Exposure of the mouse perinatal testis to radiation leads to hypospermia at sexual maturity

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Abstract

The first round of mouse spermatogenesis begins from 3 to 4 days after birth through differentiation of gonocytes into spermatogonial-stem cells and type A spermatagonia. Consequently, this step of differentiation may determine generation of the original population of stem cells and the fertility potential of the adult mouse. We aimed to determine the effect of perinatal exposure to ionizing radiation on the testis at the end of the first wave of spermatogenesis and at sexual maturity. Our results show that, radiation sensitivity of the testis substantially decreases from late foetal life to the end of the first week after birth. In addition, partial or full recovery from radiation induced testicular weight loss occurred between the first round of spermatogenesis and sexual maturity, and this was associated with the stimulation of spermatogonial proliferation. Exposure of mice at 17.5 days after conception or at 1 day after birth to γ-rays decreased the sperm counts at sexual maturity, while exposure of 8 day-old mice had no effect. This suggests that irradiation of late foetal or early neonatal testes has a direct impact on the generation of the neonatal spermatogonial-stem cell pool.

Introduction

The first round of mouse spermatogenesis begins shortly after birth, when gonocytes migrate towards the periphery of the seminiferous tubules, resume proliferation and differentiate into spermatogonial-stem cells and type A spermatagonia (de Rooij & Grootegoed 1998). These primitive spermatagonia produce differentiated progenitor cells in a stepwise, synchronous manner. The seminiferous epithelial cycle in young mice is much shorter than in adults (7.5 days between 10 and 30 days of age versus 8.6 days in the adult; Kluin et al. 1982). By the time the first mature mouse spermatozoa appear (~35 days postpartum), spermatogenic cells have established a unique cellular organization in the seminiferous tubule. Yoshida et al. (2006) have recently shown that spermatozoa generated from the first round of spermatogenesis do fertilize eggs in vitro but are not efficient in vivo. At 2 months of age mice are sexually mature.

Early neonatal gonocytes do not exhibit features of stem cells (McLean et al. 2003) but can generate pluripotent stem cells (Kanatsu-Shinohara et al. 2004). These gonocytes constitute a heterogeneous population of cells with distinct fates (Orwig et al. 2002). In this context, if early neonatal germ cells are the direct precursors of spermatogonial-stem cells, then they may be either prone to die when too severely damaged or particularly protected from DNA damage in order to ensure establishment of a damage-free pool of stem cells.

Many studies have addressed the effect of either X- or γ-irradiation in foetal, pre-pubertal and adult rodents in terms of cell death (Moreno et al. 2001) or repopulation indices (van der Meer et al. 1992a, 1992b, Vergouwen et al. 1994). Radiosensitivity of foetal gonocytes to death peaks during mitotic arrest (Vergouwen et al. 1995, Moreno et al. 2001), and a few studies in the rat have shown that postnatal testis is more radioresistant than late foetal testis (Hughes 1962, Erickson & Blend 1976). Unfortunately, none of these studies analyzed the outcome of radiation exposure beyond the first round of spermatogenesis.

The purpose of this study was to characterize the changes in testicular radiosensitivity of the neonatal mouse more precisely, over the medium term. The ages at irradiation were chosen to compare germ cells at different stages of their development: during gonocyte quiescence (17.5 days post conception; E17.5), when gonocytes resume mitosis to further differentiate into spermatogonia (1 day post partum; P1), and after the establishment of both stem and type A spermatogonial cells (8 days post partum; P8). Indeed, we asked to what extent ionizing radiation affects testicular...
development, sperm production and fertility of the mouse according to the developmental stage of germ cells at the time of irradiation.

Results

Partial or total recovery of adult testis weight after acute irradiation during perinatal life

Regardless of the dose (2 or 3 Gy), when mice were irradiated at E17.5 or P1, body weight at both P35 and P60 was significantly reduced by 12–20% compared with the control (data not shown). A dose-dependent decrease in body weight was only observed at P60 for mice irradiated at E17.5 (data not shown). Whatever the dose and the age at analysis, the body weight was not different from the control when mice were irradiated at P8 (data not shown). In all cases, no mouse died during the experiments.

Mice irradiated at a dose of 2 Gy at E17.5 or P1 showed testis weights decreased by half compared with the control at P35 (Fig. 1). At P60, the decrease was less pronounced, being about 30%. By contrast, irradiation of P8 mice at the same dose had no effect (Fig. 1).

Increasing the dose of radiation from 2 to 3 Gy led to a greater decrease in testicular weight at both P35 and P60 ($P=0.0083$ at P35; $P=0.0003$ at P60) when mice were irradiated at E17.5 (Fig. 1). By contrast, no dose-effect response was observed in mice irradiated at P1. Irradiation at 3 Gy of P8 mice decreased their testis weight at P35, by 29% ($P=0.0016$). At P60, the recovery of testis weight was complete upon irradiation at P8, but only partial after irradiation at E17.5 and P1. Altogether, these results clearly indicate the substantial decrease in radiosensitivity of the testis between late foetal life and the end of the first week after birth in the mouse.

Contrast in total spermatozoa reduction at P60 following irradiation at E17.5 and at P1

Epididymis weight at P60 was significantly decreased (by 10–20%) compared with the control ($P<0.05$), except when mice were irradiated at 2 Gy at P8. Whatever the dose, there was no significant difference between epididymis weight of E17.5 and P1 irradiated mice (data not shown), suggesting that at these ages the epididymis exhibits similar radiosensitivity.

Upon irradiation at E17.5, sperm counts were reduced by about 40% and 90% compared with controls at 2 and 3 Gy respectively (Fig. 2). Irradiation at P1 reduced the sperm counts by 25%, independently of the dose (2 or 3 Gy). By contrast, there was no decrease in total spermatozoa counts of P60 mice irradiated at P8 regardless of dose.

At P60, whatever the age at irradiation and the radiation dose (2 or 3 Gy), delay in conception, fertility, litter size and sex ratio were all unaffected, except the litter size from males irradiated at E17.5 (Table 1). However, mating of these males (irradiated at E17.5) at P102 with the same non-irradiated females did not show any alteration of all the features described above, even litter size (not shown).

To search for the dose that could actually lead to mouse sterility upon irradiation at E17.5, we further increased the radiation dose to 4 Gy. We found that only one out of four irradiated male mice was fertile at P60 and P120 (Table 2) but died at P168 (24 weeks of age). Among the three sterile males, one died at P98 (14 weeks of age) and the two others at P168. For comparison, when P1 male mice ($n=4$) were exposed at 4 Gy, all were fertile at both P60 and P120 (Table 2), with no mortality observed even at P168. Accordingly, sperm counts tended to be higher in P1 ($n=5$) rather than in E17.5 ($n=4$) irradiated mice even if the difference was not significant ($P=0.3$; Fig. 2). The increased rate of...
sterility in E17.5 mice irradiated at 4 Gy compared with those irradiated at 3 Gy was not related to decreased sperm counts (Fig. 2).

Seminiferous tubules with Sertoli cells (SCO) appear only after exposure to 3 Gy

The marked decrease in testis weight and sperm counts in mice irradiated specifically at E17.5 and P1 was an indication of overall loss of germ cells including spermatogonial-stem cells. Therefore, we characterized the histological changes occurring in P35 and P60 mice irradiated at E17.5, P1 and P8 (Fig. 3).

At both P35 and P60, irradiated testes showed various tubule alterations from the presence of vacuoles in the cytoplasm of Sertoli cells to the complete absence of germ cells (SCO) in cross-sections of seminiferous tubules (Fig. 3). At a dose of 2 Gy, a mean of 70 ± 10, 7 ± 5 and 21.4 ± 9% of tubules exhibited vacuoles in P35 testes when irradiation exposure occurred at E17.5, P1 and P8 respectively.

However, SCO tubules were only observed after a radiation dose of 3 Gy. Using DDX4 (mouse vasa homologue, MVH) as a specific marker of germ cells (Fig. 3D), we calculated the percentage of SCO tubule cross-sections at both P35 and P60 (Fig. 4). At P35, whereas 71.4 ± 4.6% of the seminiferous tubules were SCO upon irradiation at E17.5, this percentage decreased to 4.87 ± 1.82% and 0.24 ± 0.05) (Fig. 4). At P60, similar results were obtained (P > 0.05) (Fig. 4).

Delay in elongated spermatid production during the first wave of spermatogenesis

Kinetics of spermatid production during the first wave of spermatogenesis was estimated by counting at P35 the percentage of non-altered tubule cross-sections (i.e. with no vacuole) containing either elongated spermatids, or differentiated cells as far as the round spermatid stage or no round spermatids at all (Fig. 5). At the dose of 2 Gy, the percentage of tubules containing elongated spermatids was markedly decreased compared with the control (by 64%) only when irradiation occurred at E17.5 (P < 0.05). Although, this percentage tended to decrease for P1 and P8 irradiated mice (by 20% for both) it was not significantly different from the control. At a dose of 3 Gy, whatever the age at irradiation, a significant delay in elongated spermatid production was observed compared with the control (P < 0.05).

Increased spermatogonial-proliferative activity at P35 may explain testis weight recovery at P60 after irradiation at 3 Gy

Because either partial or total recovery of the testis weight of irradiated mice was observed between P35

Table 1 Fertility features at P60 of mice irradiated (3 Gy) at E17.5, P1 and P8.

<table>
<thead>
<tr>
<th>Age at irradiation</th>
<th>Number of pregnant females (%)</th>
<th>Delay for conception &lt;4 days</th>
<th>Delay for conception 4–8 days</th>
<th>Delay for conception over 8 days</th>
<th>Litter size ± S.E.M.</th>
<th>Sex ratio (M/F) ± S.E.M.</th>
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<tbody>
<tr>
<td>0 Gy</td>
<td>8 (100)</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>14.6 ± 0.7</td>
<td>11.3 ± 1.3*</td>
</tr>
<tr>
<td>3 Gy</td>
<td>8 (100)</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>10.6 ± 1.6</td>
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<td>0 Gy</td>
<td>8 (100)</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>12.0 ± 1.2</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>3 Gy</td>
<td>8 (100)</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>14.3 ± 1.2</td>
<td>15.1 ± 0.3</td>
</tr>
</tbody>
</table>

Statistical difference compared with the control is indicated by an asterisk (*P < 0.05).

Table 2 Fertility features at P60 and P120 of mice irradiated (4 Gy) at E17.5, P1.

<table>
<thead>
<tr>
<th>Age at irradiation</th>
<th>Age at mating</th>
<th>Number of pregnant females (%)</th>
<th>Delay for conception &lt;4 days</th>
<th>Delay for conception 4–8 days</th>
<th>Delay for conception over 8 days</th>
<th>Litter size ± S.E.M.</th>
<th>Sex ratio (M/F) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>4 Gy</td>
<td>8 (100)</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>14.6 ± 0.7</td>
<td>11.3 ± 1.3*</td>
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<td>3 Gy</td>
<td>4 Gy</td>
<td>87.5</td>
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<td>0</td>
<td>10.6 ± 1.6</td>
<td>13.8 ± 0.6</td>
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<tr>
<td>0 Gy</td>
<td>4 Gy</td>
<td>87.5</td>
<td>12.5</td>
<td>0</td>
<td>12.0 ± 1.2</td>
<td>11.0 ± 0.9</td>
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<tr>
<td>3 Gy</td>
<td>4 Gy</td>
<td>87.5</td>
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<td>0</td>
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<td>15.1 ± 0.3</td>
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Statistical difference compared with the control is indicated by an asterisk (*P < 0.05).
and P60 (see above), we looked at the spermatogonial-proliferative activity by measuring the number of cyclin D1-positive cells (Beumer et al. 2000; Fig. 6A) in tubule cross-sections exhibiting no vacuole. This study allows specific analysis of tubules segments assumed to be fully functional in both irradiated and control testes. A significant increase in the number of cyclin D1-positive cells per tubular cross-section was observed in testes of P35 mice irradiated at E17.5 and P8 compared with the control (P < 0.008; Fig. 6B). Whereas, 37.4 ± 5.5% of tubular cross-sections contained more than 30 cyclin D1-positive cells in the control, this percentage increased to 69.6 ± 14.3 and 67.5 ± 23.8% in testis of mice irradiated at E17.5 and P8 respectively (χ² = 24.65; P < 0.0001; v = 2). These differences were absent in P60 mice.

The diameter of the tubules in irradiated testes was reduced by about 10% at P35 but not at P60 compared with control (Fig. 6C). To take into account tubule shrinkage in irradiated testes at P35, we determined the ratio between cyclinD1- and GATA4-positive cells (i.e. Sertoli cells; Fig. 6D). Compared with the control, the ratio of proliferating spermatogonia number on the Sertoli cell number was still increased 1.3- and 1.6-fold after irradiation at E17.5 and P8 respectively. Therefore, these data suggest that the increase in proliferative activity of spermatogonia and further differentiation of these cells may account for the testis weight recovery observed between P35 and P60 mice irradiated at E17.5 and P8.

**Figure 3** Testicular histology of control and irradiated mice at E17.5, P1 and P8. A–C: Sections of testis from P35 mice stained with hematoxylin and eosin to show morphology of the seminiferous tubules in control mice (A) and in mice irradiated (3 Gy) at P1 (B) and P8 (C). Arrows indicate vacuolization of some seminiferous tubules in irradiated mice. Arrow heads indicate interstitial-cell hyperplasia. (D). Immunohistochemistry with anti-DDX4 antibody showing SCO seminiferous tubules (asterisk) in testis from a P35 mouse irradiated at E17.5. Bar = 30 μm.

**Figure 4** Percentage of SCO seminiferous tubule cross-sections at P35 and P60 after a 3 Gy irradiation at E17.5, P1 or P8. Data are presented as means ± S.E.M. of at least three animals.

**Figure 5** Analysis at P35 of the production of spermatids after irradiation. Percentage (± S.E.M.) of tubular cross-sections containing either no spermatids (black bars), rounded spermatids only (grey bars) or elongated spermatids (white bars) were counted in sets of at least three P35 mice irradiated at E17.5, P1 or P8. The radiation dose (Gy) is indicated above each graph.

**Figure 6** Analysis of the cyclin D1-positive cell number per seminiferous tubule cross-section. Representative immunohistochemistry staining of P60 mouse testis (irradiated at E17.5 at the dose of 3 Gy) for cyclin D1, a marker of proliferating spermatogonia. Sections were counterstained with hematoxylin. Bar = 30 μm. (B) Graph represents the number (± S.E.M.) of cyclin D1-positive cells per seminiferous tubule cross-section in testis from P35 and P60 mice irradiated at E17.5 (grey bars) and P8 (white bars) respectively. Significant differences from the control (black bars) are indicated (***P < 0.01). (C) Graph represents diameter (± S.E.M.) of seminiferous tubule cross-sections of P35 and P60 mice irradiated at E17.5 (grey bars) and P8 (white bars) respectively. Significant differences from the control (black bars) are indicated (*P < 0.05; **P < 0.01). (D) Graph represents the ratio (± S.E.M.) of cyclin D1-positive over Sertoli (GATA4-positive) cells per seminiferous tubule cross-section in testis from P35 mice irradiated at E17.5 (grey bars) and P8 (white bars) respectively. Significant differences from the control (black bars) are indicated (*P < 0.05).
**Germ cell rather than Sertoli cell death after perinatal irradiation (3 Gy) accounts for sperm count loss at adulthood**

By contrast to adult Sertoli cells, the neonatal ones show proliferative activity (De Kretser & Kerr 1988). The latter have been shown to be sensitive to radiation in both mice and rats (Allan et al. 1988, de Rooij & Vergouwen 1991). Moreover, the specific depletion by antimitotic drug of neonatal rat Sertoli cell population has shown to contribute to adult testis weight loss (Orth et al. 1988). We sought to compare the contribution of germ cell and Sertoli cell death in the marked decrease in sperm count at adulthood between E17.5 and P1 mice irradiated at the dose of 3 Gy. For that, we used immunohistochemistry (Fig. 7A and B) to determine total number of germ and Sertoli cells per testis (Fig. 7C and D) 24 h after radiation exposure and the percentage of these cells that were positive for cleaved caspase-3 (Fig. 7E and F) as an indicator of apoptotic activity.

According to previous data in the rat (Moreno et al. 2001), no short term germ cell loss was observed upon irradiation of E17.5 mice when gonocytes are quiescent. By contrast, after irradiation at P1, when germ cell re-enter the cell cycle, about 40% of the gonocytes are eliminated within the first 24 h after exposure (Fig. 7C). Whatever the age at irradiation, no loss of Sertoli cells was observed in the same interval of time (Fig. 7D). Therefore, in these conditions, germ cells are more sensitive to radiation than the Sertoli cells. Apoptosis was induced by radiation exposure in both cell types (Fig. 7A, B, E and F). A similar percentage of Sertoli cells positive for cleaved caspase-3 was found upon irradiation at E17.5 and P1 (P=0.12; Fig. 7F), suggesting that their radiation sensitivity remain unchanged in the foetus and the newborn. Therefore, loss of these cells cannot fully explain the drastic loss of sperm counts at adulthood in E17.5 compared with P1 irradiated mice. Taken together, these data suggest that although Sertoli cell loss may account for loss of sperm count at adulthood after irradiation, it contributes only to a minor extent.

**Discussion**

This study shows that irradiation during germ cell development in the foetal (E17.5, during gonocyte quiescence) and early neonatal (P1, when gonocytes resume mitosis) mouse testis significantly affects production of spermatozoa at sexual maturity, even if fertility is unaffected at doses below 4 Gy. By contrast, irradiation at P8 (after the establishment of both stem and type A spermatogonial cells) has no effect.

As shown by Vergouwen et al. (1995), we found that germ cells irradiated during their mitotic arrest in the foetus (E18) are particularly sensitive to death, leading to a slowing down of the germ cell differentiation; however, their study was limited to the first round of spermatogenesis. Extending this study to sexual maturity, we observed that after irradiation at E17.5, there was a partial restoration of the testicular weight between 35 days and 60 days of age that could be due to an increase of spermatogonial proliferation in the remaining active seminiferous tubules. However, at a dose of 3 Gy, 70% of the tubule cross-sections analyzed were completely devoid of germ cells that led to a 90% decrease in sperm cell count at P60 and a 65% loss of testicular weight compared with the control. In spite of the dramatic decrease in sperm counts, the animals were fertile, consistent with the low threshold of spermatozoa required for successful fertilization in the mouse (about 10% of normal sperm count; Krishnamurthy et al. 2001).

Transition from gonocyte to spermatogonia was shown to be accompanied by a decrease in testicular radiosensitivity of the immature rat (Hughes 1962), boar (Erickson 1964) and bull (Erickson et al. 1972). In rodents, this transition is concurrent with the emergence of the germ cells from their cell cycle arrest in G0/G1 (de Rooij & Grootegoed 1998). Radiosensitivity of somatic mammalian cells has been shown to decline as they progress through G1 and enter S phase (Sinclair 1968, Hinz et al. 2005). Accordingly, we found that
in P1 mice, about 20% of germ cells are in S phase (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/). This could partly explain the marked difference in radiosensitivity of E17.5 mouse germ cells compared with that of P1 germ cells. In contrast to mice irradiated at E17.5, we found no dose-dependent decrease (between 2 and 3 Gy) in either testicular weight or sperm production of mice irradiated at P1. Recently, Yoshida et al. (2006) have hypothesized that there could exist two subpopulations of germ cells in the neonatal testis: the ngn3-negative lineage would lead to production of the first spermatozoa at 35 days post partum, and the ngn3-positive lineage would be involved in the continuous production of spermatozoa throughout the reproductive life of the mouse. We found that within the first 24 h after irradiation of P1 mice at a dose of 2 (Forand et al. 2009) and 3 Gy, about 40% of the gonocytes were eliminated. Subsequently, at P60, sperm production decreased by 25%. In this context, our results are in agreement with the presence at P1 of a subpopulation of germ cells that are radiosensitive and whose elimination directly affects the production of spermatozoa at sexual maturity. This subpopulation would thus be a part of the ngn3-positive lineage. This suggests that killing stem cell precursors may quantitatively alter the neonatal stem cell pool required at least for the first rounds of spermatogenesis.

Exposure of P8 mice to an amount of 2 Gy of γ-rays caused substantial loss of spermatogonia within the first day after treatment similar to loss of gonocytes after irradiation of P1 mice (Forand et al. 2009). However, the sperm counts at P60 were not affected (the present study). This suggests that, once established, spermatogonial-stem cells confer testicular radioresistance and ensure normal amounts of spermatozoa at sexual maturity. Interestingly, we found a significant increase compared with control in the number of mitotically active spermatogonia per Sertoli cell in P35 mice irradiated either at E17.5 or P8. We propose that induction of spermatogonial-proliferative activity and further differentiation of these cells explain the partial or complete restoration of testicular weight between P35 and P60 of mice irradiated at E17.5 or P8. Therefore, the fact that testicular weight undergoes only partial recovery at P60 after irradiation at E17.5 is likely to be a consequence of the elevated percentage of SCO tubules that is stable between P35 and P60. Interestingly, Ebata et al. (2007) showed that, in contrast to adult spermatogonial-stem cells, the balance between self-renewal and differentiation is apparently shifted toward differentiation rather than to self-renewal in pups (P6–P9). This may explain that, in the injured immature testis, the spermatogonial-proliferative activity is stimulated, restoring sperm production at P60. Because at P60, the number of mitotically active spermatogonia was similar in control and irradiated mice, we speculate that equilibrium is achieved between germ cell proliferation and differentiation during repopulation of the seminiferous epithelium between P35 and P60. Further investigations are required, however, to unravel the mechanisms involved in this regulation.

In contrast to adult Sertoli cells that are fully differentiated and do not proliferate, foetal and neonatal Sertoli cells actively proliferate (De Kretser & Kerr 1988). Moreover, whereas quiescent Sertoli cells are highly radioresistant (Vergouwen et al. 1994), proliferating foetal or neonatal Sertoli cells have been found radiosensitive (Erickson & Blend 1976, Allan et al. 1988). Because Sertoli cell number in rat neonates may determine at least in part normal sperm counts at adulthood (Orth et al. 1988), we cannot exclude that decrease in sperm counts at sexual maturity upon irradiation of foetal and neonatal mouse testes would result in part from Sertoli cell apoptosis. Twenty four hours after acute exposure of P1 mice at 3 Gy, we found substantial loss of germ cells but no significant alteration of Sertoli cell number. Moreover, at both E17.5 and P1, the proliferative activity of Sertoli cells is similar (Vergouwen et al. 1991) and we found that radiosensitivity is also similar. These results are in agreement with the known higher resistance of Sertoli cells than germ cells in the foetal testis (Vergouwen et al. 1995, Lambrot et al. 2007). Therefore, the contrasting testicular radiosensitivity of E17.5 and P1 mice might not rely predominantly on Sertoli cell loss. Nevertheless, irradiation of Sertoli cells could affect gonocyte differentiation as that of spermatogonia in the adult rat (Zhang et al. 2007). Interestingly, the in vivo inhibition of a signalling pathway between mouse neonatal Sertoli and germ cells severely reduced the number of gonocytes and their capability to differentiate into spermatozoa (Basciani et al. 2008). This lead to alteration of sperm production at adulthood without any decrease in Sertoli cell number (Basciani et al. 2008). Altogether, the possible radiation induced stem cell pool alteration suggested by our results would predominantly rely on gonocyte death with a possible contributory role of Sertoli cell exocrine function alteration.

The decrease in testicular radiosensitivity between E17.5 and P8 particularly at the dose of 3 Gy may raise an important issue considering the potential transmission of mutations to the progeny. Using the specific-locus test after irradiation of foetal and postnatal mice at 3 Gy, Selby (1973) showed a twofold increase in the mutation rate in the progeny of mice irradiated at P8 compared with progeny of mice irradiated as foetuses or neonates. Mutation frequency was comparable for P8 and adult mice. It is tempting to speculate that foetal and neonatal germ cells must be particularly protected from mutation because the original spermatogonial-stem cells derive from them and determine the adult sperm production. However, the present study shows that the high
variability of germ cell radiosensitivity according to the age at the time of irradiation could explain the results obtained by Selby (1973). Indeed, irradiation (at 3 Gy) would result in the more extensive counter selection of E17.5 and P1 germ cells carrying mutations compared with P8 germ cells as shown by the percentage of SCO tubules. Moreover, E17.5 and P1 germ cells are stem cell precursors but not true spermatogonial-stem cells that are known to be more radioresistant than differentiated spermatogonia (van der Meer et al. 1992b). Therefore, partial loss of these precursors could lead to impairment of the original pool of stem cells as suggested by the loss of sperm production in P60 mice irradiated at E17.5 and P1. The extent of this loss is related to the intrinsic radiosensitivity of the germ cells that is higher in quiescent rather than in proliferating germ cells (van der Meer et al. 1992a, Vergouwen et al. 1995). By contrast, irradiation at P8 would extensively kill differentiated spermatogonia but not the spermatogonial-stem cells that could then divide to repopulate the damaged testis. Therefore at P60, the recovery from loss of testis weight is more efficient when mice were irradiated at P8 rather than at E17.5 or P1. These spermatogonial stem cells could, however, survive in the presence of mutations in their genome, leading to a higher mutation frequency in the progeny of irradiated P8 mice than of E17.5 or P1 irradiated mice. It would be therefore interesting to estimate the heritability of radiation-induced mutations in foetal and neonatal germ cells at lower doses to avoid the ‘bias’ of their variable sensitivity to death.

Because the formation of spermatogonia in humans occurs during the foetal and postnatal life, from the 15th to the 16th week of gestation to puberty (Pauls et al. 2006), our data on rodents could have implications for male paediatric patients undergoing total body irradiation for bone-marrow ablation therapy prior to hematopoietic stem cell transplantation (Wallace et al. 2005). Indeed, these patients are at high risk of testicular dysfunction (Wallace et al. 2005). Our results in mice suggest that they would also be at risk for reduced sperm counts and possibly for hypo fertility at adulthood. Interestingly, we found that adult mice irradiated at E17.5 at doses of 3 and 4 Gy exhibited a similar reduction (90%) in sperm counts compared with non-irradiated control but sterility of some animals only occurred at the highest dose. This suggests that in mice as in humans (Andersson et al. 2008) a severe decrease in concentration of sperms does not necessarily affect fertility. Nevertheless, despite the low power of sperm count as a reliable indicator of infertility in men (Guzick et al. 2001) the threshold between subfertile and fertile men has been set at a sperm concentration of 40.10^6/ml (Bonde et al. 1998). A decrease by half of this concentration is associated with substantial infertility (oligospermia).

Human adult testis is more sensitive to radiation than rodent testis (Clifton & Bremner 1983, Meistrich & Samuels 1985, de Rooij & Vergouwen 1991), and human foetal testis is even more radiosensitive (Lambrot et al. 2007). Therefore, the dose that could affect the fertility of men irradiated as children could be much lower than that which leads to infertility of mice irradiated as foetuses or newborns. With the increased rates and durations of cancer survival, especially of children and young adults (Schover & Meistrich 2005), it becomes important to evaluate their risk of suffering from hypo fertility and/or transmitting mutations to their progeny.

Materials and Methods

Mice and irradiation

A series of at least five NMRI mice per experimental condition were whole body irradiated at 2 and 3 Gy (dose rate 0.6 Gy/min) at 17.5 days post conception (E17.5), 1 day post partum (P1) and 8 days post partum (P8), using a 137Cs source (IBL 637; CIS bio International, France). For some experiments, a series of 4 to 5 E17.5 or P1 mice were irradiated at 4 Gy. The animals were used and maintained according to French regulations (Ministry of Agriculture, decree 87–848). The animal facilities are accredited by the veterinary inspectorate (A92-032-02).

Histology

Testes from 35-days old (P35) or 60-days old (P60) mice were fixed overnight in either Bouin’s fixative or 10% formalin (Panreac, Barcelona, Spain) before being dehydrated, embedded in paraffin wax and cut into 5 μm sections that were stained with hematoxylin and eosin. At least 300 seminiferous tubules cross-sections for each category were screened to determine the percentage in which spermatogenesis had proceeded up to the appearance of round spermatids or even further.

Immunohistochemistry

Formalin-fixed testes of P35 and P60 mice were used for immunostaining with either DDX4 (Abcam, Cambridge, UK), cyclin D1 (Neomarkers, Fremont, CA, USA; RM9104) or GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-1237) using the same protocol for antigen retrieval. After testis section dewaxing and rehydration, antigen retrieval was performed in sodium citrate buffer (pH 6) in a microwave oven (5 min at 620 W and 2 × 5 min at 400 W). Sections were then washed in PBS and incubated for 10 min in 3% H2O2 at room temperature. After 30 min in 5% BSA/PBS (w/v), primary antibodies (1:50 for cyclin D1; 1:400 for DDX4) diluted in blocking buffer (5% BSA in PBS) were incubated for 1 h at room temperature. Primary antibodies were detected with a biotinylated goat anti-rabbit secondary antibody and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit,
Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized using either 3,3′-diaminobenzidine or novaaRed (Vector Laboratories) as substrate.

The percentage of SCO tubules (without DDX4-positive cells) was determined by analyzing at least 100 seminiferous tubules from four individual mice.

The number of cyclin D1- or GATA4-positive cells was counted in tubules devoid of vacuoles for four individual animals and averaged. From 50 to 100 tubules were scored per testis.

Tests from foetal and neonatal mice were fixed in 4% phosphate buffered formaldehyde overnight at 4°C, embedded in paraffin and 5-μm sections were cut. Immunohistochemistry for cleaved caspase-3 was performed essentially as described above except that antigen retrieval was carried out for 5 min at 900 W and then for 3 min at 600 W. Primary antibody was a rabbit polyclonal antibodies against cleaved caspase-3 (1:100; Cell Signalling Technology, Beverly, MA, USA), in PBS/5% BSA overnight at 4°C. Primary antibody was detected after incubation with secondary biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories) and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Peroxidase activity was visualized using 3,3′-diaminobenzidine and slides were washed in PBS for 10 min. The second staining was performed using rabbit anti-DDX4 (1:500, Abcam) primary antibody. Secondary antibodies used were the same as previously described, and bound antibodies were visualized using VIP substrate (Vector Laboratories). Sections were counterstained with hematoxylin.

Germ and Sertoli cells were counted in one of 20 sections equidistantly distributed along the testis as previously described (Delbé et al. 2004). All counts were done with Histolab software (Microvision Instruments, Evry, France).

Sperm counts
Sperm counts were performed on one total epididymis of P60 mice (n=5 per experimental condition) as described by Meistrich et al. (1978). Briefly, each frozen epididymis was homogenized in 3 ml PBS with a Dounce homogenizer and further by sonication (30 × 30 s at medium power). Sperm heads were counted under the microscope using a Malassez cell. Four counts were performed for each sample and the mean value was calculated.

Fertility test
Each P60 male from the control (n=4) and irradiated groups (n=4) was housed with two females of proven fertility from the same strain for three consecutive weeks. Females were then isolated a few days before parturition. Day of birth and litter size was recorded.

Statistical analysis
The results are presented as means ± S.E.M. Statistical analyses were performed using either the Student t-test or the non-parametric Mann–Whitney U-test. In all cases, the level of statistical significance was set at P<0.05.

Declarations of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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