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Reproduction

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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 2-fold reduction ($P<0.001$) in the frequencies of fertilized eggs compared to control B6C3F1 matings. In addition, ~22 hr 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% ± 3.8, $P=0.10$). There was a 7-fold increase in the frequencies of fertilized T-stock eggs that completed the first cell cycle of development after in vitro versus in vivo fertilization. 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 induces a hostile oviductal and uterine environment with dramatic effects on fertilization and zygotic development.

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 continuously introduced in clinical practice to improve the quality of ovulated eggs, the chance for successful fertilization and pregnancy outcome (Licciardi *et al.*, 1999). However, despite significant progress, fertilization and implantation failures remain high (Dubey *et al*, 1997; Dubey *et al.*, 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- to 6-week-old females ovulate the maximum number of eggs obtainable from a given strain. 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, SJL/J strains are among the high ovulators, while A/J, C57/L, 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 and McLaren, 1971; Beaumont and Smith, 1975; Ertzeid and Storeng, 1992, 2001; Ertzeid *et al*, 1993; Van der Auwera and 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 (Elbling and Colot, 1985; Maudlin and Fraser, 1977; Takagi and Sasaki, 1976). 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 and Armstrong, 1981a, 1981b; Walton and Armstrong, 1983; Walton *et al*, 1983). In addition, superovulation in rats often results in ovulation occurring 24 hr before the administration of hCG (Miller and 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 extensively used in the specific locus and dominant lethal tests (Russell and 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 6 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 consistently showed 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 of 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.*, 2003), 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 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). T-stock mice were produced at Taconic (Germantown, NY) using three sublines provided by ORNL. The animals were housed under 14 h dark: 10 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

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

Collection of zygotes for cytogenetic analysis

Immediately after hCG injection, females were mated with untreated B6C3F1 males and checked for the presence of vaginal plugs 8 hr later. At this time, mated females were removed

from the males and 16 hr later, i.e., 24 hr after hCG, they received an i.p. injection of 0.08 mg of colchicine in 0.2 ml of distilled water to arrest development of the zygotes at the first mitotic division. Six hr later, mated females were euthanized by CO_2 inhalation and eggs flushed from the oviducts and processed according to the mass harvest procedure (Mailhes and 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 and 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 maternal (smaller) pronuclei (Fig. 1E); and, zygotes – zygotes with mitotic chromosomes (Fig. 1F).

Collection and capacitation of spermatozoa for in vitro fertilization (IVF)

Epididymal spermatozoa were obtained from the cauda epidydimis of B6C3F1 males as described by Lowe et al. (1996). After 20 min at 37 ^{o}C in 250 µl of M16 medium (Sigma), 25 µl of sperm suspension was diluted in 475 µl of 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 of bovine sperm albumin (Sigma) under mineral oil (Sigma) to

give a final concentration of 1 x 10^6 spermatozoa per ml. The fertilization drops were kept at 37 ^oC in 5% CO_2 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 hr after hCG. Ovaries along with the oviduct and part of uterus were isolated and placed into warm Hanks balanced salt solution (HBSS, Sigma). The cumulus-oocyte-complexes (COC) were collected by tearing the ampullary region of the oviduct and washed twice in fertilizing medium. Approximately 20 COC were transferred into each fertilization drop and kept at 37 ^oC in 5% CO_2 for 5 hr. Oocytes were then washed 3 times in cultivation medium (M16 medium supplemented with BSA 4 mg/ml) (Hogan *et al*, 1994) and transferred into 100 µl of cultivation medium under mineral oil (~10 oocytes per drop). They were cultured at 37 ^oC in 5% CO_2 for 21 hours.

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 2-cell embryo stage showing two blastomeres of similar size. Eggs with three pronuclei were assumed to be 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 2-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 (vs. 82% in B6C3F1, P =0.26), and ovulated an average of 34.6 eggs per female versus 33.3 eggs per female in B6C3F1. However, there was a 2-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 hr after hCG (versus 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 eggs: 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 ~40% of the unfertilized eggs in T-stock females, while they represented only 8% of the unfertilized eggs in B6C3F1 females. Eggs with maternal meiotic chromosomes associated with sperm heads in various stages of decondensation (Fig 1 C), or sperm tail only, represented ~13% of fertilized

eggs in T-stock females but were not seen in B6C3F1 females (historic frequency of these eggs in B6C3F1 females is 0.5%). Degenerated fertilized eggs with no recognizable chromosomes, fragmented or damaged pronuclei (Fig. 1D) represented more than 65% of the fertilized eggs in T-stock females vs. 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 ~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 ~87% seen in B6C3F1 females.

To rule out the possibility of physiological incompatibility between T-stock eggs and B6C3F1 sperm, 12 T-stock 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). 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 hr after administration of hCG. No eggs were found up to 12 hr 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 hr 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, that ovulated oocytes are at the normal stage of meiotic maturation and have a normal haploid count 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 estrus induction, as indicated by the fact that only 42% (15 out 36) of females mated, and the number of ovulated eggs (average11.5 per female). However, there was no improvement in fertilization rate (33.9% ± 2.9 vs. 40.3 ± 6.3 after regular hormonal dosage) and in the frequency of 1-Cl zygotes (9.3% ± 4.0 vs 6.0% ± 3.8 after regular hormonal dosage).

In vitro fertilization

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% vs. 27.5%), while they differed in the incidence of degenerated oocytes/embryos (21.8% vs. 8.0%, respectively). There was also a statistical significant difference between T-stock and B6C3F1 females in the frequencies of 2-cell embryos (42.6% vs. 62.7%, respectively, $P<0.05$). Nevertheless, IVF resulted in a 7-fold increase ($P<0.001$) in the frequencies of T-stock eggs that completed the first cell cycle of development and produced 2-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. When the frequencies of 2-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% vs, 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. In vitro fertilization of superovulated eggs improved to near normal levels both the frequencies of fertilized eggs (~1.5 fold) and the frequencies of fertilized eggs that completed the first cell cycle of development and formed 2-cell embryos (7-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 as 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 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 result in the activation of the egg and initiation of mammalian embryonic development (Abbott *et al*, 1999; Ducibella, 1998). 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, 1996; Ducibella and Buetow, 1994; Eppig *et al*, 1994). 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 calcium. Because the two phenomena may be independent, oocytes that have completed nuclear maturation can still be deficient in cytoplasmic maturation and viceversa (Eppig *et al*, 1994). Thus, although nuclear maturation was not affected in T-stock eggs, as indicated by the fact that over 94% of the oocytes recovered 16 hr 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 properly respond 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 hr after hCG, over 34 eggs per females were ovulated and their chromosomal constitution was normal. Secondly, the observation that 30 hr 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, in vitro fertilization 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, in vitro fertilization 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 oestrogenic secretion after ovulation (Ertzeid and Storeng, 1992; Miller and Armstrong, 1982; Walton and Armstrong, 1981). Elevated blood levels of steroids result in alterations of the uterine mileu that can produce an environment unsuitable to sustain embryonic development (Elmazar *et al*, 1989; Ertzeid and Storeng, 200 1; Ertzeid *et al.*, 1993; Van der Auwera *et al.*, 1999). 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+} ions concentration, epithelial secretions, etc) and creting a hostile environment which negatively affects sperm progression, capacitation and/or fertilization processes (Hunter, 1994; Hunter *et al.*, 1999). Following fertilization, the unsuitable oviductal environment manifested a negative influence on zygote development arresting development of the new embryos around 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 day after PMSG and was associated with excessive serum oestradiol levels (Miller and Armstrong, 1981b).

It is interesting to note that the incidence of spontaneous postimplantation mortality is considerably higher in T-stock females than in other mouse strains (Larsen and 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 in vitro fertilization. Degenerated eggs were significantly more in T-stock females. It is possible that these eggs represent immature eggs that degenerated during the culturing time. Also, the frequencies of 2-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 and Whitten, 1980a, 1980b). 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 and Generoso (Larsen and 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 2-cell stage at later times. When the frequencies of pronuclear eggs and 2-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. Because in vitro fertilization reversed the effects induced by superovulation, the data suggest that superovulation in T-stock females results in a hostile uterine environment with dramatic effects on sperm function and embryo development. It 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, early embryonic development, implantation and gestation in humans.

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Table 1. Fertilization rate and development in matings using B6C3F1 males

	Females	
	B6C3F1	T-stock
% Matings	81.7 ± 5.4	63.6 ± 4.8
No. mated females	67	34
No. eggs per female	33.3 ± 1.6	34.6 ± 3.7
Total eggs analyzed	1126	618
% Fertilized eggs	80.1 ± 2.4	40.3 ± 6.4[d]
% Zygotic development[b]	86.6 ± 2.8	14.9 ± 8.2[d]
% 1-Cl metaphase[c]	69.4 ± 3.0	6.0 ± 3.8[d]

[a]Percentage ± Standard Error.

[b]Number of 1-Cl metaphase / fertilized eggs

[c]Number of 1-Cl metaphase / total eggs

[d]$P < 0.001$ (Chi-square)

Table 2- Types of unfertilized and fertilized eggs in matings using B6C3F1 males

	Females[a]	
	B6C3F1	T-stock
Total eggs	1126	618
Unfertilized eggs	19.9 ± 2.4	59.7 ± 6.4[d]
Meiotic chromosomes[b]	7.6 ± 5.0	38.5 ± 14.4[d]
Degenerated[b]	92.4 ± 5.0	61.5 ± 14.4
Fertilized eggs	80.1 ± 2.4	40.3 ± 6.4[d]
Meiotic chromosomes[c]	0	12.9 ± 6.3[d]
Degenerated[c]	12.0 ± 2.6	65.9 ± 11.0[d]
Pronuclei[c]	1.5 ± 0.5	6.4 ± 3.2[e]
1-Cl metaphases[c]	86.6 ± 2.8	14.9 ± 8.2[d]

[a]Percent ± Standard Error.

[b]Percent among unfertilized eggs.

[c]Percent among fertilized eggs.

[d]$P<0.001$ (Chi-square).

[e]$P=0.02$ (Chi-square).

Table 3. - Types of unfertilized and fertilized eggs in matings using T-stock males

	Females	
	B6C3F1	T-stock
Total eggs	86	83
Unfertilized eggs	30.2	79.5[c]
Meiotic chromosomes[a]	0.0	74.2[c]
Degenerated[a]	100.0	25.8[c]
Fertilized eggs	69.8	20.5[c]
Meiotic chromosomes[b]	0.0	29.4[c]
Degenerated[b]	20.0	70.6[c]
Pronuclei[b]	8.3	0.0[c]
1-Cl metaphases[b]	71.7	0.0[c]

[a]Percent among unfertilized eggs.

[b]Percent among fertilized eggs.

[c]$P<0.001$ (Chi-square).

Table 4. Fertilization rate and development after in vitro fertilization using B6C3F1 sperm

	Source of oocytes[a]	
	B6C3F1	T-stock
Total eggs	375	317
Unfertilized	27.5 ± 2.7	26.5 ± 2.9
Degenerated	8.0 ± 2.1	21.8 ± 4.9[b]
Pronuclei	1.9 ± 1.3	9.1 ± 3.5[b]
2-cell embryos	62.7 ± 4.6	42.6 ± 5.8[b]
2-cell embryos and pronuclei	64.5 ± 3.8	51.7 ± 6.0[c]

[a]Percent ± Standard Error.

[b]$P<0.05$ (Chi-square).

[c]$P=0.10$ (Chi-square).

Figure Legends

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. **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) prunuclei. **F.** Zygote at the metaphase stage of the first meiotic division.

Figure 2. Comparison of embryonic development after in vivo matings and in vitro fertilization with B6C3F1 sperm for T-stock and B6C3F1 females. Bars represent the standard error.

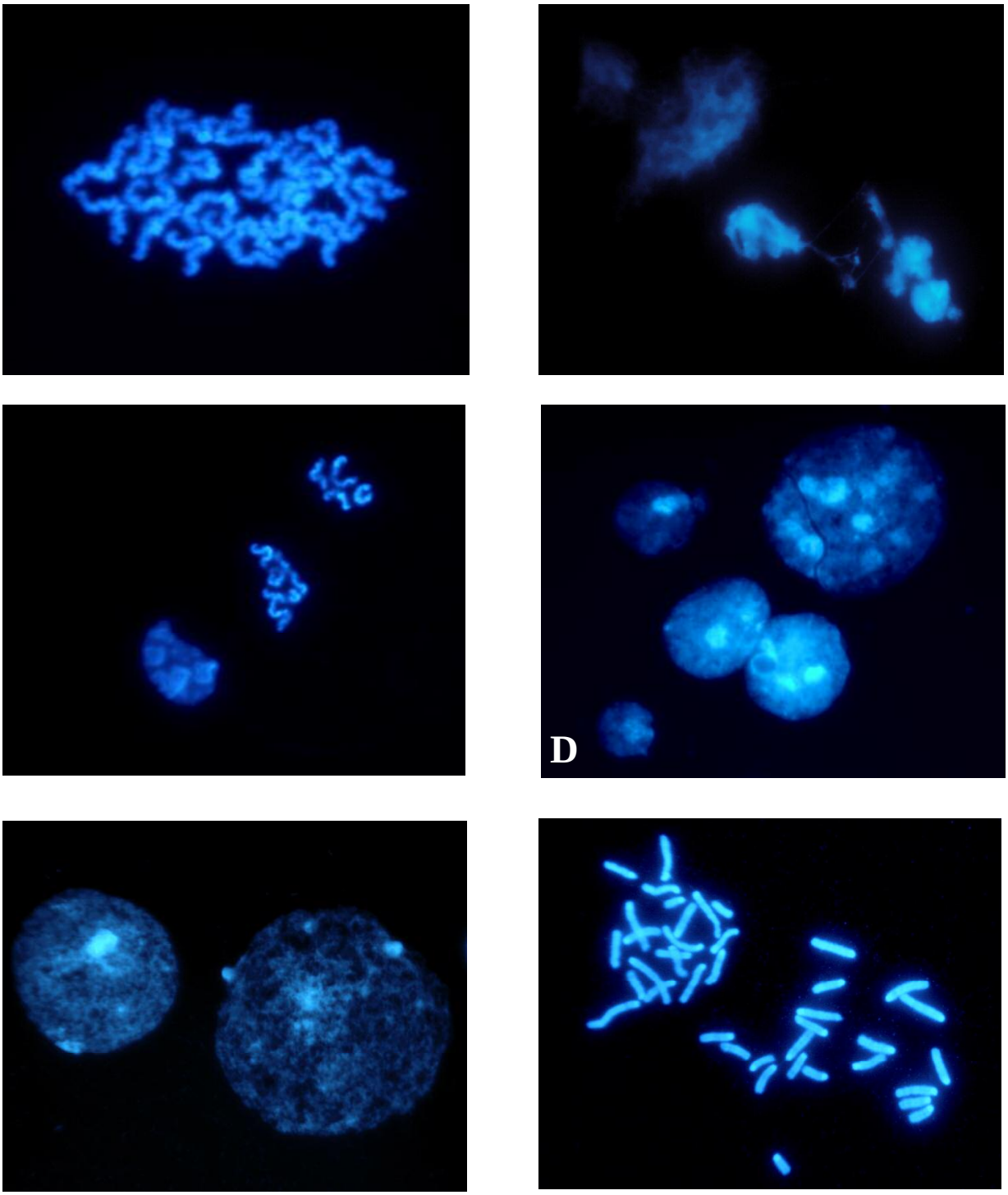

Figure 1

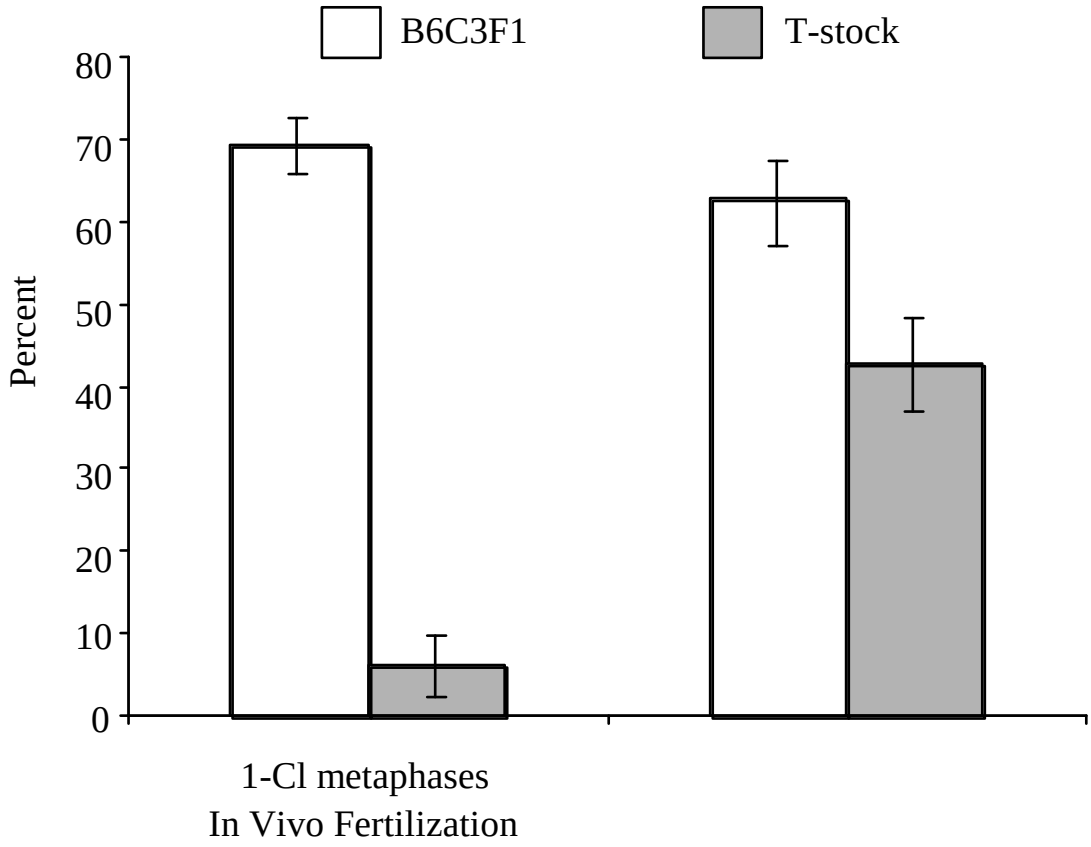
B6C3F1
T-stock
80
70
60
50
40
30
20
10
0
Percent
1-Cl metaphases
In Vivo Fertilization

Figure 2