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Cisplatin-induced alterations in the functional spermatogonial stem cell pool and niche in C57/BL/6J mice following a clinically relevant multi-cycle exposure

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Abstract

A typical clinical *cis*-diamminedichloroplatinum (II) (cisplatin) dosing regimen consists of repeated treatment cycles followed by a recovery period. While effective, this dosing structure results in a prolonged, often permanent, infertility in men. Spermatogonial stem cells (SSCs) are theoretically capable of repopulating the seminiferous tubules after exposure has ceased. We propose that an altered spermatogonial environment during recovery from the initial treatment cycle drives an increase in SSC mitotic cell activity, rendering the SSC pool increasingly susceptible to cisplatin-induced injury from subsequent cycles. To test this hypothesis, the undifferentiated spermatogonia population and niche of the adult mouse (C57/BL/6J) were examined during the recovery periods of a clinically-relevant cisplatin exposure paradigm. Histological examination revealed a disorganization of spermatogenesis correlating with the number of exposure cycles. Quantification of Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-dUTP Nick End Labeling (TUNEL) staining indicated an increase in apoptotic frequency following exposure. Immunohistochemical examination of Foxo1 and incorporated BrdU showed an increase in the undifferentiated spermatogonial population and mitotic activity in the recovery period in mice exposed to one cycle, but not two cycles of cisplatin. Immunohistochemical investigation of glial cell line-derived neurotrophic factor (GDNF) revealed an increase in production along the basal Sertoli cell membrane throughout the recovery period in all treatment groups. Taken together, these data establish that the impact of cisplatin exposure on the functional stem cell pool and niche correlates with: (1) the number of dosing cycles; (2) mitotic activity of early germ cells; and (3) alterations in the basal Sertoli cell GDNF expression levels after cisplatin-induced testicular injury.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have disclosed all financial sources and confirm that there are no conflicts of interest.

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Keywords

cisplatin; GDNF; spermatogonia; SSC niche; Sertoli cell; stem germ cell

1. INTRODUCTION

The chemotherapeutic compound *cis*-diamminedichloroplatinum (II) (cisplatin, CDDP) was first introduced in 1972 for the treatment of various solid tumors and results in a dramatic improvement in the cure rate and survivorship of patients suffering from testicular, head and neck, and ovarian cancer (Highley and Calvert, 2000). Remarkably, cisplatin-based therapy increased the five-year survival rate of patients diagnosed with testicular germ cell tumors to greater than 95% (Biggs and Schwartz, 2007; Einhorn, 1990; Highley and Calvert, 2000), the most common malignancy in males aged 15–35 years. An unfortunate side effect of cisplatin therapy in males is a lengthy, sometimes permanent, infertility (Pont and Albrecht, 1997); a consequence of particular concern to men during their reproductive period. Therefore, a critical need exists to develop effective strategies that will preserve male fertility while maintaining effective tumor elimination.

Cisplatin exposure results in the formation of cisplatin-DNA intra- and inter-strand adducts. Failure to repair these lesions initiates cell apoptosis (Highley and Calvert, 2000), leading to substantial cell elimination in populations of rapidly dividing cells (Highley and Calvert, 2000; Kelland, 2007). In adults, spermatogenesis proceeds from spermatogonial stem cells (SSCs) through multiple mitotic and meiotic divisions to produce mature spermatozoa. Under normal circumstances, a steady state ratio of each germ cell sub-population is established (Russell *et al.*, 1990). It is thought that in this steady state, a small percentage of SSC progeny progress to produce mature spermatozoa and very few SSCs undergo self-renewing divisions to maintain the SSC population (Kostereva and Hofmann, 2008). By virtue of rarely entering the cell cycle, SSCs are thought to be largely insensitive to DNA damage (Meistrich, 1984; Meistrich *et al.*, 1982). This pool of surviving SSCs would theoretically be capable of re-initiating spermatogenesis following treatment. It has been suggested that a loss of germ cells may prompt SSCs to enter the cell cycle at a higher frequency than occurs under steady state spermatogenesis as a mechanism to repopulate the testis after injury (Meistrich, 1986). Although the full complement of factors responsible for mediating such a shift in SSC kinetics are not fully understood, glial cell line-derived neurotrophic factor (GDNF) has been implicated in SSC regulation in many studies, proving to be critical for SSC self-renewal and maintenance of stem cell identity (Bugeaw *et al.*, 2005; He *et al.*, 2008; Meng, 2000; Simon *et al.*, 2007).

The consequences of exposure to cisplatin are well documented in many organ systems (Einhorn, 1990; Einhorn and Donohue, 1998) including the testis. However, a vast majority of these studies are based on a single acute dose of relatively moderate to high concentrations. While these reports provide useful information, they do not satisfactorily approximate the conventional therapeutic regimen of human cisplatin treatment. We have previously developed a low-dose, sub-chronic, multi-cycle cisplatin dosing paradigm (Sawhney *et al.*, 2005) intended to replicate human clinical cisplatin-based chemotherapeutic regimens (Reed *et al.*, 1988). Here, we examine the state of the

seminiferous epithelium of C57/BL/6J mice exposed to a repeated, low-dose cisplatin treatment, including assessment of SSC mitotic activity, undifferentiated spermatogonial population size, and GDNF expression during the recovery period. These perturbations in spermatogenesis were interpreted in the context of cisplatin exposure cycle number, and compared to a single cycle of an equivalent cumulative dose.

2. MATERIALS AND METHODS

2.1. Animals

Male C57BL/6J mice (nine to eleven weeks of age) were purchased from the Jackson Laboratory (#000664, Bar Harbor, ME). This strain was chosen based on our previously established mouse model of repeated cisplatin exposure cycles (Sawhney, Giammona, Meistrich and Richburg, 2005). The animals were housed at a constant temperature (22°C +/– 0.5°C) at 35–70% humidity with a 12 h light:12 h dark photoperiod. Mice were given standard lab chow and water *ad libitum* and allowed to acclimate for one week prior to experiments. 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. Each cisplatin group comprised at least five mice unless otherwise noted. Each age-matched vehicle control group contained three mice (Table 1).

2.2. Cisplatin Exposure Protocol

Mice were administered cisplatin (#P4394, Sigma-Aldrich, St. Louis, MO) dissolved in phosphate buffered saline (PBS, #10010-023, Life Technologies, Grand Island, NY) as per our previously established mouse model of a clinically-relevant cisplatin exposure (Sawhney, Giammona, Meistrich and Richburg, 2005). The biological activity of a cisplatin solution is determined by a number of critical variables in preparation, including the use of 0.9% NaCl as a solvent (Greene *et al.*, 1979; Sigma-Aldrich, 2007), protection from light (Macka *et al.*, 1994; Zieske *et al.*, 1991), and the avoidance of the addition of DMSO (Fischer *et al.*, 2008; Kerrison and Sadler, 1977; Sundquist *et al.*, 1986). Mice received one cycle (a single cycle consisting of five consecutive daily ip injections followed by a 16-day recovery period) of 2.5 mg/kg/day, two cycles of 2.5 mg/kg/day, or one cycle of 5.0 mg/kg/day cisplatin followed by one cycle of an equivalent volume of PBS (Figure 1). This dosing paradigm and group naming convention (dose/cycle number/day of sacrifice) are summarized in Table 1. Mice were sacrificed on: day 6 (group 2.5/1/6), day 21 (group 2.5/1/21), day 27 (groups 2.5/2/27 and 5.0/1/27), and day 42 (groups 2.5/2/42 and 5.0/1/42). Animals were sacrificed by CO₂ inhalation followed by cervical dislocation at the time points indicated, at which point both testes were rapidly removed and processed for analyses. One testis was flash frozen in liquid nitrogen and stored at –80°C and the other was immersion fixed in Bouin's solution (#16045-1, Polysciences, Inc., Warrington, PA) overnight.

2.3. Testicular Histopathology

Bouin's solution-fixed testes were washed in Li₂CO₃-saturated (#H300, Mallinckrodt, Inc., Paris, KY) 70% ethanol (#111000200, Pharmaco-AAPER, Brookfield, CT), and embedded in paraffin for histological examination. Testis cross sections (5 µm) were stained with

periodic acid-Schiff-hematoxylin (PAS-H) according to standard protocols (#SH30-500D, ThermoFischer Scientific, Waltham, MA) (Sawhney, Giammona, Meistrich and Richburg, 2005).

2.4. Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-dUTP Nick End Labeling (TUNEL) Assay

Apoptotic fragmentation of DNA in paraffin-embedded testes cross sections was visualized by TUNEL analysis using the ApopTag kit (#S7100, EMD Millipore, Billerica, MA). Seminiferous tubules (100 essentially round tubules counted per mouse) containing four or more TUNEL-positive germ cells were classified as positive tubules and expressed as an apoptotic index (AI) (Seaman *et al.*, 2003).

2.5. 5-bromo-2-deoxyuridine (BrdU) Incorporation

The incorporation of BrdU (#858811-500, Sigma-Aldrich), a thymidine analog, into the synthesizing DNA of dividing cells was measured to assess the level of mitotic activity (Ballow *et al.*, 2006; Grisanti *et al.*, 2009; Naughton *et al.*, 2006). BrdU dissolved in PBS (#10010, Life Technologies) was administered (100 mg/kg) by ip injection 1.5 h prior to sacrifice and incorporation was detected by immunohistochemistry, a protocol developed to label spermatogonia (Ballow, Meistrich, Matzuk and Rajkovic, 2006). BrdU-positive cells were not observed to be evenly distributed throughout the testis, but rather were concentrated in certain tubules. In order to capture this tubule specific uptake of BrdU, seminiferous tubules (100 essentially round tubules counted per mouse) possessing six or more BrdU-positive cells were scored as positive tubules and expressed as a mitotic index (MI).

2.6. Immunohistochemistry

Expression and localization of Foxo1, Sox9, incorporated BrdU, and GDNF were analyzed by immunohistochemistry according to standard protocols. Briefly, cross sections (5 μ m) of paraffin-embedded testes were subjected to xylene deparaffinization (#X5, ThermoFischer Scientific), rehydration, antigen unmasking (boiling in 10 mM sodium citrate solution for Foxo1, Sox9, and GDNF, #54641, Sigma-Aldrich; 1:10 0.25% trypsin-EDTA (1X):PBS for 10–20 min at 37°C for BrdU #25200-072, Life Technologies), denaturing of DNA (2N HCl for 10–20 min at 37°C for BrdU HX0603-3, EM Science, Gibbstown, NJ) and blocking of endogenous peroxidase activity (10 min, 3% H₂O₂ #BP2633, ThermoFischer Scientific) prior to incubation in a 10% horse serum blocking buffer (30 min, #S-2000, Vector Laboratories, Inc., Burlingame, CA). Cross sections were incubated with a primary antibody, diluted in 10% horse serum blocking buffer, specific for Foxo1 (1:100, #2880, Cell Signaling Technology, Inc., Beverly, MA, overnight at 4°C), Sox9 (1:500, #AB5809, EMD Millipore, overnight at 4°C), incorporated BrdU (1:50, #ab2284, Abcam, Cambridge, ENG, overnight at 4°C) or GDNF (1:100, #sc-328, Santa Cruz, Dallas, TX, 2 h at 25°C), followed by incubation with a rabbit secondary antibody (1:5000 in 10% horse serum blocking buffer, #PK-6101, Vector Laboratories, Inc.) for 30 min at 25°C. Pre-absorption of the GDNF antibody with a GDNF blocking peptide (Santa Cruz) indicated that interstitial staining of Leydig cells was non-specific (Supplemental Figure 1).

Foxo1 staining was used as an indicator of undifferentiated spermatogonia (Figure 2) (Goertz *et al.*, 2011). Foxo1-positive cells were counted and expressed as the average number of positive cells per tubule cross section (100 essentially round tubules counted per mouse).

Sox9 was utilized as a marker for Sertoli cells. Sox9-positive cells were counted and expressed as the average number of positive cells per tubule cross section (100 essentially round tubules counted per mouse).

Protein expression and BrdU incorporation were detected using the VECTASTAIN Elite ABC kit (30 min at 25°C, #PK-6101, Vector Laboratories, Inc.) and the 3,3'-diaminobenzidine substrate (30 s to 3 min at 25°C, #SK-4100, Vector Laboratories, Inc.), counterstained with hematoxylin (1:10 ddH₂O, 2 min, ThermoFischer Scientific). All cross sections were viewed using a Nikon Eclipse Ni-U microscope (Nikon Instruments, Inc., Melville, NY) and images captured with a Nikon DS-Vi1 camera (Nikon Instruments, Inc.) and Nikon Elements software (V3.22.11, Nikon Instruments, Inc.).

2.7. Statistical Analysis

Data were visualized as a box plot and subjected to a Shapiro-Wilk W test for normality. If a given data set was found to deviate from the normal distribution, a log(1+x) transformation was performed (Foxo1 and BrdU IHC quantification). Parametric analyses were then undertaken and tests utilized were the Student's *t*-test for comparisons between two groups and Analysis of Variance for multiple comparisons, followed by a Tukey's Honestly Significant Difference for those qualifying for post-hoc analysis. All statistical procedures were conducted using JMP software (Version 10, SASS, Cary, NC) and statistical significance was considered to be achieved when $p < 0.05$.

3. RESULTS

The variables of the cumulative cisplatin dose and increasing animal age were controlled for throughout these multi-cycle treatment studies by treating age-matched mice with a single cycle of an equivalent cumulative dose. The experimental design utilized in this study facilitated a number of meaningful comparisons. First, the state of spermatogenesis in animals directly following one cycle of cisplatin treatment were compared to animals after the full recovery period, corresponding to when a subsequent cycle of cisplatin would commence. Second, mice given two cycles of 2.5 mg/kg/day cisplatin (2.5/2/27 and 2.5/2/42) were contrasted to those that only received one cycle of 2.5 mg/kg/day (2.5/1/6 and 2.5/1/21). Most importantly, however, mice that sustained two cycles of 2.5 mg/kg/day cisplatin (2.5/2/27 and 2.5/2/42) were compared to mice of the same age which received an equivalent cumulative dose of the drug (5.0/1/27 and 5.0/1/42) in a single cycle, rather than divided into two cycles. The morbidity induced by 5.0 mg/kg/day was considerable, with one mouse from group 5.0/1/42 perishing during the second recovery period. This prompted a limitation in the use of the 5.0 mg/kg/day dose to only the most crucial comparisons; that is, age-matched mice receiving one cycle of a dose equivalent to the cumulative amount of two cycles of 2.5 mg/kg/day.

3.1. Testis and Body Weights

The body weights of control animals increased slightly, but significantly, over the course of the experiment (Table 2). Mouse body weights decreased during the dosing period in all treatment groups reflecting the generalized toxicity of this compound, corroborating previous reports concerning cisplatin-induced toxicity (DeSantis *et al.*, 1999; Marcon *et al.*, 2008; Meistrich *et al.*, 1989; Pont and Albrecht, 1997; Sawhney, Giammona, Meistrich and Richburg, 2005). Mice in group 2.5/1/21 regained body weight similar day zero animals. Mice from exposure group 2.5/2/42 experienced a decline in body weight significantly greater than age-matched mice in group 5.0/1/42.

Mice that underwent cisplatin exposure suffered a reduction in testis mass, the most dramatic decline occurring by day 27 (Table 2). All treatment groups, except 2.5/1/6, proved to be significantly different from controls, but not from each other. Testis to body weight ratios reflected these fluctuations in testis and body weight (Table 2). Interestingly, 16 days following the first cycle of cisplatin dosing, mice exhibited a reduced testicular weight (2.5/1/21), even though their average body weight had rebounded to control levels. This incongruity did not manifest in age-matched mice receiving an equivalent cumulative cisplatin dose (5.0/1/21), where body weights remained depressed (Table 2). Mice in exposure group 2.5/2/42 presented with a decreased testis weight similar to mice in cisplatin group 5.0/1/42; yet mice in the former group remained at a depressed body weight while those in the latter had returned to near day zero levels. Appraisal of body and testis weight indicate an association between the number of cycles administered and the extent of toxicity sustained.

3.2. Histopathology of Cisplatin-Exposed Mouse Testes

PAS-H staining of cross sections of testis from cisplatin-exposed mice showed atrophy and germ cell loss, correlating with reduced testicular weight (Figure 3). These data are very similar to previous results obtained following exposure to multiple cycles of cisplatin treatment, reported in detail by Sawhney, Giammona, Meistrich and Richburg (2005). The frequency and extent of testicular injury were dose dependent. The testis of mice in the 2.5/1/6 group (Figure 3B) exhibited a mild loss of cellularity and a retraction of Sertoli cell cytoplasm. These indicators of damage were increased in the 2.5/1/21 treatment group (Figure 3C) and showed no evidence of recovery. In fact, the histopathology of the testis at the conclusion of the recovery period presented with a more pronounced state of disruption than testes 24 h after the cessation of exposure (Figure 3C vs. 2B).

Mice in treatment group 2.5/2/27 harbored extensive cell loss and disorganization of the seminiferous epithelium (Figure 3D). Apical sloughing and shedding of cellular material were also present. At this time point and cycle frequency, spermatogonia and Sertoli cells account for nearly all of the cells present in the seminiferous tubules, indicating a near total disruption of normal spermatogenesis. These manifestations of testicular injury persisted, and in fact increased, in group 2.5/2/42 (Figure 3E). Interestingly, age-matched mice in exposure group 5.0/1/42 had returned to apparently normal spermatogenesis by day 42 (Figure 3G). Two cycles of 2.5 mg/kg/day cisplatin revealed a markedly greater frequency of indicators of toxicity than detected after the first cycle. Furthermore, testicular injury

caused by two cycles of 2.5 mg/kg/day was more substantial than that seen in mice receiving one cycle at an equivalent cumulative dose (Figure 3D and 2E, vs. 2F and 2G). Histopathological analysis affirms an exacerbation of injury correlating with an increase in treatment cycle number.

3.3. Analysis of Apoptotic Index

Apoptosis plays an important role in normal spermatogenesis, accounting for the basal-level AI recorded in control mice (Blanco-Rodriguez, 1998). Apoptotic cells were frequently observed in the spermatogonia and spermatocyte populations (Figure 4A and 3B); a finding that parallels previous reports (Seaman, Sawhney, Giammona and Richburg, 2003). The testis of mice from treatment group 2.5/1/6 displayed an increase in AI (Figure 4C). This initial peak decreased during the first recovery period (2.5/1/21) and was maintained throughout the second dosing period (2.5/2/27). This group (2.5/2/27) presented with an elevated AI over controls and over cumulative dose-matched, age-matched animals (5.0/1/27). The AI in mice from cisplatin group 2.5/2/42 was similar to that of cumulative dose- and age-matched mice from group 5.0/1/42 (Figure 4D) and controls. However, none of the groups proved to be significantly different from each other, due to the wide variation recorded between individuals within each group. Collectively, these observations reveal that multiple cycles of cisplatin result in an increase in apoptosis that is roughly equivalent to, and sustained for a greater period of time, than that seen after a single cycle of equivalent cumulative dose.

3.4. Quantification of the Undifferentiated Spermatogonia Population

The observed alterations in germ cell histology and AI prompted an investigation of changes in the population of germ cells capable of repopulation of the seminiferous epithelium. It has recently been reported that the capacity to repopulate depleted seminiferous tubules does not belong exclusively to SSCs; and in fact, may be a trait of the undifferentiated spermatogonia population (A_s , A_{pr} , and A_{al}) as a whole (Dann *et al.*, 2008; Nakagawa *et al.*, 2007). Appreciating this importance, the entirety of the population of germ cells posited to possess regenerative capacity was examined; namely, the undifferentiated spermatogonia (Nakagawa *et al.*, 2010). The undifferentiated spermatogonia population is recognized to fluctuate in size over the course of the spermatogenic cycle; yet at all times constitutes a small proportion (~0.3%) of the total number of germ cells in the testis (Oatley *et al.*, 2007). Foxo1 has been shown to be a reliable and robust marker of this functional spermatogonial stem cell (FSSC) pool (Figure 2) (Goertz, Wu, Gallardo, Hamra and Castrillon, 2011).

Foxo1-positive cells were detected in all mice, appearing in groups of one, two, and four cells, and exhibited characteristic spermatogonia morphology (Figure 5A and 4B). Staining did not reveal any alterations in the number of Foxo1-positive cells in treatment group 2.5/1/6 (Figure 5C). In group 2.5/1/21, the Foxo1-positive population had significantly increased. The number of positive cells in mice from group exposure 2.5/2/27 was far lower than cumulative dose- and age-matched animals in group 5.0/1/27. The Foxo1-positive spermatogonia population size continued to decrease, although not significantly, in treatment group 2.5/2/42 to a value roughly half of that seen in the testis of mice from group 5.0/1/42. (Figure 5D). Surprisingly, the Foxo1-positive population did not decrease during

either the first or the second 2.5 mg/kg/day dosing cycles. This observation is particularly meaningful in the context of changes in testis weight, histology, and AI at these time points. Testis weight was seen to decrease substantially at the beginning of the recovery periods in all exposure groups, commensurate with a considerable loss of germ cells. Histological analysis corroborates that this decrease in mass is largely due to the loss of more mature germ cells. Furthermore, the AI was found to peak at the initiation of the recovery periods and decline by the conclusion, though the Foxo1-positive population remained unchanged throughout the dosing period. This indicates that although a large percentage of germ cells were eliminated in the first cycle, the undifferentiated spermatogonia accounted for a small proportion of these lost cells. It is interesting that mice undergoing two cycles of 2.5 mg/kg/day did not experience an increase in the number of Foxo1-positive cells during the recovery period following the second cycle of dosing, and in fact remained slightly below controls (2.5/2/42). This behavior differs drastically from age-matched mice receiving an equivalent cumulative dose of cisplatin (5.0/1/27 and 5.0/1/42). Mice in these exposure groups (5.0/1/27) also exhibit a super-physiological increase in the Foxo1-positive cell population, but at time points dissimilar from 2.5/1/21, before decreasing to control levels by the conclusion of the experiment (5.0/1/42). Quantification of the FSSC pool reveals a greater reduction in population size after two cycles of dosing than following one cycle or a single cycle of an equivalent cumulative dose.

3.5. Quantification of Sertoli Cell Population

Cells expressing Sox9, a protein transcribed and located exclusively in Sertoli cells in the testis (Ballow, Meistrich, Matzuk and Rajkovic, 2006), were quantified (Figure 6A and 5B). During the first recovery period, the number of Sox9-positive cells did not significantly change (Table 3). Similar values were recorded for mice undergoing two cycles of 2.5 mg/kg/day and mice that received a single cycle of 5.0 mg/kg/day and (data not shown).

3.6. Quantification of Mitotic Activity

Incorporated BrdU staining, an indication of mitotic activity, was generally restricted to germ cells located in the basal compartment, frequently correlated with stage IX-III tubules, and predominately localized to groups of cells; all are indicative of spermatogonial cell labeling. Examination of control animals revealed a high basal MI value (Figure 7C). The seminiferous epithelium of mice in treatment group 2.5/1/6 exhibited a precipitous and significant drop in positive tubules, before rebounding to near controls in group 2.5/1/21. Mice in exposure group 2.5/2/27 presented with very few mitotic tubules and did not recover during the following 16 days (group 2.5/2/42). Conversely, cumulative dose-matched, age-matched animals in treatment groups 5.0/1/27 and 5.0/1/42 (Figure 7D) were capable of maintaining near control levels of mitotic activity. Evaluation of proliferative activity attests to a reduction in proliferation rate corresponding with the number of dosing cycles.

3.7. Analysis of GDNF Expression

Extensive investigations into the composition of the FSSC niche have yielded a number of factors thought to modify the behavior of spermatogonia; the most convincing of which is GDNF (Ebata *et al.*, 2011; Meng, 2000; Tadokoro *et al.*, 2002). A low but detectable degree

of GDNF expression was detected in the testis of control mice (Figure 8A). Staining manifested in a spoke-like pattern indicative of Sertoli cell production, emanating from the entirety of the cell, including both the basal and luminal portions. Interstitial staining of Leydig cells was non-specific. In the 2.5/1/6 treatment group, robust GDNF protein levels appeared along the Sertoli cell basal membrane, waxing in the 2.5/1/21 group (Figure 8B and 7C). Staining was very low in group 2.5/2/27, but returned to near controls in exposure group 2.5/2/42 (Figure 8D and 7E). IHC investigation of GDNF production revealed a considerable decrease in expression along the basal Sertoli cell membrane during the second recovery period in all treatment groups. This decline was likely a reflection of the expanded size of the FSSC pool late in the first recovery period rather than a direct consequence of the second cycle of cisplatin exposure. Diametrically, mice receiving one cycle of an equivalent cumulative cisplatin dose presented with higher expression of GDNF, FSSC pool size, and proliferation at these age-matched time points. Cumulative dose-matched, age-matched mice in groups 5.0/1/27 and 5.0/1/42 appeared to exhibit markedly stronger GDNF production at these time points (Figure 8F and 7G).

In all instances, peak intensity of expression was seen at the basal membrane, where the most immature germ cells, including SSCs, are proposed to reside (Oatley and Brinster, 2012). Analysis of GDNF production in response to cycles of cisplatin administration demonstrates disparate expression levels throughout the recovery periods.

4. DISCUSSION

Here we present data indicating disturbances to spermatogenesis during the recovery periods in C57/BL/6J mice after a multi-cycle cisplatin exposure. Most published studies seeking to recapitulate a clinical cisplatin treatment paradigm have employed an acute exposure model (Bieber *et al.*, 2006; Delbes *et al.*, 2009; Favareto *et al.*, 2011; Ilbey *et al.*, 2009a; Ilbey *et al.*, 2009b; Marcon, Hales and Robaire, 2008; Seethalakshmi *et al.*, 1992). The treatment model in this study differs from the reports that do use a multi cycle regimen in a number of critical variables: the species and age of the model organism, the daily dose, the cumulative dose, the length and number of exposure cycles and recovery periods, the vehicle, the administration route, and other compounds given in concert with cisplatin (Bieber, Marcon, Hales and Robaire, 2006; Delbes, Chan, Pakarinen, Trasler, Hales and Robaire, 2009; Favareto, Fernandez, da Silva, Anselmo-Franci and Kempinas, 2011; Ilbey, Ozbek, Cekmen, Simsek, Otuncemur and Somay, 2009a; Ilbey, Ozbek, Simsek, Cekmen, Otuncemur and Somay, 2009b; Marcon, Hales and Robaire, 2008; Seethalakshmi, Flores, Kinkead, Carboni, Malhotra and Menon, 1992). Chiefly, the multi-cycle cisplatin paradigm utilized in this study closely simulates the manner in which cisplatin is administered clinically, conforming to prescribed daily and cumulative dose, length and timing of dosing and recovery periods, and number of cycles (Sawhney, Giammona, Meistrich and Richburg, 2005) (Figure 1). The concentration of cisplatin in a given tissue, neoplastic and normal, is directly related to the number of cycles administered (Reed, Ozols, Tarone, Yuspa and C., 1988; Stewart *et al.*, 1988). Moreover, it has been established from patient samples that the concentration of cisplatin in tumor tissue correlates well with positive disease response (Reed, Ozols, Tarone, Yuspa and C., 1988; Stewart, Mikhael, Nair, Kacew, Montpetit, Nanji, Maroun and Howard, 1988). The design of this study allowed us to examine the impact of multiple cycles of

cisplatin while controlling specifically for the effects of age and cumulative cisplatin dose (Table 1).

We hypothesized that the first cycle of cisplatin would eliminate the highly proliferative cells of the testis via apoptotic cell death. The ensuing loss of differentiating spermatogonia and spermatocytes would elicit an increase in the mitotic activity of the FSSC pool. This elevated rate of proliferation would render the FSSC pool more susceptible to cisplatin during the second cycle of treatment, manifesting in an exacerbation of damage following the second dosing period.

Testis weights and testicular histopathology were utilized to appraise the extent of cell death resulting from one cycle of cisplatin treatment; revealing a widespread loss of mitotically active spermatogonia, becoming more pronounced throughout the recovery period (Figure 3B-2G). These findings largely agree with previous reports from studies on cisplatin exposure (DeSantis, Albrecht, Holtl and Pont, 1999; Marcon, Hales and Robaire, 2008; Meistrich, Chawla, Da Cunha, Johnson, Plager, Papadopoulos, Lipshultz and Benjamin, 1989; Pont and Albrecht, 1997; Sawhney, Giammona, Meistrich and Richburg, 2005). The failure of recovery was not due to the loss of Sertoli cells as no changes in the population size of this cell type were detected following repeated cisplatin treatment (Table 3); a finding that mirrors our previous reports as well as that of others (Huang *et al.*, 1990; Kangasniemi *et al.*, 1996; Sawhney, Giammona, Meistrich and Richburg, 2005).

TUNEL staining was employed to characterize the nature of germ cell loss after the first cycle of cisplatin dosing and apoptosis was observed to be moderately, though not significantly, increased over physiological levels (Figure 4C and 3D). However, it is certainly appreciated that large amounts of cells have undergone apoptotic elimination after the initial 24 h of treatment as indicated by previous reports by our laboratory and those of others following acute cisplatin exposure (Delbes, Chan, Pakarinen, Trasler, Hales and Robaire, 2009; Seaman, Sawhney, Giammona and Richburg, 2003; Zhang *et al.*, 2001). Therefore, the incidence of germ cell apoptosis measured after the first cycle reflects a low degree of ongoing germ cell death in the context of a significantly reduced population of cells by this time point.

Of considerable importance to this study is the regenerative capacity of spermatogonia germ cell sub-types after cytotoxic insult. It is predicted that the loss of more mature germ cells would prompt an increase in the mitotic activity of the FSSC pool. Traditionally, this attribute was assumed to be exclusive to SSCs, but recent evidence contradicts this notion (Dann, Alvarado, Molyneux, Denard, Garbers and Porteus, 2008; Nakagawa, Nabeshima and Yoshida, 2007). Informed by this understanding, the entirety of undifferentiated spermatogonia sub-types (A_s , A_{pr} , and A_{al}) were examined in response to cisplatin dosing by Foxo1 staining, rather than a limited scope of inquiry focused on SSCs exclusively (Figure 5). The size of the FSSC pool was unaffected by a single cycle of cisplatin treatment, but reached a supraphysiological level by the conclusion of the first recovery period, reflecting an expansion of this collective population of undifferentiated spermatogonial cell sub-types.

In the literature, conclusions regarding the kinetics of spermatogenesis after cytotoxic insult to the testis are somewhat conflicting. Early reports suggested that there is no change in the mitotic rate following exposure to several types of damage (Meistrich *et al.*, 1978; Oakberg, 1956; van Keulen and de Rooij, 1975), though more recent observations challenge this assertion (Shah *et al.*, 2009; Tadokoro, Yomogida, Ohta, Tohda and Nishimune, 2002; Zohni *et al.*, 2012). In light of the results from the quantification of the Sertoli cell population (Table 3), the undifferentiated spermatogonia pool (Figure 5), testis mass (Table 2), and histology (Figure 3), we were able to confidently infer that mitotically active cells in the seminiferous epithelium during the recovery period were predominantly immature spermatogonia. Therefore, BrdU incorporation was utilized to test for an increase in mitotic activity and tubule recovery at these time points (Figure 7). It is crucial to note that the recovery of mitotic activity perceived in animals whose testis mass was severely depressed (Table 2), suggesting that the mitotically active germ cells at these periods of recovery accounted for a greater proportion of the total germ cells than they do in the control mouse. This finding, coupled with the supraphysiological undifferentiated spermatogonia population expansion (Figure 5) indicated a substantial increase in the mitotic activity of this crucial cell type during the recovery period after one cycle of cisplatin.

The results of the experiments pertaining to the kinetics of the spermatogonial population following cisplatin exposure prompted a consideration of the state of the spermatogonial niche during the recovery periods. Published reports evaluating the components of the SSC niche have identified a large number of important factors that contribute to governing SSC behavior (Ebata, Yeh, Zhang and Nagano, 2011; Hofmann *et al.*, 2005; Oatley *et al.*, 2009; Ohta *et al.*, 2000; Tadokoro, Yomogida, Ohta, Tohda and Nishimune, 2002). GDNF, in particular, has proven to be crucial for regulating the self-renewing divisions of SSCs (Ebata, Yeh, Zhang and Nagano, 2011; Meng, 2000; Tadokoro, Yomogida, Ohta, Tohda and Nishimune, 2002). In the testis, GDNF is thought to be expressed solely by Sertoli cells (Johnston *et al.*, 2011; Sato *et al.*, 2011) and signals undifferentiated spermatogonia, including SSCs, to undergo self-renewing cell divisions; promoting proliferation and inhibiting apoptosis (Oatley and Brinster, 2008). Examination of GDNF expression after repeated cisplatin dosing indicated that production was heightened during the recovery period (Figure 8). These observations provide preliminary evidence to indicate that increases in Sertoli cell-expressed GDNF is closely correlated with the expansion of the undifferentiated spermatogonial cell population in the testis after cisplatin treatment. Although Western blotting did not indicate any differences in overall testicular GDNF production (Supplemental Figure 2), meaningful fluctuations in expression are likely too subtle to be detected in an analysis of total testis protein. Furthermore, the localization of GDNF production is crucial to the proper understanding of the nature of the recovery of the testis following cisplatin exposure. Even if recovery from cisplatin treatment did not alter GDNF expression levels at the testis-wide scale, a redistribution of production favoring the physical location of the SSC niche would, in all likelihood, encourage FSSC self-renewal.

At this halfway point through the treatment paradigm, mice had received one cycle of cisplatin followed by one recovery period (Figure 1). All evidence indicates that the testis was in a highly proliferative mode of recovery from the first cycle of cisplatin, rather than at

steady state spermatogenesis at this crucial moment of administration of the second cycle. Elevated GDNF expression in the SSC niche (Figure 8) and increased mitotic activity of the FSSC pool (Figure 5 and 6) are critical facets of this attempt to repopulate the seminiferous tubules, that paradoxically result in an increased susceptibility of the undifferentiated spermatogonial population to cisplatin exposure at the beginning of the second cycle of dosing.

We proposed that the second cycle of cisplatin would induce more extensive cell death than observed following the first cycle, or from a single cycle of an equivalent cumulative dose, and testis weights (Table 2), testicular histopathology (Figure 3), and TUNEL staining (Figure 4) were again utilized to test this assertion. These findings agree with the overall consensus of the literature focused on multi-dose cisplatin exposure (Favareto, Fernandez, da Silva, Anselmo-Franci and Kempinas, 2011; Ilbey, Ozbek, Cekmen, Simsek, Otuncemur and Somay, 2009a; Marcon, Hales and Robaire, 2008), and suggest that two cycles of cisplatin results in toxic consequences distinct from the widely used research model of a single cycle of cisplatin at an equivalent cumulative dose.

We predicted that the mitotic activity of the FSSC pool would be differentially affected by two cycles of cisplatin as compared to one cycle or a single cycle of an equivalent cumulative dose, and a quantification of the Foxo1-positive cell population (Figure 5) and BrdU incorporation were undertaken (Figure 7). These results revealed that the undifferentiated spermatogonia population is depleted to a far more extensive degree following two cycles as compared to one cycle or a single cycle of an equivalent cumulative dose.

The response in GDNF production after two cycles of cisplatin closely parallels the observed alterations in the FSSC pool and mitotic activity (Figure 8). The dissimilar response of GDNF to one versus two cycles of cisplatin can be explained by alterations in the FSSC pool size. Sertoli cell GDNF expression has been observed to decrease when spermatogenic cells are present in the seminiferous epithelium (Johnston, Olivas, DiCandeloro and Wright, 2011). It is likely that the inflated FSSC pool at the conclusion of the first recovery period drove a down regulation in GDNF expression through a negative feedback mechanism. Therefore, GDNF production receded wholly independent of the administration of the second cycle of cisplatin. Further supporting this interpretation, GDNF returned to slightly greater than control intensity by the conclusion of the second recovery period. The possibility that cisplatin may diminish the health and function of Sertoli cells cannot be discounted and toxicities sustained by this cell group could result in decreased niche factor production, culminating in a reduction in FSSC pool size and proliferation. This explanation is less parsimonious however, in that it entails a single cycle of cisplatin instigating an increase in GDNF expression by Sertoli cells but two cycles of cisplatin inducing a decrease in production. Furthermore, this model requires that Sertoli cells respond differently to a single cycle of an equivalent cumulative cisplatin dose.

Taken together, these results indicate that disturbances to the FSSC pool population, mitotic activity, and SSC niche are more dependent on treatment cycle number, rather than cumulative dose of cisplatin administered. However, it is certainly possible that other factors

contribute significantly to the behavior of undifferentiated spermatogonia during the recovery from cisplatin exposure and characterizing the alterations in various other components of the niche will be an important focus of future studies.

In light of these results, we propose that the first cycle of cisplatin treatment targets the more mature, rapidly dividing germ cells. The resulting loss of differentiating cells prompts an increase in GDNF expression from Sertoli cells. This elevated GDNF production directs the FSSC pool to enter into the cell cycle to repopulate the testis. This heightened mitotic activity renders the undifferentiated spermatogonia more susceptible to subsequent cycles of cisplatin. Exposure to cisplatin during this period of recovery results in a substantial loss of FSSC pool, rendering the organism infertile for an extended period of time.

5. CONCLUSIONS

Indicators of toxicity and evaluation of the FSSC cell pool during the recovery periods in mice subjected to a clinically-relevant cisplatin treatment paradigm indicated that multiple cycles of cisplatin cause greater damage than a single cycle of an equivalent cumulative dose. GDNF production increased along the basal Sertoli cell membrane throughout the recovery period in all treatment groups. The impact of cisplatin exposure on the FSSC pool and niche correlates with: (1) the number of dosing cycles; (2) mitotic activity of early germ cells; and (3) alterations in the basal Sertoli cell GDNF expression levels after cisplatin-induced testicular injury.

It must be emphasized that multiple cycles of cisplatin chemotherapy correlate with a higher level of cisplatin-DNA adducts and a more positive tumor response (Poirier *et al.*, 1992; Reed, Ozols, Tarone, Yuspa and C., 1988; Stewart, Mikhael, Nair, Kacaw, Montpetit, Nanji, Maroun and Howard, 1988). Therefore, it is not suggested that an increase in dose or a reduction in treatment cycle number be implemented to preserve long-term fertility. Rather, it is our hope that the insights from these important findings will provide insights that will allow for the design of new treatment strategies that will continue to promote the efficient elimination of the tumor cells while at the same time limiting the effect on normal spermatogenesis and male fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Testicular toxicity increases with treatment cycle number
- Multiple treatment cycles negatively impact early germ cell population
- Fluctuations in GDNF expression correlate with number of treatment cycles

FIGURE 1. Multi-cycle cisplatin exposure regimen

One course of treatment consisted of two cycles of cisplatin exposure. Each cycle was composed of five daily intraperitoneal injections (days 1, 2, 3, 4, 5, 22, 23, 24, 25, and 26) of cisplatin dissolved in PBS followed by 16 days of recovery days 6–21 and 27–42). Animals were sacrificed at the beginning (days 6 and 27) and end (days 21 and 42) of each recovery period.

FIGURE 2. Identification of the FSSC pool with Foxo1 labeling

Expression of Foxo1 has been shown to be restricted to the cohort of spermatogonia capable of regenerating spermatogenesis in the event of germ cell loss. Expression of Foxo1 was used as a marker of this functional spermatogonial stem cell (FSSC) pool.

FIGURE 3. Histopathological evidence of cisplatin-induced damage in the seminiferous epithelium

Histopathology for vehicle controls (A) and exposure groups 2.5/1/6 (B), 2.5/1/21 (C), 2.5/2/27 (D), 2.5/2/42 (E), 5.0/1/27 (F), and 5.0/1/42 (G). Cisplatin toxicity results in indicators of damage, including retraction of Sertoli cell cytoplasm due to loss of spermatocytes and spermatids (arrows, B–G), progressing to general loss of cellularity and disorganization of spermatogenesis (arrowheads, C, D, G), culminating in apical sloughing of cellular material and widespread atrophy (large arrows, E, F). Magnification bar equals 50 μ m.

FIGURE 4. Increase in apoptotic index (AI) during the recovery periods

TUNEL analysis of germ cell apoptosis in vehicle control and C57 mice exposed to one cycle of 2.5, 5.0, or two cycles of 2.5 mg/kg/day cisplatin followed by 16 days recovery. Arrows indicate representative TUNEL positive cells in control mice (A) and those exposed to one cycle of 2.5 mg/kg/d cisplatin (2.5/1/6, B). Magnification bar equals 50 μ M (A, B). Values (C, D) represent the mean AI $\pm$ SM.

FIGURE 5. Decrease, recovery, and subsequent increase in Foxo1-positive undifferentiated spermatogonia population in vehicle controls and C57 mice exposed to one cycle of 2.5, 5.0, or two cycles of 2.5 mg/kg/d cisplatin

Arrows indicate representative Foxo1-positive cells in control mice (A) and those exposed to one cycle of 2.5 mg/kg/d cisplatin (2.5/1/21, B). Quantification of Foxo1-positive cells by IHC, expressed as the average number of positive cells per tubule cross section following a $\log(1 + x)$ transformation. Values (C, D) represent the mean $\pm$ SM; groups not connected by shared letters are significantly different ($p < 0.05$).

FIGURE 6. Representative staining from Sox9 analysis

Arrows indicate representative Sox9 positive cells and magnification bar equals 50 μm .

FIGURE 7. Increase in germ cell mitotic rate following cisplatin treatment

IHC analysis of BrdU incorporation in vehicle controls and C57 mice exposed to one cycle of 2.5, 5.0, or two cycles of 2.5 mg/kg/d cisplatin. Arrows indicate representative BrdU-positive cells in control mice (A) and those exposed to one cycle of 2.5 mg/kg/d cisplatin (2.5/1/21, B). Magnification bar equals 50 μ m (A, B). Quantification of BrdU incorporation presented as the mitotic index (MI) of tubule cross sections containing 6 BrdU-positive cells (C, D). Values represent the mean MI $\pm$ SM, groups not connected by shared letters are significantly different ($p < 0.05$).

FIGURE 8. Immunohistochemical detection of altered GDNF expression following cisplatin treatment

Analysis of GDNF expression in vehicle-treated control C57 mice (A) and exposure groups 2.5/1/6 (B), 2.5/1/21 (C), 2.5/2/27 (D), 2.5/2/42 (E), 5.0/1/27 (F), and 5.0/1/42 (G). Arrows indicate spoke-like staining, indicative of Sertoli cell expression, and arrowheads signify basal distribution in the region of the FSSC pool niche. The magnification bar equals 50 μ m.

TABLE 1

Labeling convention.

Treatment Group (dose/cycle/experimental day)	Cisplatin Dose (mg/kg/day)	Exposure Cycles (#)	Recovery Period (d)	Cumulative Cisplatin Dose (mg/kg)	n
Vehicle Control	0.0	0	0	00.0	12
2.5/1/6	2.5	1	1	12.5	6
2.5/1/21	2.5	1	16	12.5	5
2.5/2/27	2.5	2	1	25.0	5
2.5/2/42	2.5	2	16	25.0	5
5.0/1/27	5.0	1	1	25.0	5
5.0/1/42	5.0	1	16	25.0	4

Adult C57 mice received one cycle of 2.5, one cycle of 5.0, or two cycles of 2.5 mg/kg/day cisplatin in PBS. The cumulative dose administered to each group and the length of the recovery period (days) following the final injection varied as indicated. Control animals were given an equivalent volume of vehicle (PBS).

TABLE 2

Reduction in body weights and testicular weights resulting from cisplatin exposure.

Treatment Group		Day 0	Day 6	Day 21	Day 27	Day 42
Vehicle Control	Body Weight (g)	24.3 ± 0.2	25.0 ± 0.1	26.1 ± 0.3	26.8 ± 0.8	27.6 ± 0.5
	Testis Weight (g)	--	1.09 ± 0.0	1.03 ± 0.1	1.07 ± 0.1	1.06 ± 0.1
	Testis: 100 g BW Ratio	--	4.27 ± 0.1	3.90 ± 2.2	4.00 ± 0.4	3.84 ± 0.3
2.5 mg/kg/day	Body Weight (g)	25.4 ± 0.4	20.9 ± 0.7 *	25.8 ± 0.4	23.7 ± 0.4	20.6 ± 1.1 *
	Testis Weight (g)	--	0.91 ± 0.0	0.54 ± 0.0 *	0.38 ± 0.0 *	0.37 ± 0.1 *
	Testis: 100 g BW Ratio	--	4.36 ± 0.2	2.22 ± 0.2 *	1.61 ± 0.2 *	1.78 ± 0.2 *
5.0 mg/kg/day	Body Weight (g)	24.4 ± 0.2	19.8 ± 0.6	18.7 ± 0.8	19.9 ± 1.4 *	23.0 ± 1.1 *
	Testis Weight (g)	--	--	--	0.38 ± 0.0 *	0.40 ± 0.1 *
	Testis: 100 g BW Ratio	--	--	--	1.87 ± 0.2 *	1.72 ± 0.2 *

Body weights and testicular weights are expressed in absolute and testicular weight per 100 g body weight ratios for C57 mice exposed to one cycle of 2.5, 5.0 or two cycles of 2.5 mg/kg/day cisplatin and vehicle controls. Values represent the mean ± SM, asterisks indicate denote in significant differences in comparison to controls ($p < 0.05$).

TABLE 3

Sertoli cell numbers are unaffected by cisplatin treatment.

Treatment Group	Day 6	Day 21
Vehicle Control	21.8 ± 0.8	20.3 ± 0.8
2.5 mg/kg/day	21.4 ± 1.3	21.2 ± 0.8

Quantification of Sox9 staining by IHC in testis from vehicle controls (A) and C57 mice receiving one cycle of 2.5 (B) mg/kg/day cisplatin followed by 16 days recovery. Values represent the mean number of Sox9 positive cells per tubule cross section ± SM (C).