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Cisplatin-Induced Long-term Failure of Spermatogenesis in Adult C57/Bl/6J Mice

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ABSTRACT: Exposure to cisplatin results in impaired spermatogenesis, azoospermia, and, sometimes, permanent infertility in male patients. The mechanism(s) by which cisplatin induces damage to testicular cells is poorly understood. We previously reported that acute exposure to cisplatin results in elevated germ cell apoptotic rates and that this indicates long-term damage to the seminiferous epithelium. Here, we present data that implicate an injury to Sertoli cells as a possible mechanism to explain an elevated rate of germ cell apoptosis and consequent infertility. Normal adult C57/Bl/6J mice were exposed to 1, 2, or 4 rounds of 1, 2.5, or 5 mg/kg cisplatin in a regimen designed to resemble clinical chemotherapeutic exposure (1 injection daily for 5 days with a recovery phase of 16 days between cycles). A dose-dependent reduction in testicular weight due to germ cell loss was observed. While exposure to 1 mg/kg caused only temporary germ cell depletion, higher doses (2.5 and 5 mg/kg) revealed widespread testicular atrophy as evidenced by gaps in the epithelium due to cytoplasmic vacuolization and loss of differentiating germ cells. Although the acute loss of germ cells by apoptosis can result in temporary infertility, the testis has the ability to repopulate itself with mature cells, provided the stem germ cell population remains unharmed. Here, we demonstrate that a sustained disruption of spermatogenesis occurs despite the continued presence of stem spermatogonia in the seminiferous epithelium. These results suggest that cisplatin-induced germ cell loss may occur, in part, as a result of Sertoli cell injury-dependent alterations in germ cell microenvironment.

Key words: Apoptosis, testis, germ cell, Sertoli cell.
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Testicular germ cell tumors (TGCTs) are the most common malignancy in adult men between 15 and 45 years of age (Fossa et al, 1988). Most germ cell malignancies, even post metastasis, are efficiently eliminated by a combination of cisplatin-based chemotherapy, surgery, and radiotherapy, leading to an overall cure rate of about 95% (Einhorn, 1990). Patients undergoing chemotherapy for TGCTs are usually of reproductive age, and a large proportion remain azoospermic for prolonged periods or are rendered permanently infertile. It has been suggested that long-term infertility, or permanent azoospermia, due to chemotherapeutic treatment will occur in more than 50% of patients receiving a cumulative dose of more than 600 mg/m^2 cisplatin (Pont and Albrecht, 1997). Although the acute loss of germ cells by apoptosis can result in temporary infertility, the testis has the ability to repopulate itself with mature cells, provided the stem germ cell population remains unharmed. Permanent infertility is generally believed to be the result of loss of stem spermatogonia, and the duration of azoospermia is believed to be related to the numbers of stem cells killed (Meistrich et al, 1989). According to Schrader et al, most stem spermatogonia survive chemotherapy if the cumulative dose of cytotoxic drugs does not surpass 600 mg/m^2, and spermatogenesis recovery is then imminent (Schrader et al, 2002), although it can take several years (Pogach et al, 1989; Petersen et al, 1998) or may even persist indefinitely (reviewed in Meistrich, 1998). At higher doses of chemotherapy, gonadal toxicity is severe and persistent (Ishikawa et al, 2004), and the destruction of type A spermatogonia accounts for the sustained (or irreversible) loss of sperm production (Schrader et al, 2002; Seaman et al, 2003).

The cytotoxic effects of cisplatin and other chemotherapeutic drugs result, in part, from their interactions with DNA (Zamble and Lippard, 1995; Wozniak and Blasiak, 2002; Lee and Schmitt, 2003). DNA-platinum covalent adducts inhibit fundamental cellular processes including replication, transcription, translation, and DNA repair (Wozniak and Blasiak, 2002). In experiments with testicular germ cell tumor lines, apoptosis was a common response to cisplatin treatment (Barry et al, 1990; Huddart et al, 1995; Sark et al, 1995; Burger et al,

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1997), but the mechanisms by which germ cells undergo apoptosis are not understood at the molecular level. It is believed that the recognition of cisplatin-DNA adducts by nuclear proteins and the formation of DNA-protein cross links is responsible for the anticancer activity of cisplatin (Wozniak and Blasiak, 2002). This sensitivity of testicular germ cells to cisplatin may be related to their inherent ability to undergo apoptosis (Huddart et al, 1995; Seaman et al, 2003; Spierings et al, 2003). Effects of cisplatin exposure on somatic cells of the testis (Leydig and Sertoli) have been evaluated, but the mechanism(s) remains to be elucidated. Disruption of the blood-testis barrier and decreased secretion of inhibin-B and transferrin from the Sertoli cells have been frequently reported after cisplatin injury (Pogach et al, 1989; Gotoh et al, 1990; Huang et al, 1990; Kopf-Maier, 1992; Nambu and Kumamoto, 1995; Nambu et al, 1995; Aydiner et al, 1997; Brennemann et al, 1997; Monsees et al, 2000). However, these studies have been performed after acute exposure to cisplatin, do not discuss long-term functional insufficiency of Sertoli cells, and are not reflective of cisplatin doses and toxicity encountered during clinically relevant chemotherapeutic regimens. In the case of Leydig cells, only compensatory functional insufficiency has been reported without any known clinical significance of this dysfunction (Hansen et al, 1990; Hansen, 1992; Hansen and Hansen, 1993; Fossa et al, 1995; Palmieri et al, 1996; Brennemann et al, 1997, 1998; Jakob et al, 1998; Howell and Shalet, 1998, 2001, 2002), except in 1 report that demonstrated a persistent failure of Leydig cell function following exposure to high acute doses of cisplatin (Gerl et al, 2001). From these investigations it is easily appreciated that an understanding of the mechanisms of testicular injury during chemotherapy-related subchronic exposure is required to ensure the design of optimal chemotherapeutic regimens based on individual patient prognoses and for ensuring postsurvival quality of life.

Here, we characterize a mouse exposure model designed to evaluate alterations in testicular histopathology and to study the pathogenesis of testicular germ cell loss after subchronic cisplatin exposure that closely mimics that encountered during chemotherapeutic regimens in the clinic. We report that cisplatin-induced germ cell loss is likely due to both direct effects on germ cells and their consequent death by apoptosis and Sertoli cell injury-dependent alterations in germ cell development, differentiation, and release of spermatozoa.

Materials and Methods

Animals

Adult male C57/BL/6J (C57) mice (11-week-old) (Jackson Laboratories, Bar Harbor, Me) were acclimatized 1 week prior to experiments (3 mice/cage in standard rodent cages). The animal room climate was kept at a constant temperature (23°C ± 1°C) at 30%–70% humidity with a 12 h alternating light-dark cycle. Animals were given water and standard lab chow ad libitum. All procedures involving animals were performed in accordance with the guidelines of the University of Texas at Austin's Institutional Animal Care and Use Committee (IACUC) in compliance with guidelines established by the National Institutes of Health (NIH).

*Exposure sets for cisplatin treatment**

Sets	Cisplatin Dose, mg/kg	Exposure Cycles	Recovery Period		Cumulative Dose of Cisplatin, mg/kg
			Cycles	Days	
Set 1.0/A	1.0	1	0.5	17	5.0
Set 1.0/B	1.0	2	1	34	10.0
Set 2.5/A	2.5	1	0.5	17	12.5
Set 2.5/B	2.5	1	1.5	55	12.5
Set 2.5/C	2.5	2	1	34	25.0
Set 2.5/D	2.5	2	2.7	94	25.0
Set 2.5/E	2.5	2	4	136	25.0
Set 2.5/F	2.5	2	4.5	157	25.0
Set 2.5/G	2.5	4	2	70	50.0
Set 2.5/H	2.5	4	4	142	50.0
Set 5.0/A	5.0	1	1.5	55	25.0
Set 5.0/B	5.0	2	1	34	50.0

* Adult C57 mice were given either 1, 2, or 4 rounds of 1, 2.5, or 5 mg/kg cisplatin in 0.9% saline. Total cumulative dose received by each group of mice and the recovery period after the last injection varied between groups as indicated. Control animals in each group received a similar volume of 0.9% saline vehicle. Each exposure set consisted of at least 3 vehicle control and 6 cisplatin-treated animals.

Cisplatin Exposure Protocol

Adult male C57 mice (mean weight 27 g) received 1, 2, or 4 rounds (1 intraperitoneal injection daily for 5 days with a recovery phase of 16 days between cycles) of 1, 2.5, or 5 mg/kg cisplatin in 0.9% saline (Sigma Aldrich, St Louis, Mo). In this regimen, the 2.5 mg/kg dose was the maximal dose compatible with complete mouse survival and not overt toxicity (death, loss of weight). The 5 mg/kg dose resulted in high animal mortality and data from these exposure sets is presented here in order to emphasize the severity of systemic and testicular injury. Intraperitoneal injections resulted in greater damage to the seminiferous tubules at the periphery of the testis due to direct exposure from the drug in the peritoneal cavity; however, damage was observed throughout the testis. Furthermore, intraperitoneal injections for the evaluation of cisplatin-induced testicular toxicity in rodents have been frequently used in previous investigations (Gotoh et al, 1984, 1990; Pogach et al, 1989; Huang et al, 1990; Nambu and Kumamoto, 1995). Control animals received a similar volume of 0.9% saline. Exposure sets have been summarized in the Table. Cumulative mouse doses, comparative with clinical human doses in mg/m^2, can be calculated using a factor of 3.3, which is the ratio of weight to surface area for a 26-g mouse (Freireich et al, 1966). During the course of this work, total cumulative cisplatin doses ranged between 16.5 and 165 mg/m^2. Injections were initiated for all exposure sets on day 1, and sub-

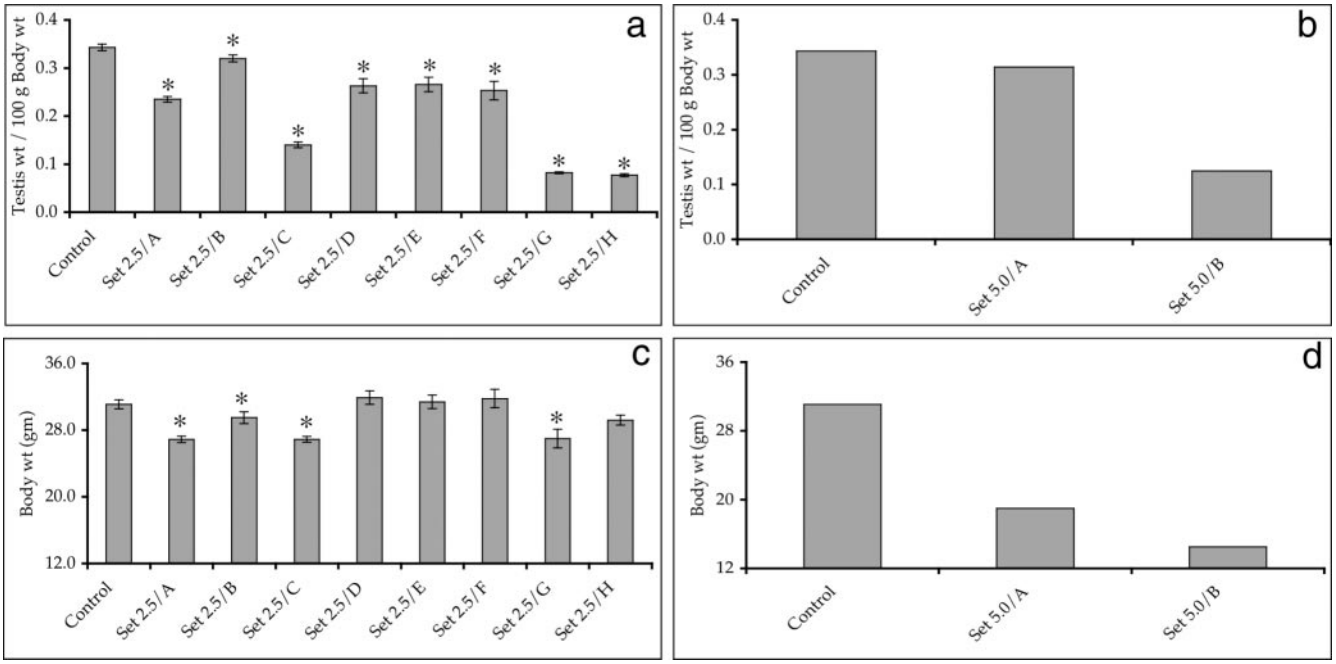

Figure 1. Testicular weight per 100 g body weight ratios **(a and b)** and body weights **(c and d)** for C57 mice exposed to **(a and c)** 2.5 mg/kg or **(b and d)** 5 mg/kg cisplatin ($n = 2$). Exposure sets have been summarized in the Table. Asterisks represent statistically significant differences in comparison to controls ($P \leq .05$).

sequent rounds of exposure were initiated on days 22, 43, and 64. Groups of 6 cisplatin-treated and 3 vehicle-treated mice were sacrificed by CO_2 inhalation at the time points indicated, and both testes were rapidly removed and processed for analyses. The duration of 1 complete spermatogenesis cycle, that is, the number of days required for a type A_1 spermatogonium to be transformed into an elongate spermatid, is 35 days in mice. Thus, after receiving the last injection, animals were allowed to recover for multiples of this time period (Table). Exposure sets are defined based on the number of rounds of cisplatin exposure and the day of sacrifice, at which time the right testis was flash frozen in liquid nitrogen and stored at −80°C and the left testis was immersion fixed overnight in Bouin solution (Polysciences, Inc, Warrington, Pa), washed in 70% EtOH-Li_2CO_3 (saturated solution, Mallinkrodt, Paris, Ky), and embedded in paraffin.

Histology and Immunohistochemistry

For evaluation of testicular histopathology Bouin fixed paraffin embedded testis cross sections (5 μm) were transferred onto poly-L-lysine coated slides and deparaffinized in CitruSolv (Fisher Scientific, Pittsburg, Pa) for 10 minutes, followed by rehydration in a graded alcohol series. For periodic acid–Schiff (PAS-H) staining, tissue cross sections were stained in periodic acid (0.5% wt/vol in distilled water) for 10 minutes, rinsed several times in water, and stained in Schiff reagent (Fisher Scientific) for 30 minutes at room temperature. After several washes in distilled water, the tissue was counterstained with a dilute solution of hematoxylin in water to provide a light background for histological evaluation. Immunohistochemical localization of both GATA-4 (a Sertoli cell nuclear antigen) and GCNA1 (a germ cell nuclear antigen) was performed according to our previously described protocols (Giammona et al, 2002) using a goat polyclonal primary antibody for GATA-4 (C-20, sc-1237; Santa Cruz Biotechnology, Santa Cruz, Calif) and a rat monoclonal antibody for GCNA1 (10D9G11; a gift from Dr George C Enders, University of Kansas Medical Center, Kansas City, Kan). Slides were viewed with a Nikon E800 microscope and images were captured using a Nikon CoolSNAP digital camera and processed using MetaMorph Imaging System (v 4.1) and Adobe PhotoShop 7.0 (Adobe, San Jose, Calif).

Statistics

Significance between groups ($P < .05$) was evaluated using parametric single factor analysis of variance (ANOVA) with Fisher protected least significance differences (PLSD) test comparison using Statview software (SAS Institute Inc, Cary, NC).

Results

Testis and Body Weights

A significant reduction in testicular weight (as a function of total body weight) and total body weight was observed after subchronic exposure of C57 mice cisplatin at 2.5 and 5.0 mg/kg doses (Figure 1; testicular weights are represented in grams per 100 g of body weight). After a single round of exposure, C57 mice demonstrated a 31% decrease in average testicular weight (set 2.5/A, Figure 1a, see the Table for description of exposure sets). This loss in testicular weight was partially recoverable after the animals were allowed to recover for a complete sper-

matogenesis cycle (set 2.5/B). An increase in cisplatin dose, after a second round of cisplatin, resulted in a much more dramatic decrease in testicular weight (set 2.5/C; 57% reduction, Figure 1a), and testicular recovery was only partially reversible. Testis weights for these mice climax at about 80% of controls, despite nearly complete recovery in body weight (Figure 1c), even after being allowed to recover for 2, 4, or more spermatogenesis cycles (sets 2.5/D, E, and F, Figure 1a). A further increase in cisplatin dose, after 4 rounds of exposure, results in far greater damage to the seminiferous epithelium with the testicular weights reduced to about 25% in comparison with age-matched controls. This injury appears irreversible, and the mice demonstrate no evidence for recovery in testicular weight even after recuperating for 4 spermatogenesis cycles (sets 2.5/G and H, Figure 1a). These mice also show a slight, though statistically significant, reduction in body weight (Figure 1c) (set 2.5/H 29.2 ± 0.6 $P = .058$).

At 5 mg/kg, each 5-day round of cisplatin at 5 mg/kg equaled a total cumulative dose of 25 mg/kg (82.5 mg/m^2), and resulted in a drastic reduction in body weight (Figure 1d) and about 66.6% animal mortality (4 out of 6 animals in each exposure set). Evaluation of testis and body weights for this dose was thus performed for 2 surviving mice from each exposure group, and is presented here, without statistical significance, in order to emphasize the severity of systemic and testicular injury, especially after 2 rounds of exposure (set 5.0/B; Figure 1b and d). At 1 mg/kg, cisplatin did not appear to cause any significant alterations in testicular weights (data not shown).

Histopathology of Cisplatin-Exposed Mouse Testes

Evaluation of testicular histopathology of cisplatin-exposed testis cross sections revealed severe and dose-dependent atrophy and germ cell loss, which correlated with testicular weight. At the lowest dose (1 mg/kg), cisplatin did not cause any significant alterations in testicular histopathology at all the time points evaluated and is not presented here. The most interesting results were observed after exposure to 1 or more rounds of cisplatin at 2.5 mg/kg. After a single round of cisplatin (set 2.5/A, Figure 2b), in comparison with vehicle-treated mice (Figure 2a), a decreased cellularity was observed coupled with a reduction in the height of the seminiferous epithelium. Sertoli cell vacuoles, apical sloughing and shedding of cellular material, and absence of specific cell populations were seen in as many as 90% tubules in a given testis cross section. For example, the stage VII–VIII tubule shown in Figure 2b has a relatively normal complement of preleptotene spermatocytes and step 16 spermatids but shows a complete absence of pachytene spermatocytes and contains very few round step 7–8 spermatids. After the mice were allowed to recover for 1 complete spermatogenesis cycle (set 2.5/B), approximately half the tubules in a cross section demonstrated normal spermatogenesis, which would account for the recovery in testicular weight (Figure 1a). However, the remaining tubules, approximately 55%, continued to show decreased cellularity, Sertoli cell vacuolation, sloughing of cellular material, and missing generations of cells, indicating only partial recovery. The stage I tubule depicted in Figure 2c shows relatively normal complements of early pachytene spermatocytes and condensing step 13 spermatids, but it shows a dramatic reduction in step 1 round spermatids, which are completely absent over half the circumference of the tubule and in Figure 2d. The stage V tubule shows a drastic reduction in step 15 spermatids and has a reduced number of pachytene spermatocytes and step 6 round spermatids.

Increasing the exposure to 2 rounds of cisplatin at 2.5 mg/kg results in an exacerbation of these symptoms. Even after recovery for a complete cycle of spermatogenesis, a reduction in seminiferous tubule diameter is accompanied by widespread atrophy, evidenced by gaps in the epithelium due to cytoplasmic vacuolization and failure of development of later stage differentiating germ cells (round and elongating spermatids; set 2.5/C, Figure 3a) in most tubules. After recovery for 2 spermatogenesis cycles (set 2.5/D), the presence of severely atrophied tubules provided evidence for long-term damage to some of the tubules (Figure 3b). Only as few as 7% to 8% tubules in these mice presented normal cellular associations due to the absence of specific germ cell subpopulations, such as the absence of an entire generation of spermatocytes, round spermatids, or mature spermatids. Furthermore, recuperation of these mice for 4 or more cycles of spermatogenesis (sets 2.5/E and F) did not provide any significant advantage. Testicular recovery in these mice was only partially reversible, which correlates with the testicular weight, which climaxed at about 80% with respect to controls.

Histopathological evaluation of mice that were exposed to 4 rounds of 2.5 mg/kg cisplatin, resulting in a total cumulative dose of 50 mg/kg (165 mg/m^2), revealed extremely severe injury to the seminiferous epithelium. A drastic reduction in tubular diameter was evident, which correlated with the drop in testicular weight. Testicular cross sections consisted of mostly small ''Sertoli cell only'' tubules with large cytoplasmic vacuoles (Figure 3c). When the same cumulative dose of cisplatin (50 mg/kg; 165 mg/m^2) was administered over a shorter time period (5 mg/kg, 2 rounds), it was highly deleterious to the testis and also to the overall well being of the animal, resulting in about 66.6% animal mortality. Evaluation of the testes of the 2 surviving mice from this exposure set revealed a similar reduction in seminiferous tubule di-

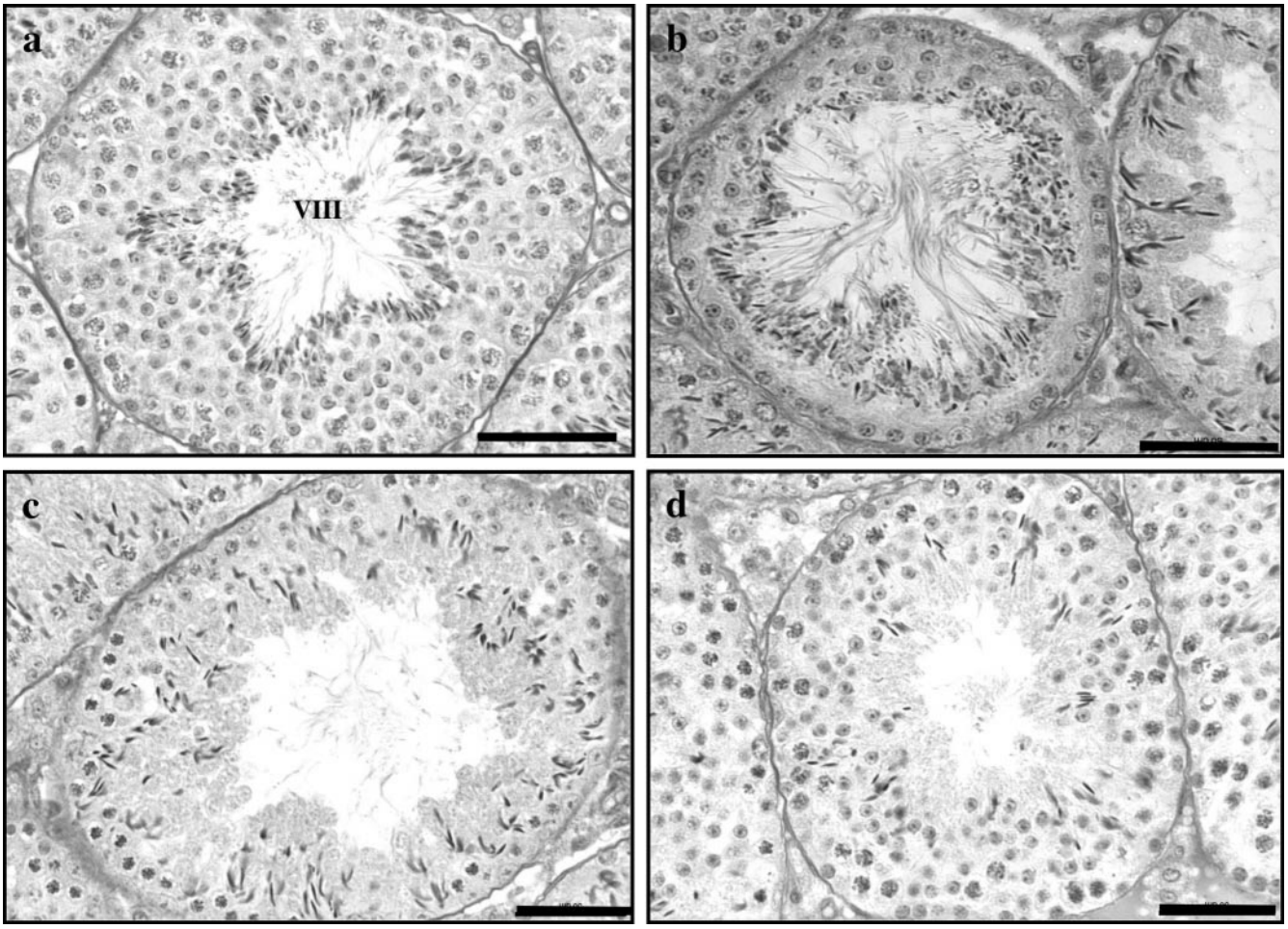

Figure 2. Histopathology for C57 mice exposed to 1 round of chemotherapy at 2.5 mg/kg. **(a)** Vehicle control (saline 0.9%), stage VIII; **(b)** set 2.5/A, increased luminal size, reduced cellularity due to absence of pachytene spermatocytes, and reduced numbers of round spermatids; **(c)** set 2.5/B, reduced cellularity due to markedly reduced numbers of step 1 round spermatids; **(d)** set 2.5/B, overall reduction in cellularity and drastic reduction in step 15 spermatids; bar equals 50 μm.

ameter and atrophy to the seminiferous epithelium (Figure 3d, set 5.0/B). This was accompanied by extensive vacuolation of the cytoplasm (Figure 3d).

Immunohistochemistry

In order to determine the cell types lost after cisplatin exposure, immunohistochemical localization of GATA-4 (a Sertoli cell nuclear antigen) and GCNA1 (a germ cell nuclear antigen), was performed on 5-μm cross sections of cisplatin-exposed mouse testes. Immunostaining for GCNA1 revealed the abundant presence of spermatogonia and differentiated germ cells in mice exposed to 1 or 2 rounds of cisplatin at both 2.5 (Figure 4b) and 5 mg/kg (Figure 4c). In contrast, after exposure to 4 rounds of cisplatin at 2.5 mg/kg, only /5% tubules showed the presence of differentiating spermatogenic cells (Figure 4d, sets 2.5/G and H) and hence there must be surviving, functional stem cells in this region of tubules. Immunostaining for GATA-4, a Sertoli cell nuclear antigen, revealed large numbers of Sertoli cell nuclei in testis cross sections from cisplatin-treated mice, indicating that there was no loss of Sertoli cells as a consequence of cisplatin exposure (Figure 5).

Discussion

Male patients receiving cisplatin-based chemotherapy for testicular cancer and other solid tumors sustain severe and sometimes irreversible damage to the seminiferous epithelium (Brennemann et al, 1997; Pont and Albrecht, 1997; Howell and Shalet, 2001). Since many of these patients are treated with chemotherapeutic agents before and during their reproductive years, and cure rates for several types of cancer are high, sterility caused by treatment is a very significant concern (reviewed in Meistrich, 1998). To understand the sequence of events leading to cisplatin-induced damage and the cellular mechanism(s) of this

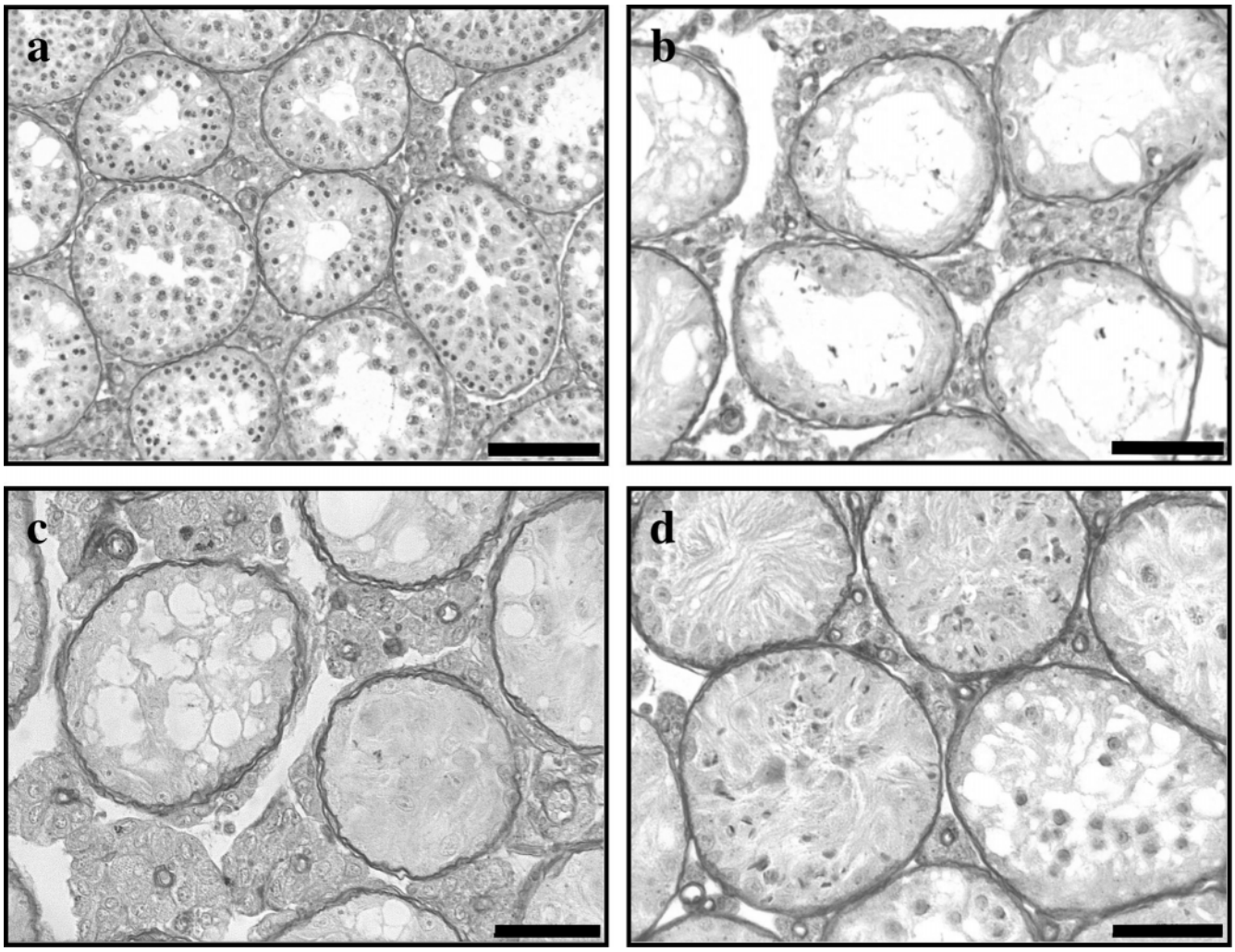

Figure 3. Histopathology for C57 mice exposed to cisplatin at 2.5 and 5.0 mg/kg. **(a)** Set 2.5/C, widespread damage, extensive vacuolation, and absence of spermatids in this field; **(b)** set 2.5/D, complete atrophy and loss of cellularity in the tubules shown here; **(c)** set 2.5/G, widespread atrophy, reduced tubular size, and loss of all germ cells; **(d)** set 5.0/B, failure of recovery of spermatogenesis and extensive vacuolation. Only a few spermatids remain; **(a and b)** bar equals 100 μm and **(c and d)** bar equals 50 μm.

damage, a comprehensive study of the impact of drug exposure on all components of spermatogenesis is required. To date, no such comprehensive study has been conducted. Elucidation of the nature of permanent testicular injury will assist the task of developing cisplatin-based chemotherapeutic treatment strategies aimed at minimizing this damage.

In this study, we characterized the pathology of cisplatin-induced damage following a subchronic exposure regimen designed to resemble clinical exposure in an attempt toward understanding the mechanisms of irreversible testicular injury. This is a first step toward testing whether a toxicity threshold exists between permanent damage versus eventual recovery. The pathological differences between the exposure sets described in the ''Results'' section demonstrate the existence of this toxicity threshold. While the 5 mg/kg dose is extremely lethal to the animal, resulting in the death of 4 out of 6 animals in each exposure set, survival of stem spermatogonia (Figure 4c) indicates a possibility for the recovery of spermatogenesis after an almost complete depletion of differentiating germ cells (Figure 3d, sets 5.0/A and B). Similarly, up to 2 rounds of cisplatin at 2.5 mg/kg allowed spermatogonial survival (Figure 4b) and recovery of spermatogenesis and testicular weight even after extensive germ cell depletion and epithelial atrophy. However, when exposed to 2.5 mg/kg cisplatin for a prolonged period at 4 rounds of exposure, animals lost a substantial percentage (/95%) of their stem spermatogonia, indicated by 95% of the tubules being devoid of germ cells (Figure 4d). The presence of even 5% stem spermatogonia provides evidence for their survival after cisplatin exposure, as has been suggested before (Meistrich, 1998; Schrader et al, 2002). It is intuitive to reason that with fewer sper-

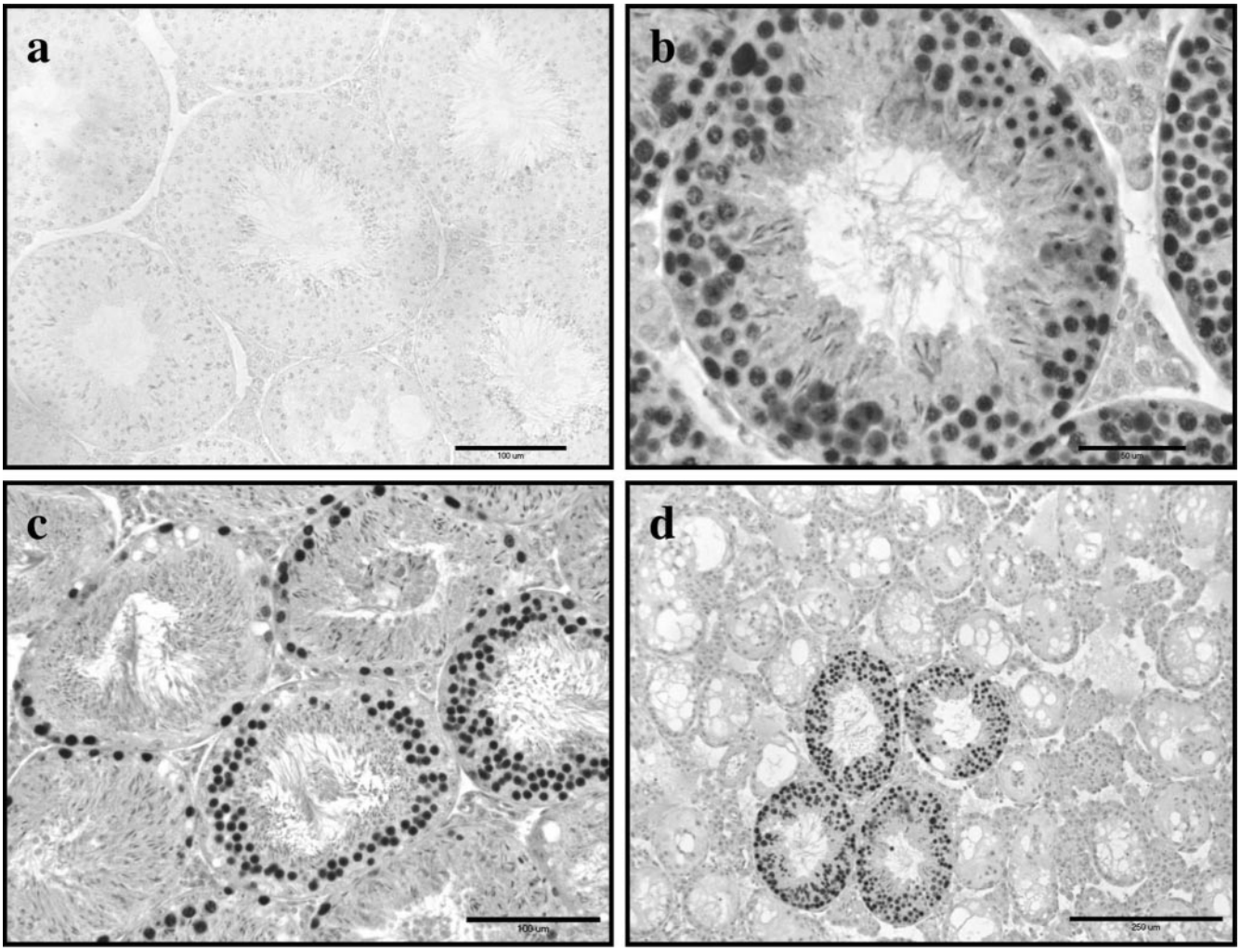

Figure 4. Immunohistochemical localization of GCNA1 in seminiferous tubules of mice exposed to cisplatin. Incubation with primary antibody (10D9G11) revealed specific staining of spermatogonia and differentiating germ cell nuclei, which were abundantly present **(a)** assay control (no primary antibody), bar equals 100 μm **(b)** set 2.5/C, bar equals 50 μm **(c)** set 5.0/A, bar equals 100 μm **(d)** set 2.5/H, bar equals 250 μm.

matogonia, the restoration of small foci of functional spermatogenesis may require several years (Petersen et al, 1998) or may even result in permanent infertility (Meistrich, 1998; Petersen et al, 1998). It has previously been shown, during evaluation of testicular biopsies from azoospermic men with Sertoli cell only syndrome, that mature sperm can be isolated from small foci of spermatogenesis within the testicular parenchyma (Mulhall et al, 1997; Howell and Shalet, 1999). In a time frame relative to humans (5–10 years), these foci of spermatogenesis could in fact result in an eventual recovery of fertility.

Previously, we have shown that exposure to an acute dose of cisplatin resulted in an elevated germ cell apoptotic rate (Seaman et al, 2003). While this elevation in germ cell apoptotic rate is observed in the initial days (days 14–21) after subchronic cisplatin exposure, a decline is evident soon after (data not shown). However, this decline in germ cell apoptotic rate occurs not as a result of a reduced level of apoptosis within the testis but as a result of the absence of germ cells themselves. Exposure to both 2.5 and 5.0 mg/kg cisplatin resulted in seminiferous tubule atrophy characterized by the appearance of cytosolic vacuoles, apical sloughing, shedding of cellular material into the lumen, and a massive loss of germ cells, which are all manifestations of Sertoli cell injury (Boekelheide, 1993). Further recovery resulted in the appearance of tubules that lacked standard stage-specific cellular associations because of the absence of specific germ cell subpopulations, such as an entire generation of spermatocytes, round spermatids, and mature spermatids (Figures 2 and 3). This could be the consequence of either the loss of those specific populations via apoptosis or a failure of the previous generation to differentiate. Although the continued incidence of germ cell apoptosis is observed at all recorded time points, it is not high enough to account for missing generations in the germ cell population (data not

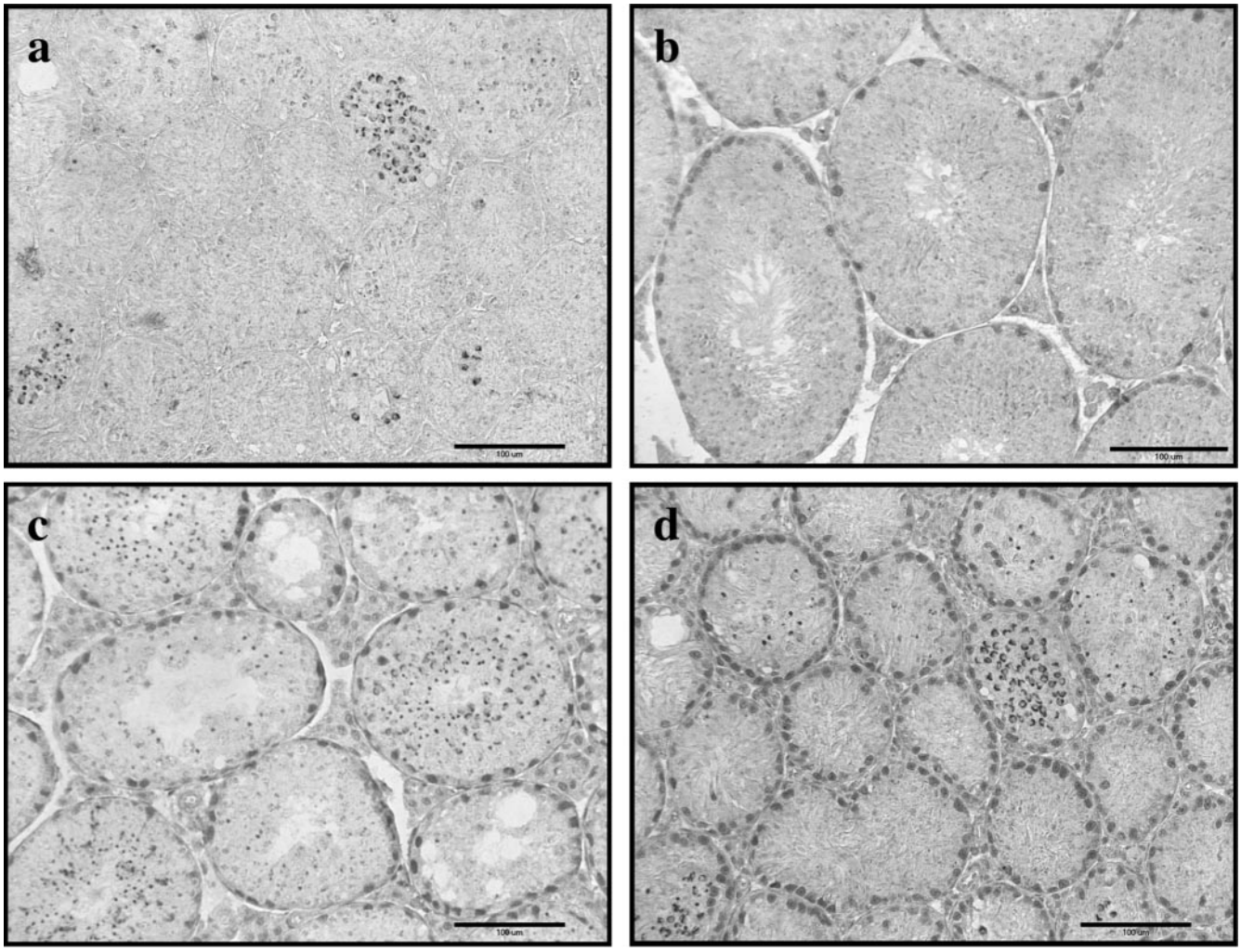

Figure 5. Immunohistochemical localization of GATA4 in seminiferous tubules of mice exposed to cisplatin. Incubation with primary antibody (sc-1237) revealed specific staining of equivalent numbers of Sertoli cell nuclei in cisplatin and vehicle-treated animals **(a)** assay control (no primary antibody) **(b)** vehicle control (saline 0.9%) **(c)** set 2.5/C **(d)** set 5.0/B; bar equals 100 μm.

shown). Thus the latter probability of germ cells failing to differentiate appears more plausible. For example, if a set of proliferating type A spermatogonia underwent a doubling division and none of them entered differentiation, the result would be a gap in differentiating germ cell stages. Absence of 1 or more germ cell generations due to the failure of previous stages to differentiate has been reported before in mice that carry mutations in the spermatogonial gene, *Plzf* (Buaas et al, 2004) and in mice that have decreased expression of GDNF (+/−) (Meng et al, 2000). Glial cell line–derived neurotrophic factor (GDNF), secreted by Sertoli cells, is known to influence the cell fate of undifferentiated spermatogonia. This observation adds further credence to our suggestion that cisplatin-induced injury to Sertoli cells results in alterations in spermatogenesis and testicular histopathology.

After exposure to 2 rounds of cisplatin, the initial loss of germ cells and testicular weight was only partially reversible, and the average testicular weights of these mice climaxed at about 80% of controls, even after recovery for 4 or more spermatogenesis cycles (Figure 1). Many of the seminiferous tubule cross sections of these animals are abundantly populated with stem spermatogonia and differentiating germ cells. The stagnation in recovery of spermatogenesis could be due to several factors. One possible factor is that the migration of surviving stem cells to populate other regions of the same tubule can be limited (van den Aardweg et al, 1983), and another is that some of the approximately 20% tubules within the testis may be completely devoid of stem cells. Also the observation of tubules with missing germ cell stages, long after cisplatin treatment has been concluded, indicates that there is a permanent dysregulation of the regularity of stem cell self-renewal and differentiation. This failure of

complete recovery of spermatogenesis could be the consequence of damage to somatic cells of the seminiferous epithelium, that is, the Sertoli cells.

The dose-dependence of our observations agrees with the suggestions of Schrader et al (Schrader et al, 2002) wherein as long as defined cumulative doses of cytotoxic drugs are not surpassed (600 mg/m^2), human stem cell spermatogonia survive chemotherapy and form the basis for the recovery of spermatogenesis. A destruction of type A spermatogonia at higher doses leads to a sustained or irreversible loss of sperm cell production (Schrader et al, 2002). While mice appear to be about threefold more sensitive to the effects of cisplatin-induced testicular toxicity, based on histopathology and ultrastructure, our results do provide indications of the effects of cisplatin on the human testis. We report that prolonged exposure at a systemically less toxic dose (2.5 mg/kg) may result in sustained injury to the seminiferous epithelium, and consequently infertility, due to the loss of stem spermatogonia. On the other hand, exposure to high doses over brief periods of time may elicit systemic toxicity but allows for the survival of spermatogonia. The loss of spermatogonia at the highest dose is also consistent with our previous observations demonstrating spermatogonial apoptosis only at high acute doses of cisplatin (Seaman et al, 2003). Our most compelling observations have arisen from the 2.5 mg/kg exposure sets that illustrate the distinct time and dose dependence of cisplatin-induced toxicity. Based on our observations in mice it can be deduced that a decision regarding a patient's chemotherapeutic regimen should take into account not just systemic toxicity of the drug, but also its potential to cause infertility after exposure for prolonged periods. Our observations indicate that a moderate dose given over a couple of cycles could result in the effective elimination of the tumor while preserving fertility. While these data provide the groundwork for understanding the molecular mechanisms responsible for the long-term testicular injury, predictions as to the fertility prognoses of patients undergoing chemotherapeutic treatment may be premature.

In summary, the results of the present study indicate that exposure to cisplatin results in long-term testicular injury in a dose-dependent manner in the testis of adult mice. These present findings suggest that cisplatin-induced Sertoli cell injury may play a role in the pathogenesis of germ cell loss from the testis and long-term testicular injury. However, at the present time, the exact contribution of Sertoli cell injury versus direct cisplatin-induced germ cell injury cannot be discerned. Regardless, this study provides the impetus for future investigations exploring the mechanism(s) of cisplatin-induced long-term failure of spermatogenesis.

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