The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice

K. Zohni1, X. Zhang1, S.L. Tan1, P. Chan2, and M.C. Nagano1,*

1Department of Obstetrics and Gynecology, McGill University, Montreal, QC, Canada H3A 1A1 2Department of Surgery, McGill University, Montreal, QC, Canada H3A 1A1

*Correspondence address. Makoto Nagano, Royal Victoria Hospital, F3.07, 687 Pine Avenue West, Montreal, QC, Canada H3A 1A1. Tel: +1-514-934-1934, ext. 35250; Fax: +1-514-843-1662; E-mail: makoto.nagano@muhc.mcgill.ca

Submitted on July 22, 2011; resubmitted on September 15, 2011; accepted on September 28, 2011

BACKGROUND: Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and represent a crucial resource for male fertility restoration. It has not been well documented, however, whether the recovery of SSC population size after cytotoxic damage associates with the kinetics of male fertility restoration. We addressed this issue using the mouse as a model.

METHODS: Following single injections of busulfan at 15, 30 or 45 mg/kg into male mice, we examined their ability to sire offspring at different times by natural mating and determined SSC numbers using spermatogonial transplantation. We measured testis physiological parameters (testis weights, sperm counts, serum and intratesticular testosterone levels, and histological assessments of spermatogenic recovery) and quantified the expression of glial-cell-line-derived neurotrophic factor (GDNF) transcripts.

RESULTS: Regardless of busulfan doses, fertility was lost within 4 weeks after treatment, while more than 95% of SSCs were lost within 3 days. Fertility and SSC numbers gradually recovered with time, but the recoveries were delayed at higher busulfan doses. Interestingly, SSC numbers reached \( \approx 30\% \) of before-treatment levels by 4 weeks prior to the time of fertility restoration, across the dose groups. Sperm counts were \( \approx 20\% \) of before-treatment levels at the onset of fertility restoration, regardless of busulfan doses. We detected a significant increase in total GDNF mRNA per testis immediately after busulfan treatment.

CONCLUSIONS: The loss and restoration of fertility after busulfan treatment are direct consequences of SSC loss and expansion. Our data suggest that there is a threshold in SSC numbers that allows for male fertility restoration and that the testicular somatic environment responds rapidly and temporarily to the loss of spermatogonia, including SSCs, by altering GDNF mRNA levels. This study provides fundamental information to clinically apply SSCs for male fertility restoration in the future.

Key words: spermatogonial stem cells / busulfan / fertility / GDNF

Introduction

Spermatogonial stem cells (SSCs) are the stem cells of the male germ line and are the foundation of spermatogenesis. They are present on the basal membrane of the seminiferous tubules in the testis and surrounded by Sertoli cells, which function as an important component of the SSC niche. Recent studies have demonstrated that a Sertoli-cell-derived growth factor, glial-cell-line-derived neurotrophic factor (GDNF), plays a key role to promote SSC survival and self-renewal, thereby stimulating SSC proliferation in vivo and in vitro (Meng et al., 2000; Tadokoro et al., 2002; Kanatsu-shinohara et al., 2003a; Kubota et al., 2004; Ebata et al., 2011). SSCs are detected by their function to regenerate and maintain spermatogenesis. The unequivocal assay to identify SSC function is spermatogonial transplantation (Brinster and Zimmermann, 1994; Nagano et al., 1999). In this assay technique, donor testis cells are injected into the seminiferous tubules of a recipient male in which endogenous germ cells have been depleted. SSCs present in the injected cell suspension colonize the recipient seminiferous epithelium and establish colonies of donor-derived spermatogenesis. As each colony is derived from a single SSC...
the evidence that SSCs can expand damage induced by a single dose of busulfan (15 mg/kg) and provided spermatogonial transplantation to quantify SSCs after cytotoxic damage. SSCs following cytotoxic damages induced by busulfan reflects the aimed to provide direct functional evidence that the recovery of resource for male fertility preservation and restoration (Kubota and Brinster, 2006), such a linkage needs to be determined in order to address such a concern is to harvest SSCs before the treatments and autologously transplant them into a patient’s testes later. Since SSCs are present in the testis from the time of birth, this strategy should be beneficial to men of any age.

Germinal epithelial damage is a recognized consequence of certain chemotherapeutic agents and radiotherapy. This was first reported in 1948 where the absence of spermatogenesis in the seminiferous tubules was observed in 27 of 30 men treated for lymphoma with nitrogen mustard (Spitz, 1948). By the late 1960s there were similar reports of testicular toxicity due to alkylating agents, such as busulfan, chlorambucil and cyclophosphamide (Schilsky et al., 1980).

Busulfan (1,4-butane diol dimethanesulfonate) is often used as a conditioning regimen prior to progenitor cell transplantation for treatment of chronic myelogenous leukemia and can induce prolonged azosper- mia (Sanders et al., 1996). It shows cytotoxic effects through the formation of DNA–DNA cross-links, DNA–protein cross-links and single strand breaks. Busulfan exerts its toxic effects on cells that are at the G1 phase at the moment of treatment. These cells are killed in the following mitosis, while those that are in S or G2 phase are killed in the subsequent mitosis (Bucci and Meistrich, 1987). In the testis, busulfan preferentially kills spermatogonia of several species, leading to male infertility (Kramer and de Rooij, 1968). When administered to pregnant animals, busulfan produces germ-cell-free gonads in the offspring (Hemsworth and Jackson, 1962; Heller and Jones, 1964).

Bucci and Meistrich (1987) showed that the duration of male infertility was dependent on the extent of stem cell depletion that occurred in a dose-dependent manner, using spontaneous regeneration of spermatogenesis after busulfan treatment as a functional measure of SSCs. This observation indicated that SSCs play critical roles not only in maintaining steady-state spermatogenesis but also in restoring spermatogenesis and male fertility. Kanatsu-shinohara et al. (2003b) used spermatogonial transplantation to quantify SSCs after cytotoxic damage induced by a single dose of busulfan (15 mg/kg) and provided the evidence that SSCs can expand in vivo.

These studies indicate that SSC numbers are crucial to confer fertility; however, some important questions still remain to be addressed. First, to what extent does the size of the SSC population need to recover to confer fertility in a male? Second, do the SSC recovery kinetics influence the process of fertility restoration? In addition, does the degree of killing of SSCs affect their expansion during the course of male fertility restoration? Therefore, the linkage between SSC recovery kinetics and the patterns of male fertility restoration has not been well documented. Since SSCs are expected to be an important resource for male fertility preservation and restoration (Kubota and Brinster, 2006), such a linkage needs to be determined in order to lay a foundation for clinical applications of SSCs. In this study, we aimed to provide direct functional evidence that the recovery of SSCs following cytotoxic damages induced by busulfan reflects the restoration of male fertility using the mouse as a model. We also analyzed multiple physiological parameters related to male reproduction (testis weights, sperm counts, testosterone levels, histological analyses of spermatogenesis and levels of GDNF transcripts in the testis) in an attempt to identify a parameter that could allow for monitoring the degree of SSC recovery.

**Materials and Methods**

**Donor mice and busulfan treatment**

Adult (>6 weeks of age) B6ROSA transgenic mice, F1 hybrids of C57BL/6 (B6) and ROSA26 (B6; 129-S-Gi(ROSA26Sor/l), Jackson Laboratory), were used for mating and transplantation experiments. These mice express the LacZ gene ubiquitously, including all types of germ cells (Zambrowicz et al., 1997, Nagano et al., 1999). Five mice per dose (in mating experiments) and three to five mice per dose (in transplantation experiments) were treated with a single intra-peritoneal injection of 15, 30 or 45 mg/kg of busulfan (Sigma), which was first dissolved in dimethyl sulfoxide (Sigma) before equal volume of distilled water was added to provide the desired final concentration.

In mating experiments, each male was mated with two B6 females for 5 days every other week, starting from 2 weeks after busulfan injection. Mated females were kept to observe pregnancy.

In transplantation experiments, a single cell suspension of donor cells was prepared using a two-step enzymatic digestion of the testis as described previously (Ogawa et al., 1997). The number of cells recovered was determined using a hemocytometer, and cell viability determined by Trypan blue exclusion, which was 96.3 ± 0.7%.

All animal procedures were approved by the Institutional Animal Care and Use Committee of McGill University.

**Recipient mice and transplantation procedure**

Recipient mice were F1 hybrids of 129/SvEv x B6, which are immunocompatible with donor mice. Recipient mice were treated with 50 mg/kg of busulfan at 4 weeks of age to destroy endogenous spermatogenesis and used in spermatogonial transplantation 4 or more weeks later (Ogawa et al., 1997). Donor testis cells were resuspended in Dulbecco modified Eagle medium (DMEM) at a concentration of 100 × 10⁶ cells/ml, and 6–7 µl of cell suspension were injected into the seminiferous tubules through the rete testis (Ogawa et al., 1997). Cells from each donor were transplanted into at least five recipient testes. Recipient testes were analyzed for SSC quantification 2 months post-transplantation by staining with 5-bromo-4-chloro-3-indolyl β-galactosidase (X-gal) (Ogawa et al., 1997). The number of donor-derived spermatogenic colonies, thus the number of SSCs, was obtained for each donor male as a mean of values collected from all recipient testes used for a specific donor mouse; i.e. although multiple recipients were used, only one number of SSCs was derived for one male treated with each dose of busulfan and at each time point. Results were expressed as numbers of colonies (i.e. functional SSCs) per donor testis, using the number of cells transplanted and that of total cells recovered from a donor testis. The expansion rate of SSCs was calculated by dividing the increase in SSC numbers in a given time interval by the number of days in the same time frame (i.e. cells per day).

**Testis weight and sperm count**

Testis weights were measured at the time of euthanasia without removing the tunica. To determine sperm counts, sperm were collected from the
cauda epididymis during the course of fertility recovery, as follows. Briefly, the epididymis was clamped in each mouse, and the cauda was dissected and transferred to DMEM supplemented with 0.5% bovine serum albumin at 37 °C. The epididymis was then minced and sperm allowed to disperse for 5 min. Cauda sperm counts per mouse were determined using a hemocytometer. At least three mice were analyzed for each time point at each dose.

Histological analyses

Testes of busulfan-treated mice were fixed in Bouin’s solution (Sigma-Aldrich) and embedded in paraffin. Sections of 5 μm thickness were taken in 25 μm section steps (i.e. 1 in every 5 sections was examined) and were stained with hematoxylin and eosin and observed under a light microscope. To determine the proportion of tubules showing spermatogenesis, we counted the number of seminiferous tubule cross-sections with or without spermatogenesis, using the method reported by Kanatsu-Shinohara et al. (2003b) as follows. Tubule sections were judged to be positive for spermatogenesis when germ cells occupy the basal membrane along the entire tubule circumference and when at least two layers of germ cells are found; otherwise, the sections were identified as negative for spermatogenesis (Kanatsu-shinohara et al., 2003b). This method can reflect changes in the population of spermatogenesis, which are the first cell type that are lost after busulfan treatment and are also regenerated after the loss of spermatogenesis. Then, the proportion of the sections positive for spermatogenesis was recorded. The values for each time point at each dose group were determined in three replicates, in each of which at least five sections and an average of 50 tubules/section was examined.

Serum and intratesticular testosterone levels

To determine serum testosterone levels, 1–2 ml of blood was collected from the jugular veins of donor mice before euthanasia, centrifuged to isolate plasma and stored at −80 °C until analyses. To measure intratesticular testosterone levels, testes were poked with a needle and centrifuged for 30 min at 500 g, and the intratesticular fluid collected was stored at −80 °C. Testosterone levels were measured using the Testosterone ELISA kit (Immuno-Biological Laboratories Inc., USA, catalog No.: IB79106) according to the manufacturer’s instructions.

Quantitative reverse transcriptase-polymerase chain reaction

GDNF mRNA levels were examined using qRT–PCR in testes of mice treated with 15 and 30 mg/kg busulfan. Total RNA was prepared using PicoPure RNA isolation kit (Arcturus) according to the manufacturer’s instructions. Complementary DNA was synthesized using Superscript III reverse transcriptase (Invitrogen) with random hexamers. Primer sequence used for transcriptional analysis of GDNF was: (Forward) 5′-TAAATGCTCAACAGGGGTCT-3′ and (Reverse) 5′-CGCCTCAGAAGGCCTCTTC AC-3′. qPCR was performed with QuantItect SYBR Green PCR kit (Qiagen) on a Rotogene 6000 (Corbett Research) with the program: 94°C for 15 min followed by 40 cycles of 94°C for 15 s/58°C for 25 s/72°C for 35 s. Samples were run in triplicate, and the average Ct (threshold cycle) values for GDNF were normalized to those for GAPDH. To generate fair comparisons (see Results), the GDNF transcript levels were normalized to a whole testis level for each time point and in each dose group, using the following formula: (G, GDNF/Ct, GAPDH) × (Total RNA per testis/Total RNA per reaction). Then, the data were expressed relative to the Day 0 value.

Figure 1 Number of fertile males after treatment with 15, 30 or 45 mg/kg of busulfan. Five mice were treated per dose, and each mouse was mated with 2 females for 5 days. Note that all mice lost fertility by 4 weeks post treatment, regardless of busulfan doses. After treatment with 15 and 30 mg/kg of busulfan, all mice regained fertility at 12 and 26 weeks, respectively, and remain so for at least another 6 weeks. After treatment with 45 mg/kg busulfan, three out of five mice regain fertility at 30 weeks while the other two do not by at least 36 weeks.

Statistical analyses

Statistical analyses were done using t-test or, for multiple comparisons, analysis of variance followed by Fisher’s Test for Least Significant Difference post hoc test. Significance was determined when P < 0.05. All data are expressed as the mean ± SEM.

Results

Fertility recovery after busulfan treatment

We first determined the profile of fertility loss and recovery over time after a single injection of busulfan at 15, 30 or 45 mg/kg (Fig. 1). Five mice were used for each dose-group and the fertility capacity was determined as the number of males that sired pups in each dose-group and at each time point. Results showed that all males were fertile across groups at 2 weeks after busulfan injection. At 3 weeks, all males still remained fertile in the 15 mg/kg group, whereas four and two out of five males sired pups in the 30 and 45 mg/kg groups, respectively. By 4 weeks, all mice lost fertility regardless of busulfan doses. Following a period of infertility, all males regained fertility by 12 weeks and 26 weeks in the 15 and 30 mg/kg groups, respectively, and remained fertile for at least the next 6 weeks until mating was terminated. In the 45 mg/kg group, three out of the five mice regained fertility by 30 weeks, while the other two remained infertile until the end of the study (36 weeks).

The changes in the litter size were similar to those of fertile males (Supplementary data, Fig. S1 versus Fig. 1). When male fertility was restored at 12, 26 and 30 weeks in the 15, 30 and 45 mg/kg groups, respectively, the litter size showed no significant differences compared with that observed before busulfan treatment. Male and female offspring in the first litter born at the time of fertility recovery were kept in all busulfan dose groups and examined for their fertility.
No defects were observed in their gross appearance during post-natal development. When mated with wild-type mice, all male and female offspring produced pups with a normal litter size, indicating that SSCs recovering after busulfan treatment were capable of generating functional gametes and offspring with normal fertility.

The recovery of SSC numbers after busulfan treatment

To quantify functional SSCs during the fertility recovery period, male mice were injected with 15, 30 or 45 mg/kg of busulfan, and each was used as a donor for the spermatogonial transplantation assay. As shown in Fig. 2A, nearly all SSCs were lost by Day 3 after busulfan treatment. On Day 3, SSC numbers per testis were 4.0, 0.8 and 0.4% of the pretreatment level (i.e., determined on Day 0) after 15, 30 and 45 mg/kg busulfan, respectively; a significantly higher number of SSCs was detected with the 15 mg/kg group than with the others (Fig. 2B). Thereafter, SSC numbers gradually increased (Fig. 2A), and in the 15 mg/kg group, they reached 28.6% of the pretreatment level by 8 weeks and 70.5% by the time of fertility recovery (12 weeks). In the 30 mg/kg group, 28.5% of SSC restoration was observed by 22 weeks, while it reached 81% of the pretreatment level by the time of fertility recovery (26 weeks). Similarly, in the 45 mg/kg group, 32.7 and 77.7% of SSC were restored by 26 weeks and by the time of fertility recovery (30 weeks), respectively.

Interestingly, these data indicated that regardless of busulfan doses, SSC numbers reached ~30% of the pretreatment level 4 weeks before the time of fertility recovery, while the numbers were 70–80% at the time of fertility recovery; no significant differences were detected in both times among the dose groups (Fig. 2A and C). From these data, we calculated that SSCs expanded at 3 cells/day in the 15 mg/kg group and 1.2 cells/day in the 30 and 45 mg/kg groups until 4 weeks prior to fertility recovery, when SSC numbers reached 30% of the pretreatment level. Once this level of recovery was achieved, the SSC expansion accelerated to 10–12.5 cells/day in all groups until the time of fertility recovery.

Testis weight and sperm count

Since we noted a correlation between kinetics of SSC recovery and those of fertility restoration, we examined whether there is a physiological parameter that allows us to monitor SSC recovery kinetics and thus, male fertility restoration, without using spermatogonial transplantation. To this end, we first assessed testis weights and sperm counts. Testis weights declined after busulfan treatment to 50% of the pretreatment level by the time of fertility loss (4 weeks) in the 15 and 30 mg/kg groups (Fig. 3A). Thereafter, the weights gradually increased in the 15 mg/kg groups but decreased further in the 30 mg/kg group until 8 weeks before initiating recovery. In the 45 mg/kg group, testis weights were 36% of control level at 4 weeks and also at 26 weeks. At the time of fertility recovery, animals in the 15 and 30 mg/kg groups showed a full recovery of testis weight, whereas those in the 45 mg/kg group reached 73% of pretreatment level (Fig. 3A), which was significantly lower than in the other two groups (Fig. 3B).

We collected sperm from the caudal part of the epididymis, as described in Materials and Methods. Sperm counts declined until 4 weeks after busulfan treatment in all groups (Fig. 4A). At this time point, sperm counts were 8.8% of the pretreatment level in the

![Figure 2](image-url)

Figure 2. SSC numbers determined by spermatogonial transplantation after treatment with busulfan at 15, 30 and 45 mg/kg. (A) Kinetics of SSC recovery during the study periods. The timing of fertility loss and restoration is indicated by arrowhead and arrow, respectively. Most SSCs are lost after treatment with busulfan (95, 99.2 and 99.6% at 15, 30 and 45 mg/kg busulfan, respectively) but their numbers increase gradually with time. At the onset of fertility recovery, 70–80% of pretreatment levels are present (472, 542 and 520 in 15, 30 and 45 mg/kg busulfan at 12, 26 and 30 weeks, respectively) with no significant difference detected among the three groups. Five donors were used in control (Day 0), at Day 3 and 12 weeks at 15 mg/kg, and Day 3 at 30 mg/kg, while four donors at 14 weeks at 15 mg/kg, 8 and 18 weeks at 30 mg/kg, and Day 3 at 45 mg/kg. Three donors were used in all others. (B) Three days after busulfan treatment, significantly higher SSC numbers are detected with the 15 mg/kg group than the others. (C) Four weeks before fertility recovery, SSC numbers reach ~30% of pretreatment levels regardless of busulfan doses. No significance is detected across the groups. Asterisks (A and B) indicate significant differences, and those in A, from pretreatment values.
15 mg/kg group, and further declines were observed with higher busulfan doses (2.3% of pretreatment level in the 30 mg/kg group and undetectable levels at 45 mg/kg groups). Later, sperm counts increased with time in all groups. At the time of fertility recovery, sperm counts were ≏20% of the pretreatment level regardless of busulfan doses. Although there was a trend at the time of fertility restoration that mice treated with 30 and 45 mg/kg busulfan produced somewhat lower numbers of sperm than those treated with 15 mg/kg busulfan, we did not detect significant differences (Fig. 4B; $P \leq 0.136$ in 15 versus 30 mg/kg and $P \leq 0.061$ in 15 versus 45 mg/kg). These results suggest that ≏20% of normal sperm counts may represent a threshold value that confers the fertility to a male mouse.

Although the recovery kinetics of testis weights and sperm counts appeared to correspond to those of SSCs generally, we did not observe a clear shift in the kinetics in both parameters as we did with SSC recovery.

**Histological analysis of spermatogenesis**

To evaluate the relationship between the recovery of SSC numbers and that of spermatogenesis, we analyzed the histology of the seminiferous tubules in paraffin sections (Fig. 5). Consistent with the results of past studies (de Rooij and Kramer, 1970; Bucci and Meistrich, 1987), we observed that cells in the basal compartment of the seminiferous epithelium were the first to disappear by 3 days after busulfan treatment, regardless of busulfan doses (Fig. 5A and B). By 4 weeks, a significant number of the tubule sections lost germ cells in all treatment groups (Fig. 5C and D). Thereafter, the epithelium was reconstituted gradually and continuously with time (Fig. 5E–H).

To give a quantitative measure to the qualitative observations described earlier, we determined the proportion of seminiferous tubules with spermatogenesis compared with pretreatment values, as defined by Kanatsu-Shinohara et al. (2003, b) (see Materials and...
Methods). As shown in Fig. 6, the proportion continuously declined by 4 weeks to 24% of pretreatment levels in the 15 mg/kg group and to 14% in the 30 and 45 mg/kg groups. Thereafter, it increased until the time of fertility recovery where complete spermatogenesis was observed in ≏70 and ≏90% of tubule sections at 4 weeks before and at the time of fertility recovery, respectively, across the dose groups.

**Testosterone levels**

The morphological parameters examined earlier (testis weights, sperm counts and testis histology) can be affected by testosterone. We thus assessed levels of serum and intratesticular testosterone in the mice treated with 15 or 30 mg/kg busulfan. The results showed that both levels remained constant throughout the study period (Fig. 7). These results are consistent with previous reports (Bucci and Meistrich, 1987; O’Shaughnessy et al., 2008) that busulfan treatment does not alter significantly the steroidogenic environment in mice. Our data thus confirm that testosterone does not affect the restoration of male fertility and the kinetics of SSC recovery in mice.

**GDNF expression patterns**

Since GDNF, which is expressed by Sertoli cells in the testis, is known to be a critical paracrine factor that promotes SSC self-renewal, we measured its mRNA levels using qRT–PCR after 15 and 30 mg/kg busulfan treatments. The expression levels were initially measured in comparison with those of a house-keeping gene (GAPDH). As shown in Supplementary data, Fig. S2, the relative abundance of GDNF transcripts increased up to 4 weeks after transplantation, during which germ cells are gradually lost (Figs 5 and 6), and returned thereafter to the pretreatment level. However, busulfan is known to eliminate germ cells but not affect testicular somatic cell numbers, including Sertoli cells (Bucci and Meistrich, 1987); therefore, our data of GDNF transcripts may be biased because of a fluctuation of the Sertoli cell concentration in a testis when germ cell numbers decline or increase during the study periods. To circumvent this problem, we normalized the qRT–PCR results to total levels of GDNF transcripts in an entire testis (see Materials and Methods). Since Sertoli cell numbers have been reported to not change after busulfan treatment (Bucci and Meistrich, 1987), this normalization should provide more accurate analyses.

Total GDNF mRNA levels per testis increased significantly by Day 5 after treatment in both dose-groups (1.23 ± 0.03-fold and 1.81 ± 0.09-fold at 15 and 30 mg/kg busulfan, respectively versus the pretreatment level) (Fig. 8). By 4 weeks and afterwards, the mRNA level showed no significant difference compared with the pretreatment level. Thus, the data showed that the increase in GDNF mRNA levels coincided with the period of rapid loss of SSCs and spermatogonia after busulfan treatment (Figs 2 and 5), suggesting that Sertoli cells may respond rapidly and temporarily to the loss of spermatogonia, including SSC, by increasing the expression of GDNF transcripts.

**Discussion**

In this study, we demonstrated that kinetics of fertility restoration correlated with those of SSC recovery in mice. Importantly, we found that SSCs restored their population size to ≏30% of the pretreatment level, regardless of busulfan doses examined, by 4 weeks before males became capable of siring offspring by natural mating. Although the 30% level was determined based on pretreatment levels, rather
Figure 6  Quantitative measurements of reconstitution of the seminiferous epithelium, as observed in Fig. 5. The proportion of tubules with spermatogenesis was measured using the method of Kanatsu-shinohara et al. (2003b), as described in Materials and Methods. Scores after treatment with 15 mg/kg of busulfan are presented in (A), those with 30 mg/kg in (B) and with 45 mg/kg in (C). Asterisks indicate significant differences from pretreatment values.

Figure 7  Intratesticular and serum testosterone levels after busulfan treatments at 15 mg/kg (A) and 30 mg/kg (B). Data are represented as percentages of pretreatment levels, compared with which no significant differences are detected for both levels and in both dose groups.

Figure 8  GDNF mRNA expression levels per testis after busulfan treatments at 15 mg/kg (A) and 30 mg/kg (B), measured by qRT–PCR and expressed as relative to pretreatment levels (Day 0). GDNF mRNA levels increase significantly in both dose groups only at Day 5 after treatment, compared with pretreatment levels. Asterisks mark significant differences.
than those of age-matched controls, this finding suggests that there may be a threshold in the SSC population size that allows for eventual restoration of male fertility in a defined time frame (~4 weeks after the 30% threshold is reached in mice). Further, SSCs shifted their expansion kinetics around the time of the 30% threshold, and the expansion accelerated once the threshold level was achieved. These results demonstrate that following cytotoxic effects of busulfan, the restoration of male fertility is closely linked to the recovery kinetics of the SSC population size, further emphasizing the fundamental role of SSCs in male reproduction.

It was unexpected to us that there was a shift in SSC expansion kinetics during fertility restoration where SSCs initially increased their numbers more slowly but accelerated later. A previous study showed a linear increase in SSC numbers without a threshold following busulfan treatment, although the dose of busulfan examined was only 15 mg/kg (Kanatsu-shinohara et al., 2003b). The cause of this discrepancy is unclear, but the difference in strains of donor and recipient mice between the two studies could have contributed to it; the H2 haplotypes of donors and recipients were different in the previous study while they were identical in ours. Nonetheless, it is notable that we observed a near-identical shift of SSC expansion kinetics with all busulfan doses examined. The results thus suggest that even though SSCs do expand following the initial massive reduction of the stem cell pool induced by cytotoxic effects of busulfan, they may commit to differentiation more preferentially until the population size reaches a threshold level; our observation that regeneration of spermatogenesis was seen in 70–80% of tubules at the time of the 30% threshold also corresponds to this notion (Figs 2 and 6). Thereafter, SSCs may accelerate their expansion to regenerate an appropriate size of the stem cell pool to sustain steady-state spermatogenesis. In this regard, we reported previously that when mouse SSCs derived from 1-week-old pups or adult males with experimental cryptorchidism were transplanted into recipient testes, more committed daughter cells were produced, compared with when SSCs derived from adult intact testes with steady-state spermatogenesis were transplanted (Ebata et al., 2007). Together with the results of the current study, these observations suggest that even though SSCs continue to proliferate during the study periods, SSCs tend to produce differentiated cells and functional gametes initially at the expense of robust SSC proliferation, compared with the later stages of SSC recovery; i.e. SSC fate decision appears to be skewed towards differentiation until the population size reaches the threshold level. Such a SSC behavior could be beneficial to rapidly produce functional gametes and efficiently achieve male fertility restoration.

Our data of initial SSC killing and physiological parameters are generally in agreement with those reported in the past (de Rooij and Kramer, 1970; Bucci and Meistrich, 1987; Kanatsu-shinohara et al., 2003a,b). Contrary to our results, however, Bucci and Meistrich (1987) did not observe fertility restoration when the busulfan dose exceeded 28 mg/kg. The difference in mouse strains used may have caused this discrepancy. Nonetheless, we found that more SSCs survived at a lower busulfan dose (15 mg/kg) than at higher doses (30 and 45 mg/kg) (Fig. 2), a trend that was observed in the previous study (Bucci and Meistrich, 1987). Interestingly, our data show that a higher survival of SSCs is associated with a greater SSC expansion rate until SSC recovery reaches the 30% threshold (3 cells/day at 15 mg/kg versus 1.2 cells/day at 30 and 45 mg/kg; Fig. 2A and B).

These observations suggest that an initial population size of surviving SSCs may influence a later trajectory of SSC expansion. It is also possible, however, that higher doses of busulfan may have damaged SSCs more significantly, and thus, a longer time was necessary for SSCs to recover. Another possibility is that a high dose of busulfan might have affected some actions of the somatic environment. For example, after treatment with 45 mg/kg busulfan, SSC numbers recovered to a level comparable to those seen in other dose groups at the time of fertility restoration (Fig. 2A), but testis weights were significantly lower (Fig. 3B). Furthermore, the recovery of sperm counts showed remarkably contrasting kinetics between busulfan doses at 15 mg/kg and 30 and 45 mg/kg (Fig. 4A). Hence, we cannot rule out the possibility that busulfan could affect functions of the somatic environment to support spermatogenic recovery. Further studies are necessary to address these possibilities.

Since the restoration of the SSC population associates with the recovery of male fertility after cytotoxic damage, a physiological parameter that correlates with SSC expansion should provide an approach to monitoring the process of male fertility recovery and could perhaps predict the timing of fertility restoration. In this study, we examined various physiological parameters but were not able to identify those that can faithfully correlate with the recovery of the SSC population, particularly the shift of SSC expansion kinetics. Since GDNF plays a critical role in promoting SSC self-renewal, we reasoned that its transcript levels could reflect SSC behaviors more faithfully. Although the data did not support our reasoning, they showed that the expression of GDNF transcripts was temporally elevated soon after busulfan treatment (Fig. 8), suggesting that Sertoli cells may promptly respond to the loss of spermatagonia and drive the survival and expansion of SSCs. Notably, a magnitude of increase in GDNF transcripts on Day 5 was greater when the busulfan dose was 30 mg/kg than 15 mg/kg (1.2-fold versus 1.9-fold after 15 and 30 mg/kg, P ≤ 0.0001) (Fig. 8), raising the possibility that Sertoli cells may have expressed GDNF transcripts according to the degree of damage inflicted on the population of spermatagonia. During SSC expansion periods, however, GDNF levels did not change in both busulfan dose groups. This finding suggests that a basal level of GDNF transcription may be sufficient to provide an environment that is permissive for SSC self-renewal and expansion.

In this regard, O’Shaughnessy et al. (2008) recently examined mRNA levels of 26 Sertoli-cell-specific genes for up to 50 days after germ cell depletion induced by 30 mg/kg busulfan. They reported that the loss of germ cells led to varied responses of Sertoli cells in gene expression patterns and that most changes were associated with the loss of spermatids. Although GDNF was not included as a target gene of the study, the authors further showed a rapid increase (within 5 days) in expression of five genes by Sertoli cells, namely Cst9, Shbg, Inhbb, Wnt5a and Clu. This pattern of gene expression was similar to what we observed with GDNF in the present study. The rapid increase in Wnt5a expression is intriguing, as we have recently shown that WNT5A promotes self-renewal of mouse SSCs, and this effect is exerted in part by stimulating SSC survival (Yeh et al., 2011). As GDNF also promotes SSC survival (Lee et al., 2007; Oatley et al., 2007), it appears that the loss of early spermatagonia, including SSCs, may stimulate the activity of Sertoli cells to encourage SSC survival and self-renewal, which could be mediated in part by
soluble growth factors, such as GDNF and WNT5A. Further investigations are necessary to address such a possibility.

To analyze GDNF transcript levels, we normalized data of qRT–PCR to the whole testis level, assuming that Sertoli cells do not change in number after busulfan treatment (Bucci and Meistrich, 1987). A caution is necessary, however, because it is unknown whether the expression of GAPDH, an internal control chosen for our assay, is not affected by busulfan treatment. To overcome the same issue associated with PCR-based quantification of GDNF transcripts in young and old mouse testes, Ryu et al. (2006) used the Sertoli-cell-specific GATA4 gene as an internal control. While this approach eliminates the necessity to convert data to those at the whole testis level, it is unknown whether GATA4 expression remains constant as the mice and Sertoli cells age. It appears, therefore, that assessing the transcript levels by qRT–PCR bears an inherent problem when the number of a given cell type fluctuates in a target organ or tissue. To solve such a problem in the future, it may be necessary to measure the absolute number of target mRNA molecules using synthetic complementary RNA as an internal control (Nagano and Kelly, 1994). In this regard, it may also be useful to examine GDNF expression at the protein level.

In conclusion, this study provides functional evidence that restoration of male fertility results from that of the SSC population after cytotoxic damage. Our study also proposes that there may be a threshold in the size of the SSC population that is required for the onset of male fertility restoration in a given time frame.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

**Authors’ roles**

M.C.N., P.C. and K.Z. designed research; K.Z. and X.Z. performed research; S.L.T., P.C., K.Z. and M.C.N. contributed with critical discussion; K.Z. and M.C.N. analyzed data and wrote the paper.

**Acknowledgements**

We thank Trang Luu for her help in testosterone measurements. We also thank Drs Riaz Farookhi and Bernard Robaire for suggestions and discussions, and Jonathan Yeh for his critical reading of this manuscript.

**Funding**

This study was supported by CIHR (MOP86532, OBM101392, SUR103396, SUR203036), Department of Obstetrics and Gynecology, RI-MUHC and CSR. K.Z. was supported by the Egyptian ministry of higher education scholarships program.

**References**


Sanders JE, Hawley J, Levy W. Pregnancies following high dose cyclophosphamide with or without high dose busulfan or total body irradiation and bone marrow transplantation. *Blood* 1996;87:3045–3052.


