynic triploidy after superovulation in mice. *Nature* **264** 278–281.
- Tarin JJ, Perez-Albala S & Cano A** 2002 Stage of the estrous cycle at the time of pregnant mare's serum gonadotropin injection affects the quality of ovulated oocytes in the mouse. *Molecular Reproduction and Development* **61** 398–405.
- Tiveron C, Marchetti F, Bassani B & Pacchierotti F** 1992 Griseofulvin-induced aneuploidy and meiotic delay in female mouse germ cells. I. Cytogenetic analysis of metaphase II oocytes. *Mutation Research* **266** 143–150.
- Van der Auwera I & D'Hooghe T** 2001 Superovulation of female mice delays embryonic and fetal development. *Human Reproduction* **16** 1237–1243.
- Van der Auwera I, Pijnenborg R & Koninckx PR** 1999 The influence of in-vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. *Human Reproduction* **14** 2570–2574.
- Walton EA & Armstrong DT** 1981 Ovarian function and early embryo development in immature rats given a superovulatory dose of PMSG, later neutralized by antiserum. *Biology of Reproduction* **25** 272–280.
- Walton EA & Armstrong DT** 1983 Oocyte normality after superovulation in immature rats. *Journal of Reproduction and Fertility* **67** 309–314.
- Walton EA, Evans G & Armstrong DT** 1983 Ovulation response and fertilization failure in immature rats induced to superovulate. *Journal of Reproduction and Fertility* **67** 91–96.

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