Reproductive Toxicology 59 (2016) 31–39

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Gonadotoxic effects of busulfan in two strains of mice

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ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form 31 August 2015
Accepted 15 September 2015
Available online 30 October 2015

Keywords:
Busulfan
Spermatogonial stem cell
Sperm morphology
Fertility
Mice

ABSTRACT

Busulfan is a chemotherapy drug that has side effects on spermatogonial stem cells (SSC). The effects of bulsufan treatment on male germ cells and fertility vary significantly between individuals. In this study, we have used molecular, cellular and histopathology approaches to investigate the effects of a single intraperitoneal dose of busulfan (40 mg kg^{-1}) in two mice strains, Balb/C and Swiss, at two different periods after treatment, 30 and 90 days. Testicular degeneration was observed in both Balb/C and Swiss mice after busulfan injection. Interestingly, testicular functions and fertility recovered spontaneously post busulfan treatment in Swiss mice, but not in Balb/C mice. Abnormal fertility induced by busulfan in Balb/C mice was associated with altered seminiferous tubules, sperm morphology and transcript levels of *Nanos2, Nanos3*, *Gdnf* and *Plzf* genes. These findings revealed that SSC of Balb/C mice are more sensitive to the toxic effects of busulfan then those of Swiss mice.

1. Introduction

Chemotherapy drugs are known to have side effects on different cell types including the reproductive system [1–4]. The side effects of chemotherapies are known to be influenced by a number of conditions including the chemotherapeutic agent, dosage and patients age [5,6]. The antineoplasic and DNA alkylating agent busulfan (1,4-butanediol dimethanesulphonate) has long been used in the treatment of chronic myeloid leukemia [7], and in preparative regimens prior to hematopoietic stem cell transplantation [8]. Busulfan has also been used to deplete germ cells in mice and to study SSC kinetics and fertility recovery [9–11]. Indeed, busulfan, alone or in association with other alkylating agents, was shown to cause spermatogonial cell death [9], prolonged azoospermia [2,9,12], and, consequently, male infertility [4,9,11]. While alkylating agents may also affect Sertoli cells [12], it has been reported that busulfan treatment did not cause degeneration of Sertoli cells [9]. Although the effects of busulfan on spermatogenesis can be irreversible [2,9], the cell and molecular consequences at different stages following treatment have not been thoroughly investigated.

Spermatogenesis is a complex process where many spermatozoa are produced from a small number of SSC, which occurs during the entire reproductive life of males [13]. Similar to other tissue-specific stem cells, SSC are rare, representing approximately 0.03% of the total germ cells in mice [14]. In adult mice, spermatogonial stem cells are restricted to the undifferentiated type A spermatogonia [15]. This subset includes A_{single} (A_s) spermatogonia and their progeny A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia [15,16].

Although the molecular regulation of SSC development and differentiation has yet to be completely elucidated, genes involved in the maintenance and self-renewal of spermatogonial cells have been identified [17–21]. One of the genes implicated in germ cell development is Nanos, which encodes an evolutionarily conserved RNA-binding protein [22]. In mice, *Nanos1* was shown to be dispensable for germ cell development [23], while *Nanos2* and *Nanos3* are expressed in primordial germ cells and are necessary for the maintenance, development and survival of these cells [21,24,25]. In spermatogonial stem cells, *Nanos2* expression is greater in A_s and A_{pr} cells, while *Nanos3* is predominantly expressed in A_{al} cells [21,26].

Another gene required for SSC self-renewal and maintenance is the transcription factor of promyelocytic leukemia zinc-finger (*Plzf*), which acts by repressing the transcription of genes involved in the differentiation of these cells [17,27]. Similarly, the glial cell

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http://dx.doi.org/10.1016/j.reprotox.2015.09.002
0890-6238/

Table 1
List of sequences and accession number of the analyzed mRNA gene expression.

Gene	Primer forward	Primer reverse	Accession number
Nanos2	AGGTAGCTGAGGAGCCCAACTC	TGCTTGCAGAAGTTGCATATGG	NM_194064.2
Nanos3	CTACTGCTACACCACCCGGAAT	AGACACCTGCTGCTGCTTCTC	NM_194059.2
Gdnf	GATTCGGGCCACTTGGAGTT	GACAGCCACGACATCCCATAA	NM_010275.2
Plfz	CGAGCTTCCGGACAACGA	AAATGCATTCTCAGTCGCAAAC	NM_001033324.2
Gapdh	CAGCCTCGTCCCGTAGACAA	GTAGACCATGTAGTTGAGGTCAATGAA	NM_008084.2

line derived neurotrophic factor (*Gdnf*) gene, which is expressed in Sertoli cells, was also identified as essential in the regulation of SSC development and maintenance [18].

To gain additional insights into the gonadotoxic effects of busulfan, Balb/C and Swiss mice were used in the present student to (i) assess testicular cell parameters including testes histopathology, and sperm morphology and motility, (ii) evaluate the recovery of fertility and production of live offspring, and (iii) determine the expression of genes involved in SSC development and maintenance in samples collected at 30 and 90 days after busulfan treatment.

2. Materials and methods

2.1. Animals

Adult (eight weeks old) Balb/C (crossing inbred) and Swiss (heterogamic crosses) male mice were used in this study. Mice were kept in polypropylene cages with water and food *ad libitum,* and under controlled temperature ($23 \pm 2\,^{\circ}C$) and light–dark cycle of 15 h and 9 h, respectively. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (number 095/2011).

2.2. Experimental protocol

Mice from each strain were randomly divided into control (C) and busulfan treated (BUS) groups. Animals were intraperitoneally injected with vehicle or 40 mg kg^{-1} body weight busulfan (Sigma, St. Louis, MO, USA). The busulfan dose was determined based on previous studies using Balb/C and C3H/Kam mice, which reported depletion of testicular germ cells and infertility caused by this dose [9,28]. Busulfan was dissolved in 50% of the injection volume in dimethyl sulfoxide (DMSO-Sigma), and then in equal volume of ultrapure water. The solution was prepared immediately before use. Control mice received the same volume of vehicle (DMSO + water).

Thirty days following busulfan (BUS30) or vehicle (C30) injection, animals were weighted, anesthetized with 20 mg kg^{-1} xylazine and 50 mg kg^{-1} ketamine, and had one of the testes and epididymides surgically removed and allowed to recover. The testes were weighted and then used in histological and gene expression analyses. Spermatozoa were recovered from the epididymides to evaluate their motility, vigor and morphology. At day 90 after treatment (BUS90 or C90), animals were weighed again, anesthetized, and then euthanized to remove the remaining testicle and epididymis. The analyses performed in samples collected at day 90 were the same as in those collected at day 30. The two time points (30 and 90 days) were elected to cover more than one spermatogenic cycle, which is to know to take approximately 35 days in mice [29]. The same analyses were performed in the control group and busulfan-treated group.

2.3. Sperm recovery and analyses

The epididymides that were recovered both at day 30 or 90 after treatment were excised and freed from the adherent and fat tissues. Cauda epididymis was separated from the rest of the epididymis and transferred to a pre-warmed (37 °C) petri dish close to a 200 μl drop of Fert medium [30]. Longitudinal incisions were performed using a fine needle and a scalpel blade to release the spermatozoa. Sperm motility was immediately evaluated by placing 3 μl of Fert medium and sperm on a slide at 37 °C followed by visual observation using an optic microscope (Olympus CX40) at 100× magnification. Sperm motility (% motile sperm) was estimated by assessing three different fields in each slide and the mean value was recorded. Sperm vigor (% progressive motile sperm) was subjectively graded on a scale of zero (without motility) to five (maximum progressive motile sperm).

Sperm morphology was assessed using a Leica microscopy (DMI 4000B) equipped with differential interference contrast (DIC). Samples were stored in formaldehyde-citrate solution and then two hundred spermatozoa from each sample were evaluated. Numbers of normal and abnormal spermatozoa were counted in each sample and sperm defects were classified as head, middle piece, tail or proximal cytoplasmic droplets. In the few samples where it was not possible to count two hundred spermatozoa, the values were normalized to percentage in relation to the counted spermatozoa. Because sperm were recovered from the cauda epididymis, the presence of distal cytoplasmic droplets was not considered a defect.

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from the testes using Trizol (Invitrogen, Brazil) according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm and RNA integrity was verified electrophoretically by ethidium bromide staining. Total RNA was treated with 0.1 U DNase (Invitrogen) at 37 °C for 5 min to digest contaminating DNA. Reverse transcriptase reactions were performed using 1 μg total RNA, 1 μM oligo-dT primer, 4U omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 mM dNTP's mix and 10U RNase inhibitor (Invitrogen), in a final volume of 20 μl.

The relative mRNA abundance was determined by qRT-PCR using the StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems). Variability in the amount of mRNA was corrected by amplification of *Gapdh* as the internal control gene. The relative expression of each gene was determined as recommended by Pfaffl et al. [31]. Specific primers for *Nanos2, Nanos3, Gdnf, Plzf* and *Gapdh* (Table 1) were designed using the Primer Express software v 3.3 (Applied Biosystems) based on sequences available in GenBank or Ensembl. Oligonucleotides were synthesized by Invitrogen.

2.5. Western Blot

Total proteins were isolated from mice testes using RIPA buffer (Sigma–Aldrich). Protein concentration was determined using the Bradford assay. Equal amounts of protein (30 μg) were loaded in each well and resolved in 10% SDS-PAGE polyacrylamide gels. Samples were run at 130 V for 1 h and then electroblotted onto nitrocellulose membranes. Membranes were incubated in block-