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ORIGINAL ARTICLE

Male Reproduction

Establishing a nonlethal and efficient mouse model of male gonadotoxicity by intraperitoneal busulfan injection

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An ideal animal model of azoospermia would be a powerful tool for the evaluation of spermatogonial stem cell (SSC) transplantation. Busulfan has been commonly used to develop such a model, but 30%–87% of mice die when administered an intraperitoneal injection of 40 mg kg⁻¹. In the present study, hematoxylin and eosin staining, Western blot, immunofluorescence, and quantitative real-time polymerase chain reaction were used to test the effects of busulfan exposure in a mouse model that received two intraperitoneal injections of busulfan at a 3-h interval at different doses (20, 30, and 40 mg kg⁻¹) on day 36 or a dose of 40 mg kg⁻¹ at different time points (0, 9, 18, 27, 36, and 63 days). The survival rate of the mice was 100%. When the mice were treated with 40 mg kg⁻¹ busulfan, dramatic SSC depletion occurred 18 days later and all of the germ cells were cleared by day 36. In addition, the gene expressions of glial cell line-derived neurotrophic factor (*GDNF*), fibroblast growth factor 2 (*FGF2*), chemokine (C-X-C Motif) ligand 12 (*CXCL12*), and colony-stimulating factor 1 (*CSF1*) were moderately increased by day 36. A 63-day, long-term observation showed the rare restoration of endogenous germ cells in the testes, suggesting that the potential period for SSC transplantation was between day 36 and day 63. Our results demonstrate that the administration of two intraperitoneal injections of busulfan (40 mg kg⁻¹ in total) at a 3-h interval to mice provided a nonlethal and efficient method for recipient preparation in SSC transplantation and could improve treatments for infertility and the understanding of chemotherapy-induced gonadotoxicity.

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INTRODUCTION

With the development of cancer treatment, the long-term survival rate of cancer patients has been considerably improved. Despite improvements in chemotherapy regimens, radiotherapy practices, and surgical options, cancer treatments still have gonadotoxic side effects that can result in loss of fertility, particularly in young cancer survivors.¹ Recently, freezing immature testicular tissue before cancer treatment and then implanting germ cells has been proposed as an optimal protocol for the treatment of prepubertal boys who do not yet have spermatozoa but need their fertility to be preserved. However, to accelerate its clinical translation, more studies should be focused on this protocol, especially spermatogonial stem cell (SSC) transplantation. Before an SSC transplantation can be performed, an animal model that simulates the clinical condition is necessary. SSC transplantation was first reported in mice.^{2,3} Briefly, testicular cells from a fertile male were microinjected into the seminiferous tubules of an infertile recipient, in which donor-derived spermatogenesis occurred. The restoration of fertility by germ cell transplantation requires efficient preparation of the

recipient.⁴ The recipient's endogenous germ cells need to be depleted, so that donor cells can colonize the free germ cell niches.⁵

To deplete all of the germ cells in the recipient testes, several techniques, including local irradiation, heat shock treatment, experimental cryptorchidism, and cold ischemia, have been developed.^{6–10} However, the efficiencies of these methods are inadequate.¹¹ Busulfan, which is an alkylating agent, can inhibit cell division by binding to one of the DNA strands, so that germ cells, with their high cell division rates, are susceptible to busulfan treatment.¹² A single intraperitoneal injection of busulfan has been reported to eliminate almost all endogenous germ cells and create an SSC niche in mice; this has become the most commonly used technique.¹³ To sterilize mice, a single dose of busulfan for intraperitoneal injection should exceed 30 mg kg⁻¹.^{14,15} However, when the dose is higher than 30 mg kg⁻¹, the mortality rate of the mice is notably increased.¹⁶ Therefore, a safer and more effective method of delivering busulfan that balances the sterilization and survival rate in mice and mimics the gonadotoxicity induced by the systemic administration of

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chemotherapeutic agents is greatly needed. Moreover, recent studies have only shown the final status of the germ cells that were cleared from the mouse seminiferous tubules by busulfan, and the loss of germ cells in the seminiferous epithelium cycle remains unknown.

Thus, we designed this study to establish a nonlethal and efficient method for the recipient preparation of SSC transplantation in mice and to analyze the loss of germ cells in the seminiferous epithelium cycle to provide a better understanding of chemotherapy-induced gonadotoxicity.

MATERIALS AND METHODS

Animals

A total of 76 C57BL/6 male mice aged 12 weeks (25–35 g) were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China) and maintained under standard laboratory conditions. The mice were given food and ultraviolet-sterilized tap water *ad libitum*. The animal experimental protocol was approved by the Committee for Animal Care and Use of Sun Yat-sen University.

Study design

First, 28 mice were randomly divided into four groups: vehicle control group, 20 mg kg⁻¹ group, 30 mg kg⁻¹ group, and 40 mg kg⁻¹ group; the four groups were administered two intraperitoneal injections of vehicle or busulfan at a total dose of 20, 30, or 40 mg kg⁻¹ body weight with a 3-h interval between injections, respectively. The effects were investigated 36 days later.

Second, some groups were treated with two intraperitoneal injections of busulfan at a total dose of 40 mg kg⁻¹ body weight at an interval of 3 h. In general, the cycle of the seminiferous epithelium (34.4 days) in mice can be divided into four stages (one every 8.6 days).¹⁷ Therefore, we observed the dynamic changes in the testes at an interval of 9 days after the administration of 40 mg kg⁻¹ busulfan. Forty-two mice were randomly divided into seven groups and further analyzed at different time points (0, 9, 18, 27, 36, 45, and 63 days) after busulfan treatment. In addition, six mice were randomly divided into two groups for long-term observation after busulfan treatment at 90 days and 126 days.

In these experiments, the main outcome measures were animal survival rate; testicular and caput epididymal weights; testicular histology; Western blot analysis and immunofluorescence of ubiquitin C-terminal hydrolase L1 (UCHL1, an SSC marker), sex-determining region Y box 9 (SOX9, a Sertoli cell marker), cytochrome P450 family 11 subfamily A member 1 (CYP11A1, a Leydig cell marker), and α -smooth muscle actin (α -SMA, a peritubular myoid cell marker); levels of serum inhibin B; and the expression of growth factor genes in the testes, including glial cell line-derived neurotrophic factor (*GDNF*), fibroblast growth factor 2 (*FGF2*), chemokine (C-X-C Motif) ligand 12 (*CXCL12*), and colony-stimulating factor 1 (*CSF1*).

Administration of busulfan

Dimethyl sulfoxide (DMSO), the solvent for busulfan, should not exceed a concentration of 10% (*v/v*), due to its toxicity.¹⁸ Our previous experiment indicated that the maximal solubility of busulfan (Cat. No. HY-B0245/CS-2224, MedChemExpress, Monmouth Junction, NJ, USA) in DMSO (Cat. No. 196055, MP Biomedicals, Santa Ana, CA, USA) is 20 mg ml⁻¹. An intraperitoneal administration volume of 20 ml kg⁻¹ is considered good practice by Diehl *et al.*¹⁹ Therefore, after diluting the DMSO to a concentration of 5% (*v/v*) with physiological saline, we separated the total liquid in half to ensure the administration volume for a single intraperitoneal injection was 20 ml kg⁻¹. Because the half-life of busulfan is nearly 3 h in mice, the interval between the two intraperitoneal injections was set at 3 h. Busulfan was dissolved in DMSO at a concentration of 20 mg ml⁻¹ and then diluted with

physiological saline to a concentration of 5% (*w/v*) DMSO. The total dosage of busulfan was equally divided into two intraperitoneal injections at 3 h apart. The detailed administration volumes of busulfan at the total dose of 40 mg kg⁻¹ are presented in **Table 1**.

Testicular weight and hematoxylin and eosin staining

At the time of sacrifice, the testis and caput epididymidis were weighed without the removal of the tunica, fixed in a solution of 4% formaldehyde (*w/v*, Cat. No. G1101, Servicebio, Wuhan, China), and embedded in paraffin. Tissue sections of 3-mm thickness on paraffin slides were deparaffinized in xylene and rehydrated in graded alcohol solutions. The slides were then stained with hematoxylin and eosin (H and E) and examined under a light microscope (BX53, OLYMPUS, Tokyo, Japan). To determine the proportion of tubules with spermatogenic cells, we randomly counted the number of seminiferous tubule cross-sections with or without spermatogenic cells in 200 fields of view on the H and E slides. Tubule sections were considered positive when germ cells and Sertoli cells were found in the tubule. Otherwise, the sections were identified as negative for Sertoli cells only. Then, the proportion of the sections that were positive for spermatogenic cells was recorded. The values for each dose group were determined from three replicates, in which at least five sections and at least three fields per section were randomly examined.

Immunofluorescence staining

Tissue sections that were embedded in paraffin slides were deparaffinized in xylene and then rehydrated in graded alcohol solutions. The endogenous peroxidase activity was inhibited by incubating the slide in 3% (*v/v*) hydrogen peroxide, and the antigenicity was recovered with a 0.01 mol l⁻¹ sodium citrate buffer (pH 6.0, Cat. No. AR0024, Boster Biological Technology, Wuhan, China). The samples were incubated overnight at 4°C with antibodies for UCHL1 (dilution: 1:100, Cat. No. 13179, Cell Signaling Technology, Danvers, MA, USA), SOX9 (dilution: 1:100, Cat. No. AB5535, Millipore, Burlington, MA, USA), and α -SMA (dilution: 1:100, Cat. No. ARG52485, Arigo, Hsinchu, Taiwan, China); washed with PBS; and then incubated with Cy3-conjugated goat anti-rabbit (dilution: 1:100, Cat. No. CW0159S, CWBIO, Beijing, China), Cy3-conjugated goat anti-mouse (dilution: 1:100, Cat. No. CW0145, CWBIO), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (dilution: 1:100, Cat. No. CW0114S, CWBIO) at room temperature for 30 min. Subsequently, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 10 μ g ml⁻¹, Cat. No. C0065, Solarbio, Beijing, China). Immunofluorescence signals were detected by fluorescence microscopy (IX71, OLYMPUS).

Western blot analysis

Total protein was extracted from the frozen testicular tissues into RIPA buffer (Cat. No. CW2333S, CWBIO) that contained a phosphatase inhibitor cocktail (100 \times , Cat. No. CW2383S, CWBIO) and a protease inhibitor cocktail (100 \times , Cat. No. CW2200S, CWBIO). The protein concentration was quantified by the Pierce BCA Protein Assay Kit (Cat. No. 23227, Thermo Fisher Scientific, Waltham, MA, USA). Protein lysates (20 μ g) were separated with 10% (*w/v*) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, IPVH00010, Millipore). Then, the membranes were blocked in a 5% (*w/v*) nonfat milk solution and incubated with antibodies for UCHL1 (dilution: 1:1000), CYP11A1 (dilution: 1:1000, Cat. No. GTX56293, Genetex, Irvine, CA, USA), SOX9 (dilution: 1:500), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; dilution:

1:4000, Cat. No. T0004, Affinity, Cincinnati, OH, USA) overnight at 4°C. Finally, the membrane was incubated with the appropriate secondary antibody (goat anti-rabbit IgG, 1:5000, CW0103S, CWBIO; goat anti-mouse IgG, 1:5000, CW0102S, CWBIO) and visualized with an enhancer (ChemiDoc Imaging System, BIO-RAD Laboratories, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA) of inhibin B

A mouse inhibin B ELISA Kit (Cat. No. CSB-E08151m, CUSABIO, Wuhan, China) was used to detect the level of inhibin B in the mouse serum. A blank well without any solution and a standard line were included on each plate. Mouse serum samples were diluted 1:2 in physiological saline and added (50 µl) to duplicate wells. Then, 50 µl HRP conjugate and 50 µl antibody were added to each well, followed by mixing and incubation for 1 h at 37°C. Subsequently, 50 µl of substrate A and 50 µl of substrate B were added to each well. After incubation for 15 min at 37°C in the dark, the chromogenic reaction was stopped by adding 50 µl stop solution to each well. The absorbances were read in a microplate reader (Multiskan FC, Thermo Fisher Scientific) that was set to 450 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total tissue RNA was extracted with TRIzol Reagent (Code No: 9109, Takara, Shiga, Japan). The concentration and integrity of the RNA were analyzed in a NanoDrop 2000 (Thermo Fisher Scientific). The total RNA (3 µg) was then used as a template to synthesize the cDNA with the High Capacity cDNA Archive Kit (Code No: RR036A, Takara). The primer sequences that were used for the transcriptional analyses are presented in **Table 2**. The primers were synthesized by Sangon Biotech (Shanghai, China). Then, qPCR was subsequently performed on the ROCHE LightCycler 480II Real-Time PCR Machine (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The RNA levels were normalized to the endogenous control gene (β -actin). The experimental cycle threshold (Ct) was calibrated against the β -actin control product.

All amplifications were performed in triplicate on fifteen samples that were obtained from fifteen mice.

Statistical analyses

All data are presented as the mean \pm standard deviation (s.d.). Statistical comparisons between two groups were performed with Student's *t*-test. In addition, multigroup comparisons of the means were analyzed by a one-way analysis of variance (ANOVA), followed by a *post hoc* Bonferroni test. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Depletion of germ cells in mice 36 days after the administration of different doses of busulfan

We first determined the testicular changes over time after two intraperitoneal injections of busulfan at 20, 30, or 40 mg kg⁻¹ or after the injection of a vehicle control (**Figure 1**). H and E staining showed that, in mice treated with different doses of busulfan, histological changes in the testes were characterized by a reduced height of the seminiferous epithelium, compared with those in the control group at day 36 (**Figure 1a**). It is important to note that almost all of the spermatogenic cells were depleted within the seminiferous tubules, with only Sertoli cells remaining in the mice that were treated with 40 mg kg⁻¹ busulfan. However, in mice that were treated with a lower dose of busulfan (20 or 30 mg kg⁻¹) or the vehicle control, several layers of seminiferous epithelial cells with spermatogonia in the outer layer could be observed (**Figure 1a**). The quantitation of the proportion of tubules with spermatogenic cells showed that, compared with those in the control group, only mice that were treated with 40 mg kg⁻¹ busulfan showed an almost complete depletion of all of their spermatogenic cells within the seminiferous tubules (0.016 \pm 0.033 vs 0.991 \pm 0.018, $P < 0.001$). In addition, the ratio of testicular to body weight was

Table 1: Administration volumes of busulfan (40 mg kg⁻¹)

Body weight (g)	Busulfan (mg)	DMSO (ml)	Total liquid diluted by NS (ml)	A single intraperitoneal injection (ml)
25	1.00	0.050	1.00	0.50
26	1.04	0.052	1.04	0.52
27	1.08	0.054	1.08	0.54
28	1.12	0.056	1.12	0.56
29	1.16	0.058	1.16	0.58
30	1.20	0.060	1.20	0.60
31	1.24	0.062	1.24	0.62
32	1.28	0.064	1.28	0.64
33	1.32	0.066	1.32	0.66
34	1.36	0.068	1.36	0.68
35	1.40	0.070	1.40	0.70

DMSO: dimethyl sulfoxide; NS: physiological saline

Table 2: List of the polymerase chain reaction primers

Gene	Forward primer	Reverse primer
GDNF	5'-GCTGTCTGCCTGGTGTGCTC-3'	5'-GGCATATTGGAGTCACTGGTCAGC-3'
FGF2	5'-AAGCGGCTCTACTGCAAGAAGC-3'	5'-CAGCCGTCCATCTCTCCATAGC-3'
CSF1	5'-CCAATGCTAACGCCACCGAGAG-3'	5'-GCCTTGTCTGCTCCTCATAGTCC-3'
CXCL12	5'-GCCACATCGCCAGACCAAC-3'	5'-GTTCTTCAGCCGTGCAACAATCTG-3'
β -actin	5'-TGCTGTCCCTGTATGCCTCT-3'	5'-TTTGATGCACGCACGATT-3'

GDNF: glial cell line-derived neurotrophic factor; FGF2: fibroblast growth factor 2; CSF1: colony-stimulating factor 1; CXCL12: chemokine (C-X-C Motif) ligand 12

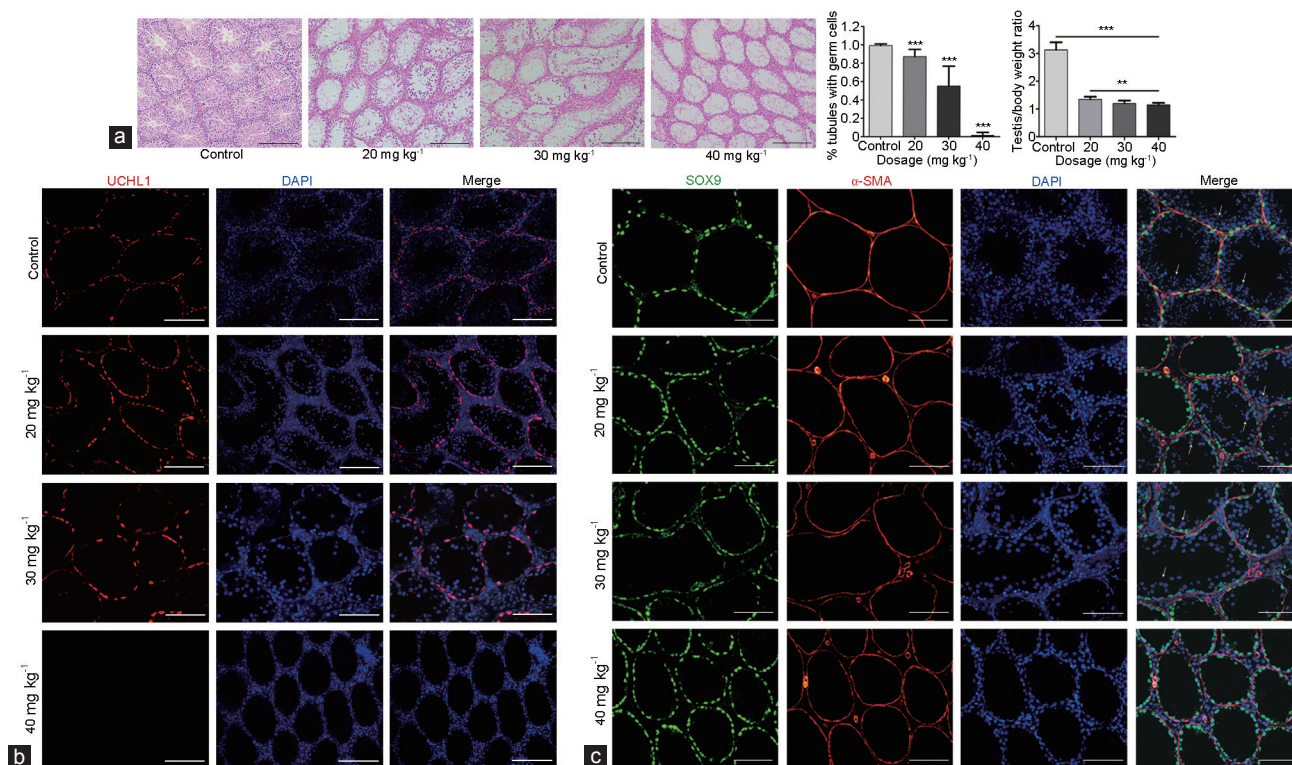


Figure 1: Depletion of mouse germ cells 36 days after the administration of different doses of busulfan. (a) H and E staining showed histological changes in the testes of mice at day 36 after vehicle (control) or 20 mg kg⁻¹, 30 mg kg⁻¹, or 40 mg kg⁻¹ busulfan treatment. The testicular-to-body weight ratio was decreased in the mice that were treated with 40 mg kg⁻¹ busulfan, compared with those treated with the vehicle control (*** $P < 0.001$) or the 20 mg kg⁻¹ dose of busulfan (** $P < 0.01$). Scale bars = 200 μ m. $n = 7$ per group. (b) The immunofluorescence staining of the testicular tissues revealed that few SSCs expressed UCHL1 (red) in the groups that were administered lower doses of busulfan (20 or 30 mg kg⁻¹), while no UCHL1 positive SSCs were found in the seminiferous tubules in the 40 mg kg⁻¹ busulfan-treated group. Scale bars = 100 μ m. (c) The testicular tissues were double immunofluorescence stained with the Sertoli cell marker SOX9 (green) and the peritubular myoid cell marker α -SMA (red). Some spermatogenic cells (arrow) could be observed in the groups that were administered the lower doses of busulfan (20 or 30 mg kg⁻¹). Scale bars = 100 μ m. H and E: hematoxylin and eosin; SSC: spermatogonial stem cell; UCHL1: ubiquitin C-terminal hydrolase L1; DAPI: 4',6-diamidino-2-phenylindole; SOX9: sex-determining region Y box 9; α -SMA: α -smooth muscle actin.

decreased in the mice that were treated with 40 mg kg⁻¹, compared with those of the vehicle control group (1.145 ± 0.074 vs 3.128 ± 0.277 , $P < 0.001$) or the group that received the lower dose of busulfan (20 mg kg⁻¹, 1.145 ± 0.074 vs 1.345 ± 0.094 , $P < 0.01$) (Figure 1a).

Further analysis by immunofluorescence staining supported these results. The seminiferous tubules primarily consist of SSCs that express UCHL1, Sertoli cells that express SOX9, and other spermatogenic cells. To confirm that no SSCs remained in the lumen, the tissues were stained with a UCHL1 antibody (Figure 1b). Moreover, the tissues were double stained with a Sertoli cell marker (SOX9) and a marker for the basement membrane of the peritubular myoid cells (α -SMA) to confirm whether any cells other than Sertoli cells remained within the lumen (Figure 1c). From the results, we found that 36 days after busulfan treatment, some spermatogenic cells remained in the groups that were given the lower doses of busulfan (20 or 30 mg kg⁻¹). However, almost all of the germ cells were depleted within the seminiferous tubules of the 40 mg kg⁻¹ group.

Dynamic changes of the testes at different stages of the seminiferous epithelium cycle after administration of busulfan at a dose of 40 mg kg⁻¹

Because the dose of 40 mg kg⁻¹ of busulfan resulted in adequate depletion of the host germ cells, we monitored the dynamic changes in the testes at different stages of the seminiferous epithelium cycle. H and E staining and immunofluorescence staining (Figure 2a–2c) showed that there

were no visible histological changes of the testes on day 9. However, the testicular histology was dramatically different on day 18, with progressive atrophic lesions, frequent disarrangement of spermatogenic cells, and reduced height of the seminiferous epithelium. Only a few germ cells were found in the seminiferous tubules (Figure 2b and 2c). Furthermore, the testis/body weight ratio was decreased on day 18, as compared with that on day 0 (1.913 ± 0.247 vs 3.255 ± 0.305 , $P < 0.001$) (Figure 2a). On day 36, almost all of the germ cells had disappeared, and the ratio of testicular to body weight was decreased, compared with that on day 0 (1.110 ± 0.072 vs 3.255 ± 0.305 , $P < 0.001$) (Figure 2a–2c).

To evaluate the SSC niche at day 36 after the administration of 40 mg kg⁻¹ dose busulfan, we analyzed the levels of inhibin B in the serum and the gene expression of growth factors in the testes. The results demonstrated that the level of inhibin B in the serum fluctuated after the administration of busulfan at different time points (0, 9, 18, 27, and 36 days), but did not display any trends (day 0: 3.553 ± 0.083 pg ml⁻¹; day 36: 3.641 ± 0.298 pg ml⁻¹, $P > 0.05$) (Figure 2d). In addition, quantitative real-time PCR showed that the expressions of *GDNF* (2.992 ± 0.926 vs 1.000 ± 0.577 , $P < 0.05$), *FGF2* (1.718 ± 0.579 vs 1.000 ± 0.297 , $P > 0.05$), *CXCL12* (6.007 ± 0.331 vs 1.000 ± 0.573 , $P < 0.001$), and *CSF1* (4.734 ± 0.355 vs 1.000 ± 0.575 , $P < 0.001$) were gradually increased in the testes on day 36 after the injection of busulfan, as compared with those of the control group (day 0) (Figure 2d).

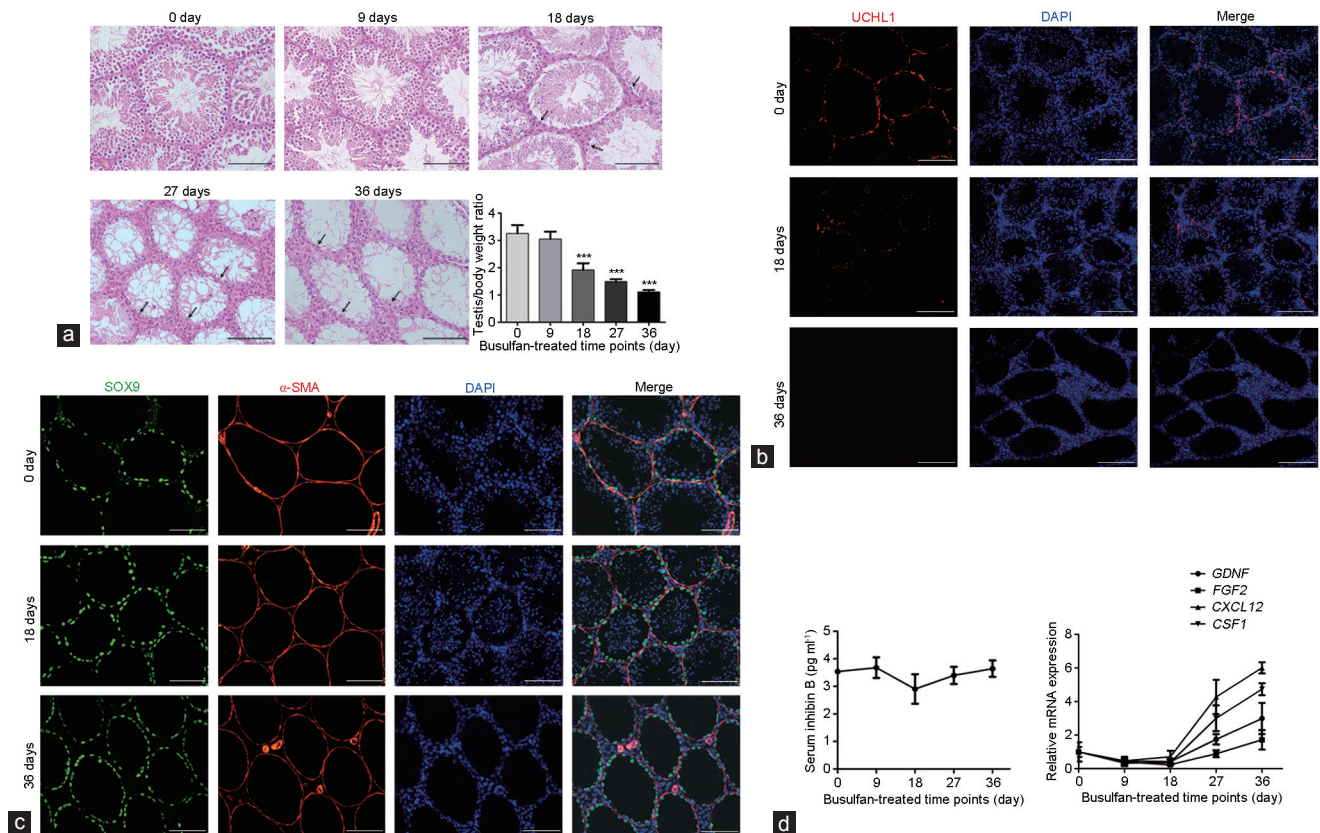


Figure 2: Dynamic changes of the testicular cycle of the seminiferous epithelium after the administration of 40 mg kg⁻¹ busulfan. (a) The testicular histology showed dramatic changes on day 18. On day 27, only a few Sertoli cells (arrow) remained in the seminiferous tubules. On day 36, almost all of the germ cells had disappeared. Compared with day 0, the testicular-to-body weight ratio was decreased on day 36 (***) ($P < 0.001$). Scale bars = 100 μ m. $n = 6$ per group. (b) The immunofluorescence staining of the testicular tissues showed that a few SSCs expressed UCHL1 (red) on day 18, while almost all of the UCHL1-positive SSCs had disappeared from the seminiferous tubules on day 36. Scale bars = 100 μ m. (c) The testicular tissues were double immunofluorescence stained with SOX9 (green) and α -SMA (red), which showed few spermatogenic cells remaining on day 18, while the spermatogenic cells were adequately depleted within the seminiferous tubules on day 36. Scale bars = 100 μ m. (d) The level of inhibin B in the serum fluctuated after the administration of busulfan at different time points (0, 9, 18, 27, and 36 days) but did not display any trends ($P > 0.05$). The total *GDNF*, *FGF2*, *CSF1*, and *CXCL12* mRNA levels were gradually increased at day 36 in the testes after the injection of busulfan, as compared with those of the control group (day 0). $n = 3$ per group. SSC: spermatogonial stem cell; UCHL1: ubiquitin C-terminal hydrolase 1; DAPI: 4',6-diamidino-2-phenylindole; SOX9: sex-determining region Y box 9; α -SMA: α -smooth muscle actin; GDNF: glial cell line-derived neurotrophic factor; FGF2: fibroblast growth factor 2; CXCL12: chemokine (C-X-C Motif) ligand 12; CSF1: colony-stimulating factor 1.

In addition, we noted that the caput epididymidis also showed histological changes after the administration of busulfan, including a decreased number of the ductus epididymidis (Figure 3). At day 36, there was also a decrease in the ratio of caput epididymal to body weight, compared with that on day 0 (0.440 ± 0.030 vs 0.582 ± 0.036 , $P < 0.001$).

Long-term observations of spermatogenic restoration and the evaluation of survival rates

Because the 40 mg kg⁻¹ dose of busulfan depleted all of the host spermatogenic cells in the mice by day 36, this was the time point at which the spermatogonial transplantation was performed. Then, we conducted a long-term observation of spermatogenic restoration. The results demonstrated that on day 63 after the administration of a busulfan dose of 40 mg kg⁻¹, rare spermatogenic cells could be observed in the testes by H and E staining, and the testis/body weight ratio was decreased, compared with that on day 0 (0.823 ± 0.132 vs 3.255 ± 0.305 , $P < 0.001$) (Figure 4a). Further analysis of the protein expression of an SSC marker (UCHL1), a Sertoli cell marker (SOX9), and a Leydig cell marker (CYP11A1) in the testes on different days after the administration of

40 mg kg⁻¹ busulfan was performed via Western blot (Figure 4b). We found that there was a very low expression of UCHL1 in the testes on day 63. Furthermore, we evaluated the changes on days 90 and 126 (Figure 4c). The results showed that, compared with that on day 63, the testicular-to-body weight ratio was gradually increased from those of day 90 (1.717 ± 0.120 vs 0.823 ± 0.132 , $P < 0.001$) and day 126 (1.957 ± 0.025 vs 0.823 ± 0.132 , $P < 0.001$). Finally, a 100% survival rate was observed in all mice after exposure to all doses of busulfan.

DISCUSSION

In this study, we first demonstrated that two intraperitoneal injections of busulfan (40 mg kg⁻¹ in total) at 3 h apart to C57BL/6 mice were a nonlethal and efficient method for recipient preparation for SSC transplantation and had a 100% rate of animal survival. Moreover, we found that the mice showed substantial depletion of all spermatogenic cells by day 36 and that a rare spermatogenic restoration occurred at day 63, indicating that the potential transplantation time window for SSCs is from day 36 to day 63 after busulfan administration. In addition, the kinetics of the germ cells showed that spermatogenesis was sharply decreased at day 18, suggesting that half of the seminiferous epithelium

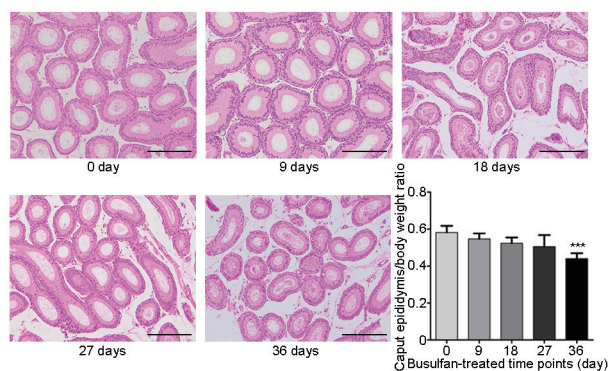


Figure 3: Dynamic changes of the caput epididymidis in a cycle of the seminiferous epithelium after the administration of 40 mg kg⁻¹ busulfan. On day 36, H and E staining showed a decreased quantity of ductus epididymidis and a decrease in the ratio of the caput epididymidal to body weight, compared with that of day 0 (***P* < 0.001). Scale bars = 200 μm. *n* = 6 per group. H and E: hematoxylin and eosin.

cycle consists of an intervention threshold for fertility preservation and minimization of chemotherapy-induced gonadotoxicity in young men.

Administration of busulfan has been widely used to establish animal models for the preparation of recipients for SSC transplantation or for studying chemotherapy-induced gonadotoxicity, but the safety and efficiency for the model animals have to be considered.^{11,20} A previous report showed that a single intraperitoneal injection of 30–40 mg kg⁻¹ busulfan leads to an 8%–87% death rate in BALB/c mice,¹⁶ and when the dose of busulfan is >30 mg kg⁻¹, approximately half of the immunodeficient mice die.²¹ More importantly, in C57BL/6 mice, which are the most commonly used mouse species in our institute, a single intraperitoneal injection of 40 mg kg⁻¹ busulfan is also associated with a 30% mortality rate.⁶ In our preliminary experiment, the mortality rate was 25% after a single intraperitoneal injection of 40 mg kg⁻¹ busulfan in eight adult C57BL/6 mice (data not shown). Recently, Qin *et al.*^{22,23} established a novel method to administer busulfan at 4 or 6 mg kg⁻¹ per side by intratesticular injection; no ICR mice died in this group, compared with a mortality rate of 32% in intraperitoneally injected group. The researchers proposed that one benefit of using an intratesticular injection was that the busulfan concentration in the circulation was extremely low and that busulfan damage to hematopoietic organs was light. However, gonadotoxicity resulting from chemotherapy in the clinic primarily occurs from chemical agents in the circulation. Therefore, on the basis of the reduced toxicity in that report, we modified our current single intraperitoneal injection protocol to contain a double intraperitoneal injection of busulfan (40 mg kg⁻¹ in total) at an interval of 3 h.

In our modified scheme, no mice died after the double intraperitoneal administration of busulfan, even at a total dose of 40 mg kg⁻¹. Because busulfan must be dissolved in DMSO, we stressed the safety of using 5% (*v/v*) DMSO as a solvent for *in vivo* administration.¹⁸ *In vitro*, DMSO concentrations >10% (*v/v*) have been reported to cause cellular toxicity through plasma membrane pore formation.²⁴ In a previous study, the final concentration of DMSO was 50% (*v/v*), which can cause toxicity *in vivo*.¹⁶ This finding may be an explanation for the high death rate in the current methods that use busulfan to create azoospermia in mice. Moreover, the half-life time of busulfan (intravenous injection) is 3.0 ± 0.7 h according to a previous report.²⁵ In the consideration of this half-life and the toxicity of DMSO, we changed the present dosing scheme of a single intraperitoneal injection to two intraperitoneal injections that were spaced 3 h apart.

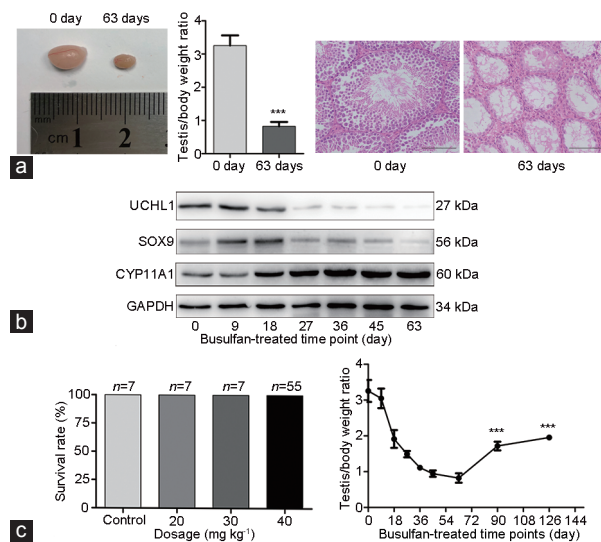


Figure 4: Long-term observations of spermatogenic restoration and the evaluation of survival rates. (a) Rare spermatogenic cells were observed in the testes by H and E staining, and the testicular-to-body weight ratio was decreased on day 63 compared with day 0 (***P* < 0.001). Scale bars = 100 μm. *n* = 6 per group. (b) The protein expression of an SSC marker (UCHL1), a Sertoli cell marker (SOX9), and a Leydig cell marker (CYP11A1) in the testes at different days after the administration of 40 mg kg⁻¹ busulfan. (c) A 100% survival rate was observed in all mice that were administered different doses of busulfan. Compared with day 63, the testicular-to-body weight ratio was gradually increased at day 90 (*n* = 3, ***P* < 0.001) and day 126 (*n* = 3, ***P* < 0.001). H and E: hematoxylin and eosin; SSC: spermatogonial stem cell; UCHL1: ubiquitin C-terminal hydrolase L1; SOX9: sex-determining region Y box 9; CYP11A1: cytochrome P450 family 11 subfamily A member 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

With a total dose of 40 mg kg⁻¹, the administration of busulfan could fully sterilize the mice with no unexpected death, indicating that this method can balance the side effects of busulfan and the depletion of germ cells to create an ideal gonadotoxic mouse model. These results are consistent with those of the previous report, in which mice receiving two injections of 22 mg kg⁻¹ busulfan exhibited similar engraftment kinetics compared with those treated with a single dose of 35 mg kg⁻¹, but those receiving two doses had greatly improved survival rates.²⁶ The nonlethality of this modified method ensures the 100% survival rate of the mice and effectively reduces the number of mice that must be used and remarkably improves animal welfare.

In mammals, the time required to generate spermatozoa from SSCs is called the seminiferous epithelium cycle, and it averages 74 days in humans, 52 days in rats, and 34.4 days in mice.^{27–29} Among these species, mice are the most commonly used in research, and their cycle is divided into four regular intervals (stages) with an average of 8.6 days each.¹⁷ Hence, we continually monitored the kinetics of the seminiferous epithelium in mice during an interval of 9 days (approximately 1 stage). Our study showed that on day 18 (approximately two stages, *i.e.*, half a seminiferous epithelium cycle) after busulfan administration, almost all of the seminiferous tubules had dramatic changes, including progressively atrophic lesions, frequent disarrangement of spermatogenic cells, and a thin seminiferous epithelium. For an easier translocation of donor cells from the seminiferous tubule lumen to the basal compartment, the endogenous germ cells of recipients should be destroyed. Furthermore, we found that almost all of the spermatogenic cells in the seminiferous tubules were eliminated by day 36 (approximately four stages) and did not recover by

day 63 (approximately seven stages). There was a partial recovery at 90 days. Donor SSCs usually require 4 weeks to attach to the basal lamina and then proliferate. Therefore, 8 weeks in total is needed to produce a complete seminiferous epithelium with spermatids and spermatozoa.¹⁷ Our results demonstrated a potentially broad period for transplantation that was between 36 days and 63 days after busulfan administration.

Spermatogenesis relies on the self-renewal and differentiation of SSCs, which are influenced by a niche growth factor milieu that is provided by several cell populations that provide testicular somatic support, including Sertoli cells and peritubular myoid cells.³⁰ Emerging evidence suggests that Sertoli cells and peritubular myoid cells are key support cell populations that influence the formation and function of niches by secreting soluble factors, including inhibin B, GDNF, FGF2, CXCL12, and CSF1.^{31–33} If the SSC niche is destroyed, the transplantation of SSCs will not be sufficient to restore spermatogenesis. In this study, the double immunostaining of Sertoli cell markers and peritubular myoid cell markers showed that there were almost no cells other than Sertoli cells within the lumen. In addition, the expression of Sertoli cell markers did not decrease, as shown by the histological and Western blot analyses. A previous study revealed that busulfan treatment did not notably alter the steroidogenic environment in mice.¹⁴ Our data confirm that the inhibin B levels in the serum also do not change markedly and that the expressions of *GDNF*, *FGF2*, *CXCL12*, and *CSF1* were gradually increased by day 36 after busulfan administration, which may be responsible for the rapid and temporary response of the testicular somatic environment to the loss of spermatogenic cells and suggests that our modified scheme would not affect SSC transplantation.

The kinetics of the seminiferous epithelium in mice may provide an animal model for exploring the mechanism of azoospermia in addition to clues for studying male infertility that is induced by chemotherapy. For example, spermatogenesis was sharply decreased at day 18, indicating that half of a seminiferous epithelium cycle may be an intervention threshold to minimize chemotherapy-induced gonadotoxicity.

CONCLUSIONS

In this study, two intraperitoneal injections of busulfan at a total dose of 40 mg kg⁻¹ could substantially deplete testicular germ cells in mice without decreasing the survival rate or damaging the SSC niche. This process could be a nonlethal and efficient method for preparing recipients for SSC transplantation. The kinetics of the seminiferous epithelium revealed that the potential transplantation time window in mice is 36–63 days after busulfan administration. Moreover, half of a seminiferous epithelium cycle may function as an intervention threshold for the preservation of male fertility and the minimization of chemotherapy-induced gonadotoxicity.

AUTHOR CONTRIBUTIONS

YX carried out the administration of busulfan, performed the data analysis, participated in the study design and coordination, and helped draft and revise the manuscript. CCD collected the samples and performed the H and E staining, immunofluorescence, and RT-qPCR and participated in the busulfan treatment. BO carried out the ELISA measurement of inhibin B and the Western blot, participated in the study design, and helped draft and revise the manuscript. LYL performed the histopathological analysis and participated in the data analysis. JHY helped with the image processing. CZ helped search and collect related papers. HCC participated in the data analysis. XYL provided the

antibodies and analytic methods. XZS participated in the design of the experiments. CHD and DHL conceived and designed the experiments, analyzed data, and revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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