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# Irradiation Causes Acute and Long-Term Spermatogonial Depletion in Cultured and Xenotransplanted Testicular Tissue from Juvenile Nonhuman Primates

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**Infertility is a serious late effect in childhood cancer survivors. Little is known about acute irradiation effects in immature primate testis. Radiation defects have previously only been studied in postpubertal primates. Here we use the juvenile rhesus monkey as a preclinical model. We expose fragments of testicular tissue to 0, 0.5, 1.0, and 4.0 Gy irradiation *in vitro*. We then maintain the fragments in organ culture for 24–48 h or xenograft the fragments into nude mice for 4 months. Histological endpoints were determined to explore the cellular responses to the irradiation. At the highest dose, irradiation provoked an acute depletion of A-spermatogonia and a rise of apoptotic germ and Sertoli cells in organ culture. A dose-dependent decrease in the number of seminiferous tubules containing type A dark and type A pale spermatogonia was observed in irradiated xenografts. The number of Sertoli-cell only tubules increased respectively. Outgrowth of grafts was affected by the 4-Gy dose. Our observations reveal that irradiation evoked an immediate and sustained depletion of A-spermatogonia. We conclude that spermatogonia in the juvenile primate testis are highly sensitive to irradiation and that spermatogonial depletion and cessation of proliferation is an acute response. In contrast to adult testes, where such damage is immediately visible, this damage in immature testes becomes apparent only when spermatogonial insufficiency leads to spermatogenic failure, and thus infertility, at the onset of puberty. Our methods are applicable to immature human testis and might serve as powerful tool to study irradiation toxicity in the juvenile human testis. (*Endocrinology* 148: 5541–5548, 2007)**

IT HAS BEEN KNOWN for many years that, in the primate testis especially, the rapidly dividing germ cells are a highly sensitive target for irradiation. Low doses of irradiation deplete the differentiating spermatogonia, whereas less sensitive spermatogonial stem cells survive, and spermatocytes and spermatids continue their maturation to sperm (1, 2). Studies exposing the testes of healthy adult men to testicular radiation cannot be performed for ethical reasons today but have previously generated a highly valuable resource for our understanding of irradiation on the human testis (2). Testicular involution is a slow process lasting several weeks until temporary or permanent azoospermia is attained (2, 3). In the human and primate testis, the exact mechanism of depletion and recovery from this damage is uncertain but appears to involve a block of spermatogonial development, a depletional wave of differentiating germ cells, a focal activation of surviving spermatogonial stem cells, and a slow recolonization of seminiferous tubules. The speed of recovery is dependent on the dose and fractionation of irradiation therapy (2–4). In the spermatogenic epithelium of humans, recolonization of surviving spermatogonia can first be detected 6 months after exposure to an irradiation dose of 0.2 Gy (2), 9–18 months after 1 Gy, and more than 4 yr after a dose of 10 Gy (2, 4, 5). Repopulation of the seminiferous epithelium in adult rhesus monkeys has been reported to occur from d 75 after irradiation onward after doses 0.5, 1, and 2 Gy irradiation (3).

It is generally accepted that the major reason for permanent absence of spermatogenic recovery is the lack of germline stem cells. This finding is contrary to findings derived from studies involving rats, where cytotoxic insult induces a block to spermatogonial differentiation that can be reversed by hormonal treatment after genotoxic insult (6). Observations in adult monkeys suggest that testicular irradiation with doses of 0.5–4 Gy first decrease the number of type A pale (Ap) spermatogonia, whereas the number of type A dark (Ad) spermatogonia remains initially unchanged (1). After radiation, the usually quiescent Ad spermatogonia start to proliferate, which, at higher doses of radiation, is followed by a decrease in number of both Ap and Ad spermatogonia.

The spermatogonial stem cells appear to be susceptible to this type of damaging insult at all ages (7). However, the impact of irradiation on the fertility potential in boys manifests itself only at the onset of puberty when FSH levels increase above normal and germ cell differentiation is di-

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Abbreviations: Ad, Type A dark; Ap, type A pale; BrdU, bromodeoxyuridine; RT, room temperature; SCO, Sertoli cell only.

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minished (7, 8). Analysis of spermatogenesis in adult monkeys who had been exposed to testicular irradiation during the juvenile period revealed that recovery of fully normal spermatogenesis occurs focally in a few seminiferous tubules. At higher doses, permanent infertility is associated with hyalinization of seminiferous tubules and complete Sertoli cell-only (SCO) pattern (9, 10).

Pediatric patients undergoing total body irradiation for marrow ablation therapy before hematopoietic stem cell transplantation are at high risk for testicular dysfunction (11). Because this strategy is successfully used on increasing numbers of young male patients who survive their oncological diseases, the number of infertile men facing a serious risk of permanent infertility in adulthood is rising. Despite the fact that infertility presents one of the most serious concerns in tumor survivors, very little is known in respect to acute effects of radiation on the immature human testis. For ethical reasons, experimental studies exploring irradiation effects in healthy children cannot be carried out. The best and closest model organisms to obtain clinically relevant data are therefore nonhuman primates. Macaques show close anatomical and endocrinological similarities to men before and after puberty and have therefore been used in previous approaches to explore irradiation defects on the testis (1, 3, 9). As yet, the analysis of acute irradiation-induced damage has not been performed, and the analysis was restricted to adult monkeys. Here we present a new approach combining organ cultures and xenografting (12) to explore acute and long-term effects of three doses of irradiation on the immature primate testis. We were able to determine the initial target cells affected by irradiation and to describe the consequences of this initial damage on subsequent testicular development.

## Materials and Methods

### *Animals and graft preparation*

Testicular tissue was obtained from two juvenile male rhesus monkeys (16 and 19 months of age). In this species, the onset of puberty, as reflected by the initiation of nocturnal testosterone secretion, occurs at approximately 30 months of age (13). Monkey testes were decapsulated and cut into 50–80 fragments. The fragments of tissue (about 0.5–1.0 $mm^3$) were dissected and maintained in ice-cold sterile Leibovitz-L15 medium (Life Technologies, Inc., Paisley, UK) until *in vitro* irradiation and grafting or short-term culture at 35 C. Tissue pieces were exposed to external irradiation by using laboratory irradiation equipment (Gammacell 40; Atomic Energy of Canada Ltd., Kanada, Ontario, Canada). The tissue fragments were exposed to doses 0, 0.5, 1, and 4 Gy irradiation.

### *Short-term culture*

Sterile, 24-well plastic plates (NUNC Brand Products, Roskilde, Denmark) were used as culture dishes. Each well received an insert that was inserted with the membrane facing the top of the well (Isopore membrane filters; Millipore, Billerica, MA). One milliliter of Leibovitz-L15 medium was added until the inserts floated. The medium was primed in the incubator for 30 min before initiating the culture. Four grafts were placed on each filter and cultured at 35 C and 5% $CO_2$ for 2, 24, and 48 h. Bromodeoxyuridine (BrdU, 100 $\mu$M) was added to the culture medium 1 h before termination of the culture.

### *Xenografting*

Five- to 7-wk-old intact immunodeficient male Nude mice (Crl:Nu/Nu-nuBR; Charles River Laboratories, Wilmington, MA) were used as recipients (n = 24). Recipient mice were randomly distributed among the four experimental groups. Eight testicular fragments of monkey tissue exposed to either no or the three different doses of irradiation *in vitro* were placed under the dorsal skin on either side of the dorsal midline by using cancer implant G13 needles (Popper Precision Instruments, Lincoln, RI). In each experimental group, six mice were transplanted. Similar numbers of recipients were transplanted with tissue from both donors for all experimental groups. Mice were maintained in groups of three to six per cage, with food and water available *ad libitum*.

The experiment was terminated 4 months after transplantation. Mice received an injection of BrdU (ip, 100 mg/kg) 2 h before being killed, were anesthetized and weighed, and blood was collected by cardiac puncture. The seminal vesicles were dissected and weighed, the back skin was removed and photographed, and the number of visible grafts was recorded. The grafts were dissected from the skin and fixed in Bouin solution. All animal experiments were approved by and performed under the guidance of the Animal Care and Use Committee at the University of Pittsburgh, School of Medicine.

### *Histology, immunohistochemistry, and statistical analysis*

The xenografts were fixed for 18–24 h in Bouin's solution, weighed, transferred for storage into 70% ethanol, and embedded in Technovit (Kulzer, Germany) for sectioning at 2 $\mu$m. Short-term cultured grafts were fixed for 18–24 h in 4% paraformaldehyde solution, transferred for storage into 70% ethanol, and embedded in paraffin for sectioning at 2 $\mu$m. All tissue sections were stained with periodic acid-Schiff's reagent/Gill's hematoxylin and examined with oil immersion light microscopy.

After dewaxing and rehydration of sections from cultured grafts, the slides were penetrated in 10 mM sodium citrate buffer (0.05% Tween 20, pH 6.0) in a microwave oven (600 W) for 5 min to retrieve antigenicity. Slides were allowed to cool for 10 min and rinsed with $H_2O$ for 2 min. After washing slides in Tris-buffered saline and in 0.85% NaCl, sections were blocked for 60 min with 5% BSA in PBS and incubated with the primary antibodies against the cleaved form of caspase 3 (1:200; Cell Signaling Technology, Inc., Beverly, MA) and MAGE-A4 (1:200). This monoclonal antibody (purified from mouse hybridoma 57B by Dr. Giulio C. Spagnoli, University of Basel, Switzerland, and kindly provided to us by Dr. Victoria Keros) was diluted in 1% BSA in PBS overnight at 4 C. After washing sections twice for 5 min in PBS, they were incubated with the secondary antibodies goat antirabbit IgG (1:500) (Alexa Fluor 594; Invitrogen, Carlsbad, CA) and goat antimouse IgG (Alexa Fluor 488; Invitrogen) for 45 min at 37 C. Finally, slides were incubated in 4′,6-diamidino-2-phenylindole (1:10,000 in PBS) for 5 min at room temperature (RT) and examined under fluorescence microscopy (Leica DM RBE). Other samples incubated with cleaved caspase 3 antibody were labeled with PowerVision immunohistochemistry kit (PowerVision+ Poly-HRP IHC Kit Biotin-free, antimouse/rabbit; ImmunoVision Technologies Co., Brisbane, CA) as instructed by the manufacturer and analyzed under a light microscope.

BrdU immunohistochemistry was performed following the manufacturer's instructions (Roche Diagnostics Corp., Indianapolis, IN). Briefly, the slides were incubated in 2 N HCl for 60 min at 37 C, neutralized with 0.1 M borate buffer (pH 8.5) for 10 min and thereafter washed with PBS for 10 min and incubated with anti-BrdU antibody (Roche) diluted (1:100) in PBS with 0.1% BSA for 60 min at RT. Subsequently, the sections were washed with PBS and incubated with goat antimouse IgG monoclonal antibody (1:500) (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 45 min at RT. Slides were then washed with PBS and incubated with avidin-biotin peroxidase complex solution for 30 min at RT, washed with PBS, and finally exposed to color reaction with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO) and evaluated using light microscopy.

Xenograft survival was defined by light microscopic observation of seminiferous tubules in the retrieved grafts. All cross-sections of seminiferous tubules from smaller grafts were analyzed. If the grafts were large, 50 tubular cross-sections were selected for analysis by random systematic sampling. Each cross-section was scored for the presence of germ cell types (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round and/or elongating spermatids), absence of germ cells (SCO), or complete hyalinization. The identification of Ad and Ap spermatogonia followed the scheme of Clermont and Leblond (29). Each of the short-term cultured grafts were morphologically analyzed by evaluating 50 randomly selected microscopic fields (0.0025 $mm^2$).

Numbers of Sertoli cells, Ad and Ap spermatogonia, and caspase 3-positive cells were scored across entire cross-sections of fragments, and these data are presented as number of cells per square millimeter. Numbers of Ad and Ap spermatogonia were also presented in relation to Sertoli cell numbers. Numbers of BrdU-positive cells identified as germ and Sertoli cells according to morphology were determined likewise. The percentages of caspase 3-positive cells that were negative or positive for the germ cell marker MAGE A4 (14) were analyzed in slides subjected to double-immunofluorescent staining. In total, 14 grafts representing all four experimental groups were scored for this analysis. The light microscopic determinations were conducted by one observer (K.J.).

Parametric data are presented as mean ± SEM and nonparametric data as median, 10th, 25th, 75th, and 90th percentiles. The one-way ANOVA with Tukey test for multiple comparisons of independent groups of samples was employed for parametric data and the Kruskal-Wallis analysis with Dunn's *post hoc* test for nonparametric data. A $P$ value of $<0.05$ was considered to indicate a statistically significant difference.

## Results

### *Analysis of irradiation effects on survival of xenografts*

In all experimental groups, survival of xenografted monkey testicular tissue was similar (67–78%). A summary of the host and xenograft descriptives 4 months after *in vitro* irradiation is presented in Table 1. Although xenograft survival was not affected, the weight of grafts was significantly lower in the group irradiated with the 4-Gy dose when compared with the controls. The pregrafting exposure of testicular tissue to irradiation had no effect on body, testis, or seminal vesicle weights of the recipients (Table 1).

### *Spermatogonial development in xenografts*

At the time of grafting, all testicular grafts consisted of seminiferous cords with spermatogonia as the most advanced germ cell type (Fig. 1A), and no morphological difference was detected between control (Fig. 1A) and irradiated grafts (Fig. 1B). Four months after grafting, a dose-dependent decline in the number of grafts containing any type of spermatogonia was recorded. Ninety percent (27 of 30) of control grafts and 90% (28 of 31), 79% (19 of 24), and 53% (17 of 32) of the grafts irradiated with doses 0.5, 1, and 4 Gy, respectively, contained germ cells. The analysis of the distribution of Ad or Ap spermatogonia in individual seminiferous tubules showed a similar dose-dependent decline that reached statistical significance even at the lower irradiation doses (Fig. 2). The decline of spermatogonia can also be analyzed by the number of grafts showing exclusively SCO pattern [0% (zero of 30), 6% (two of 31), 21% (five of 24), and 47% (15 of 32) at irradiation doses 0, 0.5, 1, and 4 Gy, respectively] and the relative number of SCO tubules in xenografts without germ cells (Fig. 2). We did not observe any fully hyalinized seminiferous tubules in the xenografts. In addition to quite abundant A-spermatogonia, few B-spermatogonia (10%, three of 30, Fig. 1E) were encountered focally in control grafts and grafts irradiated with the dose of 0.5 Gy (B-spermatogonia: 13%, three of 24; Fig. 1F). Aside from a few A-spermatogonia, no B-spermatogonia were seen in the grafts irradiated with doses of 1 Gy (Fig. 1G) and 4 Gy (Fig. 1H).

In all grafts, a morphologically normal interstitium with blood vessels, macrophages, and peritubular and Leydig cells was observed. We did also not detect any qualitative irradiation-dependent morphological change of Sertoli cell nuclei or other histological features of Sertoli cells.

### *Effects of in vitro irradiation on spermatogonia and Sertoli cells in cultured tissue fragments*

The number of Sertoli cells per square millimeter of fragmental cross-section was identical (range, 6056–6516) in all groups 2 h after irradiation. Likewise, the absolute numbers of Ad and Ap spermatogonia as well as their relative number, in respect to Sertoli cells, were identical between groups. Although we encountered between 0.9 and 1.5 Ad spermatogonia per 100 Sertoli cells, Ap spermatogonia were more frequent with 3–6.5 cells per 100 Sertoli cells (Table 2). At 24 h after irradiation, a significant decrease in absolute and relative numbers of both spermatogonial subtypes was detected in fragments irradiated with the highest irradiation dose (Table 2). The number of Sertoli cells decreased also in fragments irradiated with 4Gy (Table 2).

When the culture period was extended to 48 h, severe tissue necrosis and disintegration of tissue morphology was detected both in control and irradiated grafts, and no further analysis was performed.

### *Effects of in vitro irradiation on the number of proliferating and apoptotic cells in cultured tissue fragments*

Analysis of the percentages of germ cells (MAGE-A4 positive) and Sertoli cells (MAGE-A4 negative) among the population of caspase 3-positive cells revealed a similar proportion in all experimental groups; 69.9 ± 5.3% of the caspase 3-positive apoptotic cells costained for germ cell marker MAGE-A4, indicating a constant ratio of apoptotic cells between germ cells and Sertoli cells. However, because Sertoli cells are about 20-fold more abundant than germ cells, it appears that apoptotic cell death is a rather frequent event in germ cells and a rather rare event in Sertoli cells under normal conditions. As can be seen in Fig. 3, exposure to irradiation evoked an increase of apoptotic cell death in the cultured fragments after 24 h,

**TABLE 1.** Host and graft descriptive and number of surviving grafts per host when *in vitro* irradiated testicular grafts were used for xenotransplantation

| Dose of graft irradiation (Gy) | No. of recipients | Body weight (g) | Testis weight (mg) | Seminal vesicle weight (mg) | No. of retrieved grafts | No. of grafts per host | Graft survival (%) | Graft weight (mg) |
|---|---|---|---|---|---|---|---|---|
| 0 | 5 | 28 ± 3 | 99 ± 17 | 219 ± 42 | 30 | 8 ± 2 | 75% (30/40) | 3.8 ± 0.7 |
| 0.5 | 5 | 30 ± 3 | 113 ± 26 | 259 ± 59 | 31 | 8 ± 1 | 78% (31/40) | 5.0 ± 0.9[a] |
| 1 | 4 | 29 ± 5 | 99 ± 6 | 234 ± 60 | 24 | 7 ± 1 | 75% (24/32) | 3.5 ± 0.8 |
| 4 | 6 | 33 ± 2 | 114 ± 9 | 327 ± 64 | 32 | 6 ± 1 | 67% (32/48) | 2.2 ± 0.5[a] |

[a] $P < 0.05$ compared with the control value.

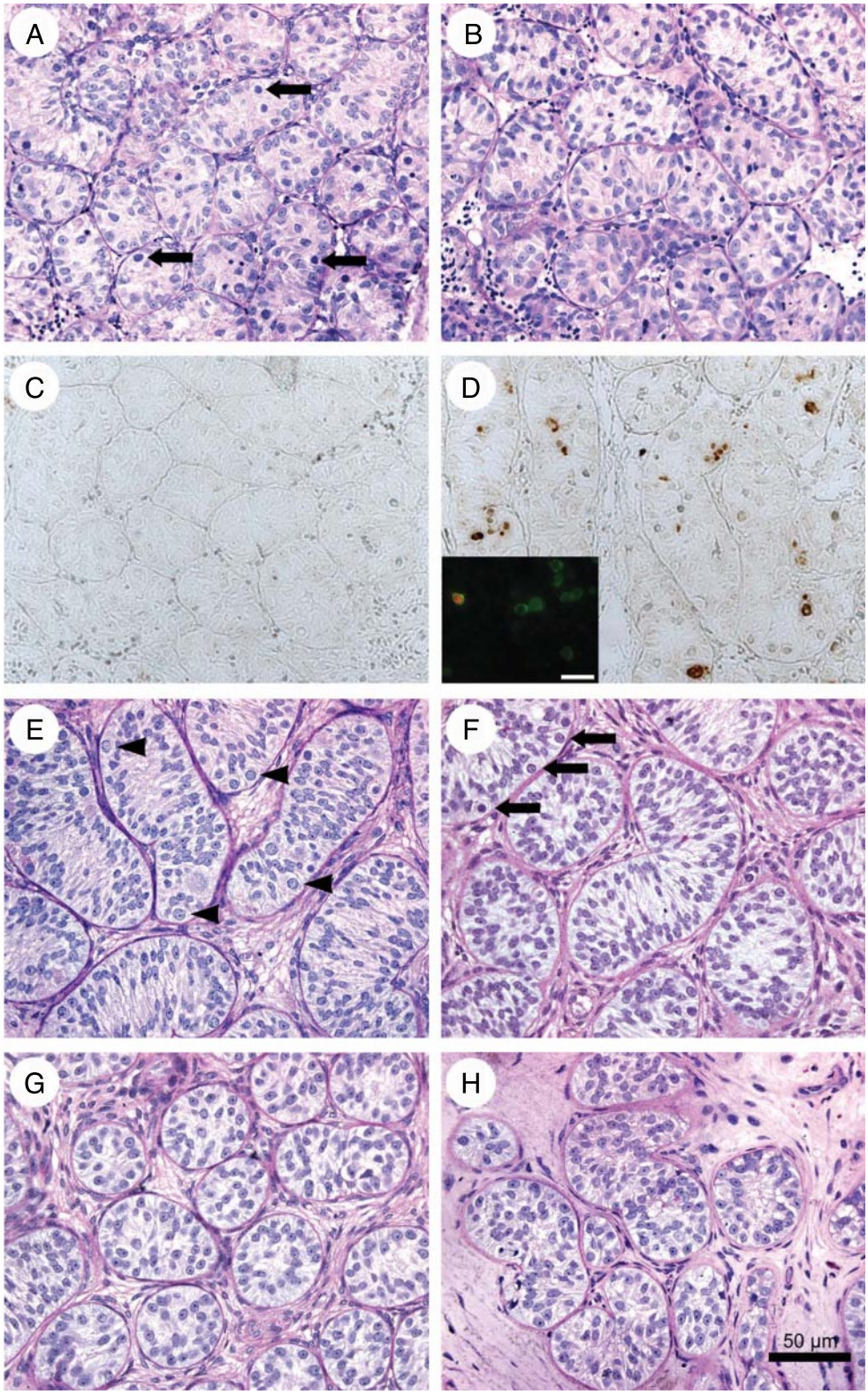


FIG. 1. Representative light micrographs of immature monkey testicular grafts 24 h after short-term culture (A–D) and 4 months after xenografting into immunodeficient host (E–H). After short-term culture, several A-spermatogonia (A, *arrow*) and no caspase 3-positive cells (C) are detected in control tissue. The number of A-spermatogonia (B) is decreased and the number of caspase 3-positive cells (D) is increased in tissue fragments exposed to a single dose of irradiation of 4 Gy before organ culture (D). *Inset*, Immunofluorescent micrograph showing a caspase 3/MAGE-A4 double-positive apoptotic germ cell (*red* and *green* fluorescence) and several MAGE-A4-only positive germ cells (*green*) in testicular tissue that was cultured for 24 h after exposure to 4 Gy irradiation. *Scale bar* of *inset*, 25 μm. Four months after xenografting, numerous A-spermatogonia and focal spermatogenesis up to level of B-spermatogonia (*arrowhead*) can be seen in the control grafts (E). In the grafts exposed to a dose of 0.5 Gy (F), A-spermatogonia are detected (*arrow*). The grafts exposed to 1 Gy (G) and 4 Gy (H) of irradiation contain no germ cells. Morphologically normal testicular interstitium and Sertoli cells are observed in all samples.

which reached statistical significance at the highest dose. This apoptotic increase affected both Sertoli and germ cells.

Sertoli cells and germ cells showed a different response with respect to proliferation. Although we did not obtain any statistically significant changes (Fig. 3), it was obvious that no BrdU-labeled germ cells were encountered among spermatogonia after exposure to 1 and 4 Gy irradiation. BrdU-positive Sertoli cells, however, were encountered in all experimental groups.

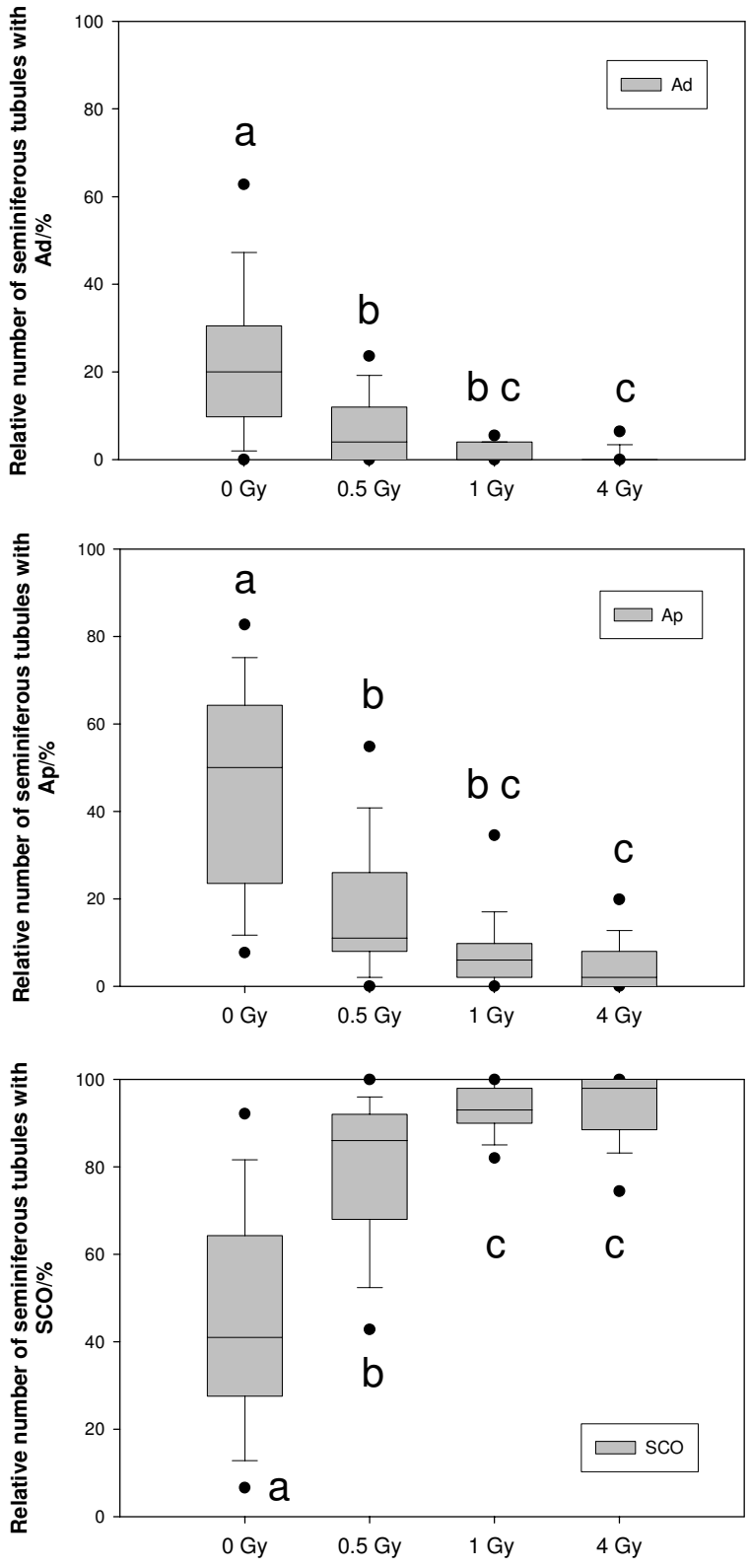


FIG. 2. Dose-dependent decrease in relative number of seminiferous tubules containing Ad and Ap spermatogonia and increase in SCO pattern after increasing dose of *in vitro* irradiation in xenografted immature primate testicular grafts. *Lines within the boxes* depict the median and the *boundaries of the boxes* 25th and 75th percentiles, *whiskers* indicate the 10th and 90th percentiles, and *points* refer to the 95th and 5th percentiles. *Different letters* indicate statistically significant differences ($P < 0.05$) between the different treatment groups.

## Discussion

This study presents a new experimental model using 1) fragment culture to reveal short-term and 2) xenografts to depict long-term effects of irradiation on immature testicular tissue. We show that *in vitro* irradiation of testicular tissue before organ culture and/or xenografting offers a valid toxicological approach to study acute and long-term effects of gonadal toxicity.

**TABLE 2.** Number of Ad and Ap spermatogonia per square millimeter calculated area and per 100 Sertoli cells and number of Sertoli cells per square millimeter calculated area in irradiated grafts after culture for 2 and 24 h

| Dose of irradiation (Gy) | 2 h | | | | | 24 h | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ad | | Ap | | Sertoli cells/mm$^2$ | Ad | | Ap | | Sertoli cells/mm$^2$ |
| | Cells/mm$^2$ | Per 100 Sertoli cells | Cells/mm$^2$ | Per 100 Sertoli cells | | Cells/mm$^2$ | Per 100 Sertoli cells | Cells/mm$^2$ | Per 100 Sertoli cells | |
| 0 | 83 ± 32 | 1.3 ± 0.7 | 278 ± 10 | 6.5 ± 0.3 | 6056 ± 235 | 88 ± 19 | 2.0 ± 0.4 | 178 ± 36 | 4.0 ± 0.9 | 5432 ± 108 |
| 0.5 | 68 ± 13 | 0.9 ± 0.5 | 291 ± 42 | 5.5 ± 0.6 | 6304 ± 250 | 65 ± 8 | 1.4 ± 0.1 | 185 ± 19 | 4.1 ± 0.4 | 5167 ± 171 |
| 1 | 76 ± 13 | 1.5 ± 0.9 | 271 ± 99 | 3.0 ± 0.3 | 6516 ± 188 | 70 ± 10 | 1.7 ± 0.3 | 192 ± 6 | 4.7 ± 0.3 | 4574 ± 359 |
| 4 | 73 ± 23 | 1.3 ± 0.4 | 316 ± 16[c] | 5.1 ± 0.7 | 6246 ± 101 | 16 ± 7[a,b] | 0.5 ± 0.2[a,b,c] | 33 ± 10[a,b,c] | 0.9 ± 0.3[a,b,c] | 3249 ± 199[a] |

[a] $P < 0.05$ compared with the control value.
[b] $P < 0.05$ compared with the corresponding value after 0.5 Gy irradiation.
[c] $P < 0.05$ compared with the corresponding value after 1 Gy irradiation.

The profound germ cell depletion in xenografts revealed that spermatogonia in the juvenile primate testis have a similar sensitivity to irradiation as was earlier reported for human and nonhuman primates (1–4, 9). In immature monkeys, focal recovery of spermatogenesis was frequently observed at single doses of less than 8 Gy (9). Because the repopulated focal areas showed normal spermatogenesis, the major obstacle for initiation of fertility after prepubertal irradiation is the spermatogonial colonization of the testis from the focal areas with surviving stem cells (9).

In mature testes, irradiation doses of more than 0.5 Gy efficiently block the development of B-spermatogonia and initiate a depletional wave of spermatogenesis leading to a successive disappearance of the differentiating germ cells. In men, this process continues for 46 d after irradiation, and azoospermia is achieved at doses above 0.78 Gy (2). In addition to the loss of differentiating germ cells, doses above 0.5 Gy also diminish the number of seminiferous tubules with Ad and Ap spermatogonia (1–3). As in immature monkeys, spermatogenic recovery after the depletional wave depends on the slow recolonization of A-spermatogonia to overcome the dose-dependent degree of spermatogonial depletion.

Our observations here show that the acute depletion of spermatogonia is due to apoptotic cell death. Although the effect becomes immediately detectable in the adult testes, the consequences of spermatogonial depletion in immature monkeys are hidden until puberty. This suggests that neither the stem cell niches nor the spermatogonial stem cells show an age-related difference in their sensitivity to irradiation in postnatal primate testis. This conclusion is not supported by previous studies on rodents where significantly increased spermatogonial toxicity is associated with cytotoxic drug and irradiation treatment at early postnatal days (15–17). Interestingly, a recent report describes a high sensitivity of cultured fetal human testicular tissue to germ cell apoptosis after very low irradiation doses 0.1–0.2 Gy (18). These observations suggest that significant difference in radiosensitivity may exist between fetal and juvenile testes, whereas it is lacking between pre- and postpubertal primate germ cells.

In a previous study, we exposed the recipients of xenografted immature testicular monkey tissue to busulfan (12). Our observation here after low-dose exposure to radiation is comparable to the toxic effect we observed in xenografts when the recipients were exposed to 100 mg/m$^2$ (38 mg/kg) busulfan. This let us suggest that our lowest irradiation dose of 0.5 Gy had a similar effect compared with a busulfan dose of 100 mg/m$^2$. Both treatments had no effects on the number of Ad spermatogonia in xenografts. We as-

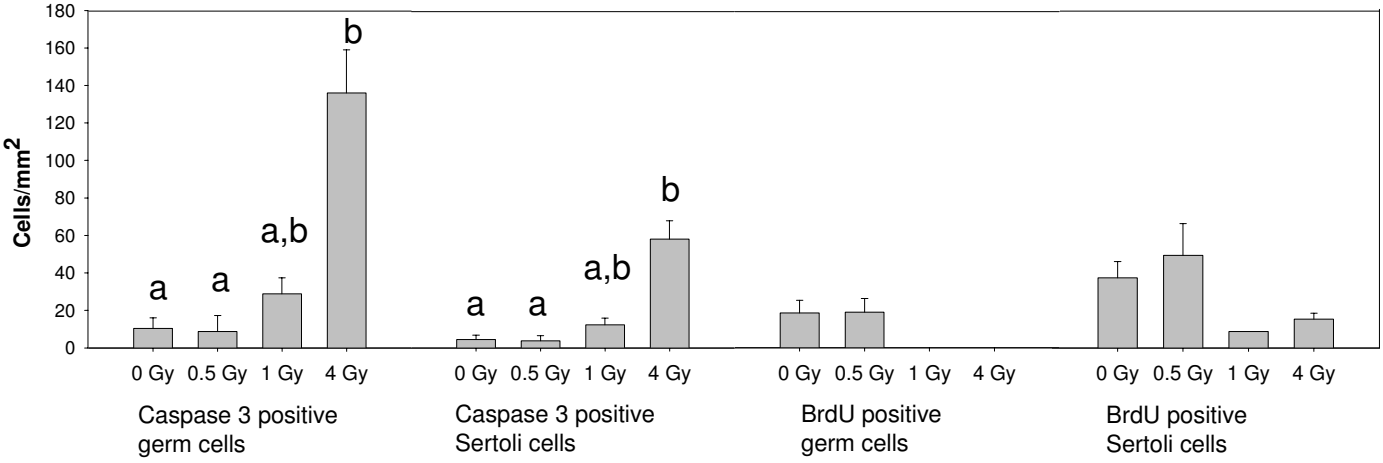


FIG. 3. Dose-dependent changes in apoptotic and proliferating Sertoli and germ cells after increasing dose of *in vitro* irradiation in cultured immature primate testicular grafts after 24 h. Caspase 3 immunostaining indicates apoptotic cells and BrdU incorporation cells in S-phase of the cell cycle. A significant increase of caspase 3-positive germ cells and Sertoli cells is encountered at the highest irradiation dose. No significant changes are determined for BrdU-positive cells. However, no BrdU incorporation is detected in germ cells after exposure to irradiation of 1 and 4 Gy. *Different letters* indicate statistically significant differences ($P < 0.05$) between the different treatment groups.

sume that this weak damage will allow an efficient recovery of spermatogenesis after sexual maturation of the testicular tissue.

Using a culture period of 24 h, we were able to explore the cellular mechanisms responsible for the acute effects of testicular irradiation. Organ cultures of immature testes have been used in earlier studies to explore the effects of hormones and growth factors on testicular development (19, 20) as well as to screen for effects of factors like environmental toxicants and cytotoxic and physical exposures (18, 21–23). This approach has recently been used to evaluate the effect of cryopreservation on spermatogonial survival and steroidogenic capacity of human testicular tissue (24, 25). Here we detected significant changes of cell numbers and of functional markers for apoptotic and proliferating cells in response to irradiation. We showed that 80% of A-spermatogonia were depleted already 24 h after irradiation with 4 Gy and that the death of these cells is through apoptotic pathways. In addition, spermatogonia also ceased to proliferate after 1 and 4 Gy irradiation. These findings let us conclude that we detected an acute effect of spermatogonial depletion that we later revisited by observing a significant depletion of germ cells in xenografts. It is an obvious disadvantage that organ culture of juvenile primate testis allows only a very short window to study physiological responses in seminiferous tubules. We experienced a severe disintegration of the morphology in the primate testicular fragments after 48 h in culture. It has been previously suggested that culture times of less than 3 d represent a reasonable reflection of the *in vivo* situation (20). Although the limited time window for evaluation of short-term effects limits the applicability of organ culture, the combination of organ culture and xenografting offered a unique opportunity to explore both acute and long-term effects of irradiation exposure.

Only one experimental study has evaluated irradiation exposure of Sertoli cells during prepubertal ages (9). This experimental study with single or fractionated total body irradiation applying doses of 4–8.5 Gy reported a depletion of Sertoli cells leading to low testis weights in adulthood. These findings are consistent with our findings in xenografts that indicate a diminished growth of xenografts exposed to 4 Gy irradiation. They are also consistent with our finding in cultured fragments that Sertoli cells undergo apoptotic cell death at higher doses of irradiation. We conclude that irradiation doses of at least 4 Gy affect the somatic environment in the juvenile primate testis.

Although xenografting of testicular tissue is less successful in adulthood (26), the xenograft model has some advantages. In the clinical situation, an estimate of the fertility option of an irradiation-exposed boy can only be made after pubertal initiation, when the loss of Sertoli cells and germ cells incurs a diminished growth of the testis and a focal to complete absence of spermatogenesis. Testicular tissue xenotransplantation of juvenile testicular tissue into immunodeficient mouse allows an accelerated pubertal development and an early initiation of spermatogenesis (12). In the present study, the accelerated pubertal development in irradiated xenografts allowed us to detect long-term effects of irradiation after only 4 months, whereas detection of this outcome *in vivo* would have lasted several years. In addition, long-term observations are important because previous studies revealed that histological detection of spermatogonial numbers in testicular tissue does not necessarily imply that these spermatogonial stem cells are functional (27, 28).

In conclusion, our novel approach to combine organ culture and xenografting for analysis of a single *in vitro* exposure to an irradiation insult offers a simple and reliable tool to explore acute and long-term effects on the juvenile primate testis. This study reveals that toxicological manipulation and evaluation of its consequences on primate spermatogonia and their somatic microenvironment can be performed *ex vivo* without treatment of the nonhuman primates or the mouse host. We assume that this technique can serve as a powerful experimental tool to study irradiation toxicity in spermatogonial stem cell function in immature human testis.

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