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Functional Analysis of the Cooled Rat Testis

Z. ZHANG,* R.V. SHORT,*† T. MEEHAN,‡ D.M. DE KRETSER,‡§ M.B. RENFREE,* AND K.L. LOVELAND‡§

*From the Departments of *Zoology and †Obstetrics and Gynaecology, University of Melbourne, and ‡Monash Institute of Reproduction and Development, Monash University, Victoria, and §Australian Research Council Centre of Excellence in Biotechnology and Development, Australia.*

ABSTRACT: Direct cooling of the testis results in the depletion of most germ cells in vivo. Germ cell–depleted testes are now commonly used to investigate spermatogenic regeneration and can serve as recipients for germ cell transplantation. The present study explored the effects of cooling rat testes on the depletion of endogenous germ cells, spermatogenic regeneration, and Sertoli cell function. Adult rat testes were cooled with iced Ringer's solution for 60 minutes, which results in the initiation of apoptotic germ cell loss within 8 hours. Pachytene spermatocytes at stages XII–I were the cells most sensitive to cooling. In 46%–67% of seminiferous tubule cross-sections, only Sertoli cells remained in the cooled testes 3–10 weeks after treatment. Germ cell loss was accompanied by a significant decrease in circulating inhibin B and an increase in follicle-stimulating hormone concentrations, which indicated a change in Sertoli cell function. Quantitative analysis of mRNA expression associated with apoptotic signals showed no significant uniform changes among the cooled testes, although some individuals had a distinct up-regulation of *FAS* mRNA at 24 hours. Attempts to use the cooled testes as recipient testes for mouse-to-rat germ cell transplantation were undertaken, but none of the mouse germ cells transplanted into the testes 15–34 days after cooling appeared to have undergone spermatogenesis 64–92 days after transplantation. These data suggest that modifications to Sertoli cell function resulting from testicular cooling create an environment that is unable to support spermatogenesis by donor germ cells.

Key words: Spermatogonia, testis, apoptosis, cooling, and transplantation.

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Spermatogenesis requires both spermatogonial stem cells, which have the capability to self-renew and commence differentiation, and an optimal spermatogenic environment that is directly governed by Sertoli cells (Russell et al, 1990). Spermatogonial stem cells are only a small proportion of total spermatogenic cells in testes (Meistrich and Van Beek, 1993). Because of the paucity of both morphological and cell surface markers available for unequivocal identification of germline stem cells (Shinohara et al, 1999; Orwig et al, 2002), the assessment of spermatogonial stem cell potential currently relies on both in vivo and in vitro functional assays (Meistrich and Van Beek, 1993). Since testicular germ cell transplantation was successfully developed in 1994 (Brinster and Avarbock, 1994), it has increasingly been used as the in vivo functional assay for spermatogonial stem cells. Similarly, the presence of an optimal spermatogenic environment also can be tested using germ cell transplantation (Ogawa et al, 1999; Ohta et al, 2000; Creemers et al, 2002).

The ideal recipient testis for male germ cell transplantation would be one in which there was a maximal depletion of endogenous germ cells while maintaining a normal environment within the testis that allows donor germ cells to colonize and regenerate spermatogenesis (Shinohara et al, 2002). Several approaches have been used to deplete testicular germ cells from immature and adult animals, including treatment with cytotoxic agents such as busulfan, an alkylating agent (Jackson et al, 1962), radiation (Dym and Clermont, 1970), induction of cryptorchidism (Moore, 1924), hyper- or hypothermia (Gronsky 1930; Steinberger and Dixon 1959), ischemia (Steinberger and Tjioe, 1969), and vitamin A deficiency (Mitranond et al, 1979). Testicular germ cell loss caused by these treatments usually involves germ cell apoptosis (Blanchard et al, 1996; Blanco-Rodriguez and Martinez-Garcia 1997; Turner et al, 1997; Yin et al, 1997).

Mice are commonly used as recipients in allo– or xeno–germ cell transplantation. This may reflect the lower spermatogenic recovery observed in rats after testicular damage in comparison to mice (Meistrich, 1998).

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Correspondence to: Kate Lakoski Loveland, Monash Institute of Reproduction and Development, Monash Medical Centre, 27–31 Wright Street, Clayton, Victoria 3147, Australia (phone: 6-13-9594-7125; fax: 6-13-9594-7111; e-mail: kate.loveland@med.monash.edu.au).

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After administering most treatments to rats, spermatogenic recovery is characteristically slow or incomplete, even when endogenous spermatogonial stem cells remain in the testes (Meistrich, 1993). Providing the appropriate spermatogenic environment in the recipient testis appears at present to be more difficult in rats than in mice (Ogawa et al, 1999), as was demonstrated by the more efficient production of live young after mouse-to-mouse germ cell transplantation compared with rat-to-rat germ cell transplantation (Jiang and Short, 1995; Nagano et al, 2000; Kanatsu-Shinohara et al, 2003; Zhang et al, 2003). We recently reported that, after mouse-to-rat transplantation, mouse spermatogonial stem cells could undergo complete spermatogenesis in the testes of wild-type rats that had been treated prepubertally with busulfan, and rat donor–derived offspring were repeatedly produced after rat-to-rat germ cell transplantation (Zhang et al, 2003). However, germ cell transplantation methods in rats are under development, and continued modifications to improve the success rate of donor cell colonization remain desirable.

Busulfan can effectively deplete most spermatogonia after 1 or 2 intraperatoneal injections (Jackson et al, 1962; Jiang, 1998), and it has been used to produce recipient testes for germ cell transplantation (Brinster and Avarbock, 1994; Jiang and Short 1995), but its myelogenic toxicity usually has an adverse effect on the health of the treated animal, and bone marrow transplantation may be required (Clouthier et al, 1996). In addition, rats are more sensitive to the toxicity of busulfan than mice are (Ogawa et al, 1999). Recipient mouse testes have been generated through the use of genetic mutations affecting germ cells (Brinster and Avarbock, 1994; Boettger-Tong et al, 2000) and radiation (Creemers et al, 2002), although to date, alternatives to busulfan treatment to produce recipient testes are not available for rats. Optimization of the recipient testis preparation remains under development for this species, although cryptorchidism in combination with busulfan treatment of rat has been explored (Ogawa et al, 1999).

To identify a suitable method for the preparation of recipient rat testes, we did a pilot study to compare the degree of germ cell depletion produced by testicular heating, cooling, ischemia, or cooling plus ischemia. On the basis of histological results (data not shown), cooling and cooling plus ischemia produced greater germ cell depletion while keeping the gross morphology of the Sertoli cells relatively intact. We therefore chose testicular cooling for further study.

A mild hypothermia (10°C for 30 minutes) given to rat testes was reported to result in germ cell loss via a significant increase in the number of apoptotic germ cells (Blanco-Rodriguez and Martinez-Garcia, 1997), but whether the germ cell loss caused by longer exposure of the testes to a lower temperature (Gronsky, 1930; Macdonald and Harrison, 1954) was due to a similar apoptotic mechanism has not been explored. The germ cell–depleted testis produced by testicular cooling approaches have yet to be examined as recipients in male germ cell transplantation. Therefore, the present study provides an investigation of the germ cell–depleted rat testes produced by 1 hour of direct cooling. The spermatogenic environment of the cooled testes was evaluated by histological examination, measurement of Sertoli cell function, cell apoptotic assays, and germ cell transplantation.

Materials and Methods

Animals and Experimental Design

For testicular cooling, 133 male Sprague-Dawley rats (8 weeks old) were divided into 10 groups. Group 1 animals were untreated controls (n = 7). Animals in groups 2–10 (G2–G10) were killed at 2, 8, and 24 hours and 3, 7, 14, 21, 35, and 70 days, respectively, after surgery. Each of these groups was further divided into cooling and sham-cooling subgroups, with 7 animals in each subgroup.

For germ cell transplantation, testicular cooling was applied to an additional 11 Sprague-Dawley rats (4.5–8 weeks old) that were used as recipients 15–34 days later. They were divided into 2 groups, one being given the immunosuppressive drug cyclosporin (CS) after germ cell transplantation. H253 *lacZ* transgenic male mice (background: F_1 of C57/b × DBA_2) were used as the source of donor cells.

Animals were maintained in the animal house, Department of Zoology, Melbourne University, at 21°C ± 1°C, under a 14-hour light and 10-hour dark regimen with food and water available ad libitum. Experimental procedures followed the National Health and Medical Research Council Guidelines, and approval was obtained from the University of Melbourne Animal Ethics Committee. All surgical procedures were conducted under sterile conditions.

Testicular Cooling Procedure

Three to four animals underwent surgery simultaneously. Rats were intraperitoneally injected with a dose of 50 mg/kg body weight of Nembutal that contained 60 mg/mL of sodium pentobarbitone (Boehringer Ingelheim, New South Wales, Australia). Both testes were withdrawn from the scrotum via a lower midline abdominal incision, then put through a hole in the base of a plastic weighing boat, which was lined with 4 layers of gauze. In 2 rats from each group, a fine catheter probe (diameter 0.7 mm; YSI Inc., Yellow Springs, Ohio) connected to an electronic thermometer (Cole-Parmer; Extech Equipment PTY Ltd., Victoria, Australia) was inserted into the left testis at its lower pole using a 26-gauge needle as a guide. Ringer's

solution was autoclaved and then frozen. The testes were covered with 0°C to 1°C crushed iced Ringer's solution for 60 minutes, and the core temperature of the left testis was maintained at around 4°C ± 1°C by frequent additions of crushed Ringer's ice. Rats in the sham-cooling group underwent the same surgery, but Ringer's solution at room temperature replaced the crushed ice slush, and no thermometer probe was inserted. After cooling, the testes were returned into the scrotum, and the abdominal cavity was closed by 2 layers of sutures with 4/0 plain gut (Ethicon, Sydney, Australia).

Sample Collection

At each designated time point, animals were killed by an overdose of nembutal. Blood was collected by a cardiac puncture with an 18-gauge needle attached to a 10-mL sterile syringe coated with heparin (DBL; David Bull Laboratories, Victoria, Australia). Plasma was collected from the blood samples and stored at −20°C for subsequent hormone assays.

The left testis of each rat was snap frozen in liquid nitrogen and then kept at −80°C prior to the extraction of RNA. The right testis was perfused in situ with Bouin's fixative solution via the aorta, then excised and fixed by immersion in Bouin's solution for another 6 hours. Testes were washed in 70% ethanol, and then a portion was processed, embedded in paraffin, and sectioned at 7 μm for hematoxylin and eosin staining and at 5 μm for immunohistological staining. Another part of the perfused testis was cut into 2-mm^3 blocks and fixed in 1% osmium tetroxide in 0.2 mol/L cacodylate buffer (pH 7.2) for 2 hours and cut into 1-μm sections for toluidine blue staining (Jiang 1998).

Evaluation of In Situ Germ Cell Apoptosis

Cell apoptosis was identified with an in situ terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) technique, according to the supplier's instructions (Boehringer Mannheim, Germany). In brief, dewaxed tissue sections were blocked with a mixture of methanol/30%H_2O_2 (9 : 1) for 30 minutes, followed by incubation with DNA labeling mixture for 30 minutes at 37°C. The sections were treated with 20% normal rabbit serum in phosphate-buffered saline (PBS; KH_2PO_4 2 mmol/L, Na_2HPO_4 10 mmol/L, NaCl 137 mmol/L, and KCl 2.7 mmol/L) for 10 min, followed by incubation with sheep anti-digoxigenin antibody (Boehringer Mannhem, Germany; 1 : 100 in 20% normal rabbit serum PBS) for 60 minutes at room temperature. Sections were incubated with rabbit anti-sheep IgG-biotinylated antibody (Silenus, Melbourne, Australia) 1 : 500 in Tris-buffered saline (TBS, TrisHCl 54 mmol/L and NaCl 150 mmol/L) for 30 minutes. Sections were next incubated with a streptavidin-horseradish peroxidase (HRP)–conjugated complex (Silenus; 1 : 100 in TBS) for 30 minutes. Antibody binding was finally detected using the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark), and the sections were lightly counterstained with hematoxylin. Criteria previously established for counting apoptotic germ cells in the ischemic rat testis were used (Turner et al, 1997). The percentage of cross-sections of seminiferous tubules having any positive-staining nuclei and having ≥3 positive-staining nuclei were recorded by counting either all tubules in a testis cross-section or in >200 tubules in each section after TUNEL staining.

Classification of Germ Cell Complement in Seminiferous Tubules

For the testes with few spermatogenic cells remaining ≥1 week after cooling, the percentage of seminiferous tubule cross-sections having spermatogenic cells at varying stages was assessed. Tubule cross-sections were scored in 2 categories, according to the contents of the seminiferous epithelium: (1) those with only Sertoli cells or those with Sertoli cells plus the spermatogenic cells matured only to the leptotene spermatocyte stage and (2) those with at least 3 pachytene spermatocytes or more advanced cells. A small proportion of atrophic tubule cross-sections was present in the cooled testes, which appeared as described elsewhere (Steinberger and Tjioe, 1969; Miller et al, 1990). These tubules are characterized by the complete or partial absence of seminiferous epithelium, including Sertoli cells, and contain necrotic debris that are stained deep purple by hematoxylin.

RNAse Protection Analysis (RPA)

RNA was isolated by the acid–guanidinium thiocyanate–phenol-chloroform method (Chomczynski and Sacchi 1987). RPA was done using a commercially available Multi-Probe RNase Protection Assay system with the rAPO-1 multiprobe template set (PharMingen, San Diego, CA) specific for detection of rat mRNAs, according to the manufacturer's instructions. The apoptosis-associated mRNA target molecules were FAS, bcl-x, FASL, Caspase-1, −2, −3, bax, and bcl-2. Two housekeeping genes, L32 and GAPDH, were included as loading controls. In brief, 20 μg of testicular RNA was hybridized overnight with 2 μL of the ^{32}P-labeled probe mixture. The remaining single-stranded RNA was then digested with RNAse A/RNAse T_1 mix. The RNAse-protected probes were resolved by denaturing polyacrylamide sequencing gel electrophoresis. The gels were dried, exposed to a phosphoimaging screen, and the signal collected for quantitation on a Molecular Dynamics Phosphoimage Analyser (FLA2000; Fujifilm, Tokyo, Japan). The signal obtained for the L32 internal loading standard was used to calcu-

late the relative units of mRNA expression (Meehan et al, 2001).

Hormone Assays

Concentrations of follicle-stimulating hormone (FSH) were determined using radioimmunoassay (RIA) reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The iodinated preparation and the antiserum were rFSH I-8 and anti–rFSH-S-11, respectively. Results were expressed in terms of NIDDK rFSH-RP-2. Goat anti-rabbit IgG (GAR#12; Institute of Reproduction & Development, Monash University, Melbourne, Australia) was used as the second antibody. The assay buffer used was PBS that contained 0.5% bovine serum albumin (BSA; Sigma, St. Louis, MO). Samples were diluted in a dose-dependent manner that was parallel to the standard curve in the assay. The lower limit of detection of rFSH was 1.8 ng/mL. The within-assay variation in the FSH assay was 5.6%. All samples were measured in a single assay.

Testosterone concentrations were measured using a direct RIA, as described elsewhere (O'Donnell et al, 1994), but using stripped normal mouse serum. The lower limit of detection of testosterone was 0.04 ng/mL. The within-assay variation in the testosterone assay was 9.6%. All samples were measured in a single assay.

Inhibin B was measured using a specific enzyme-linked immunoassay (Oxford BioInnovation Ltd., Upper Heyford, United Kinddom), according to the manufacturer's instructions (Groome et al, 1996). Rat serum samples were diluted in a dose-dependent manner that was parallel to the standard curve in the assay. The lower limit of detection of inhibin B was 2.5 pg/mL. The within-plate variation was 4.1%. The interplate coefficient of variance for inhibin B (n = 4) was 7.6%.

Collection of Mouse Donor Germ Cells

Donor germ cells were collected from 2.5–4 week-cryptorchid mouse testes, and a 2-step enzymatic digestion was used to harvest germ cells (Zhang et al, 2003). In brief, 12–33 testes were digested with 0.05% collagenase (type IV; Sigma) in Dulbecco's PBSD (DPBS, KH_2PO_4 1.5 mmol/L, Na_2HPO_4 8 mmol/L [1.15 g], NaCl 137 mmol/L, KCl 2.7 mmol/L, $CaCl_2 2H_2O$ 0.7 mmol/L, and $MgCl_2 6H_2O$ 0.5 mmol/L) for 15 minutes at 35°C while shaking at 120 rpm. The dispersed testicular tissue was further digested with 0.0625% trypsin (porcine pancreas; Sigma) in Ca^{2+}/Mg^{2+}free-DPBS that contained 50–100 µg/mL DNAseI (Sigma), for 15 minutes. An equal volume of DPBS that contained 10% rat serum was added to inactivate the trypsin, followed by gentle centrifugation (at 500 × g for 5 min). The cell pellet was resuspended in McCoy's 5A medium (Trace Biosciences Pty Ltd., Victoria, Australia) that contained 0.1% BSA, 5 mmol/L L-lactate sodium salt (ICN Biochemicals, Aurora, Ohio), 1 mmol/L pyruvate sodium salt (Sigma), 100 IU/100 µg/mL of penicillin/streptomycin (Trace Biosciences), and 100µg/mL DNAseI, filtered through a 73-µm nylon mesh. Cell viability in the final cell suspension in the McCoy's 5A medium was 90%–99%, as determined by trypan blue exclusion, with a concentration of 77–152 × 10^6cells/mL. The suspension was stored on ice and used within 4 hours of collection.

Preparation of Recipient Rats and Germ Cell Transplantation

Testicular cooling described above was done to a group (n = 11) of 4.5–8-week-old Sprague-Dawley rats. The rats were used as recipients for donor cell transfer 15–34 days later. At that point, 110–400 µL of the donor cell suspension was injected into the rete testis via a vasa efferent insert, using a 30-gauge dental needle connected by 15-cm polyethylene tubing (inner diameter = 0.28, outer diameter = 0.61 mm; Dural Plastics & Engineering, New South Wales, Australia) to a 1-mL syringe. Simultaneous experiments were conducted using busulfan-treated rats as recipients for the same mouse donor cell population (Zhang et al, 2003).

Recipient Treatment after Germ Cell Transplantation

To reduce the immunological reaction of recipients to donor cells, CS (Novartis Pharma AG, Basel, Switzerland) was given to 6 of 11 recipient rats after germ cell transplantation, as described elsewhere (Zhang et al, 2003). CS is an immunosuppressive agent that can selectively inhibit T lymphocyte function. We have used it successfully to achieve germ cell transplantation between immunologically incompatible mice and rats. Donor mouse sperm developed in rat testes, and offspring were produced from rat donor germ cells transplanted into recipient rats (Zhang et al 2003). CS was initially dissolved in absolute ethanol, then redissolved in olive oil to a final concentration of 15 mg/mL in 4% ethanol. With slight modification to the published method (Bellgrau and Selawry, 1990), the recipient rats were injected subcutaneously with 15–20 mg/kg of CS on the day of germ cell transfer, followed by 10 mg/kg daily for 7 days, then 10 mg/kg every second day for 2 weeks, then 10 mg/kg once a week for another 1–2 weeks. Four of the 11 recipients were treated with 10 mg/kg of testosterone propionate (Jurox, New South Wales, Australia) (Seethalakshmi et al, 1990; Shetty et al, 2002), started immediately after transplantation, and repeated every third day for 6 weeks. Cells from 3 of the animals that received testosterone were transferred to CS-treated recipients, and those from the fourth were transferred to non–CS-treated recipients. Two other animals received 50 IU/kg of recombinant human FSH (Organon, Sydney, Australia) daily for the first week

Table 1. *Details of the cooled rats receiving donor germ cells from* LacZ *mouse testes*

Treatment After Transfer*	No. of Injected Testes	Age at Cooling (d)	Age at Transfer (d)	Volume (μL) Injected Into Testis	Days From Transfer to Autopsy	No. of Testes: Score ≤2	No. of Testes: Score =3
No CS	7	48 (31–54)	81 (60–88)	240 (150–400)	72 (64–136)	5	2
CS	12	39 (31–54)	61 (48–88)	200 (110–400)	73 (70–79)	10	2

* The recipient rats were divided into two groups according to whether or not CS was used. In each group, testosterone (T), or FSH, or no further treatment (None) was given to the rats. In the CS group, one rat received T, 1 received FSH, and 2 received none. In the group in which CS was not used, 3 rats received T, 1 received FSH, and 2 received none. The average value is provided with the range listed in parentheses.

(McLachlan et al, 1995) after transplantation, to improve donor spermatogenesis in the recipient testis (see Table 1).

Assessment of Recipients

For the analysis of donor cell colonization, the testes were removed and fixed in 4% fresh paraformaldehyde (BDH Laboratory Supplies, Dorset, England) for 1 hour using a standard method (Zhang et al 2002). The testes were then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal; Sigma) to detect the lacZ-positive cells and then further fixed in 10% neutral buffered formalin overnight. They were then dehydrated, processed, and embedded in paraffin. We then cut 7 μm sections for counterstaining with neutral fast red.

A semiquantitative criterion was used to judge the extent of mouse germ cell colonization in the recipient testes by recording the blue X-gal staining when viewed under a dissecting microscope (Zhang et al, 2003). The degree of staining was given a score where 1 denoted the absence of visible staining, 2–3 represented ambiguous or very weak staining, and 4–5 indicated good or very good blue staining in several seminiferous tubules.

Statistics

Data analysis was done using 1-way analysis of variance, correlation, and Student's *t* test with Systat software (SPSS, Chicago, IL). Log transformation was done for abnormal distributions of data. Significance was determined at the level $P < .05$.

Results

Cooling Leads to Testicular Weight Loss and Hormonal Changes

Cooling caused significant testicular weight loss but had no effect on body or reproductive accessory organ weight (including prostate, seminal vesicle, epididymis, and vas deferens). Testicular weight in the cooled rats decreased to 77% of that of the sham controls 3 days after cooling and remained around 44%–49% levels of the control testes 14–70 days after cooling ($P < .01$) (Figure 1). Plasma T concentrations varied over time but showed no significant difference between the sham control and treated rats (Figure 1).

Plasma inhibin B concentrations were significantly decreased to 55% of the control level 24 hours after cooling treatment ($P < .001$), a change that preceded the changes in testicular weight (Figure 1). From 7 days after treatment onward, the circulating inhibin B concentrations in cooled rats remained at 8%–29% of the control levels and were positively correlated with the changes in testicular weight ($R = 0.766$; $P < .001$).

Plasma FSH concentrations in the cooled rat testes increased to 135%–220% of the control levels ($P < .05$–.01). This was first observed 3 days after the decline in testicular weight and was maintained in all the treated animals (Figure 1). The change in FSH concentration with testicular cooling was highly negatively correlated with testicular weight ($R = -0.733$; $P < .001$) and with the decrease in the inhibin B concentration ($R = -0.676$; $P < .001$).

Histological Changes in the Cooled Testes

The cooled testes showed striking histological changes compared with the sham controls. Massive germ cell loss had occurred in the first week and progressively increased thereafter. By 35–70 days after cooling, 52%–67% of seminiferous tubule cross-sections had Sertoli cells only or germ cells up to the preleptotene stage of maturation; 20%–40% had pachytene spermatocytes or more advanced germ cells, and 2%–15% had lost cell and epithelial structures and been stained deep purple color with hematoxylin (''ghost tubules''; Table 2, Figure 2a; Steinberger and Tjioe, 1969). Most germ cell–depleted seminiferous tubule cross-sections in the cooled testes after 21 days still had spermatogonia and preleptotene spermatocytes remaining (Figure 2b). Multinuclear giant cells were found in a few tubule cross-sections of the treated testes. No abnormalities of the seminiferous epithelium were found in any of the control testes.

For TUNEL staining, a total of 35 sections from 28 different testes in G1–G5 were counted. The number of spermatogenic cells in testes 7 days after cooling (G6–G10) had been reduced to a level that was obvious by visual inspection. As a consequence, the proportion of seminiferous tubule cross-sections that contained TU-

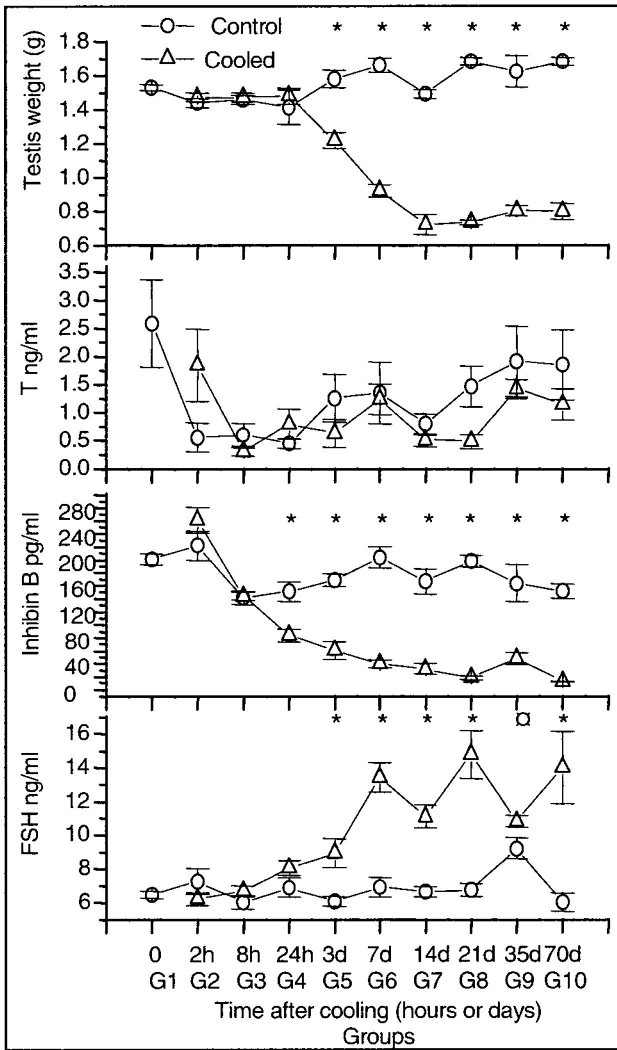

Figure 1. Testicular weight and concentrations (mean ± SEM) of plasma T, follicle-stimulating hormone (FSH), and inhibin B in rats subjected to testicular cooling (triangles) and their sham-operated controls (circles). A significant decrease in testicular weight or plasma inhibin B concentrations and an increase in plasma FSH concentrations occurred 1–3 days after cooling and onward, compared with sham controls (*$P < .01$; □$P < .05$). The significant changes in inhibin B concentrations first occurred within 24 hours after cooling, which is earlier than the changes in testicular weight.

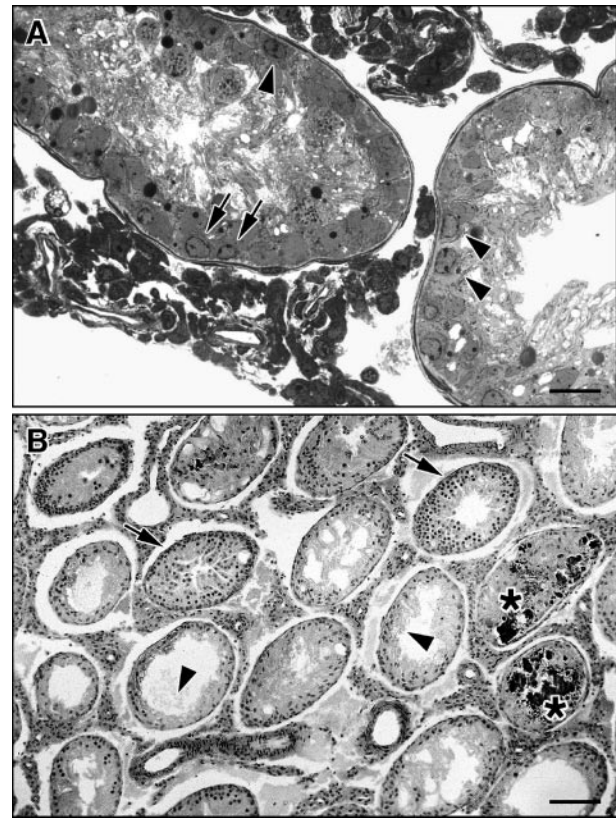

Figure 2. Variable histological changes in the cooled testes. **(A)** Seminiferous tubules of a cooled testis had spermatogonia (arrowheads) and preleptotene spermatocytes remaining (arrows) 21 days later (1-μm section for toluidine blue staining, bar = 18 μm). **(B)** Seminiferous tubules with only Sertoli cells remaining (arrowhead) or showing spermatogenic recovery (arrows) or necrosis (asterisk) present in a testis 35 days after cooling (7-μm section for hematoxylin and eosin staining, bar = 3 μm).

NEL-positive cells was also decreased in G7–G10, and reliable stage assessment was not possible. Therefore, we recorded the counting of TUNEL-positive cells with staging for G2–G6, and, for the analysis of G7–G10 samples, we counted the percentage of tubule cross-sections containing TUNEL-positive nuclei in the absence of staging.

TUNEL-positive–staining cells were found in the seminiferous tubules of both the control and cooled testes

Table 2. *Percentages of seminiferous tubules containing germ cells at various stages of spermatogenic maturation or showing atrophy after direct testicular cooling varying with time after treatment (mean ± SE, n = 7 in each group)*

Days After Cooling (Group Number)	Sertoli Cells Only or Plus Germ Cells Developed to Early Spermatogenic Stages	Germ Cell Maturation to Spermatocyte Stage or Beyond*	Tubular Destruction or Atrophy
7 (G6)	25.8 ± 5.8	67.6 ± 5.2	6.6 ± 2.8
14 (G7)	29.3 ± 7.0	69.0 ± 6.9	1.7 ± 0.9
21 (G8)	45.5 ± 7.8	39.6 ± 11.6	14.9 ± 8.4
35 (G9)	52.1 ± 7.1	40.3 ± 9.3	7.5 ± 3.1
70 (G10)	67.2 ± 5.7	19.8 ± 5.5	13.0 ± 5.6

* Tubules contained ≥3 germ cells at or beyond the pachytene spermatocyte stage of development.

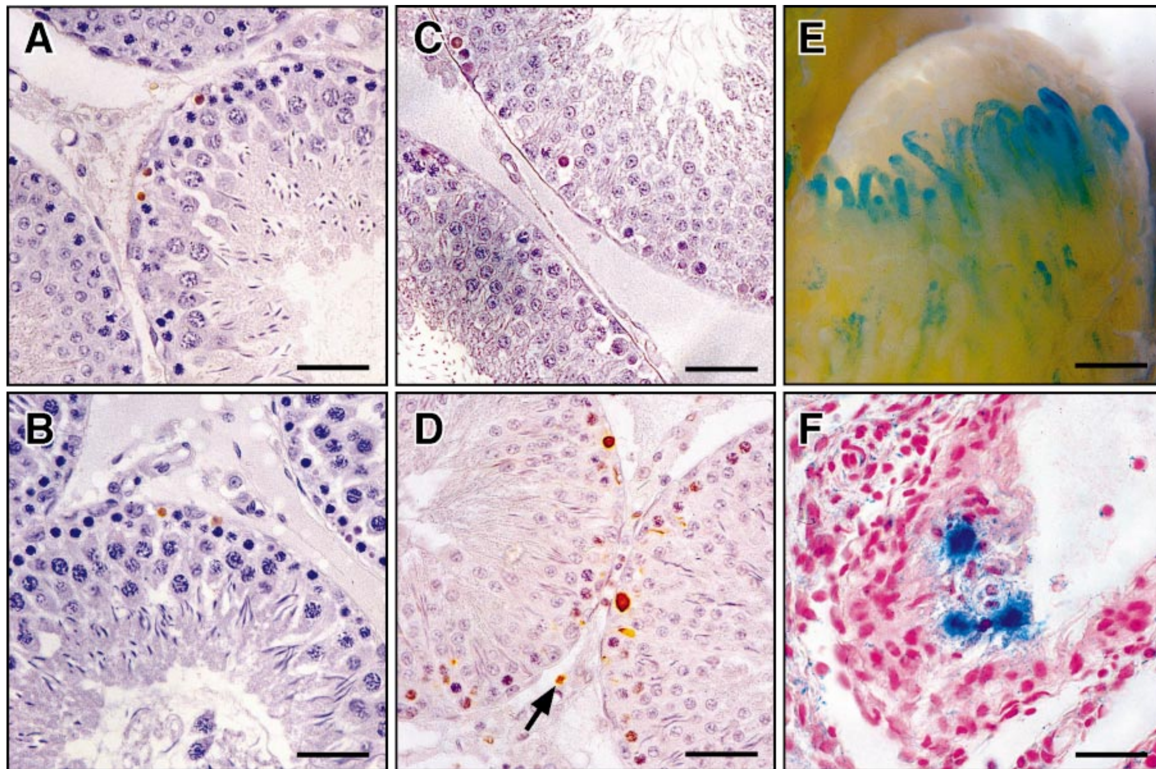

Figure 3. Cooled testes with terminal deoxynucleotidyl transferase –mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) staining **(A–D)** or X-gal staining **(E–F).** The presence of TUNEL-positive cells (brown) in either cooled testes **(A)** or sham control testes **(B)** 2 hours after cooling suggested that there is no obvious difference. Although the number of TUNEL-positive cells was significantly increased in the cooled testes 8 hours **(C)** after surgery (see Figure 4), the change was readily observed at 24 hours **(D).** Occasionally, a few TUNEL-positive intertubular cells were found (arrow in D). Blue X-gal staining showed donor mouse testicular cells in the seminiferous tubules of a cooled testis that had received donor cells from cryptorchid mouse testes 3 days earlier **(E),** but most of the donor mouse cells had disappeared and a few remaining cells were degenerating and surrounded by the host's Sertoli cells 75 days after transplantation **(F).** Bar in A–D = 4.5 μm, E = 22.6 μm, and F = 3.0 μm.

(Figure 3). There was no significant difference in the percentage of tubule cross-sections having TUNEL-positive cells between the control, sham-cooled, and cooled testes 2 hours after surgery. The proportion of tubule cross-sections with either any TUNEL-positive cells or at least 3 TUNEL-positive germ cells in the cooled testes were each significantly increased at 8 hours after surgery, compared with sham control testes (0.9% vs. 4.3%, $P = .01$; 0.2% vs. 2.2%, $P = .025$, respectively). By 24 hours, in the cooled testes, 34% of seminiferous tubule cross-sections had TUNEL-positive cells, and 28% had at least 3 positive cells, whereas the control testes had no tubules that contained ≥3 TUNEL-positive cells, and only 0.5% of tubules containing <3 positive cells. By 3 days after cooling, 62% of tubule cross-sections in the treated testes had TUNEL-positive cells, and >28% had at least 3 positive cells, whereas testes from control animals still had very few tubules containing TUNEL-positive cells and only 0.3% of tubule cross-sections had ≥3 positive cells ($P <$ .001, Figure 4).

The germ cell type most sensitive to the cooling treatment during the interval of 8 hours–3 days after cooling (G2–G5), as judged by the incidence of apoptosis, was the primary spermatocyte, followed by the round spermatid. The incidence of apoptotic spermatogonia was rare (Figure 5). Very few TUNEL-positive intertubular cells were found in the testes 1–3 days after cooling (Figure 3), and no obvious structural abnormality was found in Sertoli cells of the treated testes, with the exception of occasional cytoplasmic vacuoles.

Assessment of mRNA Molecules Associated with Apoptosis

RPAs were done on 2 independent occasions using testicular RNA prepared from 3 different rats in each of following groups: before cooling, 2 or 8 hours, and 1, 3, and 7 days after cooling (G1–G6). Not every target mRNA signal in the assayed samples was detectable or expressed consistently in each RPA experiment, although the housekeeping genes L32 and GAPDH were always readily detectable. The Bcl-xl, Bcl-xs, Bax, and Caspase-3 mRNAs were most consistently observed, reaching detectable levels in 97% and 100%, 79% and 82%, 76% and 91%, and 55% and 56% of the assayed samples in

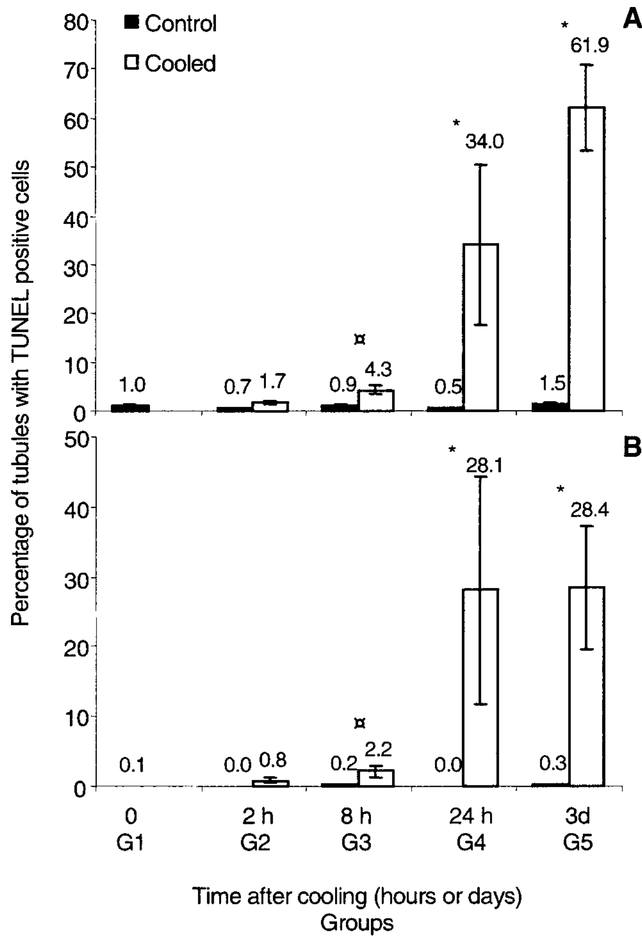

Figure 4. Percentage of seminiferous tubules with TUNEL-positive cells in normal control, sham-operated, and cooled testes from each group (G1–G5) before, 2, 8, and 24 hours after, and 3 days after cooling. **(A)** Percentage of tubules with TUNEL-positive cells. **(B)** Percentage of tubules having at least 3 TUNEL-positive cells. Cooled testes had significantly more apoptotic cells at 8 (¤$P < .05$), 24, and 72 hours (*$P < .01$), compared with sham controls.

the control and cooled testes, respectively. Some individual cooled testes showed measurable levels of Fas mRNA, whereas this signal was not observed in any control samples. The Fas-L, Caspase 2, Caspase 1, and Bcl-2 mRNAs were either not detectable or were measured in <25% of most samples assayed. Variability in the RPA results between individuals might be attributed to the fact that a thermoprobe was used in only 2 of the 3 or 4 rats that underwent surgery simultaneously, although the proportion of tissue expected to be affected by this procedure would be extremely small. Quantitative analysis of the target mRNA signals after the adjustment of their signal relative to that of the L32 housekeeping gene showed no significant up- or down-regulation in the cooled testes compared with the normal or sham controls at same time point ($P > .05$). RNA from all 14 testes in group 3 (8 hours after treatment) was assayed in duplicate. The quantitated mRNA signals were still not significantly different between the cooled and control testes (Figure 6), even though, at this time point, a significant difference in the incidence of apoptotic cells had been observed after cooling.

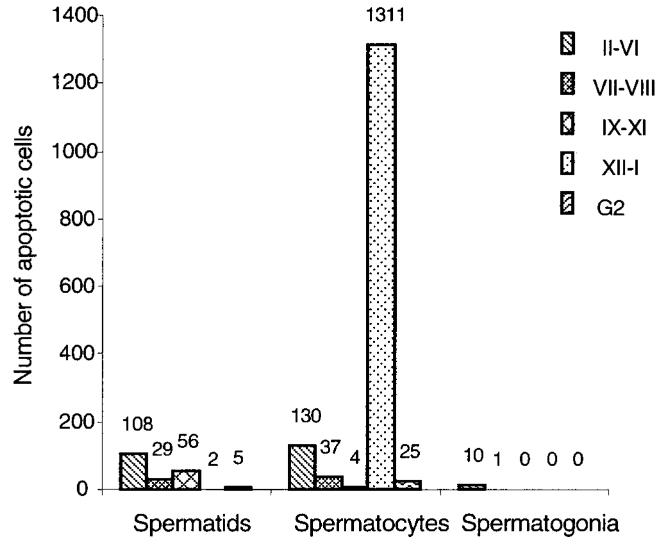

Figure 5. Number of apoptotic cells in G2 and G3–G5 cooled testes at various stages. Apoptotic spermatogonia represented <1% of all TUNEL-positive germ cells counted from a total of 18 sections from 15 testes in groups 2–5. Spermatogenesis stages II–VI (hatched bars), VII–VIII (cross-hatched bars), IX–XI (loosely hatched bars), and XII–I (dotted hatched bars), compared with G2 (wavy striped bars). The most sensitive cells were spermatocytes at stages XII–I.

Donor Mouse Germ Cells Do Not Develop in the Cooled Testes

For testing the spermatogenic environment, we transferred mouse donor germ cells into the cooled rat testes. In our previous results of mouse to rat germ cell transplantation (Zhang et al, 2003), the use of CS plus testosterone or FSH effectively improved the donor mouse spermatogonial cell colonization and regeneration. In the

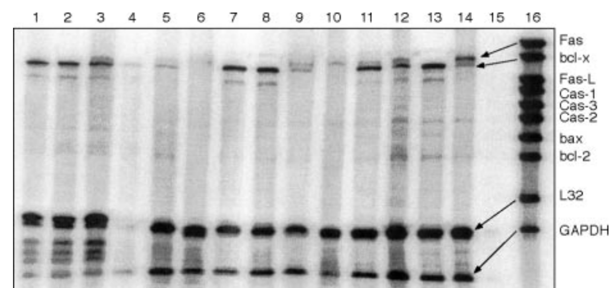

Figure 6. Representative RNase protection assay of group 3 (8 hours after cooling). Lanes 1–7 are individual testis RNA samples from the sham controls. Lanes 8–14 are individual testis RNA from the cooled testes. Lane 15 is a yeast negative control, and lane 16 contains the undigested cRNA probes.

present study, we therefore tried a similar regimen to improve the success. A total of 11 rats with 21 cooled testes received mouse donor germ cells (Table 1). One recipient rat was killed 3 days after mouse germ cell transplantation to confirm transfer success, and the remaining 10 rats were killed 64–136 days after transplantation. The testis assessed 3 days after cooling was filled with donor mouse germ cells (Figure 3e). In contrast, the testes examined 2–3 months after germ cell transplantation, with or without CS treatment, showed very few surviving mouse germ cells, and no testis had an X-gal staining score >3 (Table 1), in spite of simultaneous experiments with busulfan-treated recipients having good colonization of mouse donor cells (data not shown).

These testes showed variable histological changes in the seminiferous epithelium that were similar to those observed in cooled testes that did not receive donor germ cells. Most tubule cross-sections had only Sertoli cells, and a few had partial or full endogenous spermatogenesis. A few LacZ-positive cells (blue) were detected in 1 or 2 seminiferous tubules, indicating the presence of some remaining mouse donor cells. These donor germ cells did not appear to have expanded in numbers to form a colony, and most of them appeared to be degenerating or were being surrounded by the host's Sertoli cells (Figure 2f).

Discussion

Cooling the testis to around 4°C ±1 °C for 1 hour resulted in the significant loss of germ cells in the rat testis. The cooled testes had not recovered endogenous spermatogenesis at least for 10 weeks after cooling and were not successfully recolonized by transplanted germ cells, which suggests that irreversible changes had occurred in the testicular environment that were not conducive to germ cell survival. There was a change in the circulating hormones, inhibin B, and FSH, but testosterone levels showed no significant changes after treatment. The histological changes were variable, and seminiferous tubule cross-sections containing most germ cell types were present simultaneously adjacent to those having only Sertoli cells. Some seminiferous tubules were damaged and had destruction of epithelium in which all germ cells and Sertoli cells disappeared, as shown by deep purple hematoxylin staining.

In previous studies, the direct cooling of the rat testis to 0°C for 1 hour destroyed all germ cells and resulted in a spermatogenic epithelium with only Sertoli cells (Gronsky, 1930). Spermatogenesis was not reestablished, which suggests that the stem spermatogonia were destroyed. In contrast, after cooling to 2°C to 4°C for 1 hour, some spermatogonia survived, and spermatogenesis began to recover 28 days after cooling (Gronsky, 1930; Macdonald and Harrison, 1954). In these early studies of cooled testes (Macdonald and Harrison, 1954), the mechanism of action of the hypothermia was not clear, although angiography showed that intratesticular vasoconstriction had occurred during hypothermia, which suggests that it was due to intratesticular ischemia. This vasoconstriction occurred in fine intratesticular branches of the testicular artery only when the temperature dropped to −2°C (Macdonald and Harrison, 1954). In another study, cooling produced a protective effect on the ischemic testis (Goldstein et al, 1983; Miller et al, 1990). Ischemia caused by spermatic cord torsion through 720° for 1 hour resulted in permanent germ cell loss and no spermatogenic recovery in rats (Turner and Brown, 1993). The germ cell depletion in the ischemic testis may be due to ischemia-reperfusion injury from the endogenous production of reactive oxygen species (ROS), which causes massive germ cell apoptosis 4–24 hours after reversion of the torsion (Turner et al, 1997; Lysiak et al, 2000). The temperature of the iced Ringer's slush used in the present study did not drop below 0°C, so that the core temperature of the cooled testis was always around 4°C, a temperature at which cooling for 1 hour would not be expected to cause severe microischemia and ROS production. Germ cell apoptosis has also been previously produced by mild hypothermia to the testes (10°C for 30 minutes; Blanco-Rodriguez and Martinez-Garcia, 1997). Lower temperature from cooling, like higher temperature from cryptorchidism, may have a direct effect on the germ cells themselves (Yin et al, 1997).

In the present study, 1.7%–15% of seminiferous tubule cross-sections lost epithelial structures (both germ cells and Sertoli cells) 1–10 weeks after cooling. The cause of this tubular destruction was not clear. Direct testicular cooling depleted most germ cells by apoptosis, as demonstrated by TUNEL staining. However, the TUNEL technique can also label necrotic cells (Ansari et al, 1993).

Proteins of the Bcl-2 family are key regulators of the balance between cell survival (e.g., Bcl-2, Bcl-xl, Bcl-W, Mcl-1, and A1) and apoptosis (e.g., Bax, Bak, Bok, Bad, and Bim), and their role in testicular homeostasis has been studied (Print and Loveland, 2000; Blanco-Rodriguez, 2001). Apoptotic cell death follows 1 or both of 2 broad and convergent pathways, the death-receptor pathway and the mitochondrial pathway. When apoptosis through the death-receptor pathway is triggered by Fas receptor binding to Fas-L, caspase 8 is recruited and activated; this in turn activates downstream effector caspases and leads to cell death. The mitochondrial pathway involves cytochrome c release from mitochondria via activation of some Bcl-2 family members such as bax and bcl-x. Cytochrome c associates with Apaf-1 and then procaspase 9 to form the apoptosome. These 2 pathways converge at

the level of caspase-3 activation, which leads to activation of caspase-1 and -2 and causes cell apoptosis (Hengartner, 2000). Changes in expression of *Fas* and *FasL* appear to be useful for distinguishing germ cell death from Sertoli cell damage; Fas mRNA is up-regulated in testes with germ cell injury, whereas both Fas and FasL are up-regulated in response to Sertoli cell injury (Lee et al, 1999). In the torsion-reperfusion–injured testis, both apoptotic pathways may be involved (Lysiak et al, 2000). In the present study, the variability in RPA results indicates that this approach cannot be used to definitively determine whether germ cell apoptosis in the cooled testis involved the death-receptor, the mitochondrial pathway or both. However, in some individual animals, a distinct up-regulation of FAS mRNA was obvious at 24 hours. The absence of a common significant difference in target mRNA expression in the cooled and control testes suggests that the mechanisms of tissue damage are complex in these circumstances. The appearance of both apoptotic and atrophic cells in the cooled testis samples may underpin the variation in RPA results. Further investigation of changes in expression of downstream components is required to elucidate the mechanism of the germ cell apoptosis in cooled testes.

Just as differentiated spermatogenic cells are most sensitive to abdominal temperature (Nishimune et al, 1978), we found that spermatocytes were most vulnerable to the hypothermic insult. Their death occurred with high frequency in stage XII–I, in a pattern that was similar to that reported for the mild cooling-induced germ cell loss model (Blanco-Rodriguez and Martinez-Garcia, 1997). Very few spermatogonia were affected by cooling. Sertoli cell morphology, which was assessed by light microscopy, was not obviously affected by the cooling treatment. However, massive germ cell loss has been associated with the appearance of cytoplasmic vacuoles and Sertoli cell dysfunction as evaluated by ultrastructural changes and the measurement of androgen binding protein (Steinberger and Tjioe, 1969; Hatier et al, 1982; Pinon-Lataillade et al, 1988). The lack of spermatogenic recovery by 10 weeks after cooling suggests that the spermatogenic environment was affected by this treatment. The significant decrease in circulating inhibin B and increase in FSH after testicular cooling indicated there were specific changes in Sertoli cell function reflecting the loss of the germ cell population (Hatier et al, 1982; Sharpe et al, 1999). The permanent loss of spermatogenesis occurred in rat testes after direct cooling coupled with vascular occlusion for 90 minutes or with testicular cord torsion (720° for 60 minutes), circumstances under which no evidence of Sertoli or Leydig cell damage was detected (Young et al, 1988; Turner et al, 1997). It is important to note that the recovery of the endogenous germ cells has failed to occur, as shown by the lowered testicular weight, histological data, and the persistent elevation of FSH in accordance with low inhibin B levels. Results from the present study suggest that injury to the spermatogenic environment of the cooled testes is likely to account for the failure of mouse donor germ cells to survive in rat testes after transplantation.

The regeneration of spermatogenesis from surviving germ cells depends on a normal spermatogenic environment, in which Sertoli cells and Leydig cells play key roles in maintaining the intra-and intertubular compartments (Russell, 1979; Kangasniemi et al, 1996; Tanemura et al, 1996). Donor mouse spermatogonia can survive and undergo spermatogenesis in the testes of busulfan-treated wild-type rats treated with CS to suppress the immunological reaction and testosterone or FSH, to enhance spermatogenesis (Zhang et al, 2003). Donor rat–derived offspring have been repeatedly produced in our laboratory after rat-to-rat germ cell transplantation using a similar regimen (Zhang et al, 2003). Therefore, this rat model for germ cell transplantation should be appropriate for ascertaining whether the cooled testes provide an appropriate environment for donor germ cell colonization and regeneration. The cooled testes failed to support donor mouse germ cell spermatogenesis with or without the use of CS or exogenous testosterone, even while parallel, simultaneous experiments with busulfan-treated recipients did result in donor cell colonization (data not shown). Thus, the cooled testes do not appear to be suitable recipients for germ cell transplantation. In addition, they do not support the recovery of endogenous germ cells.

In conclusion, direct testicular cooling caused rapid and extensive germ cell loss by cell apoptosis in rat testes. Despite the loss of more advance germ cell types, spermatogonia were present in the cooled testis. Their lack of development indicated that testicular cooling can cause permanent damage to the spermatogenic environment. When our understanding of the specific cues by which spermatogonial stem cells are triggered to differentiate, returning to this cooled testis model to assess the integrity of such cues may be valuable.

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