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Postovulatory Aging of Oocytes Decreases Reproductive Fitness and Longevity of Offspring[1]

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ABSTRACT

We analyzed the long-term effects of postovulatory aging of mouse oocytes on reproductive fitness and longevity of offspring. Hybrid (C57BL/6JIco × CBA/JIco) parental generation (F_0) females were artificially inseminated at 13 h (~1 h postovulation) or 22 h (~10 h postovulation) after GnRH injection. Reproductive fitness of first generation (F_1) females was tested from the age of 28 wk until the end of their reproductive life. In males, the testing period ranged from the age of 2 yr until their natural death. Experimental F_1 females exhibited longer between-labor intervals, decreased frequency of litters, and lower total number of litters and offspring born. Experimental second generation (F_2) pups displayed teratogenic defects, higher preweaning mortality, and decreased body weight at weaning. Incidence of infertility was higher in experimental F_1 males, which translated into lower total number of offspring born when compared with the control group. Life expectancy of F_1 offspring was decreased in the experimental group. These results clearly show that postovulatory aging of mouse oocytes decreases reproductive fitness and longevity of offspring.

aging, fertilization, oviduct, ovulation, ovum

INTRODUCTION

Many mammalian species restrict sexual activity to distinct estrous periods or are induced to ovulate by coitus. In these species, therefore, ovulation and insemination are highly synchronized processes. However, like many other primates, human beings potentially engage in sexual activity at any day of the menstrual cycle. Because of this sexual behavior, ovulation and insemination may not be synchronized, resulting in either fertilization of aged oocytes if insemination is delayed with respect to the ovulation time or fertilization of fresh oocytes by aged spermatozoa if insemination takes place well in advance of ovulation. Indirect epidemiological evidence suggests that several factors likely associated with preovulatory aging of human oocytes, including longer follicular phase and temperature rise during the conceptional cycle, low and high maternal age, short preceding pregnancy or birth interval, and month of birth, may cause congenital malformations in offspring and many constitutional diseases such as diabetes mellitus, schizophrenia, and nonfamilial Alzheimer's disease (reviewed in [1]). Furthermore, these epidemiological studies suggest that preovulatory aging of human oocytes may be associated with menstrual disorders and/or decreased fecundity in daughters and likely decreased offspring longevity (reviewed in [1]). Fertilization of postovulatory aged mouse oocytes gives rise to individuals suffering from retarded sensorimotor integration during preweaning development, increased spontaneous motor activity, and higher emotionality [2]. Kimler et al. [3] observed that preovulatory aging of rat oocytes induced changes in brain cortical thickness concomitant with decreased exploratory behavior in a continuous corridor, which can be considered as analogous to cognitive function in humans. Kimler et al. then suggested that children resulting from fertilization of oocytes that undergo preovulatory aging may display behavioral alterations, learning difficulties, or even mental retardation.

The purpose of the present study was to analyze the long-term effects of postovulatory aging of mouse oocytes on reproductive fitness and longevity of first generation (F_1) offspring. Reproductive fitness was broken down into 2 major components: total number of offspring born (fertility) and quality of these offspring, measured as preweaning mortality and weaning weight. The variation in total number of offspring born was in turn attributed to other metric characters, including mating success, time to pregnancy, litter size, frequency of litters, and number of litters [4].

MATERIALS AND METHODS

Origin of F_1 Mice

A detailed description of the procedures followed to produce F_1 mice has been published previously [2]. Hybrid (C57Bl/6JIco female × CBA/JIco male) parental generation (F_0) females 9–12 wk of age were artificially inseminated at 13 h (~1 h postovulation) or 22 h (~10 h postovulation) after GnRH injection at the proestrous phase of the estrous cycle. In each session, 1–6 females were artificially inseminated using spermatozoa from 1 to 4 randomly selected hybrid F_0 males 12–14 wk of age. On Day 3 after birth, litter size and sex ratio of pups from control females were matched with litter size and sex ratio obtained in the experimental group. The surplus control pups were killed by exposure to ether. The final sample size at weaning was 115 experimental (49 females and 66 males) and 113 control (49 females and 64 males) mice.

Housing of F_1 Mice

At the age of 21 days (weaning), male and female F_1 offspring were separated and housed in groups of 10 in 35.5 × 23.5 × 18.5-cm plastic cages. Cages were examined daily and were not consolidated as mice died or after removal of a female or a male from each litter to test for reproductive fitness. Mice were weighed every 10 wk starting at the age of 10 wk. Each animal was marked by ear punching/cutting after weaning. Mice were fed a standard laboratory diet and tap water ad libitum and were

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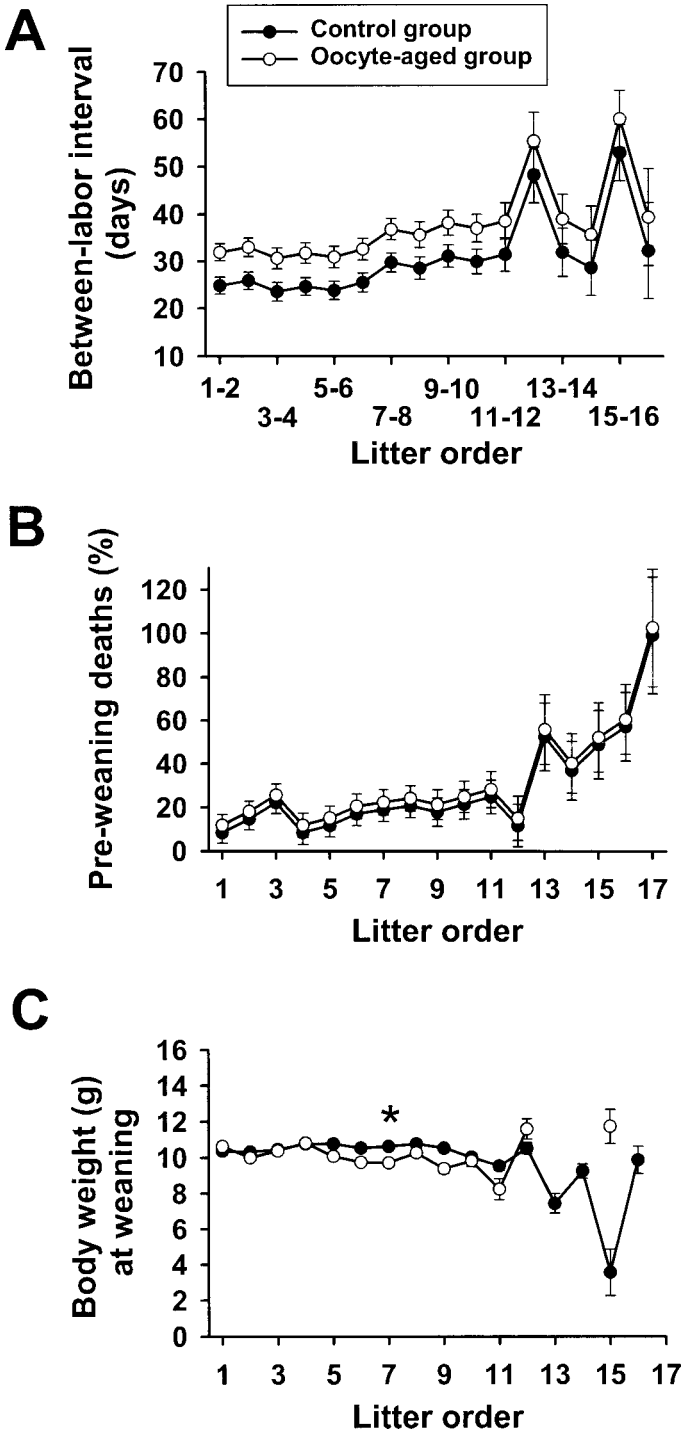

FIG. 1. Effect of postovulatory aging of oocytes on between-labor interval of F_1 female mice (**A**), preweaning mortality of F_2 pups (**B**), and body weight at weaning of F_2 pups (**C**). In **C**, the SEM of most of the points is too small to display an error bar. Furthermore, data from litters 13, 14, and 16 for the oocyte-aged group are missing because none of the newborn pups reached the weaning stage. *, $P = 0.013$.

maintained on a 14L (0800–2200 h):10D photoperiod in a temperature-controlled room at 21–23°C.

Fitness Components of Female F_1 Mice

At the age of 28 wk, 1 randomly selected F_1 female from 19 control and 18 experimental litters was housed in a 26.5- × 20.5- × 13.5-cm plastic cage with a randomly selected 14-wk-old hybrid F_0 male for the rest of her reproductive life. Cessation of a female's reproductive life was defined as the age of the last labor following which no more offspring were born for 3 mo. After this period, each cohabiting male was housed with a female 10–12 wk of age to ascertain whether he was still fertile. From Day 18 after adding the male to the cage until the end of their respective reproductive lives, females were examined once daily to determine the day of parturition and to record litter size and gender of pups at birth of each consecutive litter. At weaning, second generation (F_2) offspring were weighed and killed by exposure to ether. After cessation of reproductive life, females were returned to 35.5- × 23.5- × 18.5-cm plastic cages and housed under the same light:dark cycle and temperature conditions as their virgin siblings.

Fitness Components of Male F_1 Mice

Reproductive fitness of F_1 males was tested when males reached the age of 2 yr (104 wk). At this time, 1 randomly selected F_1 male from 18 control and 15 experimental litters was housed in a 26.5- × 20.5- × 13.5-cm plastic cage with a randomly selected 10-wk-old hybrid F_0 female for the rest of his life. When the female was 30 wk old, she was removed from the cage and replaced by a 10-wk-old F_0 female. Twenty-one days after a 30-wk-old female was separated from her respective male or after his death, each female from infertile couples was housed with a 12-wk-old F_0 male to test her fertility. Day of parturition, litter size, gender of pups at birth, and body weight of pups at weaning were recorded in each consecutive litter. All the animal experiments performed in this study were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (1996).

Statistical Analysis

A Fisher exact test was used for comparisons of frequencies in 2 × 2 contingency tables. Mixed-effects nested designs for analysis of covariance were used for comparisons of means. Nested designs were applied to control the potential correlation among observations within a particular dam and to avoid spurious inflation of the sample size [5]. A Kolmogorov-Smirnov 1-sample test was used to check whether variables were normally distributed. When the normality assumption was violated, logarithmic (body weight and between-labor interval), square root (for counts), or logit (for percentages) transformation of the variable was applied to induce normality. Differences in sex ratios (percentage of males) between groups were tested using automated binomial logistic regression based on forward stepwise variable selection. A 1-sample binomial test was utilized to test the null hypothesis that the probability of being male in each group was 0.5. Cox regression models were fitted taking into account the potential correlation among siblings (i.e., a robust variance was estimated) to examine the effect of several continuous and categorical independent variables on survival of F_1 mice. Significance was defined at $P \leq 0.05$. Unless otherwise indicated, values given are estimated marginal means ± SEMs. The analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) and the S-PLUS 2000 User's Guide (Data Analysis Products Division, MathSoft, Seattle, WA).

RESULTS

Reproductive Fitness of F_1 Females

Time to pregnancy, as estimated from between-labor interval, was significantly affected by time of artificial insemination of F_0 dams (37.9 ± 1.1 days in the experimental group vs. 30.7 ± 1.2 days in the control group; $P \leq 0.01$) (Fig. 1A), mother ($P \leq 0.0005$), and litter order ($P \leq 0.001$; Pearson correlation coefficient $r = 0.168$, $P \leq 0.01$). The longer between-labor intervals displayed by experimental females were translated into decreased frequency of litters (1 litter every 4.6 ± 0.2 wk in the experimental group vs. 3.9 ± 0.2 wk in the control group; $P \leq 0.05$), decreased total number of litters (140 litters, 7.8 ± 0.9 litters/dam in the experimental group vs. 215 litters, 11.3 ± 0.8 litters/dam in the control group; $P \leq 0.01$), and decreased total number of offspring born (837 pups, 46.5 ± 5.5 pups/dam in the experimental group vs. 1206 pups, 63.5 ± 4.6 pups/dam in the control group; $P \leq 0.05$). Experimental dams exhibited decreased total number of litters and decreased total number of offspring born even after removing from

TABLE 1. Effect of litter order and litter size at birth and at weaning on mortality rate and body weight (g) of F_1 mouse pups.

Dependent variable	Independent variable	Group	r[a]	P
Preweaning deaths (%)	Litter order	Control and experimental	0.369	≤0.01
	Litter size at birth	Control and experimental	−0.450	≤0.01
		Control	−0.382	≤0.01
		Experimental	−0.572	≤0.01
Body weight at weaning	Litter order	Control and experimental	0.112	≤0.01
	Litter size at weaning	Control and experimental	−0.328	≤0.01
		Control	−0.362	≤0.01
		Experimental	−0.264	≤0.01

[a] Pearson correlation coefficient.

the analysis 4 control and 9 experimental dams that died before the end of reproductive life (79 litters, 8.8 ± 1.2 litters/dam in the experimental group vs. 179 litters, 11.9 ± 0.9 litters/dam in the control group, $P \leq 0.05$; 414 pups, 46.0 ± 8.0 pups/dam in the experimental group vs. 1008 pups, 67.2 ± 4.4 pups/dam in the control group, $P \leq 0.05$; respectively). All the siring males were still fertile, as estimated on the basis of their reproductive performance when housed with a 10- to 12-wk-old female after their respective females either died or ceased reproductive life.

Delayed insemination of F_0 dams affected between-labor interval, total number of litters, and total number of offspring produced but also affected the quality of pups during preweaning development. Mortality rate of pups was higher in the experimental group (83 deaths, 32.4% ± 4.2%/litter in the experimental group vs. 116 deaths, 29.0% ± 3.5%/litter in the control group; $P \leq 0.005$) (Fig. 1B). Other variables affecting percentage of preweaning deaths were litter order ($P \leq 0.01$), litter size at birth (covariate, $P \leq 0.0005$), and interaction between time of artificial insemination of F_0 dams and litter size at birth ($P \leq 0.005$) (Table 1).

Body weight of pups at weaning was also affected by time of artificial insemination of F_0 dams ($P \leq 0.001$) (Fig. 1C) and by other variables, including mother ($P \leq 0.0005$), gender of pups (10.2 ± 0.1 g in males vs. 9.7 ± 0.1 g in females; $P \leq 0.0005$), litter order ($P \leq 0.0005$), litter size at weaning (covariate, $P \leq 0.0005$), and interaction between time of artificial insemination of F_0 dams and litter size at weaning ($P \leq 0.0005$) (Table 1). Interaction between time of artificial insemination of F_0 dams and litter order ($P \leq 0.0005$) was also significant. For this reason, data were analyzed taking into account each consecutive litter individually. This analysis showed that in most litters experimental F_2 pups were lighter than control pups, although differences were significant only for litter no. 7 (Fig. 1C).

No pups with morphological abnormalities were found in the control group. In contrast, several abnormalities were found in 9 pups coming from 3 experimental dams, including 1) a female pup from the third litter of dam no. 1 that had a single finger in the left hind paw and syndactyly of 2 fingers in the right hind paw, 2) 2 female pups and 1 male pup from the eighth litter of dam no. 2 that displayed bone protuberances in all fingers, 3) 1 female pup and 1 male pup from the second litter and 1 male pup from the seventh litter of dam no. 3 that exhibited a short tail, and 4) 2 female pups from the second litter of dam no. 3 with a gray coat and 2 male pups from the same litter with a coat composed of a mixture of black and gray hair.

Sex ratio (percentage of males) of offspring at birth was not affected by time of artificial insemination of F_0 dams, mother, litter order, and maternal age. In contrast, percentage of males exhibited a concomitant increase with litter size at birth ($r = 0.037 \pm 0.018$; $P \leq 0.05$). In the control group, percentage of males was significantly ($P \leq 0.05$) lower (46%, 559/1206) than 50%. In contrast, in the experimental group percentage of males (49%, 409/837) was not significantly different from 50%. Preweaning mortality was not dependent on gender of pups. Sex ratio of dead pups in both the control (48%, 56/116) and the experimental (41%, 34/83) groups was not significantly different from 50%.

Reproductive Fitness of F_1 Males

Percentage of fertile males that sired offspring from the age of 104 wk until natural death was significantly decreased in the experimental group compared with the control group (2/15, 13.2% vs. 9/18, 50%; $P \leq 0.05$). The higher incidence of infertility observed in the experimental group was translated into lower total number of offspring born (4 litters, 8 pups, 0.5 ± 0.5 pups/male in the experimental group vs. 22 litters, 204 pups, 11.3 ± 3.7 pups/male in the control group; $P \leq 0.005$). Differences between groups were still significant after removing from the analysis 13 experimental and 9 control males that did not sire any offspring during the testing period (4.0 ± 3.0 pups/male in the experimental group vs. 22.5 ± 5.1 pups/male in the control group; $P \leq 0.05$). All the females used for testing the reproductive fitness of F_1 males were fertile, as estimated on the basis of their reproductive performance when housed with a 12-wk-old male either after they were replaced by a younger 10-wk-old female or after the death of their respective males.

Differences between groups in between-labor intervals, frequency of litters, sex ratio of offspring at birth, mortality rate of pups, and body weight of pups at weaning could not be analyzed because of the small number of fertile males (2) and offspring produced (8) in the experimental group. No pups with major morphological abnormalities were found in the control or the experimental group.

Longevity of F_1 Offspring

Figure 2 shows the survival functions for control and experimental F_1 offspring. The curve for experimental mice was significantly lower than the curve for control mice (hazard ratio = 1.294, 95% confidence interval [CI]: 1.130–1.469; mean age at death of the 10th deciles of survivorship, which affords a reasonably accurate estimate of physiological aging [6]: 128.4 ± 2.4 wk and 135.9 ± 2.6 wk, respectively; $P \leq 0.050$). Gender, virginity status, interaction between time of artificial insemination of F_0 dams and gender, interaction between time of artificial insemination of F_0 dams and virginity status, and interaction between time of artificial insemination of F_0 dams, gender, and virginity status were not significant. However, inter-

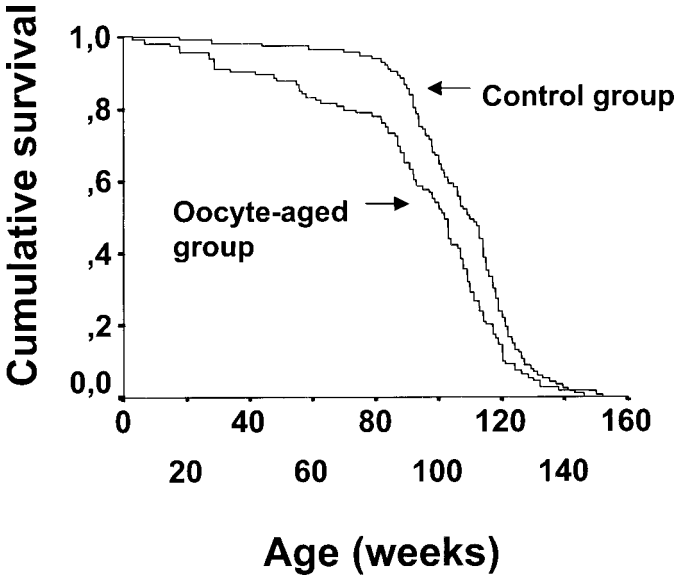

FIG. 2. Effect of postovulatory aging of mouse oocytes on expectation of survival times of offspring.

action between gender and virginity status significantly affected survival times of F_1 mice (hazard ratio = 0.637, 95% CI: 0.556–0.729). Because of this interaction, the effect of gender and virginity status on survival times of both control and experimental F_1 mice was analyzed separately. This analysis showed that mated females had survival times shorter than those of virgin females (hazard ratio = 1.46, 95% CI: 1.21–1.75; mean age at death of the 10th deciles of survivorship: 121.8 ± 2.5 wk and 143.2 ± 2.1 wk, respectively; $P \leq 0.0005$) (Fig. 3A). In contrast, survival times were longer for mated males than for virgin males (hazard ratio = 0.581, 95% CI: 0.49–0.689; mean age at death of the 10th deciles of survivorship: 132.0 ± 5.1 wk and 124.3 ± 2.5 wk, respectively; $P = 0.194$) (Fig. 3B). Further analyses showed that virgin males had both a lower survival curve (hazard ratio = 1.41, 95% CI: 1.19–1.67) and lower mean age at death of the 10th deciles of survivorship ($P \leq 0.0005$) when compared with virgin females (Fig. 3C). In contrast, mated males had longer survival times (hazard ratio = 0.552, 95% CI: 0.415–0.733) and higher mean age at death of the 10th deciles of survivorship ($P \leq 0.05$) than mated females (Fig. 3D).

DISCUSSION

The results of the present study show that postovulatory aging of mouse oocytes reduces the reproductive fitness and longevity of both male and female offspring. These results support previous epidemiological data suggesting that aging of human oocytes may decrease offspring longevity and induce menstrual disorders and/or reduce fecundity in daughters (reviewed in [1]). Our data also expand results of previous prospective studies showing that pre- and/or postovulatory aging of oocytes is associated with teratogenesis in invertebrate and vertebrate species, retarded sensorimotor integration during preweaning development of mouse pups, changes in brain cortical thickness concomitant with decreased exploratory behavior in the rat, and higher spontaneous motor activity and emotionality in the mouse (reviewed in [1]).

Although it is not clear which age-associated factor(s) modifies gene expression in progeny thus decreasing reproductive fitness and longevity of individuals, we have proposed a mechanism based on the oxygen radical-mitochondrial injury hypothesis of aging [7] to explain the effects of oocyte aging on subsequent offspring viability [8, 9].

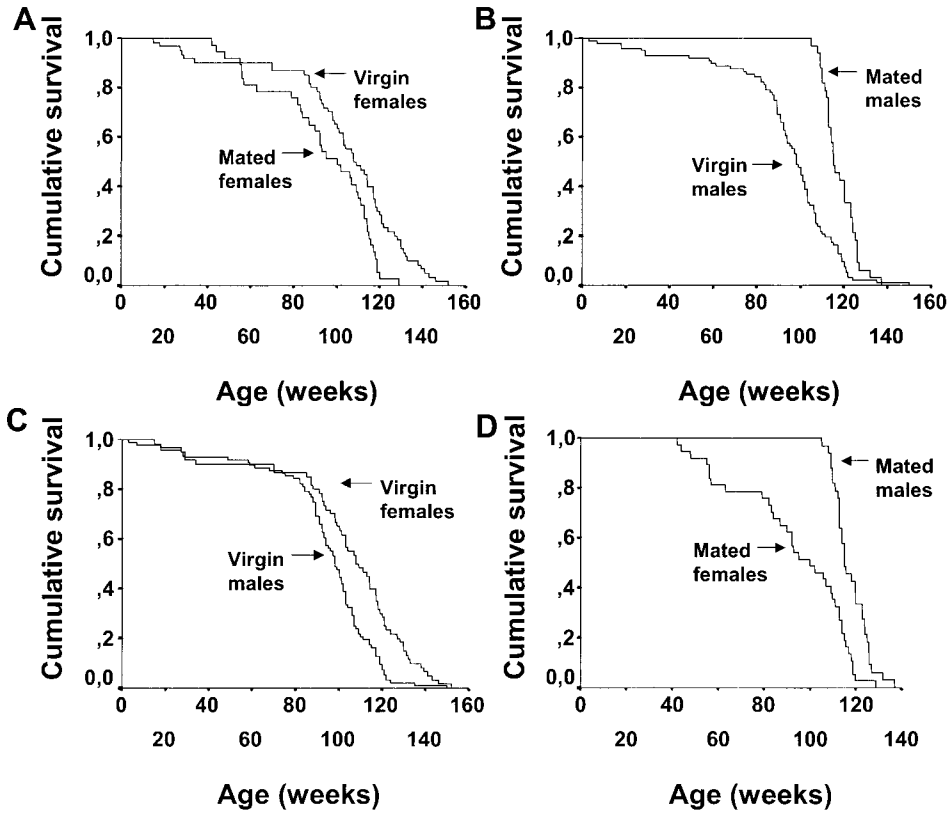

FIG. 3. Survival curves of both control and experimental F_1 mice according to their virginity status (**A** and **B**) and gender (**C** and **D**).

This mechanism ascribes a key role in the senescent process to oxygen radical damage to mitochondrial DNA (mtDNA), proteins, and lipids. Accumulated oxidative damage to mitochondria may both decrease the number of functionally intact mitochondria and thus ATP levels and raise the production of reactive oxygen species by the electron transport chain. Such generalized damage to oocyte mitochondria may increase the probability that the resulting offspring carry a subpopulation of defective mitochondria and express anomalous phenotypes linked to mtDNA and/or oxidative phosphorylation defects, including congenital malformations, type I (insulin-dependent) diabetes mellitus, Alzheimer's disease, schizophrenia, behavioral alterations, learning difficulties, male and female infertility, and decreased longevity (reviewed in [4]). This hypothesis is supported by the fact that human metaphase II oocytes aged in vitro for 24 h exhibit a significant drop in mitochondrial membrane potential [10].

However, the occurrence of age-induced oxidative damage to oocyte mtDNA is not the only mechanism capable of explaining the present results. Aging of oocytes prior to fertilization may affect many molecular, biochemical, and cellular pathways that may jeopardize not only pre- and postimplantation embryo/fetus development but also later life of the offspring. For example, postovulatory aging of mammalian oocytes is associated with alteration of the physicochemical state of the DNA-protein complex, changes in the electrophoretic pattern of synthesized proteins, decreased intracytoplasmic levels of reduced glutathione and ATP, disturbances of intracellular Ca^{2+} regulation, decreased $p34^{cdc2}$ kinase/cyclin B and mitogen-activated protein kinase activities, and presence of apoptotic traits including DNA fragmentation and caspase activity (reviewed in [1]).

The present study also shows that virgin males have a shorter life expectancy than do virgin females. Furthermore, virgin males and virgin females live longer than mated females. These results are in agreement with the general phenomenon observed in the animal kingdom of a gender gap or bias in life expectacy in favor of females [11]; and with the disposable-soma hypothesis, which explains the evolution of senescence in terms of a trade-off between the investment of metabolic resources into reproduction and into the repair/maintenance of the soma [12]. However, contrary to the disposable-soma hypothesis, we found that mated males display a longer life expectancy than virgin males. This paradoxical result is likely a direct consequence of the advanced age at which males were tested for their reproductive fitness. Males used in mating experiments were randomly selected among those that were alive at the age of 104 wk (60 of the 130 males that entered the study), i.e., mating males were selected among the most long-lived virgin males and, therefore, had a longer life span than did virgin males.

Our results clearly show that postovulatory aging of oocytes decreases reproductive fitness and longevity of offspring. These results have direct implications for human beings. Couples wishing to achieve pregnancy should be aware of the negative effects of gamete aging on offspring to take all necessary steps to guarantee a correct maturational synchronization of gametes at fertilization. Although there are many methods that couples wishing to have a baby may use to monitor ovarian activity and predict ovulation such as the thermal shift in the basal body temperature and evaluation of cervical mucus, it appears that self-determination of urinary LH levels using a home kit is the most accurate method [13, 14]. Alternatively, couples planning a pregnancy may daily engage in intercourse to avoid the uncertainty of predicting fertile days [15]. Although this strategy is likely the easiest way for couples to prevent the aging of the oocyte at fertilization, it may result in the aging of the fertilizing spermatozoon. In the rabbit, aged spermatozoa exhibit a 5% probability of fertilizing oocytes when competing directly with fresh spermatozoa [16]. This risk should not be overlooked because in vivo aging of spermatozoa in the female genital tract is associated with polyspermy, nonextrusion of the second polar body, chromosomal anomalies in zygotes and blastocysts including digynic triploidy and mosaicism, failure of sperm chromatin decondensation, abnormal male pronuclear development, and increased pre- and/or postimplantation embryo/fetus mortality (reviewed in [1]).

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