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Long-Term Effects of Mouse Intracytoplasmic Sperm Injection with DNA-Fragmented Sperm on Health and Behavior of Adult Offspring[1]

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ABSTRACT

Genetic and environmental factors produce different levels of DNA damage in spermatozoa. Usually, DNA-fragmented spermatozoa (DFS) are used with intracytoplasmic sperm injection (ICSI) treatments in human reproduction, and use of DFS is still a matter of concern. The purpose of the present study was to investigate the long-term consequences on development and behavior of mice generated by ICSI with DFS. Using CD1 and B6D2F1 mouse strains, oocytes were injected with fresh spermatozoa or with frozen-thawed spermatozoa without cryoprotector. This treatment increased the percentage of TUNEL-positive spermatozoa, tail length as measured by comet assay, and loss of telomeres as measured by quantitative PCR. The ICSI-generated embryos were cultured for 24 h in KSOM, and 2-cell embryos were transferred into CD1 females. The DFS reduced both the rate of preimplantation embryo development and number of offspring. Immunofluorescence staining with an antibody against 5-methylcytosine showed a delay of 2 h on the active demethylation of male pronucleus in the embryos produced by ICSI. Moreover, ICSI affected gene transcription and methylation of some epigenetically regulated genes like imprinting, X-linked genes, and retrotransposon genes. At 3 and 12 mo of age, ICSI with DFS-produced animals and in vivo-fertilized controls were submitted to behavioral tests: locomotor activity (open field), exploratory/anxiety behavior (elevated plus maze, open field), and spatial memory (free-choice exploration paradigm in Y maze). Females produced by ICSI showed increased anxiety, lack of habituation pattern, deficit in short-term spatial memory, and age-dependent hypolocomotion in the open-field test ($P < 0.05$). Postnatal weight gain of mice produced by ICSI with fresh or frozen sperm was higher than that of their control counterparts from 16 wk on ($P < 0.01$). Anatomopathological analysis of animals at 16 mo of age showed some large organs and an increase in pathologies (33% of CD1 females produced with DFS presented some solid tumors in lungs and dermis of back or neck). Moreover, 20% of the B6D2F1 mice generated with DFS died during the first 5 mo of life, with 25% of the surviving animals showing premature aging symptoms, and 70% of the B6D2F1 mice generated with DFS died earlier than controls with different kind of tumors. We propose that depending on the level of DFS, oocytes may partially repair fragmented DNA, producing blastocysts able to implant and produce live offspring. The incomplete repair, however, may lead to long-term pathologies. Our data indicate that use of DFS in ICSI can generate effects that only emerge during later life, such as aberrant growth, premature aging, abnormal behavior, and mesenchymal tumors.

assisted reproductive technology, behavior, DNA fragmented, early development, embryo, embryo development, ICSI, organ weight, sperm DNA fragmentation

INTRODUCTION

The integrity of sperm DNA is of crucial importance for balanced transmission of genetic information to future generations. Evidence suggests that DNA-fragmented sperm (DFS) may lead to conception failure, abortions, malformations, and genetic diseases [1, 2]. Recently, it has been recognized that human couples have a very low potential for natural reproduction if the male semen contains a high percentage of sperm cells with heavily fragmented DNA [3]. The origin of the DNA strand breaks may be multiple; rather than programmed cell death, the mechanism of DNA damage detected in ejaculated spermatozoa is thus related to abnormal cellular metabolism and oxidative stress, which affect the integrity of the sperm chromatin and cause high frequencies of single and double DNA strand breaks [4, 5]. Aging, smoking, exposure to air pollution, prolonged sexual abstinence, and abnormal testicular warming are other factors believed to increase the proportion of sperm cells with fragmented DNA [6]. A significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa [7], and it remains unclear whether assisted reproductive techniques can compensate for poor chromatin packaging and/or DNA damage. A recent meta-analysis suggested that children born following use of assisted reproductive technology (ART) are at increased risk of birth defects compared with those born following spontaneous conception (SC) [8]. Several studies have reported the negative effect on pregnancy rates of an increased proportion of spermatozoa with damaged DNA in sperm samples used for ART [9, 10]. Some examples of research describing health problems as a consequence of ICSI are the increased prevalence of sex chromosome anomalies and the high prevalence of structural and numerical chromosomal aberrations [11, 12]; infants born after use of intracytoplasmic sperm injection (ICSI) are twice as likely as naturally conceived infants to have a major birth defect and nearly 50% more likely to have a minor defect [13, 14]. Also, ICSI offspring are at risk of being infertile [15]. Several follow-up studies on the health and development of

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children born after ICSI, however, indicated that at age 8, physical examination, including a thorough neurological examination, did not reveal important differences between singletons born through ICSI and SC, but major congenital malformations were significantly more frequent in the ICSI group [16]. At age 10, ICSI and SC children showed comparable cognitive and motor development [17].

In the context of ICSI, only those spermatozoa with normal morphology and motility are selected and considered to be "good sperm cells" for clinical use. Such sperm parameters, however, do not ensure DNA integrity. A high percentage (~40%) of the men clinically considered to be subfertile, who are frequently recommended for ICSI treatment, have high proportions of DFS cells in their semen samples [18]. Sperm DNA fragmentation analysis before ICSI is not required in most fertility clinics around the world. The role of sperm chromatin integrity during ICSI is of critical importance [18, 19], because this procedure bypasses multiple, apparently redundant mechanisms that have evolved to ensure selection of high-quality sperm cells for fertilization. When ICSI is employed, even spermatozoa with severe DNA damage may undergo nuclear decondensation and pronucleus formation. Developmental abnormalities arising from such chromatin damage may not be observed until the postimplantation stages [19–21]. The biological impact of an abnormal sperm chromatin structure depends on the combined effects of the extent of DNA or chromatin damage in the spermatozoa and the capacity of the oocyte to repair that damage [22]. It has been reported in a mouse model system that spermatozoa with defective DNA can fertilize an oocyte and produce high-quality, early stage embryos, but then, as the extent of the DNA damage increases, the likelihood of a successful pregnancy to term decreases [23]. Those authors suggest that the oocyte has the capacity to repair DNA damage of sperm when it is damaged by less than 8% [23]. Depending on the level of sperm DNA fragmentation, three situations can be expected: In some cases, the oocyte repair machinery is not sufficient to repair DNA damage, and the embryo may fail to develop or implant in the uterus or may be aborted naturally at a later stage (uncompensable damage). In other cases, the oocyte repairs the DNA strand breaks before the initiation of the first cleavage division, and this sperm is then able to generate normal offspring (compensable damage). In the worst and last scenario, deletions or sequence errors may be introduced because of partial oocyte repair, and abnormal offspring may then result (partial compensable damage). It has been reported that 80% of de novo structural chromosome aberrations in human are of paternal origin [24]. If the DNA damage involves an oncogene, the result would be an increased risk of cancer in the offspring.

Although the prevalence of spermatozoa with fragmented DNA is considered to be among the most common causes of male infertility that may pass undetected [25], to our knowledge the long-term effects of using sperm cells with DNA damage for ICSI have not been evaluated. In the present study, by using the mouse ICSI model, we determined the short- and long-term impacts of using such sperm cells by analyzing level of development, implantation, and DNA methylation of embryos as well as adult animal health and behavior.

MATERIALS AND METHODS

Reagents and Media

Unless otherwise stated, all chemicals and media were purchased from Sigma Chemical Co.

Animals, Gamete Collection, and Sperm Freezing

CD1 and B6D2F1 (C57BL/6 x DBA/2) mice (Harlan Iberica SL) were used as oocyte and sperm donors. Females were 6–8 wk old at the time of the experiments, and males were at least 3 mo old. CD1 females mated with vasectomized males were used as surrogate mothers for embryo-transfer experiments. Mice were fed ad libitum with a standard diet and maintained in a temperature- and light-controlled room (23°C, 14L:10D). In all experiments, pregnant dams were allowed to deliver spontaneously. The pups were nursed by their natural dams until weaning. To ensure standardized nutrition and maternal care, all litters were redistributed (or augmented with additional pups) on the day after birth to have litter sizes of six to eight pups. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines and in adherence with guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

Metaphase II oocytes were collected from the oviducts of 6- to 8-wk-old female mice superovulated with 7.5 IU of eCG and, 48 h later, with an equivalent dose of hCG. Briefly, at 14 h post-hCG administration, oviducts were removed from superovulated female mice and placed in an M2-containing Petri dish at room temperature. After washing, collected oviducts were placed in fresh M2 medium, and cumulus-oocyte complexes were released from the ampulla with the aid of Dumont #55 forceps. Cumulus-oocyte complexes were then moved either into a fertilization drop (for in vitro fertilization [IVF] experiments) or into a dispersion drop (for ICSI experiments). In ICSI experiments, cumulus cells were dispersed by a 3- to 5-min incubation in M2 medium containing 350 IU/ml of hyaluronidase; after washing, oocytes were maintained in KSOM medium [26] at 37°C in an atmosphere of 5% CO_2 in air until use. Fresh and frozen-thawed sperm were prepared essentially as described previously [26] with minor differences. Briefly, epididymal sperm from mature (3–6 mo old) males was collected in M2 medium by excising with a pair of fine scissors and compressing with forceps blood-free and adipose tissue-free epididymal cauda. Sperm cells collected in a minimal volume, to be frozen-thawed, were placed in the bottom of a 1.5-ml polypropylene centrifuge tube and overlaid with the volume of fresh medium necessary to obtain the final concentration of 2.5 million cells/ml. The sperm extender used did not contain cryoprotecting agents, such as EDTA or EGTA. Sperm samples were frozen in liquid nitrogen and stored for periods ranging from 1 day to 4 wk at −80°C. Asepsis was maintained throughout the procedure. Both fresh and frozen-thawed sperm were mixed with 40–50 μl of a 10% polyvinyl-pyrrolidone (PVP-360) in M2 solution before being placed in the culture dish for microinjection.

ICSI with Fresh and Frozen-Thawed Sperm

The ICSI with fresh and frozen-thawed spermatozoa was performed in M2 medium at room temperature [27]. One volume of sperm was mixed with five volumes of M2 medium containing 10% PVP to decrease stickiness. The ICSI dish contained a manipulation drop (M2 medium), a sperm drop (sperm solution in M2/10% PVP), and an M2/10% PVP needle-cleaning drop. Injections were performed with a PMM-150 FU piezo-impact unit (Prime Tech) and Eppendorf micromanipulators using a blunt-ended, mercury-containing pipette (inner diameter, 6–7 μm). Individual sperm heads either mechanically decapitated with the piezo unit (for fresh sperm) or by the freezing-thawing procedure were injected into oocytes. Oocytes were injected in groups of 10. After 15 min of recovery at room temperature in M2 medium, surviving oocytes were returned to mineral oil-covered KSOM and cultured at 37°C in an atmosphere of 5% CO_2 air for up to 96 h. For embryo culture, 50-μl drops of KSOMaa medium were set up in a plastic culture dish, overlaid with mineral oil, and equilibrated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. Oocytes were scored for male and female pronucleus formation (fertilization) at 6 h after the initiation of culture, and the number of 2-cell embryos was scored after 24 h in culture. For full term development, 2-cell embryos were transferred into oviducts of recipient pseudopregnant females. Embryo transfer was performed as described previously [26].

For the IVF experiments, oocytes were obtained from superovulated female mice as above. The methodology used has been described elsewhere [28]. In the IVF assays, 2.5–10.0 μl of fresh epididymal sperm was added to each fertilization drop to achieve a final concentration of ~1–2 × 10^6 spermatozoa/ml. Four hours after oocyte and sperm coincubation at 37°C in a humidified atmosphere of 5% CO_2 in air, oocytes were washed and cultured in KSOM.

Evaluation of Sperm DNA Fragmentation by TUNEL

An aliquot of each sperm sample was diluted to 10^6 cells per ml in PBS. Gelled aliquots of 0.8% low-melting-point agarose were aliquoted in Eppendorf

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tubes. Each Eppendorf tube was placed in a water bath at 90–100°C for 5 min to fuse the agarose and then maintained in a water bath at 37°C. After a 5-min incubation for temperature equilibration at 37°C, 60 μl of the diluted semen sample were added to the Eppendorf tube and mixed with the fused agarose. Twenty microliters of the semen-agarose mix were pipetted onto an agarose-precoated slide and then covered with a 22- × 22-mm coverslip. The slide was placed on a cold plate in the refrigerator at 4°C for 5 min to allow the agarose to produce a microgel with the sperm cells trapped within. Three different microgels, each corresponding to a sample from a different treatment, were simultaneously prepared and processed on the same slide. Fragmented DNA was nick-end-labeled with fluorescein isothiocyanate-conjugated dUTP using a terminal transferase (In situ Cell Death Detection Kit; Roche Molecular Biochemicals) for 1 h at 37°C in the dark following the vendor's instructions. Positive controls were incubated in DNase I at 50 μg/ml for 20 min at 37°C and washed in PBS before TUNEL. Negative controls were incubated in fluorescein-conjugated dUTP in the absence of enzyme terminal transferase. After TUNEL, sperm cells were washed in PBS and treated with RNase (50 μg/ml) for 1 h at room temperature. Subsequently, the sperm cells were washed in PBS and nuclear stained by a 10-min incubation in PBS containing 20 μg/ml of propidium iodine. In the end of this incubation period, sperm cells were washed again in PBS, mounted on a glass slide with Vectashield (Vector), and examined under a fluorescence microscope. A total of 400 cells/sample were randomly analyzed. The proportion of sperm cells with fragmented DNA (labeled with an intense green nuclear fluorescence) was referred to as TUNEL (%).

Evaluation of Sperm DNA Fragmentation by Comet Assay

The DNA damage of spermatozoa was assessed by single-cell gel electrophoresis (comet assay). Analysis of the shape and length of "comet" tail, just like the DNA content in the tail, gives an assessment of DNA damage. Sperm suspension (30 μl) was diluted in low-melting-point agarose (70 μl, 1% w/v; LMagarose). A 100-μl mixture of sperm-agarose was immediately pipetted onto agarose-coated slides (1% w/v normal-melting-point agarose). Samples were immersed in ice-cold lysing solution (Trevigen) supplemented with 40 mM dithiotreitol and proteinase K (200 μg/ml). Incubation was performed during 1 h at 37°C. After this step, slides were rinsed in distilled water three times (5 min each time) and incubated in electrophoresis neutral buffer (Tris-borate-EDTA, pH 8) for 20 min. Electrophoresis was then performed at 25 V and 300 mA for 7 min. Following electrophoresis, the slides were neutralized with Tris-HCl buffer (pH 7.4) for 5 min and rinsed in distilled water. Samples were stained with SYBR Green (Trevigen) and analyzed under an epifluorescence microscope (Optiphot-2; Nikon). Comets were analyzed using specialized SCG analysis software (CometScore, freeware version; TriTek Corp). Tail length (μm), tail DNA (%), and tail moment were recorded for 100 cells/animal. The parameter that allowed us to describe extension of DNA damage was the tail moment, defined in this software as the product of the tail length and the fraction of DNA in the tail.

Embryo Gene Transcription Analysis

The quantitative RT-PCR methodology used the present study has been described elsewhere [29]. Briefly, poly(adenylate) RNA was extracted from four to five pools of 10 embryos using Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) following manufacturer's instructions. After reverse transcription, the quantification of all gene transcripts was carried out by real-time quantitative RT-PCR. Experiments were conducted to contrast relative levels of each transcript and mouse histone *H2afz* in every sample. Genes analyzed were two retrotransposons (intracisternal-A particle [*Iap*] and murine endogenous retrovirus-L [*Erv4*]), an X-linked gene (hypoxanthine-guanine phosphoribosyltransferase [*Hprt*]), and six imprinting genes (CD 81 antigen [*Cd81*]; solute carrier family 38, member 4 [*Slc38a4*]; insulin-like growth factor 2 [*Igf2*], *H19*; mesoderm specific transcript/paternally expressed gene 1 [*Mest*], and the growth factor receptor-bound protein 10/maternally expressed gene 1 [*Meig1*]) [29]. The PCR quantification was performed using a Rotorgene 2000 Real Time Cycler (Corbett Research) and SYBR Green (Molecular Probes) as a double-stranded DNA-specific fluorescent dye. The method used for quantification of expression was the relative standard curve method [29]. Experiments were conducted to contrast relative levels of each transcript and mouse histone *H2afz* in every sample.

Quantification of Telomere Length

Average telomere length was measured from mouse sperm DNA using a real-time quantitative PCR method described previously [30]. Each of the epididymal sperm samples collected from 10 male mice (five CD1 and five B6D2F1) was divided in two aliquots: One was kept fresh, and the second was frozen without cryoprotectant directly in liquid nitrogen. Fresh and frozen-thawed samples were subjected to an osmotic stress by adding three volumes of H_2O, and then samples were centrifuged for 10 min at 9000 × *g* to eliminate the pellet of spermatozoa. The supernatants were used for quantification of telomere length by real-time PCR performed a minimum of three times for each sample. The assay measures an average telomere length ratio by quantifying telomeric DNA with specially designed primer sequences and then dividing that amount by the quantity of a single-copy gene (*Rplp0*). The average of these ratios was reported as the average telomere length ratio. The primers used for RT-PCR are listed in Supplemental Table 1 (available online at www.biolreprod.org).

Embryo Epigenetic Analysis by 5-Methylcytosine Immunodetection and Methylation-Specific PCR by Bisulfite Analysis

Three hours after ICSI or IVF, five fertilized oocytes were processed every hour for 5-methylcytosine immunodetection. Fertilized oocytes were washed in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeated with 0.2% Triton X-100 in PBS for 15 min at room temperature. Subsequently, oocytes were treated with a 2 M HCl solution at room temperature for 30 min and later neutralized for 10 min with 100 mM Tris-HCl buffer (pH 8.5). After several washes with 0.05% Tween 20, embryos were placed for 1 h in a blocking solution containing 2% BSA/0.05% PBS-Tween 20. Methylated DNA was visualized with a mouse monoclonal antibody against 5-methylcytosine (Calbiochem NA81). Samples were incubated with this antibody at 37°C for 1 h (1:100 dilution in PBS-2% BSA) and washed with 0.05% PBS-Tween 20 for 30 min. Subsequently, samples were incubated for 1 h at room temperature with a fluorescein isothiocyanate- or sulfonated indocyanine dye three-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). After several washes in 0.05% PBS-Tween 20, samples were chromatin-labeled for 30 min with 20 μg/ml of propidium iodine, submitted to ribonuclease A treatment (1 mg/ml at 37°C for 1 h), and washed again with 0.05% PBS-Tween 20. Samples were mounted in 50% glycerol in PBS. Image analysis was carried out by measuring the level of fluorescence under a Nikon epifluorescence microscope (Optiphot-3). Images were recorded digitally with a high-resolution camera and were processed and analyzed using the Adobe Photoshop plug-in Image Processing Tool Kit 5.0 (Reindeer Games).

For the methylation-specific PCR by bisulfite analysis, the isolated DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research). Bisulfite-modified DNA was amplified by PCR. The methylated status of *Iap* long-terminal repeats (LTRs; GenBank accession no. M17551) was examined using the following primers: IAP-F1, 5′-TTGATAGTTGTGTTT-TAAGTGGTAAATAAA; IAP-R1, 5′-CAAAAAAAACACCACAAAC-CAAAAT; IAP-F2, 5′-TTGTGTTTTAAGTGGTAAATAAATAATTTG; and IAP-R2, 5′-AAAACACCACAAACCAAAATCTTCTAC. The methylated status of *Erv4* LTRs (GenBank accession no. Y12713) was examined using the following primers: RVL-F1, 5′-GTTATTATGTGATTTGAATTA; RVL-R1, 5′-ACATACAAAACCATCAATAAAC; RVL-F2, 5′-TTTATTAT-GAGTTGGGTAT; and RVL-R2, 5′-ATAAACCAAACTCTAATCTTC. The methylated status of *H19* (GenBank accession no. U19619) was examined using the following primers: H19-F1, 5′-GAGTATTTAGGAGGTATAAGAATT; H19-R1, 5′-ATCAAAAACTAACATAAACCCCT; H19-F2, 5′-GTAAGGA-GATTATGTTTATTTTTGG; and H19-R2, 5′-CCTCATTAATCCCATAAC-TAT. The PCR conditions for the three markers were as follows: first PCR (30 cycles), F1/R1; second PCR (30 cycles), F2/R2. Temperature conditions for the first PCR were as follows: 94°C for 3 min, 94°C for 20 sec, and 53°C for 30 sec. Temperature conditions for the second PCR were as follows: 94°C for 3 min, 94°C for 20 sec, 60°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. The PCR products were purified from agarose gels using ELU-QUIK DNA purification kit (Schleicher & Schuell) and transformed into XL1 *Escherichia coli* cells. Positive clones were verified by restriction enzyme analysis, and their products were sequenced using standard methods.

Behavioral Studies

Particular aspects of the behavior of adult CD1 and B6D2f1 mice generated by ICSI with frozen-thawed sperm and controls were evaluated with the open-field test, the elevated plus maze paradigm, and the free-choice exploration paradigm in Y maze as described previously [29] and in Supplemental Table 2 (available online at www.biolreprod.org). They were measured at both 3–4 and 13–15 mo of age. Particular attention was taken to analyze habituation responses, as previously described. [29]. All tests were performed by trained technicians in blind trials. The devices used for all behavioral studies were carefully cleaned with a diluted acetic acid solution between animals to prevent olfactory cues.

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TABLE 1. Percentage of fragmentation and mouse embryo development obtained with fresh and frozen-thawed sperm for the CD1 and B6D2F1 strains.

Strain cross	Percentage of TUNEL+ (5 replicates)*	Comet tail length (μM)*	Surviving oocytes/ injected (%)	Two-cell embryos transferred (%)	Live pups (% from transferred)	Surviving after 25 weeks (%)
CD1 × CD1 (frozen)	24 ± 3[a]	50.73 ± 9[a]	501/772 (69)[a]	432 (86)	54 (13)[a]	46 (85)[a]
CD1 × CD1 (fresh)	7 ± 4[bc]	33.54 ± 8[b]	101/111 (91)[b]	72 (71)	19 (26)[b]	19 (100)[b]
B6D2 × B6D2 (frozen)	16 ± 6[ab]	48.03 ± 7[a]	327/396 (83)[ab]	274 (84)	52 (19)[ab]	47 (90)[ab]
B6D2 × B6D2 (fresh)	4 ± 4[c]	27.9 ± 8[b]	73/104 (70)[a]	60 (82)	16 (27)[b]	16 (100)[b]

* Values are mean ± SEM.
[a,b,c] Different superscript letters indicate significant differences within a column ($P < 0.05$), One-way Anova.

Postnatal Growth and Histological Analysis of Aged Animals

After weaning, mice were weighed weekly until 10 wk of age and then biweekly thereafter. Twenty months after birth, some viscera, including liver, lung, heart, kidney, spleen, and testes, were excised, and body/organ weights were measured. In addition, histopathological analysis was done. Samples (of liver, lung, heart, kidney, and spleen) were fixed in 10% buffered formalin and embedded in paraffin wax. Sections (thickness, 4 μm) were stained with hematoxylin-eosin and Congo red and then examined under the microscope. Stained sections of each tissue were reviewed by an experienced pathologist. Systolic blood pressure was determined at 12 mo of age by tail-cuff plethysmography using an NIPREM model 546 blood pressure monitor (Cibertec) after 1 h of acclimatization using data-acquisition software and hardware (Powerlab; AD Instruments) following the manufacturer's instructions. Recordings (n = 3 on average) were blind to animal code.

Data Analysis

The mean telomere lengths were compared using an independent samples *t*-test. Data regarding postnatal growth and organ weight were analyzed using the SigmaStat software package (Jandel Scientific). One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using the Student-Newman-Keuls method) was used for the analysis of differences in weight. Differences of $P < 0.05$ were considered to be significant. Significance of the effects of behavioral analysis was assessed by a one-way ANOVA or a multivariate ANOVA with a Bonferroni or Newman-Keul test for post-hoc analysis. Factor analysis included culture conditions, sex, novelty/familiarity, and time points. The Student *t*-test was used for comparisons between two groups. Nonparametric data were analyzed by chi-square test. Behavioral analysis was processed by using the SPSS v.12 program.

RESULTS

DNA Fragmentation According to Sperm Treatment and Embryo Development

Both TUNEL and comet assay of CD1 and B6D2F1 mouse sperm indicated a significant difference between fresh sperm cells with low level of DNA fragmentation (DNA-intact sperm) and frozen-thawed sperm without cryoprotector, which showed high levels of DNA fragmentation (DFS) (Table 1). When sperm cells were incubated for more than 1 h at room temperature, we observed a significant increase in the percentage of DNA fragmentation in fresh, but not in frozen-thawed, sperm (in CD1 mice, the percentage of apoptosis increased from 8% to 17% in fresh sperm cells and from 24% to 27% in frozen sperm cells). To reduce the effect that released endogenous nucleases might have in the DNA fragmentation of fresh sperm cells, ICSI experiments with fresh sperm were always done during the first hour after sperm collection [31]. No differences in developmental rate were found between CD1 and B6D2F1 mice. It was interesting to observe that fresh and frozen sperm cells displayed significant differences in the average telomere length ($P < 0.002$), indicating that more telomere DNA sequences are broken by the freezing-thawing procedure.

In our ICSI experiments with CD1 mice, the percentage of surviving oocytes after injection was lower when fresh sperm cells were used compared to that when frozen sperm cells were used (69% vs. 91%, respectively) (Table 1). No differences, however, were found for B6D2F1 mice (83% vs. 70%, respectively). The proportion of transferred embryos that gave rise to live offspring was significantly higher when fertilization was done with fresh sperm cells (26% and 27% for CD1 and B6D2F1 mice, respectively) than when fertilization was done with frozen-thawed sperm cells (13% and 19% for CD1 and B6D2F1 mice, respectively). These results were, in some way, anticipated by the pregnancy rates observed in both experimental groups. Whereas 100% of the female recipients used for the embryos fertilized with fresh sperm cells got pregnant, this proportion was just 54% and 85% when transferred embryos were fertilized with frozen-thawed sperm cells in CD1 and B6D2F1 mice, respectively.

Gene Expression and Methylation Patterns in ICSI-Generated Embryos

We analyzed by real-time PCR if our ICSI protocol with DFS produced alteration in the mRNA expression of epigenetic regulated genes (two retrotransposons: *Iap* and *Erv4*; an X-linked gene: *Hprt*; and six imprinting genes: *Cd81*, *Slc38a4*, *Igf2*, *H19*, *Mest*, and *Meig1*) in mouse embryos at the blastocyst stage that were produced by ICSI with DFS or were fertilized in vivo and cultured in the same culture media as ICSI embryos. The expression was significantly different in retrotransposons, in the X-linked gene, and in three of the imprinting genes (*Cd81*, *Scl38a4*, and *H19*), indicating an ICSI-dependent epigenetic defect at the blastocyst stage (Fig. 1). Culture in KSOM for 4 days was used as a control and produced significant modification of expression in three of the genes analyzed, indicating that some of the alterations in gene expression observed in blastocysts produced by ICSI were a consequence of embryo culture.

To provide a detailed characterization of the methylation dynamics that occur after fertilization, precise timing of progression to the pronucleus stage was made possible by IVF. We used IVF instead of in vivo fertilization because IVF allowed us to determinate the moment of fertilization with more precision. The average time for completed demethylation in the paternal genome after ICSI was 8 h. Instead, the average time after IVF was 6 h, indicating that ICSI produced a delayed active demethylation of the paternal pronucleus in the zygote generated by ICSI. When 5-methylcytosine fluorescence was quantified in 2-cell, 4-cell, and morula, inner cell mass, and trophectoderm in the blastocyst, no differences were observed between embryos produced by ICSI with DFS and those produced by IVF. Small differences may not have been detected, however, because these may be masked by differences in access of antibody to the interphase nuclei.

To analyze if ICSI produced an effect at the blastocyst stage on methylation of *H19* imprinting gene and *Iap* or *Erv4* retrotransposons, we performed methylation-specific PCR by bisulfite analysis (Supplemental Figure 1 available online at

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www.biolreprod.org). We discriminated the effect of ICSI by analysis of the effect of in vitro culture on embryos fertilized in vivo or by ICSI. In vitro culture did not modify the methylation of *H19* or retrotransposon genes at the blastocyst stage; however, embryos produced by ICSI had a reduction in the methylation of *H19* and in the methylation of the two retrotransposons (Supplemental Figure 1).

Postnatal Growth and Histology of Aged Mice

Body weight data for female and male CD1 and B6D2F1 mice produced by ICSI with DFS (12 female and 15 male CD1 mice; 10 female and 31 male B6D2 mice), fresh sperm (20 female and 15 male CD1 mice), and in vivo fertilization (13 female and 12 male CD1 mice; 12 female and 10 male B6D2F1 mice) were collected weekly from Weeks 10–39. Female body weight data are presented in Figure 2. Weights were not significantly different between males for both CD1 and B6D2F1 mice, whereas from the 16th week onward, weights of CD1 female mice produced with DFS or fresh sperm were higher than those of in vivo-produced animals (Fig. 2a). The same increase happened in B6D2F1 female mice starting on Week 15 and onward (Fig. 2b). Also, for both strains of mice, a higher variability was found in animals obtained from ICSI with DFS (Fig. 2, c and d, example at 26 wk of age). No differences in systolic blood pressure were observed (Supplemental Table 3 available online at www.biolreprod.org).

The postmortem histology of selected organs (liver, heart, spleen, kidney, lungs, and testicles) showed some abnormalities when frozen sperm, fresh sperm, or both were used (Supporting Table 3). Liver and lungs of females produced by ICSI were bigger than those of in vivo-produced animals. We also found increased incidence of pneumonia (40% of ICSI cases vs. 13% of control). Through regular observation of a large group of ICSI-generated B6D2F1 mice, we also found that 20% of the mice produced with DFS died between 3 and 5 mo of age without any external symptoms (Table 1). When those animals were biopsied, some of them showed hemothorax, whereas in others, pulmonary hemorrhage was evident, suggesting fatal hemorrhagic pneumonia. Moreover, ~25% of surviving B6D2F1 mice acquired an "aged" appearance starting at ~3 mo of age (Fig. 3, g and h). The ICSI with

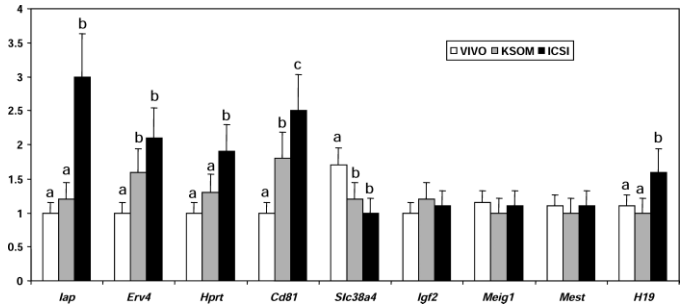

FIG. 1. Quantification by real-time PCR of unregulated mRNAs in ICSI blastocysts produced by oocyte fertilization with frozen sperm or in vivo and cultured 4 days in vitro in the same conditions. Each lane represents the results of three independent experiments expressed relative to those obtained for the group in which expression was the lowest. Bars with different lowercase letters within each gene transcript differ significantly ($P < 0.05$).

DFS produced B6D2F1 mice with premature skin symptoms of aging; mice had brittle hair, earlier and more frequent hair depigmentation, and reduced fitness. Skeletal abnormalities like curvature of the spinal column (lordokyphosis) were detectable between 12 and 14 mo of age in these animals. No aging phenotype was found in the animals generated by ICSI with fresh sperm.

With ICSI using DFS, but not using fresh sperm, 33% of CD1 females developed tumors (Fig. 3, a–d), and 15% of these females that developed tumors died between 3 and 5 mo of age. These tumors could be macroscopically observed from Week 20 onward. Tumor histological analysis showed that 50% were benign fibrous histiocytomas (Fig. 3, a and b), whereas the other half were malignant lymphoid and myeloproliferative neoplasias (mesenchymal tumors of extraordinary metastatic ability). Mesenchymal tumors are associated with early developmental problems, such as misregulation of mesenchymal cell proliferation. These tumors are not a consequence of aging, and they are very unusual in laboratory mice. Both histology and immunophenotype (data not shown) analysis of the hematological tumors showed diseases compatible with the myeloproliferative syndrome. Tumor cell infiltrations of the myeloproliferative process in lungs, kidneys, and liver of a

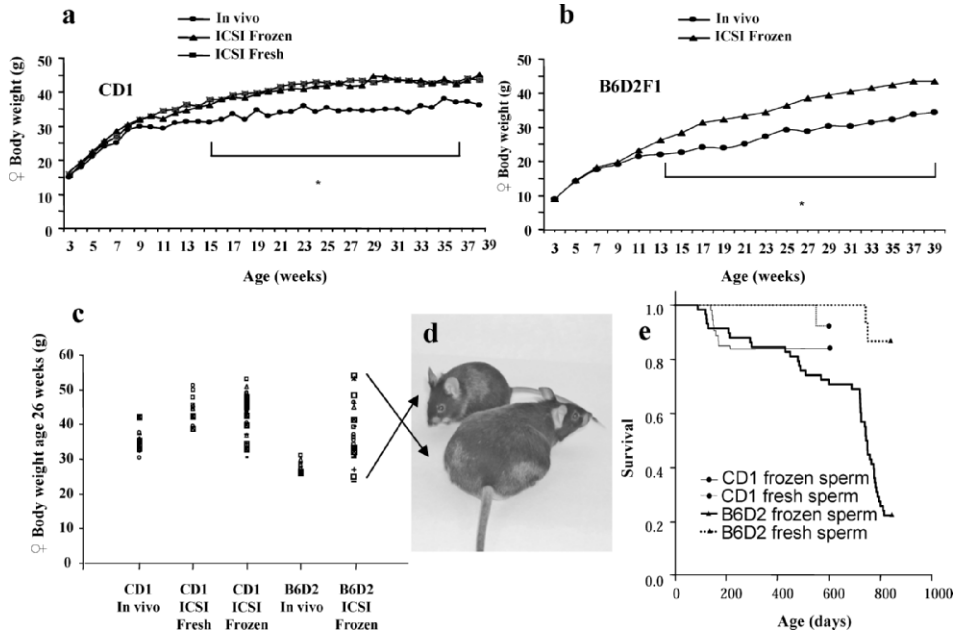

FIG. 2. **a**) Body weights of female CD1 mice produce by ICSI with frozen sperm, ICSI with fresh sperm, and in vivo fertilization. **b**) Weights of female B6D2F1 mice produced by ICSI with frozen sperm or in vivo fertilization. **c**) Individual variability observed in body weight at 26 wk of age. **d**) Example of two B6D2F1 female mice produced with frozen sperm showing the high weight variability. **e**) Survival rate of CD1 and B6D2F1 mice produced by ICSI with fresh or frozen sperm. CD1 mice were killed at 600 days for anatomical and histological analysis. B6D2F1 mice were killed at 840 days.

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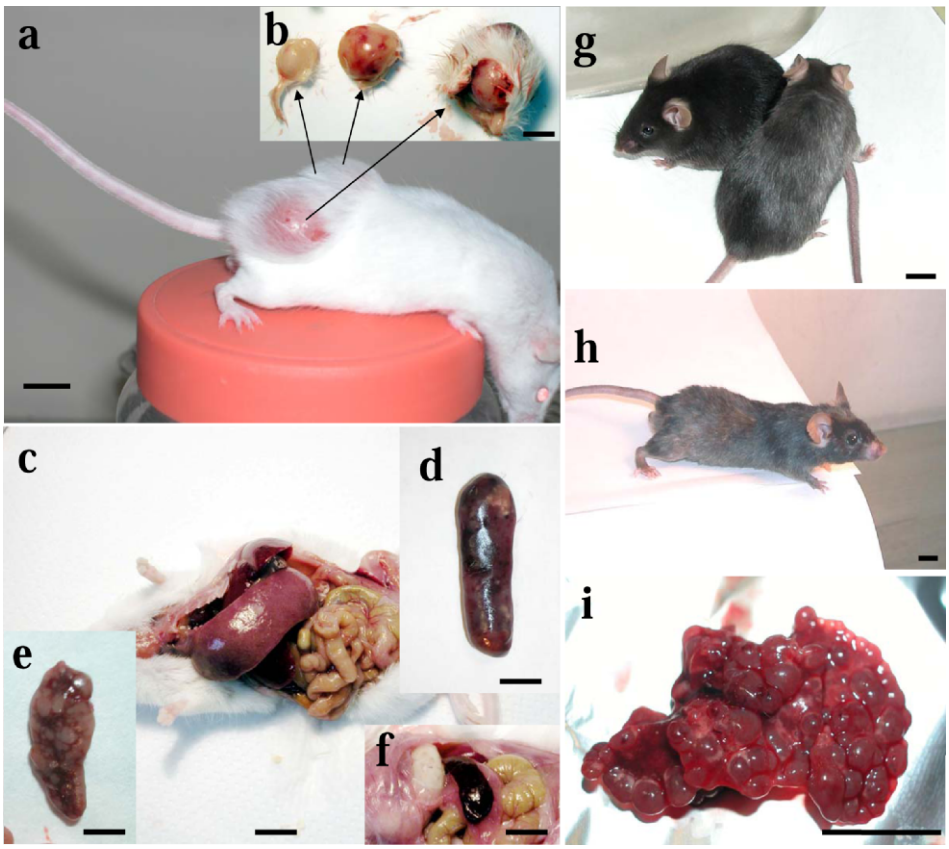

FIG. 3. Some of the ICSI-produced mice developed tumors (**a**–**f** and **i**), and some of them showed premature cutaneous symptoms of aging phenotype (**g** and **h**). Shown are an ICSI female with benign fibrous histiocytomas (**a**) and detail of the tumors (**b**), an ICSI female with a spleen tumor (**c**) and a control spleen of the same age (**f**), two different spleen tumors (**d** and **e**), a lung adenocarcinoma (**i**), a typical example of early hair depigmentation in two males produced by ICSI in comparison to an in vivo produced B6D2F1 mouse at 3 mo of age (**g**), and a 7-mo-old, ICSI-produced B6D2F1 mouse showing loss of hair and depigmentation (**h**). Bar = 1 cm.

female mouse produced by ICSI with DFS can be seen in Figure 4. Also, after 12 mo, we found tumors in B6D2F1 mice (Fig. 3, c–f and i), some of which were mesenchymal but the majority of which were adenocarcinomas and lymphosarcomas of hematopoietic organs. The different genetic backgrounds may explain the observed differences in tumors and aging between CD1 and B6D2F1 embryos.

Behavioral Analysis of Adult Mice Generated by ICSI with DFS

A complete behavioral analysis was performed in male and female CD1 and B6D2F1 mice produced by ICSI with DFS. Data were collected at 12–18 wk or 12 mo of age. Alterations in the phenotype were mainly observed in CD1 mice, with a clear sexual dimorphism. Female CD1 mice produced by ICSI with DFS presented general alterations in behavioral responses at both early and late stages of life. Animals produced by ICSI exhibited reduced exploration on the open arms of the elevated plus maze, suggesting the presence of anxiety-like reactions. They poorly habituated to both the plus maze and the open field (Supplemental Figure 2 available online at www.biolreprod.org). Most of the CD1 females of the ICSI group did not exhibit the aversive reaction observed in control animals at the second exposure to the behavioral test, something that can be related to deficits in implicit memories, as described previously in animals obtained after IVF and embryo culture with serum [29]. Memory deficits were confirmed in female CD1 mice produced by ICSI with DFS using the Y maze novelty recognition paradigm: All the animals recognized the novel arm after a short-term (2-min) intertrial interval, but female CD1 mice of the ICSI group failed to do so after long intertrial tests (30 min) (Supplemental Figure 3 available online at www.biolreprod.org). Finally, at 12 months of age, these CD1 females exhibited a clear hypolocomotion and reduced general activity in the open field under habituation conditions (Fig. 5).

DISCUSSION

Introduced in 1992, ICSI is becoming the most commonly used method for the treatment of male infertility factor. No experimental testing preceded its introduction, however, partly because animal models were thought to be unsuitable and partly because of this method's immediate and overwhelming success [32]. Many concerns exist regarding the use of ICSI in humans, because the technique bypasses the physiological interactions between the oocyte and sperm, the signaling events that occur in the sperm before and during interaction with the oocyte's vestments, and the natural selection of the fertilizing sperm [33]. In addition, ICSI may allow sperm with fragmented DNA, which cannot naturally fertilize in vivo, to fertilize in vitro. The cumulative effect of several insults during fertilization may negatively affect the development potential of ICSI-produced embryos, with perhaps the most obvious being the traumatic injury inflicted to the oocyte by the microinjection pipette. Implantation rates after ICSI-derived embryos continue to be lower than those generated through conventional oocyte insemination, suggesting diminished quality of ICSI-derived embryos [12]. A clear, sex-related growth difference was found in human blastocysts originated by ICSI but not in blastocysts originated from IVF, indicating a stress-associated ICSI artifact and raising the issue of the safety of the ICSI procedure [34, 35]. These differences between ICSI and IVF could be related with oxidative stress and the presence of cumulus cells, because sperm injection could increase the level of reactive oxygen species (ROS) in the oocyte and the antioxidant function of cumulus cells is not present in ICSI (the oocyte is completely denuded of cumulus cells before

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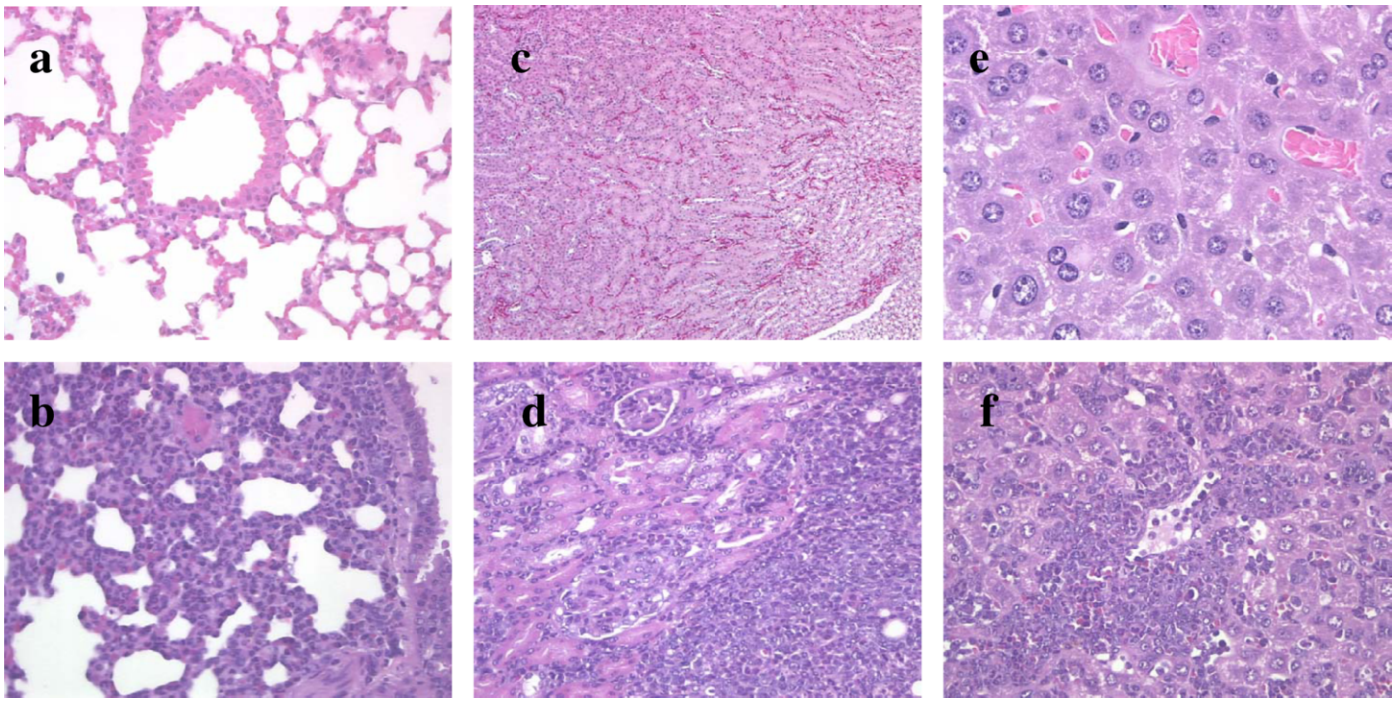

FIG. 4. Myeloproliferative processes with infiltration were observed in ICSI-produced mice. Shown are the lung of a control (**a**) or an ICSI-produced female with a tumor (**b**), a kidney of a control (**c**) or an ICSI-produced female with a tumor (**d**), and a liver of a control (**e**) or an ICSI-produced female with a tumor (**f**). Original magnification ×200.

spermatozoa are injected inside the oocyte) [35]. A mechanism has been proposed by which oxidative stress in the sperm can lead to genetic disease, such as cancer, in the offspring [36]. The present study demonstrates an ICSI-dependent, epigenetic defect in mouse blastocysts produced with DFS. In addition, we have observed that the use of ICSI with DFS may result in a long-term manifestation of a variety of deleterious phenotypes in mice.

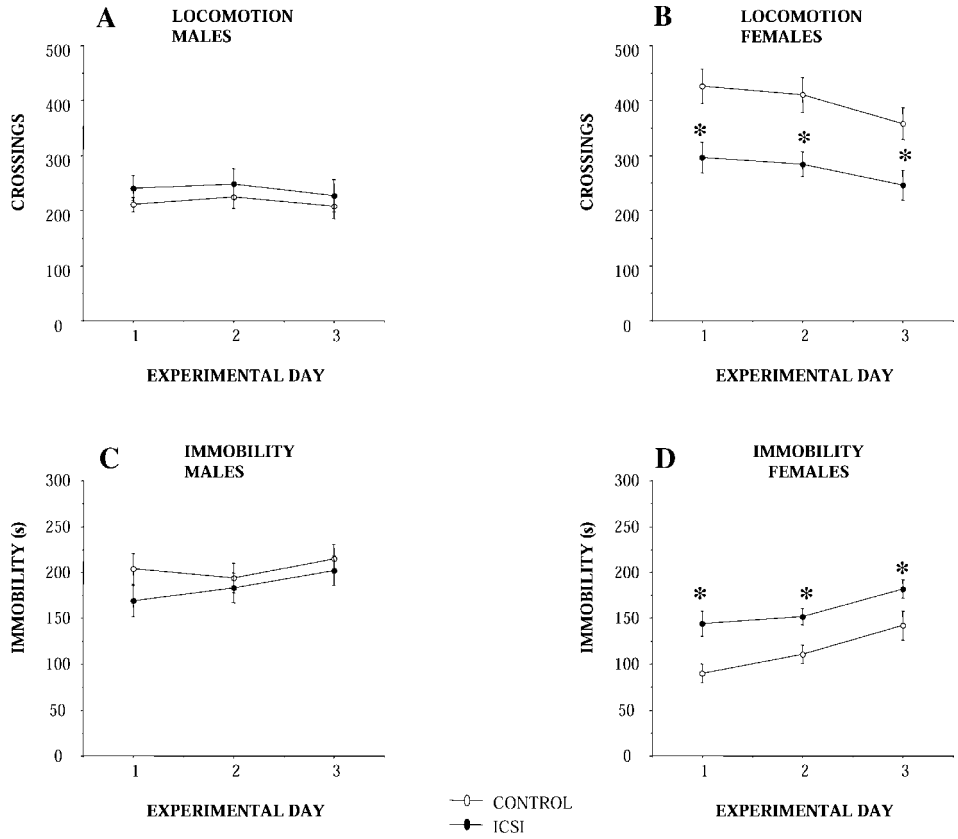

FIG. 5. Performance in the open field of 12-mo-old male and female CD1 mice produced by ICSI with frozen sperm. Animals were exposed in three consecutive days to the open field, and the total number of crossings (**A** and **B**) and time spent immobile (**C** and **D**) were recorded. Data are the mean ± SEM of 8–10 determinations per group. $^{*}P < 0.01$ versus control animals.

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The DNA damage of sperm was measured directly by TUNEL and comet assays, indicating that frozen-thawed sperm without cryoprotectants experienced fragmentation of the DNA. We also observed a loss of telomeres as a consequence of freezing without cryoprotectant. Approximately 15% of the DNA in the sperm nucleus is associated with histones, and some of these histones are associated with the telomeres. The spatial architecture of chromosomal DNA indicates that telomeres forming dimers are positioned in the nuclear periphery and are associated to histones in a nucleosomal structure [37, 38]. The telomere plays a major role in chromosomal localization within the sperm nucleus [38] and may be related to sperm chromatin remodeling and male pronuclear formation. It has been reported in mice that ICSI resulted in less synchronous chromatin remodeling than with IVF [39] and that telomeres are associated with microtubule motors during movement of the male pronucleus [40]. The repeated sequence of telomeres plays a vital role in the protection of the chromosomes, and its length has been proposed to function as a biological clock. We think that freezing mice sperm without cryoprotectant may affect preferentially the nucleosomal regions of the sperm DNA and, thus, produce telomeric DNA losses.

In animal studies, it has been demonstrated that ICSI may result in abnormal sperm head decondensation, delaying the onset of DNA synthesis [41], and that ICSI-induced intracellular calcium oscillations are not equivalent to those initiated by IVF [42]. This may have developmental consequences, because ICSI-generated mouse zygotes cleaved at a slower rate, had lower cell numbers, and had lower hatching rates than in vivo-fertilized embryos [42]. Also, in rabbits, the cell numbers of blastocysts obtained by ICSI was lower than the numbers obtained by in vivo fertilization [43]. In humans, it has been reported that embryos obtained after ICSI have a lower potential to develop into blastocysts compared with embryos obtained after IVF [44]. This probably is because the sperm used for ICSI usually is obtained from subfertile men and has a relative high risk of genetic abnormalities, such as fragmented DNA and chromosomal abnormalities [45], but the ICSI procedure itself seems to contribute to a reduced capacity of blastocyst formation in comparison with conventional IVF, as suggested by studies in which sibling oocytes were subjected to ICSI or IVF using sperm from the same semen samples [46]. Tamashiro et al. [47] found that ICSI carried out with fresh sperm obtained from fertile mice does not produce adverse effects on the growth, fertility, or behavior of mouse offspring; however, those authors did not analyze the effect of ICSI with DFS. Using fresh sperm, the present results agree regarding the growth during the first 7 wk after birth, because even though ICSI-produced animals had higher body weight, differences at this age were not significant. Tamashiro et al. [47], however, did not check the long-term consequence of ICSI and did not report the weight in adult animals, the stage at which we found the significant differences. Even when fresh sperm cells were used, ICSI may affect the quality of the oocyte, somehow reducing its ability to repair the fragmented DNA. In agreement with the present results, it has been reported that in vitro culture affects the expression of DNA repair genes in blastocysts, impairing their ability to repair DNA damage [48].

Many recent observations suggest a link between ART and epigenetic errors [49]. The preimplantation period is marked by many epigenetic changes; these changes affect processes such as gene expression, metabolism, development, and differentiation (e.g., large offspring syndrome in ruminants) [50]. We have found that ICSI produces blastocysts with alterations in the methylation pattern of some imprinting genes, genes linked to the X-chromosome, and retrotransposons. In agreement with the present results, it also has been reported that in vitro culture may affect the expression of imprinting genes [29, 51–53]. In the early embryo, X-chromosome inactivation is a developmentally regulated process that causes one of the two X chromosomes in normal female mammals to become transcriptionally silenced, thus equalizing the expression of X-linked genes between sexes. Such dosage compensation depends on dynamic genetic and epigenetic events occurring very early in development. It is possible that in addition to the decompensation expression that we have found at the *Hprt* gene, the expression of other X-linked genes may be altered. It has been reported that skewed inactivation of one of the two X chromosomes is significantly increased in women with recurrent spontaneous abortions [54]. Moreover, we have found that the expression of other epigenetically controlled genes, like the *Iap* and *Erv4* retrotransposons, is also altered. Retrotransposable elements are repetitive mobile elements that occupy more than 40% of mouse genomes. The two transposons analyzed in the present study belong to the vertebrate-specific endogenous viruses that appear to be active in the mammalian genome, *Iap* and *Erv4*. They are flanked by 5′ and 3′ LTRs that regulate transcription of the internal genes. We selected these elements because several studies concluded that they are still active elements and that their expression is genetically and epigenetically regulated during preimplantation [55–57]. The finding that ICSI can affect the methylation of imprinting, X-linked, and retrotransposon genes provides a model system for the linkage between preimplantation and the long-term effect of ICSI. Aberrant genome DNA methylation in early embryos is an important mechanism contributing to the high incidence of developmental failure in mammals [58]. Epigenetic abnormalities of the mouse embryonic genome also have been associated with microinjection of round spermatids and with somatic nuclear transfer [59]. Moreover, we cannot discard epigenetic defects in other genes, because aging has been reported to result in hypermethylation of ribosomal DNA in the sperm of rats [60].

Male mice generated by ICSI displayed only subtle changes in the pattern of exploration in the open field and the elevated plus maze, suggesting a higher emotional reactivity compared with control mice. Female mice generated by ICSI, however, showed a marked phenotype, with memory deficits, hypolocomotion, anxiety, and increased adipose mass. This phenotype resembles that described for female mice generated by IVF and embryo culture in the presence of serum [29]. The most remarkable finding is the obesity displayed by female mice. Obesity is an overweight state of sufficient magnitude to produce adverse health consequences, such as type II diabetes and hypertension. We do not know why only females have a higher body weight; however, there are many differences between sexes that can affect obesity, such as sex hormones [61]. Also, the association between body mass index and lipids/lipoproteins is stronger in females than in males [62], and leptin-deficient obesity is affected by female sex [63]. Hypolocomotion may contribute to the obese phenotype; however, it also might be a consequence of the phenotypic alterations induced by ICSI. Alteration in X-linked imprinting may be related with this phenotype as well [64]. The finding that mice generated by culture in fetal calf serum or by micromanipulation (nuclear transfer) are susceptible to behavioral alterations and obesity [29, 58, 65] is consistent with a supposed phenotypic influence by preimplantation in vitro procedures. Tamashiro et al. [58] reported that because the obese phenotype was not transmitted to offspring of the cloned

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mice produced by natural mating, it probably resulted from epigenetic errors in donor cells or from errors that arose because of inadequate nuclear remodeling. Moreover, some of the changes described in cloned mice were also present in a second control group of mice, from which zygotes were recovered and cultured in the same system as used for the cloned embryos before transfer of a few embryos and, ultimately, delivery by cesarean section. In the present study, we confirm that the well-documented perturbations in development of sheep and cattle that result from manipulating embryos are also evident in a different order of eutherian mammals (mice). Long-term postnatal survivors might have subtle epigenetic defects that are below the threshold that threatens viability but can produce long-term deleterious effects. In addition, ICSI may increase the ROS levels. Increasing evidence has indicated a role for ROS during preimplantation in the development of diabetes-induced malformations in rodent models [66, 67]. We do not know if this finding regarding weight could appear in human offspring produced by ICSI, but a medical follow-up study of 5-yr-old children has reported that more ICSI-produced children require dietary therapy [12].

Malformations in some viscera have been found in many mammalian animals produced by nuclear transfer [68] and also in some in vitro-produced animals [29]. In both reports, the authors observed that oversized calves derived from in vitro-produced embryos had abnormally large hearts compared with their control counterparts when slaughtered at the same weight and 1 yr of age, suggesting that at least for the heart, some effects of aberrant prenatal development can persist into adulthood. These findings may explain the persistence up to adulthood of a large liver in mice produced with ICSI. In addition, the postmortem anatomical and histological study indicated that ICSI with DFS produced a significant increase in tumors. Careful examination may reveal that the welfare of these animals is compromised more severely than that of control animals.

Because both DFS and fresh sperm produce female mice with higher body weight and organomegaly, we believe that the cause of this phenotype is not an attribute related only to DNA but is also a consequence of epigenetic alteration. During the period immediately following fertilization, extensive remodeling of the oocyte- and sperm-derived genomes occurs [69]. Methylation of DNA is one of the principal factors that control gene expression and epigenetic alteration during early development. The first epigenetic event is the active demethylation by the oocyte of the male. In contrast, the female pronucleus is passively demethylated. This zygotic demethylation may be necessary to reprogram the sperm genome for somatic development [70]. In the present study, we have observed that ICSI with DFS has a delay of 2 h in active demethylation. Also, in rabbit embryos, it has been reported that ICSI with intact DNA can induce aberrant DNA methylation [71]. Those authors reported that ICSI, by an unknown mechanism, may induce changes in the structure of maternal chromosomes, rendering them accessible to demethylation activity. Other events, such as sperm head decondensation, shedding of the acrosome and perinuclear theca, and onset of DNA synthesis, also appear to be delayed in ICSI fertilization in human and primates [41] and may further compromise the development of ICSI-generated embryos. An association between ART and congenital malformation syndromes with imprinting defects and rare tumors, such as retinoblastoma, has been documented [72]. Also, epigenetic differences have been reported between male and female embryos that may explain why we have found obesity only in female mice [73–76].

Our freezing-thawing protocol produces ~30% DFS. Eighty percent of the zygotes fertilized by ICSI with this sperm, however, develop to the 2-cell stage, and from them, more than 90% develop to morulae [26]. Using a similar sperm freezing-thawing protocol without cryoprotectant, it has been reported that 40% of mouse oocytes fertilized by ICSI have abnormal paternal chromosomes. Despite this, they can divide to the 2-cell stage, and from these, 77% can develop to morulae [77]. In agreement with the present results, it has been reported in B6D2F1 mice that chromosomal aberrations increased in ICSI-fertilized oocytes when spermatozoa were frozen without any cryoprotection [28]. Those authors also found 8% abnormal karyotypes when they used fresh sperm, and they suggested that the ICSI procedure per se, independent of freezing, has some adverse effect on chromosome stability. This small proportion of DFS that we have found in the fresh B6D2F1 sperm could explain these adverse effects of fresh sperm after ICSI. Freezing-thawing pretreatments caused significant DFS; however, many of the embryos produced with this DFS could still develop to morulae. Several studies have attempted to establish a correlation between sperm DNA integrity, cleavage rates, and embryo quality. The mature sperm lacks DNA repair capacity and appears to be highly vulnerable to radiation and some chemicals or their metabolites [78]; therefore, DNA damage could accumulate as sperm cells age and/or are in contact with potential exogenous damaging agents as a result of oxidative stress [25]. Whether DNA-damaged spermatozoa can impair the process of embryo development remains unclear. The DNA repair activity of the penetrated oocyte should correct for the background damage of sperm DNA used for ICSI. We do not know the limit of the oocyte, however, or whether in some cases these DNA breaks are not corrected completely; thus, these damages may have a long-term effect in the offspring. Damage to sperm DNA may be linked to an increase in early embryo death, but a greater miscarriage rate has been reported for ICSI, reflecting the use of genomically compromised spermatozoa [79]. Whereas embryo quality based on morphological criteria appeared to be unaffected by DNA fragmentation, DNA damage has been correlated with pregnancy outcome [9, 25]. Using sperm chromatin structure assay, it has been determined that men with >30% DNA fragmentation are at greater risk for low blastocyst rate and failure to initiate an ongoing pregnancy [80]. Differences are evident between species, however, because we have produced mice with sperm that have a 24% DFS subpopulatio. Also, in humans, using TUNEL, the absence of pregnancy has been reported when the sperm subpopulation showing DNA fragmentation was >20% [9], and the absence of birth has been reported when the sperm subpopulation with DNA fragmentation was >15% [81].

We believe that the increased incidence of tumors observed is related to the DNA fragmentation of the sperm used, because ICSI with fresh sperm did not produce this effect. The ICSI with DFS can also be the cause of premature aging and of the aging-associated tumors that we have found. Aging and cancer are two sides of the same coin. In one case, cells stop dividing, and in the other, cells cannot stop dividing. It has been reported that DNA damage is thought to contribute to aging [82]; however, hypomethylation also has been related with premature aging in mice [18]. In humans, it has been reported that ICSI may produce some epigenetic problems [83]. Alterations in DNA methylation are regarded as epigenetic changes, and alterations in DNA methylation might be pivotal in the development of a wide variety of tumors [84]. Epigenetic

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changes in the genome may induce health problems, such as childhood cancer or neurodevelopmental complications [85].

The safety of the ICSI procedure has been questioned [33], and the findings from the present study may give further reason for concern. In many cases of severe male factor infertility, a significant proportion of spermatozoa injected into oocytes may contain fragmented DNA. A real risk exists of accidentally selecting genetically abnormal sperm that may appear to be motile and morphologically normal. It is unlikely that ICSI helps in cases when fertilization failure is associated with sperm nuclear (chromatin or DNA) alterations [86]. The use of ICSI in case of DNA damage may result in long-term consequences regarding health and behavior of the offspring. It has been proposed that sperm DNA damage is promutagenic [4] and can give rise to mutations after fertilization (as the oocyte attempts to repair DNA damage before the initiation of the first cleavage). Mutations occurring at this point will be fixed in the germline and may be responsible for the induction of pathology, such as infertility [4], childhood cancer [4, 48, 87], and imprinting diseases [49, 88]. According to our data, DFS might cause not only an impaired embryonic development and early embryonic death [89, 90] but also an increased risk of childhood cancer in the offspring. In agreement with the present results, it has been reported that paternal smoking and oxidation of human spermatozoa, which produce DFS, are related with cancer in the offspring [4, 48]. Furthermore, the possible promutagenic effects of smoking on DNA integrity in the male germline have been reinforced by another study indicating that 15% of all childhood cancers are directly attributable to paternal smoking [91]. Perhaps problems with DNA repair will not be passed along to children conceived through ICSI, because such problems may simply result in miscarriages (defective DNA repair is also commonly found in spontaneously aborted embryos). Embryos with small DNA repair defects may survive to term, however, and according to the present results, they could theoretically then have an increased risk of defect later in life, such as early aging, tumor formation, or behavior alterations.

The results in our mice model suggest, first, that a risk is linked to the ICSI procedure itself, capable of producing alterations in the early embryo and long-term consequences, such as obesity and organomegaly, and, second, that a risk is linked to the use of ICSI with DFS, capable of producing genetic and epigenetic changes during preimplantation that may lead to altered fetal/placental development and, as a consequence, offspring with aberrant growth, behavior, early aging, and tumors. Because sperm with damaged DNA can fertilize oocytes during ICSI and no system exists to select spermatozoa with strand breaks, an evaluation of sperm DNA fragmentation level is crucial to prevent or reduce the risk of inducing genetic alterations in the offspring. Further studies in humans are needed to determine the threshold of sperm DNA fragmentation after which ICSI should not be recommended.

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