Molecular reproduction and development. (2017.7) 84(7):638-648.

Preservation of chromosomal integrity in murine spermatozoa derived from gonocytes and spermatogonial stem cells surviving prenatal and postnatal exposure to γ-rays in mice

Hiroyuki Watanabe, Atsushi Kohda, Jun-ichiro Komura, Hiroyuki Tateno

Preservation of chromosomal integrity in spermatozoa derived from gonocytes and spermatogonial stem cells surviving prenatal and postnatal exposure to γ-rays in mice

Hiroyuki WATANABE[1], Atsushi KOHDA[2], Jun-ichiro KOMURA[2], Hiroyuki TATENO[1,*]

[1]Department of Biological Sciences, Asahikawa Medical University, Asahikawa 078-8510, Hokkaido, Japan

[2]Department of Radiobiology, Institute for Environmental Sciences, Rokkasho, Kamikita, Aomori 039-3213, Japan

***Corresponding author**: H. Tateno, Ph.D.

Department of Biological Sciences, Asahikawa Medical University,

2-1-1-1 Midorigaoka-higashi, Asahikawa 078-8510, Hokkaido, Japan

E-mail: htateno@asahikawa-med.ac.jp

Fax: +81-166-68-2783

Running Head: Radiation toxicity to developing testes

Keywords: radiation, cell death, spermatogenesis, chromosome aberrations, spermatozoa

Abbreviations: BSA, bovine serum albumin; DSB, double-strand break; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; OR, odds ratio; PCC, premature chromosome condensation; pc, post-coitus; pp, post-partum

Grant sponsors: The Japan Society for the Promotion of Science; Grant numbers: JSPS, KAKENHI, 25870027 and 16K16191, and The Radiation Effects Association

Abstract

Prenatal and postnatal male mice were acutely (659−690 mGy/min) and continuously (0.303 mGy/min) exposed to 2 Gy γ-rays to evaluate spermatogenetic potential and chromosome damage in germ cells at adulthood. Acute irradiation on days 15.5, 16.5, and 17.5 post-coitus (pc) affected testicular development as the result of massive quiescent gonocyte loss. In these testes, the majority of the seminiferous tubules were devoid of germ cells. Acute irradiation on days 18.5 and 19.5 pc had less effect on testicular development and spermatogenesis, even though germ cells on these days were quiescent gonocytes. Adverse effects on testicular development and spermatogenesis were observed following continuous irradiation between 14.5 and 19.5 days pc. Upon exposure to acute and continuous postnatal irradiation after the differentiation of spermatogonial stem cells and spermatogonia, nearly all of the seminiferous tubules were engaged in spermatogenesis. Neither acute nor continuous irradiation directed at quiescent gonocytes was responsible for the increased number of multivalent chromosomes in descendent primary spermatocytes. In contrast, a significant increase in cells with multivalent chromosomes was observed following acute irradiation on days 4 and 11 post-partum (pp). However, there were no significant increases in unstable structural chromosomal aberrations and aneuploidy in spermatozoa, regardless of cell stage at irradiation or the radiation dose-rate. Thus, germ cells surviving prenatal and postnatal irradiation can restore spermatogenesis and produce viable spermatozoa without chromosome damage. These findings may provide a better understanding of reproductive potential following accidental, environmental, or therapeutic irradiation during the prenatal and postnatal periods in humans.

Introduction

In mammals, including humans, the period from the fetal stage through the neonatal stage is crucial for the development of male reproductive ability, as the germline lineage is established during this period. Specifically, gonocytes proliferate mitotically during the early fetal stage, and they differentiate into spermatogonial stem cells and spermatogonia during the neonatal stage after passing through the quiescent phase during the late fetal stage (Rugh, 1968; Vergouwen et al., 1991; Nagano et al., 2000; Culty, 2009; Manku and Culty, 2015). It is important to recognize that exposure to cytotoxic and genotoxic agents, such as ionizing radiation, during the fetal and neonatal periods is relevant to infertility at adulthood and inherited diseases in offspring. Risk estimation using animal models is valuable for understanding the magnitude of exposure from the accidental release of toxic agents and from environmental sources. This information is of great importance when considering fertility following chemotherapy and radiotherapy for cancer treatment in children and young men, and the risk of chromosomal disorders in spermatozoa when spermatogenesis is restored (Meistrich, 2009, 2013).

Studies using murine models have demonstrated that, compared with the subsequent spermatogonial stem cells and spermatogonia, quiescent gonocytes are highly sensitive to ionizing radiation-induced cell death (Vergouwen et al., 1995; Moreno et al., 2001a). The drastic reduction in the gonocyte pool following γ-ray exposure was responsible for sterility in the seminiferous tubules of the testes in prepubertal rats (Moreno et al., 2001a) and hypospermia in the epididymides of adult mice (Forand et al., 2009a). When the testes of human fetuses aborted at 6 to 12 weeks of gestation were exposed *in vitro* to a single dose of γ-rays at 0.1−1.5 Gy, the number of germ cells was dose-dependently decreased (Lambrot et al., 2007), implying that prenatal exposure to ionizing radiation can affect spermatogenesis in adulthood.

Regarding genetic damage of germ cells originating from gonocytes, spermatogonial stem cells and spermatogonia after ionizing radiation exposure, it has been reported that there was no significant increase in the mutation rate of seven specific loci in the offspring of male mice exposed

to 2 Gy X-rays on day 17.5 post-coitus (pc), compared with the offspring of adult male mice who were administered the same dose (Cater et al., 1960). This conclusion was supported by the specific-locus mutation test in the offspring of male mice postnatally exposed to 3 Gy X-rays, because the induced mutation rate was not significantly different from that in adult male mice (Selby, 1973a, 1973b). Furthermore, it has been speculated that fetal and neonatal germ cells are protected from chromosomal mutations, as there was no obvious reduction in litter size from male mice exposed to a single dose of γ-rays at 3 and 4 Gy on day 17.5 pc and days 1 and 8 post-partum (pp) (Forand et al., 2009a). In contrast, an assay using the phosphorylation of the H2AX histone (γ-H2AX) as a marker of DNA double-strand breaks (DSBs) found that DSBs increased in a dose-dependent manner in neonatal gonocytes and spermatogonia after γ-irradiation with 0.2−2 Gy in mice (Forand et al., 2004, 2009b). A dose-dependent increase in the number of chromatid breaks was also identified in murine neonatal germ cells collected from the testes 2 h after γ-irradiation with 2 Gy (Forand et al., 2004). To our knowledge, few attempts have been made to answer the question of whether induction of primary DNA/chromosome damage by perinatal irradiation can contribute to chromosomal aberrations in spermatozoa at reproductive age. In addition, the risk of generating secondary chromosomal aberrations during spermatogenesis in the testes of infertile and subfertile mice following perinatal irradiation remains unknown.

In this study, male prenatal (fetal) and postnatal (neonatal and juvenile) mice of ages corresponding to the stages of quiescent gonocytes, differentiating spermatogonial stem cells and spermatogonia, and developing primary spermatocytes in the first wave of spermatogenesis were exposed to acute and continuous γ-rays (Fig. 1). First, we estimated the age- and dose-rate-dependent effects of radiation on spermatogenesis at adulthood. Second, the frequency of multivalent chromosomes in primary spermatocytes arising from surviving gonocytes and spermatogonial stem cells was estimated to confirm the presence of translocations in the germlines. Finally, the frequency of unstable structural chromosome aberrations and aneuploidy originating from sperm cells were

measured by analyzing the chromosomes of zygotes produced by *in vitro* fertilization with epididymal spermatozoa or by intracytoplasmic sperm injection (ICSI) with testicular spermatozoa.

Results

Weights of testes derived from irradiated mice

Whole mouse bodies were exposed to acute (659−690 mGy/min) and continuous (0.303 mGy/min) 2 Gy γ-rays during the prenatal and postnatal periods. All mice, except for one exposed to acute γ-rays on day16.5 pc, grew to adulthood. When 10-week-old mice were used for experiments, their testes were weighed (Fig. 2). Prenatal exposure to either acute or continuous γ-rays significantly reduced the weight of the testes. Notably, acute irradiation on days 15.5, 16.5, and 17.5 pc affected testicular development, and the weight of the testes was less than 20% of the control testes. However, the weight of the testes following exposure to acute γ-rays on days 18.5 and 19.5 pc recovered to nearly 60% of the control testes. Continuous irradiation between 14.5 and 19.5 days pc reduced the weight of the testes to 33.4% of the controls. Neither acute nor continuous irradiation during the postnatal period had an adverse effect on testicular development.

Histological examination of testes

The total number of seminiferous tubules was counted in the largest cross section of the testes at 10 weeks of age, and the functional tubules, containing germ cells, were discriminated from sterile tubules, devoid of germ cells, to estimate the spermatogenetic efficiency (i.e., percentage of functional tubules) (Fig. 3). Regardless of radiation dose-rate, prenatal irradiation resulted in a significant reduction in the number of functional tubules. The spermatogenetic efficiency was conspicuously low in males irradiated on days 15.5 pc (13.8 ± 3.2%), 16.5 pc (2.9 ± 1.5%), and 17.5 pc (13.4 ± 1.7%). These mice were considered infertile, as there were few or no spermatozoa in the epididymides (Fig. 4). In contrast, the spermatogenetic efficiency was greatly improved in males irradiated on days 18.5 pc (93.1 ± 3.1%) and 19.5 pc (91.4 ± 3.1%). In mice exposed to continuous γ-

rays between 14.5 and 19.5 days pc, 44.3 ± 2.0% of tubules were engaged in spermatogenesis, and the epididymides contained enough spermatozoa to be used for *in vitro* fertilization (IVF) experiments. Overall, the decrease in spermatogenetic efficiency was consistent with testicular weight loss (Fig. 2). There were no apparent pathological signs of testicular cancer in any of the irradiated groups.

Multivalent chromosomes in primary spermatocytes

Multivalent chromosomes in primary spermatocytes are indicative of induction of nonhomologous translocations (to be referred to as translocations) in gonocytes, spermatogonial stem cells and spermatogonia that have survived radiation exposure. Mice exposed to acute γ-rays on day 16.5 pc, day 4 pp, and day 11 pp were used at 10 weeks of age to determine the frequency of multivalent chromosomes in primary spermatocytes, as were the mice exposed to continuous γ-rays between 14.5 and 19.5 days pc, between 2 and 7 days pp, and between 9 and 14 days pp. Although mice exposed to acute γ-rays on day 16.5 pc had only a small number of functional seminiferous tubules (Figs. 3 and 4), they were available for chromosome analysis of primary spermatocytes.

More than 300 cells per animal were recorded, and more than 1000 cells were analyzed in each group (Table 1). Neither acute irradiation nor continuous irradiation during the prenatal period was responsible for induction of multivalent chromosomes. However, the percentage of cells with multivalent chromosomes was significantly higher after the acute irradiation on day 4 pp (1.3%; odds ratio [OR] 16.71, $P < 0.05$), day 11 pp (4.9%; OR 68.61, $P < 0.05$), compared with the controls (0.1%). The value in the continuous irradiation between 2 and 7 days pp was apparently high (0.8%; OR 10.23), though the statistical significance was not determined because of the small number of animals used. In these irradiation groups, chain-type quadrivalent chromosomes were predominant over ring-type quadrivalent chromosomes (Fig. 5).

Chromosome analysis of zygotes

As with chromosome analysis in primary spermatocytes, irradiated mice were used as sperm donors to produce zygotes. Table 2 provides the success rate of IVF using epididymal spermatozoa and the incidence of chromosomal aberrations in the zygotes. The rate of oocyte penetration was significantly reduced in mice exposed to acute irradiation on day 4 pp (OR 0.14, $P < 0.05$) and day 11 pp (OR 0.13, $P < 0.05$) and was less than 50% in mice exposed to continuous irradiation between 14.5 and 19.5 days pc (OR 0.04, $P < 0.05$). Regardless of the reduction in oocyte penetration rate, almost all fertilized ova developed to the first cleavage in metaphase. The ova were prepared on chromosome slides, and only monospermic diploid (2*n*) zygotes were used for chromosome analysis. Unstable structural aberrations, including breaks, acentric fragments, dicentrics, chromatid exchanges, and aneuploidy, were detected using conventional Giemsa and C-band stains. However, no significant increase in zygotes with these chromosomal aberrations was observed in any irradiation group.

Mice exposed to acute 2 Gy γ-rays on day 16.5 pc were infertile because of the absence of spermatozoa in the epididymides (Fig. 4), thus zygotes were produced by ICSI using testicular spermatozoa. Table 3 provides technical data on the ICSI and the incidence of chromosomal aberrations in the resultant zygotes. In the irradiation group, 267 oocytes were injected with sperm nuclei, and 82.0% grew diploid (2*n*) zygotes. The others included ova with arrested development of sperm nuclei (13.9%) and triploid (3*n*) zygotes caused by suppression of the second polar body (4.1%). These frequencies were similar to those observed in the control mice (81.2%, 15.3%, and 3.5%, respectively). The incidence of chromosomal aberrations, including unstable structural aberrations and aneuploidy in the irradiation group, was higher than that in the control, though the difference did not exceed the statistically significant level (OR 1.86, P=0.059).

The incidence of structural chromosomal aberrations in the control group was higher in zygotes produced by ICSI than in those produced by IVF (Table 2). This is likely because ICSI, using testicular spermatozoa, is usually accompanied by some chromosome damage (Tateno, 2008).

Discussion

Cellular responses to ionizing radiation are highly dependent on several biological factors, including the cell type, cell differentiation, and the cell cycle. Regardless, one of the primary cellular targets of ionizing radiation is DNA. If cells cannot repair the DNA damage, they undergo apoptosis, which is intricately mediated by signaling pathways triggered by DNA damage. In addition, after radiation exposure, apoptotic pathways are also triggered by extranuclear events, including stimulation of death receptors on the cell membrane and loss of mitochondrial membrane potentials (Prise et al., 2005). The massive cell loss causes atrophy or degeneration in the tissues and organs, and atrophic gonads result in infertility. If repair is erroneous, gene mutations and chromosomal aberrations can occur. Genetic alterations in somatic cells increase the risk of carcinogenesis, and those in germ cells increase the risk of hereditary diseases (UNSCARE 2000 report).

The present study determined that testicular development and spermatogenesis were seriously affected following acute γ-irradiation with 2 Gy between 15.5 and 17.5 days pc, but the detrimental effect was considerably reduced when irradiated on days 18.5 and 19.5 pc (Figs. 2 and 3). This suggests that the sensitivity to radiation exposure is reduced between 17.5 and 18.5 days pc in murine quiescent gonocytes. Furthermore, it should be noted that continuous γ-irradiation for 5 days from day 14.5 to day 19.5 pc markedly reduced the weight of the testes and spermatogenetic efficiency, although the dose-rate of continuous irradiation (0.303 mGy/min) was much lower than that of the acute irradiation (659−690 mGy/min). It is likely that a substantial number of quiescent gonocytes suffered lethal damage as the result of cumulative doses of 2 Gy.

A comparable murine study reported that acute exposure of the NMRI strain to 2 Gy and 3 Gy γ-rays (dose rate: 600 mGy/min) on day 17.5 pc reduced the weight of the testes at 60 days of age by approximately 70% and 35% of the weight of the control testes, respectively (Forand et al., 2009a). Furthermore, sperm counts in the epididymides and vas deferens at the same age were reduced by approximately 40% and 90% compared with the controls at 2 Gy and 3 Gy, respectively. However, after mating with non-irradiated females, the fertility of these irradiated males, even by

irradiation with 3 Gy, had not deteriorated. On the other hand, when CBA/P mice were exposed to 1.5 Gy X-rays (dose rate: 300 mGy/min) on day 18 pc, corresponding to day 17.5 pc in the present study, the weight of the testes on day 31 pp decreased to approximately 30% of the controls, and the percentage of seminiferous tubules containing spermatids was sharply reduced to approximately 5% (Vergouwen et al., 1995). The present results obtained using $B6C3F_1$ mice are similar to the results obtained using CBA/P mice. As suggested in studies on adult mice (Meistrich et al., 1984) and rats (Abuelhija et al., 2012), several factors, including intrinsic DNA repair, progression of the cell cycle, and recuperation of spermatogenesis from surviving cells, may account for the strain differences in male reproductive potential following irradiation during the fetal period. Taken together, we conclude that radiation exposure of 1.5−2.0 Gy between 15.5 and 17.5 days pc greatly reduces the gonocyte pool and induces an adverse effect on murine male fertility.

It was shown that the irradiation-induced death of neonatal gonocytes on day 1 pp was accompanied by caspase activation via the pro-apoptotic BH3-only protein, PUMA, which is regulated by the tumor suppressor, p53 (Forand and Bernardino-Sgherri, 2009). However, the molecular mechanism underlying the large change in the radiosensitivity of fetal gonocytes during the quiescent phase remains poorly characterized. Intriguingly, in the quiescent gonocytes of mice, DNA hypomethylation is maintained from day 13.5 pc to day 15.5 pc, and then it is drastically hypermethylated at day 16.5 pc (Coffigny et al., 1999). It has been proposed that expression of apoptosis-related genes in rat gonocytes following exposure to γ-rays may be related to drastic changes in DNA methylation (Moréno et al., 2001b). The question of whether DNA hypomethylation or hypermethylation can modulate the radiosensitivity of fetal quiescent gonocytes remains to be answered.

Forand et al. (2009b) elucidated that murine neonatal gonocytes were 1.5-fold more sensitive to DSB induction than proliferating somatic cells, and the lesions increased in a dose-dependent manner following acute γ-irradiation with 0.2−2 Gy. Moreover, the study indicated that neonatal gonocytes repaired DSBs faster than spermatogonia using alternate DNA repair

mechanisms. When DBSs are misrepaired, chromosomal segments between two nonhomologous chromosomes are exchanged, and a translocation is formed. The occurrence of translocations in gonocytes is associated with an increase in heritable risk, because chromosomally imbalanced spermatozoa are produced by aberrant segregation of multivalent chromosomes resulting from translocations. In the present results, we observed no significant increase in the number of multivalent chromosomes in primary spermatocytes that originated from fetal quiescent gonocytes that survived acute and continuous irradiation, indicating that irradiation of fetal quiescent gonocytes has no relevance with regards to the induction of translocations. As suggested by Forand et al. (2009b), the preservation of genomic integrity following irradiation may be tightly regulated in gonocytes.

In contrast to prenatal irradiation, we observed that acute irradiation during the postnatal period caused a statistically significant increase in primary spermatocytes with multivalent chromosomes. Considering the interval of at least 8 weeks between irradiation and cell harvest, it is likely that multivalent chromosomes arose from translocations in spermatogonial stem cells that survived irradiation. When adult mice were exposed to 2 Gy X-rays at a dose-rate of 700 mGy/min, which is comparable to that in the current study (2 Gy γ-rays at a dose-rate of 659−680 mGy/min), translocation frequencies were estimated to be approximately 4% in spermatogonial stem cells at mitotic metaphase, and approximately 3% in primary spermatocytes at meiotic metaphase I after 7 weeks of irradiation (van Buul et al., 1995). These values were similar to the 4.9% observed in the primary spermatocytes that originated from spermatogonial stem cells following acute irradiation on day 11 pp. It appears that the chromosomal radiosensitivity of spermatogonial stem cells in the postnatal testes is comparable to that of spermatogonial stem cells in adulthood.

A chromosome analysis of zygotes derived from the spermatozoa of irradiated mice indicated that prenatal and postnatal irradiation, regardless of cell stage at irradiation or the dose-rate, had no relationship with the induction of unstable structural aberrations and aneuploidy in spermatozoa (Tables 2 and 3). In particular, there was no significant increase in the number of

chromosomal aberrations in the testicular spermatozoa of infertile mice following acute irradiation on day 16.5 pc. This strongly suggests that even in atrophic testes following prenatal irradiation, if a small number of seminiferous tubules are functional, chromosomally normal spermatozoa can be produced. In support of this, we confirmed that 46.5% (20/43) of 2-cell embryos produced by ICSI using testicular spermatozoa derived from infertile mice could develop into normal fetuses after embryo transfer (Fig. 6). This may raise the possibility of reproductive rescue of male infertility following prenatal irradiation. In performing assisted reproduction, sperm chromosome screening prior to embryo production would be of great help in minimizing the risk of chromosomal aberrations (Watanebe et al., 2013).

Although higher incidences of multivalent chromosomes were observed in the primary spermatocytes of mice exposed to γ-rays during the postnatal period, no occurrence of apparent translocation was found in the spermatozoa. One explanation for this discrepancy is that potential translocations could not be detected using the conventional Giemsa and C-band stains. It is also possible that spermatocytes with multivalent chromosomes could not differentiate into spermatozoa, as previous studies have indicated that certain translocations characterized by incomplete synapsis were associated with impairment of spermatogenesis (Forejt and Gregorová, 1977; de Bore et al., 1986; Homolka et al., 2012) or regulation at the pachytene checkpoint (Roeder, 2000). In addition, the incidence of cells containing multivalent chromosomes was originally less than 5% (Table 1). The values should have been reduced through alternate disjunction of quadrivalent chromosomes at meiosis I (Oliver-Bonet et al., 2004). Genetic risk still remains to be monitored in the offspring of males exposed to ionizing radiation during the prenatal and postnatal periods. This is especially pertinent given that a number of studies have shown that genomic instability can be transmitted to offspring through spermatozoa of irradiated male mice (Barber et al., 2002; Dubrova, 2003; Streffer, 2006; Barber et al., 2006; Barber et al., 2009; Asakawa et al., 2013).

In our IVF experiments, the fertilization rate was reduced significantly after mice were exposed to acute irradiation on days 4 and 11 pp and when those exposed to continuous irradiation

between 14.5 and 19.5 days pc were used as sperm donors. The reduced fertilization ability of spermatozoa was not due to morphological alterations. Although spermatozoa were cryopreserved prior to IVF experimentation, visually, they maintained good motility after thawing. The fertilization ability of murine spermatozoa is dependent upon many extrinsic and intrinsic factors (Stival et al., 2016). Further attempts should be made to determine why exposure of developing testes to ionizing radiation belatedly deteriorated the fertilization ability of spermatozoa at the reproductive age.

In the present study, we focused on the reproductive risk, including spermatogenetic failure and chromosomal defects in germ cells, when the whole body was acutely and continuously exposed to 2 Gy γ-rays during the fetal, neonatal, and juvenile periods. At the same time, testicular germ cell tumors, the most common solid malignant tumor in young men, may be another risk associated with exposure to ionizing radiation during these periods, because it has been suggested that the tumors arise from defective gonocyte differentiation (Gilbert et al., 2011). Importantly, a study using a murine model demonstrated that whole body irradiation with two doses of 0.8 Gy γ-rays on days 10.5 and 11.5 pc increased the risk of developing testicular cancer without disruption of spermatogenesis (Shetty et al., 2012). Because fetuses and children are the most vulnerable groups of humans (NCRP 2013 report; UNSCARE 2013 report), additional studies, involving developing animals, should be conducted, to more clearly understand the risks of exposure to radiation from various perspectives.

In conclusion, the exposure of fetal testes to ionizing radiation causes death of quiescent gonocytes, and the drastic reduction in the gonocyte pool deteriorates spermatogenesis at adulthood. However, chromosomal integrity is well preserved in surviving gonocytes, and it seems unlikely that spermatogenesis in infertile and subfertile testes, following perinatal irradiation, increases the risk of generating secondary chromosomal aberrations. Spermatogonial stem cells and spermatogonia in the postnatal testes are relatively resistant to radiation-induced cell death, whereas these germ cells are susceptible to radiation-induced translocations. It is likely that the spermatogonial stem cells and spermatogonia with translocations are ultimately unable to develop into spermatozoa. These findings

may improve our understanding of reproductive and heritable risks following exposure to radiation during the prenatal and postnatal periods.

Materials and methods

Reagents and media

All organic and inorganic reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated. Incubation for sperm capacitation and *in vitro* fertilization was carried out in Toyoda-Yokoyama-Hosi (TYH) medium (Toyoda et al., 1971). Fertilized ova were cultured in Chatot-Ziomek-Bavister (CZB) medium (Chatot et al., 1989), containing 5.56 mM D-glucose and 5 mg/mL lipid-rich bovine serum albumin (BSA) (AlbuMAX; Gibco, Auckland, New Zealand). Testicular spermatozoa were collected in TYH medium supplemented with 20 mM Hepes-Na, 5 mM $NaHCO_3$, and 3 mg/mL of polyvinyl alcohol (cold-water soluble; Sigma-Aldrich, St. Louis, MO, USA) instead of BSA, which was designated as H-TYH. Collection and micromanipulation of oocytes were performed in CZB medium supplemented with 5.56 mM D-glucose, 20 mM HEPES-Na, 5 mM $NaHCO_3$, and 3 mg/mL of polyvinyl alcohol instead of BSA. This medium was designated as H-CZB. TYH and CZB media were used under an atmosphere of 5% CO_2, and H-CZB and H-TYH media were used under an atmosphere of 100% air.

Animals

Hybrid mice ($B6C3F_1$) were used as the experimental model in this study. Mice 6 weeks of age were purchased from CLEA Japan, Inc. (Tokyo, Japan). When prenatal and postnatal mice were required, females (C57BL/6N Jcl) were mated with males (C3H/HeN Jcl), or pregnant females on day 7 of gestation were purchased. The day after mating and the day of birth were counted as one half day post-coitus (pc) and day zero post-partum (pp), respectively. Animals were maintained under optimal temperature in a light-controlled environment, and food and water were provided *ad*

libitum. All animal experiments were performed according to the institutional guidelines of the Asahikawa Medical University and the Institute for Environmental Sciences.

Irradiation

Acute irradiation

Prenatal mice were irradiated with a single dose of 2 Gy ^{137}Cs γ-rays at 659−690 mGy/min on days 15.5, 16.5, 17.5, 18.5, and 19.5 (the day of birth) pc, and the same dose was administered to postnatal mice on days 4 and 11 pp. According to the criteria of Rugh (1967), Vergouwen et al. (1991), Nagano et al. (2000), and Culty (2009), the germ cell stage on days 15.5−19.5 pc, day 4 pp, and day 11 pp was considered the quiescent gonocyte stage, the transitional stage from gonocytes to spermatogonial stem cells and spermatogonia, and the stage of increasing primary spermatocytes, respectively (Fig. 1). Acute irradiation was performed at the Asahikawa Medical University, Asahikawa, Hokkaido, Japan.

Continuous irradiation

Mice were continuously exposed to ^{137}Cs γ-rays at 400 mGy for 22 h/day (0.303 mGy/min) for 5 days for a total dose of 2 Gy. For the irradiation of prenatal mice, pregnant females were irradiated from 14.5 to 19.5 days of gestation (predominantly quiescent gonocytes). Irradiation of postnatal mice was performed between 2 and 7 days pp (during the formative period of spermatogonial stem cells and spermatogonia), and between 9 and 14 days pp (predominantly spermatogonial stem cells and spermatogonia and primary spermatocytes). During radiation exposure for 5 days, mice were housed with food and water *ad libitum*. For animal care, mice were removed from radiation exposure daily between 10:00 and 12:00. Continuous irradiation was performed at the Institute for Environmental Sciences, Rokkasho, Aomori, Japan.

Histological preparation of testes and epididymides

The testes were routinely fixed in Bouin's solution, dehydrated in alcohol, and embedded in paraffin wax (Waco Pure Chemical Industries, Osaka, Japan). Serial sections (5−7 μm thick) were conventionally dewaxed, rehydrated, and stained with hematoxylin and eosin (Sakura Fintech Japan, Tokyo, Japan). Finally, the slides were sealed with cover slips. In some mice with atrophic testes, the epididymides were also histologically prepared.

Induction of premature chromosome condensation (PCC) in primary spermatocytes

Calyculin-A (Waco Pure Chemical Industries), a specific inhibitor of protein phosphatase, was used to allow chromosomes to condense effectively by inducing PCC (Miura and Blakely, 2011). When mice matured (10 weeks of age), the testes were removed and minced in a small amount of medium (10% fetal bovine serum in HEPES-buffered RPMI 1640) with scissors. Additional medium (1 mL) was added to suspend the cells, and the suspension was transferred to a centrifuge tube and prepared to a final volume of 5 mL using the same medium. Calyculin-A was added at a final concentration of 50 nM, and the tube was incubated at 37ºC for 1 h to induce PCC.

The tube was centrifuged at 270 × *g* for 5 min to collect cells. The supernatant was discarded, a hypotonic solution (0.075 M KCl) was added, and then the tube was incubated for 20 min at 37ºC. Cells were subsequently fixed with an alcohol/acetic acid (3:1) solution. After centrifugation, the supernatant was discarded, and fresh fixative solution was added to the tube. This procedure was repeated twice to thoroughly fix the cells. PCC spreads were prepared on glass slides using a temperature- and humidity-controlled chamber (HANABI; ADStec Inc., Funabashi, Japan). The slides were conventionally stained with a 1% Giemsa solution (Merck Japan, Tokyo, Japan) in a phosphate buffered solution (pH 6.8) for 8 min to detect multivalent chromosomes. For ambiguous cases, the C-band technique (Sumner, 1972) was applied to the slides to stain constitutive centromeric heterochromatin.

Sperm cryopreservation

For repetitive analyses of the sperm sample from an individual donor, spermatozoa were cryopreserved in advance using the method described by Nakagata (2000) with some modifications. Briefly, the cauda epididymides of mice at 10 weeks of age were placed in 200−220 μL of cryopreservation solution containing raffinose and skim milk, and the ducts of the epididymides were cut to retrieve motile spermatozoa. The sperm suspension was subdivided into 15−20 plastic straws, each containing 10 μL of the suspension. Following incubation in the gaseous phase of liquid nitrogen for 10 min, the sample straws were plunged into liquid nitrogen.

Production of zygotes, and the chromosome analysis

In vitro fertilization (IVF) procedure

Mature females 7−12 weeks of age were hormonally stimulated to induce superovulation by injecting 7.5 IU equine chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) followed by the injection of 7.5 IU human chorionic gonadotropin (hCG; ASKA Pharmaceutical) 48 h later. Oocytes enclosed by cumulus cells were recovered from the oviducts between 14 and 16 h after hCG injection, and placed in a droplet (100 μL) of TYH medium under paraffin oil (Merck Japan, Tokyo, Japan) that had been prepared for insemination.

Frozen sperm sample straws were immersed in a water bath at 37ºC for 10 min. The straws were wiped with paper, and both ends were cut to transfer the sperm suspension to a plastic dish. The sperm suspension was added to a droplet (100 μL) of TYH medium under paraffin oil in the dish, and incubated at 37ºC under 5% CO_2 in air for 30 min to recover motility. Spermatozoa were added to the insemination droplet containing the oocytes. Sperm concentration was adjusted to 150−200/μL. After 6 h, fertilized ova with 2 or more pronuclei and a second polar body were selected and further cultured in CZB medium.

Intracytoplasmic sperm injection (ICSI) procedure

When irradiated males were infertile as the result of an absence of spermatozoa in the epididymis, zygotes were produced from testicular spermatozoa using the ICSI technique. As described previously, oocytes were harvested from female mice following hormonal stimulation, and then separated from cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma-Aldrich) in H-CZB medium. The cumulus-free oocytes were washed with CZB medium and maintained in fresh CZB medium until use.

Spermatozoa were retrieved from the testes of infertile mice at 10−11 weeks of age. Testes were minced in a small amount of H-TYH medium to prepare a cell suspension. After being appropriately diluted with H-TYH medium, the cell suspension was mixed with equal amounts of H-TYH medium containing 10% (w/v) polyvinylpyrrolidone. An aliquot (10 μL) of the mixture was placed in a culture dish and covered with paraffin oil. Then, 15 oocytes were transferred to a droplet (10 μL) of H-CZB medium under paraffin oil, which had been placed next to a sperm-containing droplet in the same dish. A spermatozoon was aspirated into the injection pipette, and the head was separated from the tail by application of a few piezo pulses. The heads were individually injected into oocytes as described previously (Kuretake et al., 1996). After the sperm injection, oocytes were thoroughly washed with CZB medium and cultured.

Zygote chromosome preparation

Six hours after insemination or sperm injection, fertilized ova were transferred to the CZB medium containing 0.02−0.04 μg/mL vinblastine sulfate (Sigma-Aldrich), and cultured in the same medium at 37ºC under 5% CO_2 in air. Eighteen to twenty hours after insemination or sperm injection, the fertilized ova (zygotes) were treated with 0.5% actinase (Kaken Pharmaceuticals, Tokyo, Japan) to loosen the zona pellucida, and then they were placed in a hypotonic solution consisting of a 1:1 mixture of 1% sodium citrate and 30% BSA for 8 min at room temperature (23−25ºC).

Chromosomal slides were produced using a gradual-fixation/air-drying method (Mikamo and Kamiguchi, 1983). The slides were conventionally stained with a 1% Giemsa solution to count

the chromosomes and to detect any breakage-type structural aberrations. C-band staining was used to detect aneuploidy and dicentric chromosomes, as previously described (Tateno et al., 2000).

Statistical analysis

The weight of the testes and the number of seminiferous tubules were compared using one-way analysis of variance, and the Dunn's test was applied for multiple comparisons. The results of histological evaluations of the testes, and chromosome analyses of spermatocytes and zygotes were analyzed by logistic regression (binominal distribution). All percentage data were analyzed using the following model: $\ln(\alpha/(1-\alpha)) = \beta$ + main factor (experimental group), where α = the frequency of the positive outcome and β = the intercept. If the main factor had a significant effect, multiple comparisons were performed using the 95% confidence interval of the odds ratio (OR). Significant differences were set at $P < 0.05$.

Acknowledgments

The authors are thankful to Dr. T. Ono at the Institute for Environmental Sciences for his valuable suggestions. This study was supported by JPSP KAKENHI Grant number 25870027 and 16K16191 (to HW) and a grant from the Radiation Effects Association (to HT).

Conflict of interest: None declared.

References

Abuelhija M, Weng CC, Shetty G, Meistrich ML. 2012. Differences in radiation sensitivity of recovery of spermatogenesis between rat strains. Tox Sci 126:545−553.

Asakawa J, Kodaira M, Cullings HM, Katayama H, Nakamura N. 2013. The genetic risk in mice from radiation: an estimate of the mutation induction rate per genome. Radiat Res 179:293−303.

Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. 2002. Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice. Proc Natl Acad Sci USA 99:6877−6882.

Barber RC, Hickenbotham P, Hatch T, Kelly D, Topchiy N, Almeida GM, Jones GDD, Johnson GE, Parry JM, Rothkamm K, Dubrova YE. 2006. Radiation-induced transgenerational alterations in genome stability and DNA damage. Oncogene 25:7336−7342.

Barber RC, Haardwick RJ, Shanks ME, Glen CD, Mughal SK, Voutounou M, Dubrova YE. 2009. The effects of *in utero* irradiation on mutation induction and transgenerational instability in mice. Mutation Res 664:6−12.

Carter TC, Lyon MF, Phillips RJS. 1960. The genetic sensitivity to X-rays of mouse foetal gonads. Genet Res 1:351−355.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 86:679−688.

Coffigny H, Bourgeois C, Ricoul M, Bernardino J, Vilain A, Niveleau A, Malfoy B, Dutrillaux B. 1999. Alterations of DNA methylation patterns in germ cells and Sertoli cells from developing mouse testis. Cytogenet Cell Genet 87:175−181.

Culty M. 2009. Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res (part C) 87:1−26.

de Boer P, Searle AG, van der Hoeven FA, de Rooij DG, Beechey CV. 1986. Male pachytene pairing in single and double translocation heterozygotes and spermatogenic impairment in the mouse. Chromosoma 93:326−336.

Dubrova YE. 2003. Radiation-induced transgenerational instability. Oncogene 22:7087−7093.

Forand A, Dutrillaux B, Bernardino-Sgherri J. 2004. γ-H2AX expression pattern in non-irradiated neonatal mouse germ cells and after low-dose γ-radiation: relationships between chromatid breaks and DNA double-strand breaks. Biol Reprod 71:643−649.

Forand A, Bernardino-Sgherri J. 2009. A critical role of PUMA in maintenance of genomic integrity of murine spermatogonial stem cell precursors after genotoxic stress. Cell Res 19:1018−1030.

Forand A, Messiaen S, Habert R, Bernardino-Sgherri J. 2009a. Exposure of the mouse perinatal testis to radiation leads to hypospermia at sexual maturity. Reproduction 137:487−495.

Forand A, Fouchet P, Lahaye J-B, Chicheportiche A, Habert R, Bernardino-Sgherri J. 2009b. Similarities and differences in the in vivo response of mouse neonatal gonocytes and spermatogonia to genotoxic stress. Biol Reprod 80:860−873.

Forejt J, Gregorová S. 1977. Meiotic studies of translocations causing male sterility in the mouse. I. Autosomal reciprocal translocations. Cytogenet Cell Genet 19:159−179.

Gilbert D, Rapley E, Shipley J. 2011. Testicular germ cell tumors: predisposition genes and the male germ cell niche. Nat Rev Cancer 11:278−288.

Homolka D, Jansa P, Forejt J. 2012. Genetically enhanced asynapsis of autosomal chromatin promotes transcriptional dysregulation and meiotic failure. Chromosoma 121:91−104.

Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. 1996. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol Reprod 55:789−795.

Lambrot R, Coffigny H, Pairault C, Lécureuli C, Frydman R, Habert R, Rouiller-Fabre V. 2007. High radiosensitivity of germ cells in human male fetus. J Clin Endocrinol Metab 92:2632−2639.

Manku G, Culty M. 2015. Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. Reproduction 149:R139−R157.

Meistrich ML. 2009. Male gonadal toxicity. Pediatr Blood Cancer. 53:261−266.

Meistrich ML. 2013. Effects of chemotherapy and radiotherapy on spermatogenesis in humans. Fertil Steril 100:1180−1186.

Meistrich ML, Finch M, Lu CC, de Ruiter-Bootsma AL, de Rooij DG. 1984. Strain differences in the response of mouse testicular stem cells to fractionated radiation. Radiat Res 94:478−487.

Mikamo K, Kamiguchi Y. 1983. A new assessment system for chromosomal mutagenicity using oocytes and early zygotes of the Chinese hamster. In Ishihara T and Sasaki MS, editors. Radiation-induced Chromosome Damage in Man. New York: Alan R Liss. p 411−432.

Miura T, Blakely WF. 2011. Optimization of calyculin A-induced premature chromosome condensation assay for chromosome aberration studies. Cytometry Part A, 79:1016−1022.

Moreno SG, Dutrillaux B, Coffigny H. 2001a. High sensitivity of rat foetal germ cells to low dose-rate irradiation. Int J Radiat Biol 77:529−538.

Moréno SG, Dutrillaux B, Coffigny H. 2001b. Status of p53, p21, mdm2, pRb proteins, and DNA methylation in gonocytes of control and γ-irradiated rats during testicular development. Biol Reprod 64:1422−1431.

Nagano R, Tabata S, Nakanishi Y, Ohsako S, Kurohmaru M, Hayasi Y. 2000. Reproliferation and relocation of mouse male germ cells (gonocytes) during prespermatogenesis. Anat Rec 258:210−220.

Nakagata N. 2000. Cryopreservation of mouse spermatozoa. Mamm Genome 11:572−576.

NCRP. 2013. Preconception and prenatal radiation exposure: Health effects and protective guidance. NCRP Report No. 174. Bethesda: National Council on Radiation Protection and Measurements.

Oliver-Bonet M, Navarro J, Codina-Pascual M, Abad C, Guitart M, Egozcue J, Benet J. 2004. From spermatocytes to sperm: meiotic behavior of human male reciprocal translocations. Hum Reprod 19:2515−2522.

Prise KM, Schettino G, Folkard M, Held KD. 2005. New insights on cell death from radiation exposure. Lancet Oncol 6:520−528.

Roeder GS, Bailis JM. 2000. The pachytene checkpoint. Trends Genet 16:395−403.

Rugh R. 1967. The mouse: Its reproduction and development. Minneapolis: Burgess Publishing. p 1−430.

Selby PB. 1973a. X-ray-induced specific-locus mutation rate in newborn male mice. Mutation Res 18:63−75.

Selby PB. 1973b. X-ray-induced specific-locus mutation rates in young male mice. Mutation Res 18:77−88.

Shetty G, Comish PB, Weng CCY, Matin A, Meistrich ML. 2012. Fetal radiation exposure induces testicular cancer in genetically susceptible mice. ProsOne 7: e32064.

Stival C, Puga Molina Lis del C, Paudel B, Buffone MG, Visconti PE, Krapf D. 2016. Sperm capacitation and acrosome reaction in mammalian sperm. Adv Anat Embryol Cell Biol 220:93−106.

Streffer C. 2006. Transgenerational transmission of radiation damage: genomic instability and congenital malformation. J Radiat Res 47 (suppl):B19−B24.

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304−306.

Tateno H, Kimura Y, Yanagimachi R. 2000. Sonication per se is not as deleterious to sperm chromosomes as previously inferred. Biol Reprod 63:341−346.

Tateno H. 2008. Chromosome aberrations in mouse embryos and fetuses produced by assisted reproductive technology. Mutation Res 657:26−31.

Toyoda Y, Yokoyama M, Hosi T. 1971. Studies on the fertilization of mouse eggs in vitro: I. In vitro fertilization of eggs by fresh epididymal sperm (in Japanese). Jpn J Anim Reprod 16:147−151.

UNSCEAR. 2000. Sources and Effects of Ionizing Radiation. Volume II. Effects. UNSCEAR 2000 Report to the general assembly, with scientific annexes. New York: United Nations.

UNSCEAR. 2013. Sources and Effects of Ionizing Radiation. Volume II. Scientific Annex B, Effects of radiation exposure of children. UNSCEAR 2013 Report to the General Assembly, with scientific annexes. New York: United Nations.

van Buul PPW, Zandman IM, Grigorova M, Boei JJWA, Natarajan AT. 1995. Mitotic and meiotic detection of radiation-induced translocations in mouse stem cell spermatogonia using fluorescence *in situ* hybridization. Chromosome Res 3:427−432.

Vergouwen RPFA, Jacobs SGPM, Huiskamp R, Davids JAG, de Rooij DG. 1991. Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fert 93:233−243.

Vergouwen RPFA, Huiskamp R, Bas RJ, Roepers-Gajadien HL, Davids JAG, de Rooij DG. 1995. Radiosensitivity of testicular cells in the fetal mouse. Radiat Res 141:66−73.

Watanabe H, Kusakabe H, Mori H, Yanagimachi R, Tateno H. 2013. Production of offspring after sperm chromosome screening: an experiment using the mouse model. Hum Reprod 28:531−537.

Figure legends

Figure 1. Schematic representation of spermatogenesis during the prenatal and postnatal periods, testicular histology, and the timeline of acute and continuous irradiation administration. Quiescent gonocytes were observed between day 14.5 post-coitus (pc) and day 1.5 post-partum (pp). They then pass through the mitotic phase and differentiate into spermatogonial stem cells and spermatogonia. From day 9 pp, the first wave of spermatogenesis begins, producing primary spermatocytes. Cross sections of seminiferous tubules on day 16.5 pc, day 4 pp, and day 11 pp depict quiescent gonocytes containing large nuclei in the tubules, spermatogonial stem cells and spermatogonia in the peripheral region of the tubules, and increasing primary spermatocytes in the tubules, respectively. Scale bars in histological illustrations stained with hematoxylin and eosin, 50 μm. Acute γ-ray irradiation (2 Gy) (arrowheads) was administered daily between 15.5 and 19.5 days pc, on day 4 pp, and day 11 pp. Continuous γ-ray irradiation, consisting of the same total dose over 5 days (0.4 Gy/day), was administered between 14.5 and 19.5 days pc, between 2 and 7 days pp, and between 9 and 14 days pp. Details of radiation exposure are described in the Materials and methods. The timeline of germ cell development was summarized according to the available information (Materials and methods).

Figure 2. The weight of the testes (mean ± SEM) at 10 weeks of age in mice exposed to acute and continuous γ-ray irradiation (2 Gy) throughout the prenatal (post-coitus, pc) and postnatal (post-partum, pp) periods. Four to ten animals per group were used. Asterisks depict statistically significant weight reductions compared with those in the control mice.

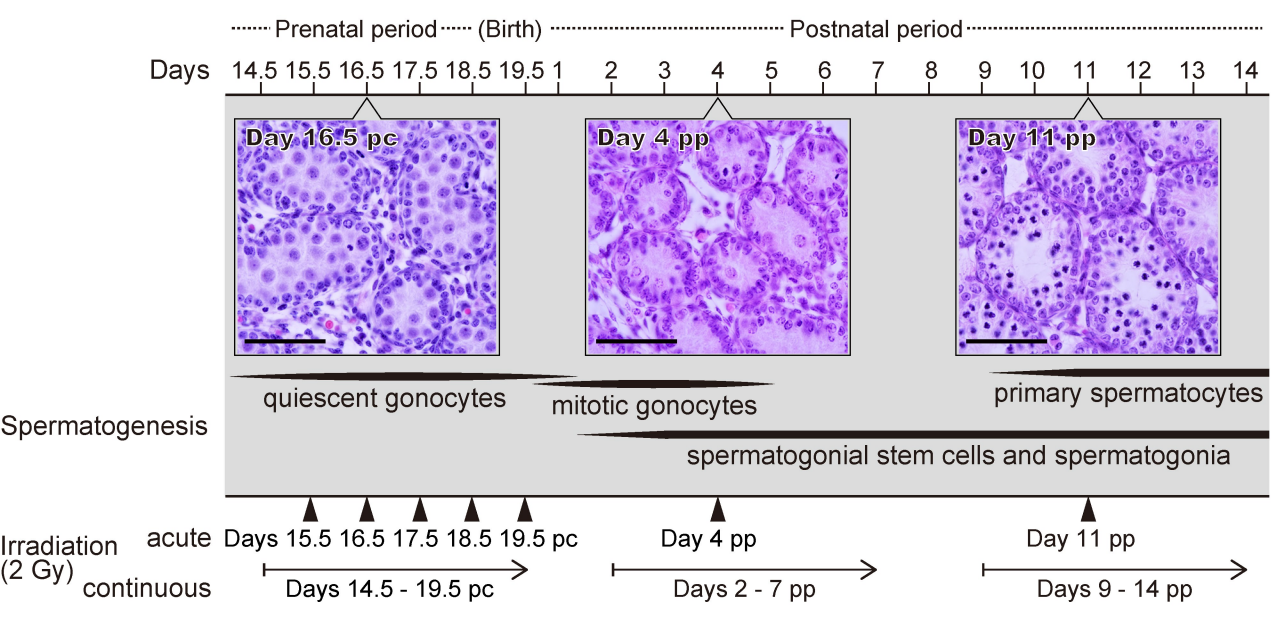
Figure 3. Number (mean ± SEM) of cross sections of seminiferous tubules in a maximal cross section of the testis at 10 weeks of age in mice exposed to acute and continuous γ-rays at 2 Gy throughout the prenatal (post-coitus, pc) and postnatal periods (post-partum, pp). Each group consisted of three mice. Black and white graphic bars indicate the number of functional tubules

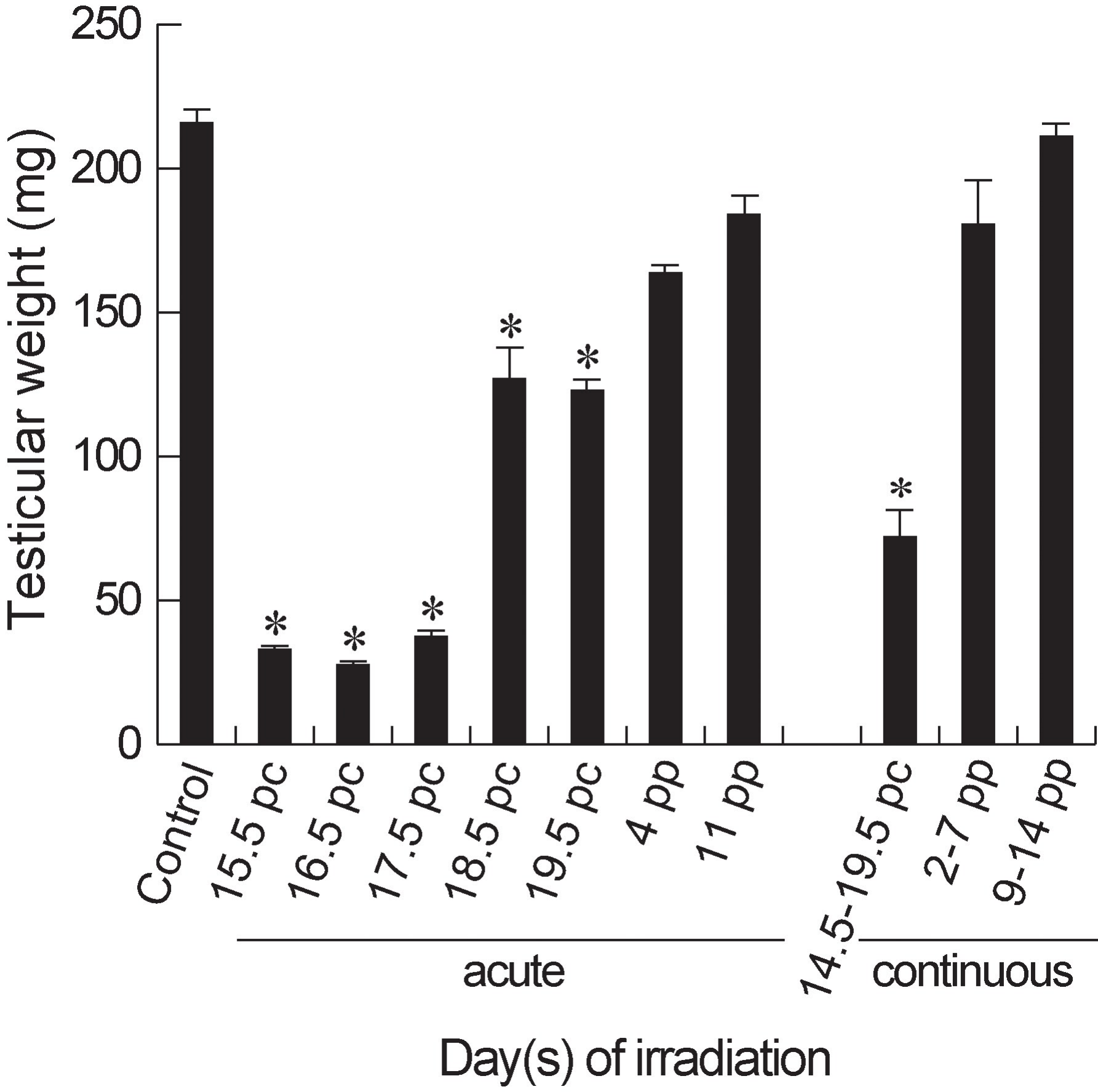
containing germ cells and sterile tubules devoid of germ cells, respectively. Asterisks depict statistically significant reductions in the percentage of functional seminiferous tubules compared with the control mice.

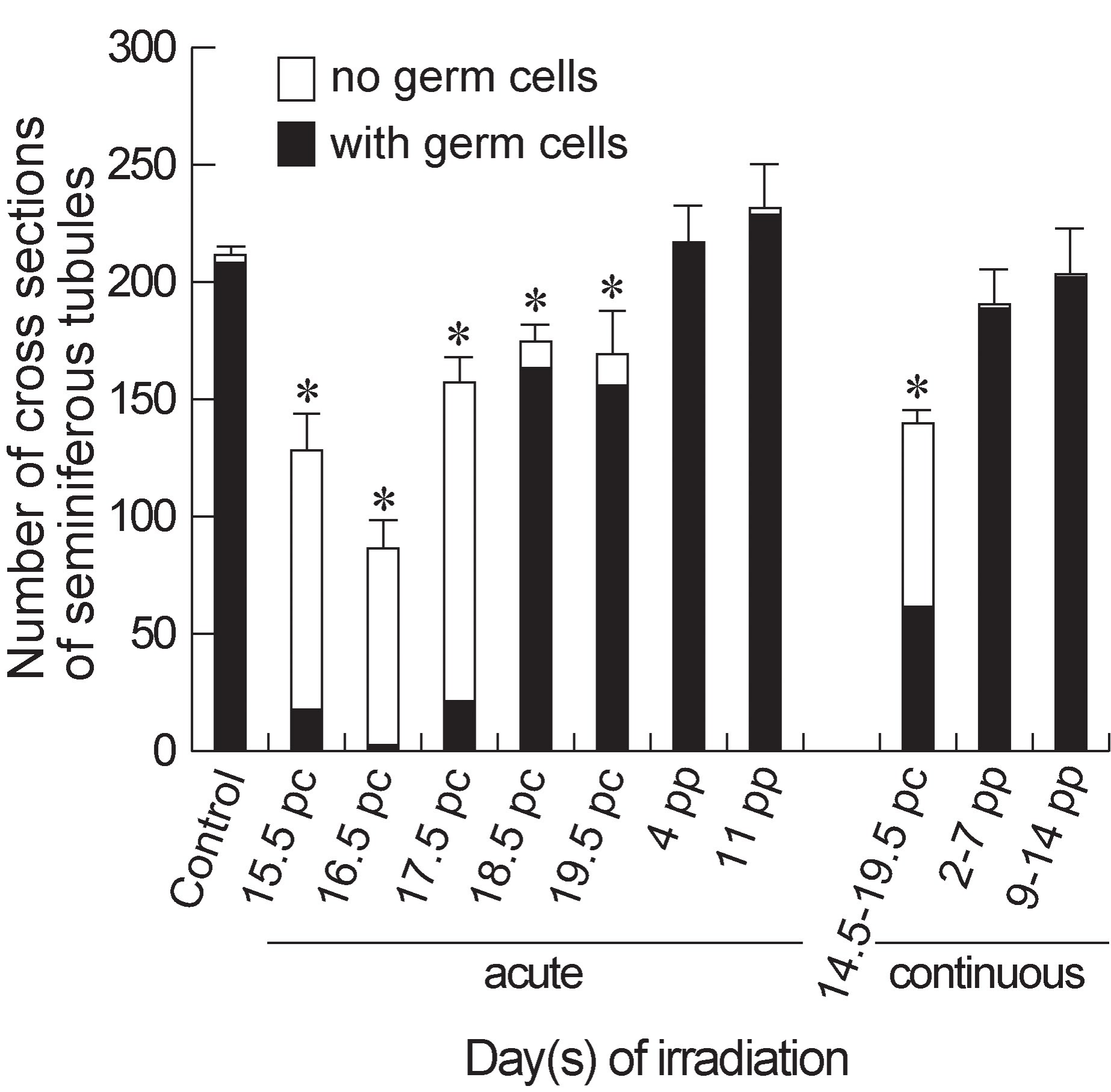
Figure 4. Histological preparations of the testes and epididymides at 10 weeks of age. **A and B.** Cross sections of the seminiferous tubules. (A) Control. (B) Exposure to acute γ-rays at 2 Gy on day 16.5 pc. Many tubules are devoid of germ cells. Asterisks depict cross sections of functional tubules demonstrating spermatogenesis. **C and D.** The caudal region of the epididymis. (C) Control. Enlarged inset presents numerous sperm heads. (D) Exposure to acute γ-rays at 2 Gy on day 16.5 pc. Enlarged inset demonstrates the absence of spermatozoa. Scale bars in A to D, 200 μm; and those in insets, 50 μm.

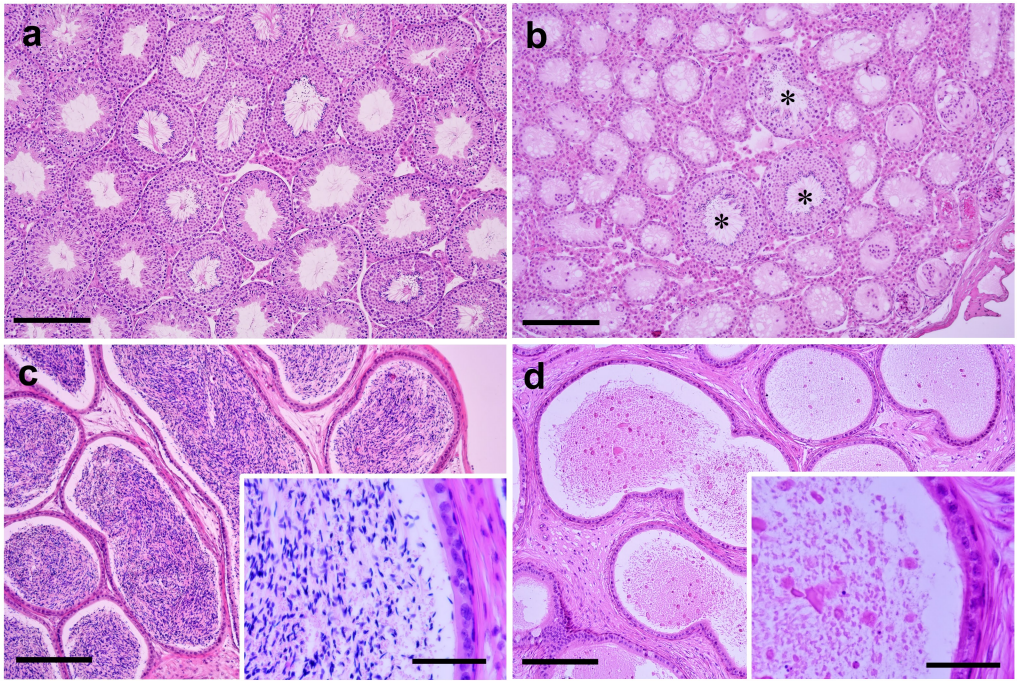
Figure 5. Quadrivalent chromosomes observed in the primary spermatocytes of mice at 10 weeks of age exposed to acute γ-rays at 2 Gy on day 11 pp. **(A) and (A')** A chain quadrivalent chromosome (arrow) observed by conventional Giemsa and C-band stains, respectively. **(B) and (B')** A ring quadrivalent chromosome (arrow) observed by conventional Giemsa and C-band stains, respectively. Scale bars, 10 μm.

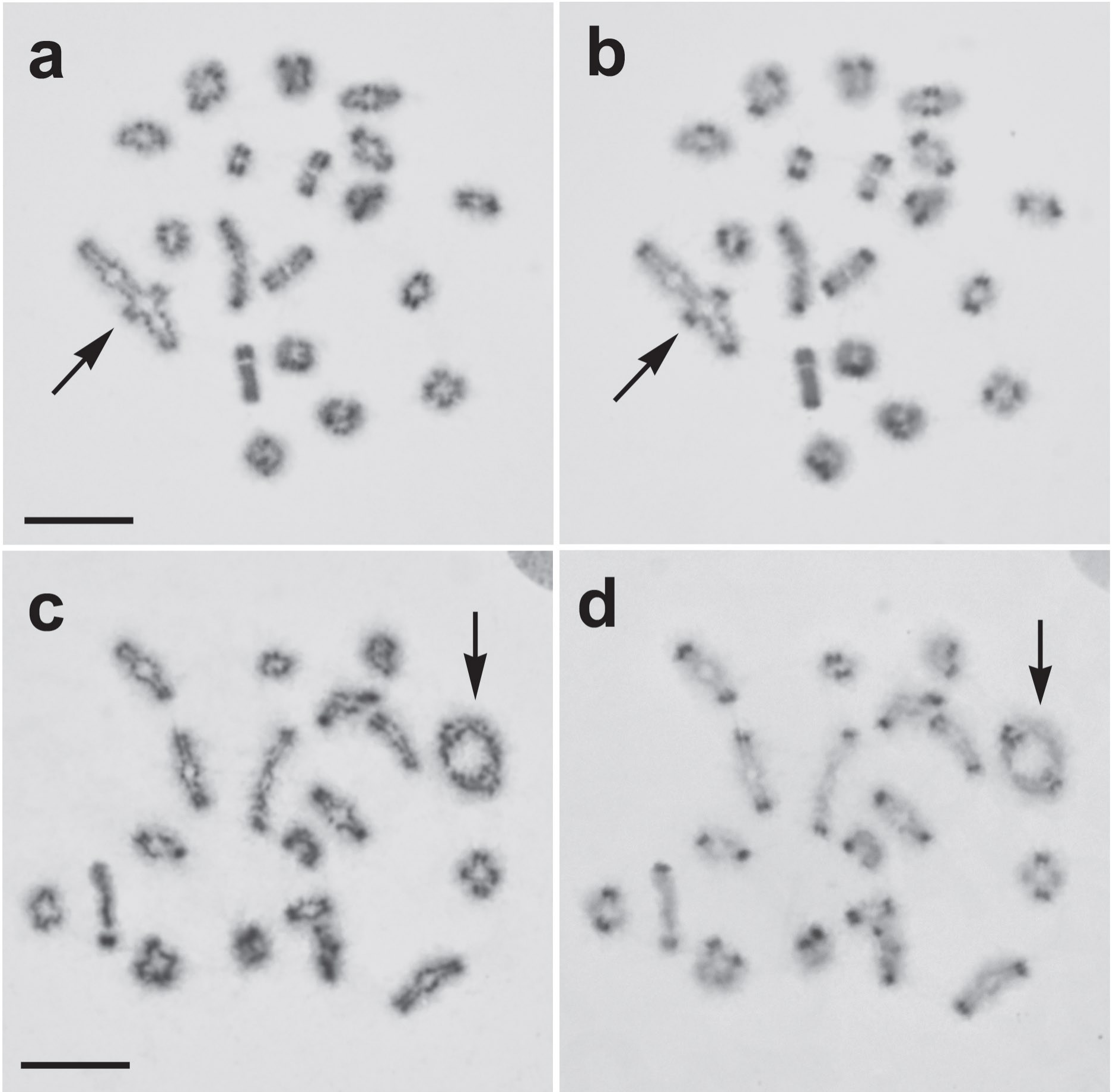
Figure 6. Fetuses derived from the testicular spermatozoa of infertile mice exposed to acute γ-rays at 2 Gy on day 16.5 pc. Image demonstrates 6 normal fetuses (lower section) and 2 dead conceptuses (upper section) harvested from one side of the uterus of a female on day 16.5 of gestation. Scale bar, 1 cm.


Prenatal period
(Birth)
Postnatal period
Days
14.5 15.5 16.5 17.5 18.5 19.5 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day 16.5 pc
Day 4 pp
Day 11 pp
quiescent gonocytes
mitotic gonocytes
primary spermatocytes
Spermatogenesis
spermatogonial stem cells and spermatogonia
Irradiation (2 Gy)
acute
Days 15.5 16.5 17.5 18.5 19.5 pc
Day 4 pp
Day 11 pp
continuous
Days 14.5 - 19.5 pc
Days 2 - 7 pp
Days 9 - 14 pp


250
200
150
100
50
0
Testicular weight (mg)
*
*
*
*
*
*
Control
15.5 pc
16.5 pc
17.5 pc
18.5 pc
19.5 pc
4 pp
11 pp
14.5-19.5 pc
2-7 pp
9-14 pp
acute
continuous
Day(s) of irradiation


300
250
200
150
100
50
0
no germ cells
with germ cells
Number of cross sections of seminiferous tubules
*
Control
15.5 pc
16.5 pc
17.5 pc
18.5 pc
19.5 pc
4 pp
11 pp
14.5-19.5 pc
2-7 pp
9-14 pp
acute
continuous
Day(s) of irradiation


a
b
c
d


a
b
c
d

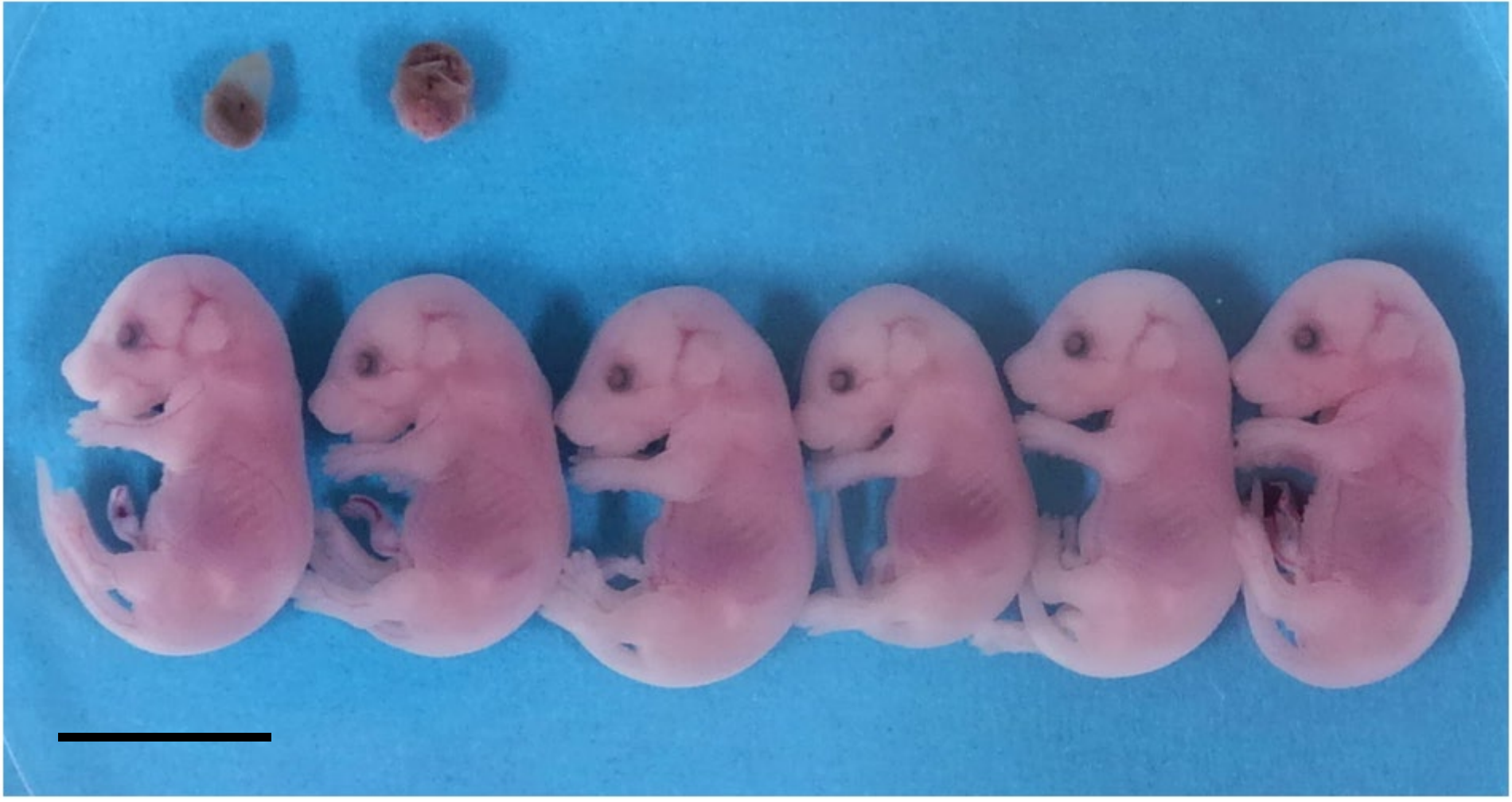


Table 1. Chromosome analysis of primary spermatocytes derived from mice exposed to acute or continuous 2 Gy γ-ray irradiation during the prenatal and postnatal periods (assay at 10 weeks of age)

Day(s) of radiation exposure	No. of males used	No. of cells analyzed	No. (%) of cells with multivalent chromosomes	No. of multivalent chromosomes	Configuration of multivalent chromosomes	
					Chain	Ring
Control	4	1320	1 (0.1)	1	1	0
Acute irradiation						
Prenatal period						
16.5 pc[1]	3	1016	1 (0.1)	1	0	1
Postnatal period						
4 pp[2]	4	1279	16 (1.3)*	17[#]	14	3
11 pp	4	1254	62 (4.9)*	62	39	23
Continuous irradiation						
Prenatal period						
14.5−19.5 pc	2	1071	1 (0.1)	1	0	1
Postnatal period						
2−7 pp	2	1039	8 (0.8)	8	7	1
9−14 pp	2	1031	3 (0.3)	3	2	1

[1]pc: post-coitus.

[2]pp: post-partum.

[#]One cell contained two multivalent chromosomes.

[*]Significant difference ($P < 0.05$) compared with the control mice.

Table 2. Chromosome analysis of zygotes produced by *in vitro* fertilization with epididymal spermatozoa derived from fertile mice exposed to acute and continuous 2 Gy γ-ray irradiation during the prenatal and postnatal periods (assay at 10 weeks of age)

Day(s) of radiation exposure	No. of males used	No. of oocytes used	No. (%) of oocyte penetration	No. of 2*n* zygotes analyzed	No. (%) of zygotes with chromosomal aberrations	Chromosomal aberrations	
						Structural aberration (%)	Aneuploidy (%)
Control	4	198	188 (94.9)	121	3 (2.5)	2 (1.7)	1 (0.8)
Acute irradiation							
Postnatal period							
4 pp[1]	4	390	283 (72.6)*	250	15 (6.0)	7 (2.8)	8 (3.2)
11 pp	4	495	353 (71.3)*	321	16 (5.0)	10 (3.1)	6 (1.9)
Continuous irradiation							
Prenatal period							
14.5−19.5 pc[2]	4	313	142 (45.4)*	133	8 (6.0)	4 (3.0)	4 (3.0)
Postnatal period							
2−7 pp	4	186	174 (93.5)	136	3 (2.2)	0 (0)	3 (2.2)
9−14 pp	4	159	158 (99.4)	142	4 (2.8)	2 (1.4)	2 (1.4)

[1]pp: post-partum.

[2]pc: post-coitus.

*Significantly lower ($P<0.05$) compared with the control.

Table 3. Chromosome analysis of zygotes produced by ICSI using testicular spermatozoa derived from infertile mice exposed to acute 2 Gy γ-ray irradiation on day 16.5 post-coitus (pc) (assay at 10−11 weeks of age)

Day of radiation exposure	No. of males used	No. of oocytes injected	No. (%) of ova with incomplete sperm nuclear decondensation	No. (%) of 3*n* zygotes	No. (%) of 2*n* zygotes	No. of 2*n* zygotes analyzed	No. (%) of 2*n* zygotes with chromosomal aberrations	Chromosomal aberrations	
								Structural aberration (%)	Aneuploidy (%)
Control	4	170	26 (15.3)	6 (3.5)	138 (81.2)	124	14 (11.3)	12 (9.7)	2 (1.6)
16.5 pc	6	267	37 (13.9)	11 (4.1)	219 (82.0)	188	36 (19.2)	31 (16.5)	6[#](3.2)

[#]one aneuploid embryo was accompanied with a chromosome fragment.