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PERFIL DAS CÉLULAS GERMINATIVAS APÓS INDUÇÃO DE DEGENERAÇÃO TESTICULAR PELO BUSULFAN

DISSERTAÇÃO DE MESTRADO

Karina Gutierrez

Santa Maria, RS, Brasil

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PERFIL DAS CÉLULAS GERMINATIVAS APÓS INDUÇÃO DE DEGENERAÇÃO TESTICULAR PELO BUSULFAN

por

Karina Gutierrez

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Orientador: Prof. João Francisco Coelho de Oliveira

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elaborada por
Karina Gutierrez

como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

PERFIL DAS CÉLULAS GERMINATIVAS APÓS DEGENERAÇÃO TESTICULAR INDUZIDA PELO BUSULFAN

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ORIENTADOR: JOÃO FRANCISCO COELHO DE OLIVEIRA
Data e Local da Defesa: Santa Maria, 21 de fevereiro de 2013.

Apesar de amplamente utilizado em estudos de cinética das células-tronco espermatogoniais ou em estudos de infertilidade masculina, não se sabe ao certo quais efeitos o quimioterápico busulfan exerce, de fato, sobre as células germinativas masculinas e em diferentes linhagens e espécies animais. Sendo assim, o objetivo deste estudo foi analisar os danos causados pelo busulfan na espermatogênese de camundongos machos de duas linhagens distintas, através do perfil de expressão de RNAm dos marcadores moleculares espermatogoniais, análises de motilidade, vigor e morfologia espermática, histopatologia do epitélio germinativo e teste de recuperação de fertilidade. Os animais receberam uma única dose de 40 mg/kg de busulfan ou do veículo. Após 30 dias da aplicação do fármaco, os camundongos foram pesados, anestesiados e tiveram um dos testículos e epidídimo cirurgicamente removidos. Este procedimento não acarretou dano algum ao outro testículo, uma vez que camundongos que tiveram um testículo e epidídimo removidos foram capazes de gerar prole após o período de recuperação cirúrgica. A partir destes testículos, foram avaliadas tanto características histológicas quanto a expressão de RNA mensageiro de marcadores de células-tronco espermatogoniais (SSC). Outros parâmetros avaliados foram a motilidade, vigor e morfologia espermática, a partir da recuperação dos espermatozoides da cauda do epidídimo. Passados 90 dias da aplicação inicial do fármaco, os camundongos foram novamente pesados e anestesiados, e tiveram o outro testículo removido para as mesmas análises. Sendo assim, as análises referentes aos 30 e aos 90 dias pertencem aos mesmos animais, minimizando a variação da resposta individual entre os períodos analisados. Foi observado que para a linhagem Balb/C, independente do período analisado, os animais tratados com busulfan apresentaram uma severa degeneração testicular, com perda de células-tronco espermatogoniais, diminuição de motilidade e vigor espermáticos e aumento no número de espermatozoides defeituosos. Com relação à expressão gênica, foi observada uma marcada redução na expressão do gene *Nanos2*, e aumento da expressão dos genes *Gdnf* e *Plzf*. Para os animais da linhagem Swiss, foi observado que aos 30 dias após a aplicação do fármaco, os resultados foram semelhantes aos obtidos para a linhagem Balb/C, com exceção da expressão gênica, a qual não apresentou variação. Porém, noventa dias após a administração do fármaco, esses animais apresentaram epitélio germinativo quase normal, com linhagem espermatogênica, aumento na massa testicular e epididimal, aumento na motilidade e vigor espermáticos e aumento na quantidade de espermatozoides normais, não diferindo dos animais do grupo controle. Além disso, essa melhora foi comprovada através da obtenção de prole após o acasalamento com fêmeas virgens, sendo que os machos utilizados neste teste não foram submetidos ao processo de remoção dos testículos. Esses resultados demonstram a variação do efeito do busulfan em duas linhagens distintas de camundongos,

sendo o fármaco eficiente para causar degeneração testicular severa por mais de 90 dias linhagem Balb/C, porém não na linhagem Swiss, provavelmente devido as variações genéticas entre as duas linhagens.

Palavras chave: Células-tronco espermatogoniais. Expressão gênica. Camundongos. Balb/C.

Swiss.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

GERM CELLS PROFILE AFTER TESTICULAR DEGENERATION INDUCED BY BUSULFAN

AUTHOR: KARINA GUTIERREZ
ADVISOR: JOÃO FRANCISCO COELHO DE OLIVEIRA
Date and Place of Defense: Santa Maria, February 21th, 2013.

The chemotherapeutic busulfan is widely used in studies of spermatogonial stem cells kinetics and studies of male infertility. However it effects on male germ cells in different animal lineages or species are unclear. The objective of this study was to assess the damage caused by busulfan in male mice spermatogenesis of two distinct lineages. The analyzed parameters were the mRNA pattern expression profile of spermatogonial stem cells (SSC) markers, analysis of spermatozoa motility, vigor and morphology, germinal epithelium histology and fertility recovery analysis. The animals received a single dose of 40 mg kg^{-1} of busulfan or vehicle. Past 30 days of the drug injection, the mice were weighted and anesthetized and had one of the testes and epididymis surgically removed. This procedure did not cause any damage to the other testis, once mice that had one of the testes removed were capable to generate offspring. From these testes, were evaluated the tissue histology and mRNA gene expression of spermatogonial stem cell (SSC) markers. The motility, vigor and spermatozoa morphology were analyzed from cauda epididymal spermatozoa. After 90 days of the drug injection, the mice were weighted, anesthetized and had the other testis removed for the same analyzes procedure. The analysis at 30 and 90 days after drug injection were from the same animals, minimizing the variation in individual response between periods. It was observed that, for Balb/C lineage, independent of the analyzed period, the busulfan treated animals showed a severe testicular degeneration, with loss of SSC, decreased motility and spermatozoa vigor and increased number of defective spermatozoa. In mRNA expression it was observed a marked reduction in *Nanos2* expression, and an increase in *Gdnf* and *Plzf* gene expression. For animals of Swiss lineage, it was observed that at 30 days after the busulfan injection, the results obtained were similar to that obtained for Balb/C lineage, except in gene expression, which remained unchanged. However, 90 days after the drug injection, these animals show germinal epithelium almost normal, with spermatogenic lineage. They also show an increase in testicular and epididymal mass, spermatozoa motility and vigor and an increase in number of normal spermatozoa, did not differing from the control group. Moreover, this recovery was confirmed by the offspring obtained after matting with virgin females. The male mice utilized in these tests were not submitted to testes removal. These results shows the variation of busulfan effects in two distinct mice lineages, being the drug effective to cause a severe testicular degeneration for more than 90 days in Balb/C lineage, but not in Swiss lineage, due to genetic variations.

Key words: Spermatogonial stem cells. Gene expression. Mice. Balb/C. Swiss.

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1. INTRODUÇÃO

A espermatogênese é um processo complexo, no qual um pequeno número de células-tronco espermatogoniais (SSC) é capaz de gerar um grande número de espermatozoides (Brinster e Zimmermann, 1994). Esse processo ocorre durante toda a vida reprodutiva de machos e é fundamental que esse processo ocorra de maneira organizada e constante.

Uma das principais causas da infertilidade masculina é atribuída a falhas na proliferação e diferenciação de células germinativas. A espermatogênese é dependente de uma população de células-tronco espermatogoniais (SSC) (De Rooij e Grootegoed, 1998), e o número destas células é muito pequeno nos testículos, aproximadamente 2-3 × 10^4 em camundongos (Kanatsu-Shinohara *et al.*, 2003). As células-tronco espermatogoniais compreendem as espermatogônias indiferenciadas, mais precisamente as espermatogônias isoladas - A_s (Brinster, 2002).

Diversos estudos são feitos atualmente para entender a cinética das SSC ou para estudar terapias/tratamentos para a infertilidade masculina. Para tanto, é necessário preparar o testículo dos animais receptores através da depleção da espermatogênese endógena. O método mais utilizado para esse procedimento é a partir da utilização do quimioterápico busulfan (Bucci e Meistrich, 1987; Lue *et al.*, 2007; Perez-Crespo *et al.*, 2011; Zohni *et al.*, 2012). Apesar de ser amplamente utilizado, ainda não se sabe ao certo o local de atuação do fármaco, duração de seu efeito, e variações de resposta entre espécies e linhagens animais. Até o momento nenhum estudo caracterizou a atuação do busulfan a partir do uso de marcadores moleculares de SSC.

Sendo assim, o objetivo deste trabalho foi verificar se existe variação da resposta ao fármaco entre duas linhagens de camundongos estudadas, utilizando parâmetros como análises de expressão gênica, morfologia do epitélio germinativo e características espermáticas. As duas linhagens escolhidas foram Balb/C, linhagem oriunda de sucessivos acasalamentos endogâmicos, e Swiss, obtidos através de cruzamentos heterogâmicos.

2. REVISÃO BIBLIOGRÁFICA

2.1. Espermatogênese em mamíferos

A espermatogênese é um processo complexo, que envolve fases de proliferação e alguns passos de diferenciação celular e que ocorre constantemente em machos, durante sua vida reprodutiva. O processo inicia a partir da diferenciação de um tipo específico de célula-tronco e culmina com a liberação de espermatozoides no lúmen do túbulo seminífero, passando por três etapas distintas: espermatogoniogênese, espermatocitogênese e espermiogênese (Ken *et al*., 2006). A espermatogoniogênese envolve o processo de surgimento das espermatogônias a partir das células germinativas primordiais e sucessivas divisões mitóticas das espermatôgonias. A espermatocitogênese é a fase onde ocorre a divisão meiótica, onde o DNA dos espermatócitos é recombinado e segregado, formando células haploides denominadas espermátides. E por fim, na espermiogênese, ocorre a diferenciação das espermátides até espermatozoides, os quais terminam o processo de maturação no epidídimo, se tornando aptos para a fecundação. O tempo necessário desde o início da divisão das células-tronco até a formação de espermatozóides é de aproximadamente 35 dias, em camundongos (Kanatsu-Shinohara *et al.*, 2003).

As células-tronco espermatogoniais (SSC) pertencem a uma população de células-tronco adultas, que possuem a função de auto-renovação, manutenção e produção contínua de espermatozoides ao longo da vida do macho (Brinster e Zimmermann, 1994). Nos testículos de camundongos adultos, a função de célula-tronco espermatogonial fica restrita à população de espermatogônias indiferenciadas do tipo A, a qual inclui as espermatogônias isoladas (A_s), espermatogônias pareadas (A_{pr}, em cadeias de duas células) e espermatogônias alinhadas (A_{al} – cadeias de quatro a 16 e ocasionalmente 32 células) (De Rooij e Russell, 2000; De Rooij, 2001). Após essa fase, as espermatogônias passam a ser consideradas diferenciadas (A_1-A_4; intermediária (In) e B), e seguem a rota de diferenciação até a formação dos espermatozoides. Uma característica da espermatogênese é a divisão celular incompleta, formando células que possuem ligações citoplasmáticas (pontes citoplasmáticas) entre si (Huckins, 1971; De Rooij e Russell, 2000; Ken *et al*., 2006), fato observado nas células A_{pr} e A_{al}. Dentre diversos modelos criados para se estudar a espermatogênese, um dos mais clássicos é o "modelo A_s". Segundo esse modelo, as espermatogônias isoladas são as células que possuem a verdadeira função de SSC (células-tronco "atuais"), ou seja, são capazes de se renovar constantemente e

manter o desenvolvimento e produção de células germinativas dentro do epitélio seminífero (Brinster, 2002), porém, elas representam apenas 0,03% de todas as células germinativas de roedores (Tegelenbosch e De Rooij, 1993). Segundo propõe esse modelo, ocorre uma sequência de desenvolvimento linear desde a espermatogônia A_s até a formação dos espermatozoides (A_s - A_{pr} - A_{al} (cadeia de quatro a 32 células) – A_1 – A_2 – A_3 – A_4 – In – B – espermatócitos primários – espermatócitos secundários – espermátides – espermatozoides). Esse modelo propõe que as células A_{pr}, A_{al} e A_1 são terminalmente diferenciadas e já estão comprometidas com a formação de espermatozoides (Huckins, 1971). A princípio acreditava-se que a diferenciação proposta pelo "modelo A_s" era unidirecional e irreversível, porém, atualmente se sabe que esse processo não é unidirecional e que é reversível, dependendo da necessidade que o epitélio germinativo apresenta (Nakagawa *et al.*, 2010; Yoshida, 2010). Em condições de falhas no processo fisiológico normal da espermatogênese, seja pela utilização de fármacos, por alterações na temperatura, danos causados por radiações ou uma desorganização do nicho celular, acredita-se que as espermatogônias pareadas ou alinhadas são capazes de formar novas células A_s através da quebra da ponte citoplasmática que une as células, assim sendo capazes de repovoar e reconstituir o epitélio germinativo danificado, sendo por isso denominadas "células-tronco potenciais" (Nakagawa *et al.*, 2007; Phillips *et al.*, 2010). Qualquer falha em alguma das fases, ou em alguma divisão/diferenciação citada, implica em perda da fertilidade por algum período de tempo, variando conforme o grau e profundidade das células afetadas. Até o momento não se sabe ao certo se a divisão celular que ocorre nas células-tronco espermatogoniais é simétrica ou assimétrica (De Rooij, 2001).

A espermatogênese envolve um balanço adequado entre auto-renovação e diferenciação das células-tronco espermatogoniais, sendo esse processo responsável pela homeostase no tecido germinativo, e constante produção de espermatozóides. Esse processo é em grande parte controlado pelo nicho celular, que nada mais é do que o microambiente celular e molecular que permite a manutenção da homeostase dos tecidos através da sinalização endócrina e/ou parácrina (De Rooij, 2001; Moore e Lemischka, 2006; De Rooij, 2009). É o nicho celular que controla os processos fundamentais de manutenção da auto-renovação, diminuindo e controlando os riscos da formação de um câncer ; diferenciação celular ; entrada no estágio de quiescência ; apoptose ou pluripotência (Oatley e Brinster, 2012). Em roedores, as espermatogônias localizam-se próximas à membrana basal dos túbulos seminíferos, principalmente próximas de vasos sanguíneos e de outros túbulos seminíferos (Ken *et al.*, 2006; De Rooij, 2009). Sendo assim, sugere-se que o nicho das células-tronco espermatogoniais seja formado pelas células de Sertoli, membrana basal na qual as SSC estão

apoiadas e elementos intersticiais como células de Leydig e vasos sanguíneos (De Rooij, 2009; Phillips *et al.*, 2010; Yoshida, 2010). A sinalização endócrina e/ou parácrina dessas células favorece a permanência e auto-renovação das células-tronco espermatogoniais neste local (preferencialmente dentro do nicho), enquanto sinalizações diferentes estimulam a diferenciação (fora do nicho), quiescência, apoptose, ou até mesmo pluripotência das espermatogônias tronco (De Rooij, 2009). Embora as células intersticiais desempenhem um papel importante no nicho celular das células-tronco espermatogoniais, as células de Sertoli parecem ser as células mais importantes para a manutenção desse nicho celular, uma vez que a maior disponibilidade de nichos de SSC não é acompanhada por aumento de vasos sanguíneos ou células intersticiais, mas somente pelo aumento de células de Sertoli (Oatley *et al.*, 2011).

2.2. Padrão de expressão gênica das células-tronco espermatogoniais

Apesar de não estar completamente estabelecido o padrão de expressão gênica das SSC, muitos autores já descreveram alguns genes fundamentais para a manutenção e auto-renovação destas células e alguns marcadores específicos. O gene *Nanos* codifica para uma proteína de ligação ao RNA conservada evolutivamente, descrita primeiramente em *Drosophila* (Lehmann e Nusslein-Volhard, 1991). O genoma de camundongos possui três genes homólogos (*Nanos1-3*), sendo que o *Nanos1* é dispensável para o desenvolvimento das células germinativas, uma vez que camundongos *knockout* pra esse gene apresentam células germinativas masculinas normais (Haraguchi *et al.*, 2003). Já os genes *Nanos2* e *Nanos3* são expressos nas células germinativas primordiais (PGC) e desempenham um papel fundamental na manutenção, desenvolvimento e sobrevivência dessas células (Tsuda *et al.*, 2003; Suzuki *et al.*, 2007; Suzuki *et al.*, 2008). Ambos os genes são expressos nas espermatogônias indiferenciadas (Tsuda *et al.*, 2003; Lolicato *et al.*, 2008). O gene *Nanos2* é expresso pelas PGC masculinas, sendo que camundongos machos *Nanos2* $^{-/-}$ apresentam testículos com tamanho reduzido e são inférteis, ao contrário das fêmeas, que apresentam desenvolvimento normal e se mantém férteis (Tsuda *et al.*, 2003). Segundo Sada *et al.*, (2009), o gene *Nanos2*, em mamíferos, atua como importante fator intrínseco da auto renovação das SSC. Esses mesmos autores demonstraram que esse gene é expresso nas células A_s e A_{pr}, e que a deleção deste gene acarreta acúmulo das espermatogônias no testículo e bloqueio da espermatogênese, ou seja, é fundamental para a manutenção das SSC e previne sua diferenciação. O gene

Nanos3 é expresso nas PGC logo após sua migração para as gônadas, tanto em fêmeas quanto em machos (Tsuda *et al.*, 2003) e volta e ser expresso novamente nos testículos de machos logo após o nascimento, preferencialmente nas células A_{al} (Sada *et al.*, 2009). Camundongos *knockout* para o gene *Nanos3* são estéreis e apresentam testículos e ovários atrofiados devido à perda das células germinativas primordiais migratórias durante a embriogênese. A perda das PGC na ausência da expressão de *Nanos3* ocorre devido a apoptose (Suzuki *et al.*, 2007; Suzuki *et al.*, 2008).

Os fatores extracelulares liberados pelas células do nicho são fundamentais na regulação das atividades das espermatogônias tronco. Um fator extremamente importante para o desenvolvimento e manutenção das SSC é o fator neurotrófico derivado das células gliais (*Gdnf*), que é liberado pelas células de Sertoli e é fundamental para a manutenção do nicho celular (Meng *et al.*, 2000). Esse fator atua por meio do complexo de receptores GFRα1/c-RET localizados na superfície das células-tronco espermatogoniais (Hess *et al.*, 2006). Camundongos com um alelo *knockout* para o gene *Gdnf* (Meng *et al.*, 2000), e camundongos *knockout* para *Gdnf/GFRα1/c-RET* (Naughton *et al.*, 2006) apresentam depleção das reservas de espermatogônias tronco devido a perda da função de auto-renovação e de manutenção de seu estado indiferenciado. Ao passo que, camundongos com elevada expressão desse gene apresentam acúmulo de espermatogônias indiferenciadas (Meng *et al.*, 2000). Isso demonstra o papel desse fator na manutenção das SSC e de seu estado indiferenciado.

Outro fator importante na espermatogênese é o *Plzf* (fator de transcrição do tipo *zinc-finger* da leucemia promielocítica), também conhecido como *Zfp 145*. Ele é um fator de repressão da transcrição e atua reprimindo a transcrição de genes relacionados à diferenciação dessas células, sendo necessário para a auto-renovação das SSC (Costoya *et al.*, 2004). Ele é expresso pelas espermatogônias indiferenciadas (Costoya *et al.*, 2004). Camundongos *knockout* para esse gene apresentam testículos visivelmente reduzidos, túbulos seminíferos degenerados e ausência de gametas (Costoya *et al.*, 2004). Camundongos com uma clássica e espontânea mutação do luxoid, inicialmente caracterizada por anormalidades nos membros de roedores, apresentam progressiva perda das SSC, sendo o gene *Plzf* associado a essa mutação (Buaas *et al.*, 2004; De Rooij, 2009).

2.3. Modelos de degeneração das células-tronco espermatogoniais

Considerando que a infertilidade masculina é um dos fatores limitantes para o sucesso reprodutivo, vários pesquisadores têm focado seus estudos no restabelecimento da fertilidade. Para isso, diversos modelos para depleção das células germinativas foram criados, entre eles o estresse térmico, exposição a agentes ionizantes ou substâncias tóxicas como os quimioterápicos, depleção hormonal ou perda da sinalização dos fatores do nicho celular (Langendorff e Stevenson, 1981; Bucci e Meistrich, 1987; Ehmcke *et al.*, 2007). Dentre os inúmeros modelos, um dos mais utilizados é a partir da administração do quimioterápico busulfan (1,4-dimetanosulfonoxibutano) (Kanatsu-Shinohara *et al.*, 2003; Nayernia *et al.*, 2006; Lue *et al.*, 2007; Perez-Crespo *et al.*, 2011; Zohni *et al.*, 2012). O busulfan é um agente antineoplásico, alquilante de DNA e ciclo celular não específico, utilizado no tratamento de leucemia granulocítica (Von Bubnoff e Duyster, 2010). É utilizado amplamente para verificar a função de SSC e para preparar testículos de camundongos para receber transplante de células germinativas para o tratamento da infertilidade masculina, ou seja, para causar depleção da espermatogênese endógena. Acredita-se que essa droga destrói as SSC de uma maneira dose-dependente, e que a degeneração parcial destas células, culmina na regeneração espontânea das espermatogônias, e consequentemente da espermatogênese (Kanatsu-Shinohara *et al.*, 2003), sendo que o reestabelecimento da fertilidade após um período de esterilidade não ocorre a menos que a contagem de espermatozoides testiculares atinja ao menos 15% dos espermatozoides presentes no grupo controle (Bucci e Meistrich, 1987). Segundo Bucci e Meistrich, (1987) roedores que receberam uma única aplicação deste fármaco na dose de 30 mg/Kg ficaram azoospérmicos 56 dias após o tratamento, sendo esse efeito remetido à morte das SSC pelo fármaco. Mesmo sendo amplamente utilizado para causar depleção da espermatogênese endógena, sabe-se que essa depleção nem sempre é irreversível, podendo haver regeneração espontânea deste epitélio após um período de tempo. Essa variação de resposta em consequência do uso do fármaco atrapalha no momento da escolha do melhor modelo a ser utilizado para causar a depleção da espermatogênese para a realização de transplantes/terapia celular para o tratamento da infertilidade masculina.

3. ARTIGO CIENTÍFICO

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

MICE BUSULFAN TESTICULAR DEGENERATION AND SPERMATOGENESIS PROFILE

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Mice busulfan testicular degeneration and spermatogenesis profile

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ABSTRACT

Spermatogonial stem cells (SSC) are responsible by the maintenance and constant sperm production throughout the male life. The chemotherapy busulfan is wildly used for SSC depletion model in studies about male infertility and SSC kinetics, but its effects in different mice lineages are not established. The aim of this study was to compare the action of busulfan in two different mice lineages, Balb/C and Swiss, evaluating SSC molecular markers mRNA expression, spermatozoa analysis and histology's injuries. For this purpose, the animals received an injection of 40 mg kg^{-1} of busulfan and had one of the testes removed at day 30 and the other at day 90 after the drug injection. Following the analysis, it was possible to observe testicular degeneration in the animals Balb/C that remained for at least 90 days, while Swiss mice presented a spontaneously regeneration 90 days after treatment. In conclusion, Balb/C mice are more sensitive to testicular degeneration caused by busulfan.

Keywords: mRNA expression; spermatozoa morphology; Balb/C; Swiss; spermatogonial stem cell.

Highlights

The effects of busulfan vary according the lineage.

Busulfan affects the spermatozoa motility and morphology.

The busulfan alters the expression of specific spermatogonial molecular markers.

1 Introduction

Spermatogenesis is a complex process where many spermatozoa are produced by a small number of spermatogonial stem cells (SSC). This process occurs during all male reproductive life [1]. Like in other tissue-specific stem cells, the SSC are rare, representing only 0,03 per cent of the germ cells in rodent testes [2]. In adult male mice testes, spermatogonial stem cell function is restrict to the undifferentiated type A spermatogonia [3]. This subset includes A_{single} (A_s) spermatogonia and their progeny A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia [3, 4].

Although, the gene expression pattern profile of these cells is not completely elucidated, some fundamental genes related to the maintenance and self-renew of these cells and some specific molecular markers are well established [5-9]. Nanos is an evolutionarily conserved RNA-binding protein, the function of which is implicated in germ cell development [10]. In mice, the gene *Nanos1* is dispensable for the germ cells development [11]. However, the genes *Nanos2* and *Nanos3* are expressed in primordial germ cells and play a crucial role in the maintenance, development and survival of these cells [5, 12, 13]. These genes are expressed in undifferentiated spermatogonia [5, 14], being the *Nanos2* more expressed in A_s and A_{pr}, and the *Nanos3* mainly expressed in A_{al}. The transcription factor of

promyelocytic leukemia zinc-finger (*Plzf*) is necessary for SSC self-renew and spermatogonia maintenance, and acts by repressing the transcription of genes related to differentiation of these cells [7]. This gene is expressed by undifferentiated spermatogonia. The niche is fundamental to SSC maintenance and decision cell fate. The Sertoli cells are one of the most important structures of testicular niche. The glial cell line derived neurotrophic factor (*Gdnf*), produced by Sertoli cells, regulates the cell fate of undifferentiated spermatogonial cells to initiate the spermatogenesis or for the renovation of stem cells reserves therefore, it is fundamental to SSC development and maintenance [8].

Considering that male infertility is one of the limiting factors for the reproductive successes, many authors have focused their studies in fertility recovery; either through the germ cell transplantation or cell therapy. For this, many models for germ cells depletion were created [15-17]. Among the various models, one of the most utilized is the administration of chemotherapic busulfan [16, 18-20]. Although this model has been sorely utilized, the validation is based only in morphological analysis and there is no study showing some spermatozoa parameters as motility and vigor and mRNA expression of SSC markers in busulfan treated animals. Thus, this study investigated the effect of busulfan on germ cells depletion in two different mice lineages (Balb/C and Swiss); analyzing the mRNA expression of specific SSC markers besides spermatozoa parameters, histology's injuries and fertility recovery.

2 Materials and methods

2.1 Animals

Adult male mice, eight weeks age, of two lineages (Balb/C – crossing inbred, and Swiss – heterogamic crosses) were utilized in these experiments. The animals were kept in

polypropylene cages under controlled temperature (23º ± 2ºC) and light-dark cycle of 15 h and 9 h respectively, with water and food *ad libitum*. All animal procedures were approved by the Animal Ethics Committe from the Federal University of Santa Maria (process 095/2011).

2.2 Experimental protocol

Balb/C male mice and Swiss male mice were randomly divided into two groups with half of animals for each lineage: control group (C) and busulfan treated group with 40 mg kg^{-1} (BUS).

Testicular degeneration was induced by a unique intraperitoneal injection (i.p.) of busulfan (Sigma, St. Louis, MO, USA) at the dose of 40 mg kg^{-1} body weight. Busulfan was first dissolved in 50% of the final volume of dimethyl sulfoxide (DMSO - Sigma St. Louis, MO, USA), and equal volume of ultrapure sterile water to reach the final concentration. The solution was prepared immediately before use. In the control groups mice received only the vehicle (DMSO + water).

After 30 days of the busulfan/vehicle (BUS30/C30) injection, all animals were weighted and anesthetized with 20 mg kg^{-1} of xylazine and 50 mg kg^{-1} of ketamine and had one of the testes and epididymis surgically removed. All the analysis of this study were performed with at least three animals for group and for lineages.The testes were submitted to histological or gene expression analysis. In all animals, the spermatozoa were recovered of epididymis for morphological, motility and vigor analysis. At day 90 after the busulfan treatment or vehicle administration (BUS90/C90), the animals were weighted again, anesthetized and euthanized for removal of the other testis and epididymis, which were immediately weighted. The same analyses were repeated for the spermatozoa and mRNA expression.

After 90 day of the busulfan injection, three animal of each lineage were allocated with virgin females to verify the fertility recovery. These animals were not submitted to testis removal.

2.3 Removal of epididymis and retrieval of spermatozoa

The epididymis were excised and freed from the adherent and fat tissues. Cauda epididymis was separated from the epididymis, transferred to a petri dish near to a drop of 200 µl of Fert's medium [21]. All reagents were adequately preheated. Longitudinal incisions were made in the cauda epididymal with a fine needle and a scalpel blade to release the spermatozoa. Sperm motility was evaluated immediately using a humid chamber placing a 3 µl drop of Fert's medium containing spermatozoa on a slide at 37ºC, and then examining it under an optic microscope (Olympus CX40) at x100 magnification. Motility estimations were performed from three different fields in each sample and the mean recorded as the final motility. The spermatozoa vigor was analyzed according to the spermatozoa propulsion force, being five the maximum value and zero the minimum.

To analyze the morphology, spermatozoa were kept in formaldehyde-citrate solution and two hundred sperm were visualized from each animal. The analysis were performed using a Leica microscopy (DMI 4000B) equipped with differential interference contrast (DIC). The amounts of normal or abnormal spermatozoa were quantified and the defects were classified as head, middle piece, tail defects or proximal cytoplasmic droplets. In animals which were not possible to count two hundred spermatozoa, the values were normalized. Distal cytoplasmic droplets were not considered as a defect, once spermatozoa were obtained from cauda epididymis.

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 DNase (Invitrogen, Brazil) at 37°C for 5min to digest any contaminating DNA. The transcriptase reverse reaction was performed with 1µM oligo-dT primer, 4U omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0,5mM dNTP's mix, 10U RNase inhibitor (Invitrogen, Brazil) in a final volume of 20µl.

The relative gene expression was performed by qRT-PCR using the StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) and variability in the amount of mRNA was corrected by amplification of *Gapdh* housekeeping gene. The calculation of relative expression was performed as recommended by Pfaffl et al. [22]. The primers for *Nanos2*, *Nanos3*, *Gdnf*, *Plzf* and for the housekeeping gene *Gapdh* (Table1) were designed in Primer Express software v 3.3 (Applied Biosystems) based in sequences available in GenBank or Ensembl and the oligonucleotides synthesized by Invitrogen.

2.5 Histological analysis

The testes were fixed with 4% paraformaldehyde and submitted to a dehydration battery. Paraplast (Sigma, St. Louis, MO, USA) was used for the confection of the tissue blocks. Histological sections with 7 µm in thickness were prepared to perform the hematoxylin-eosin (HE) staining for visualization of the structure under an optical microscope.

2.6 Fertility

Animals treated with busulfan in both lineages were allocated with female mice, from the same lineage, around 90 day after busulfan treatment. The date recovery of fertility was estimated by the time that they were put mate, subtracted the gestation time.

2.7 Statistical analysis

The regulation of gene expression and body, testes and epididymis weight were analyzed by ANOVA and means compared by a Student's *t* test. Data were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. One-way ANOVA was used for comparison of means of spermatozoa analysis. For spermatozoa motility and vigor the analyses were performed using a non-parametric test (Wilcoxom test). All analyses were performed using JMP software (SAS Institute Inc., Cary, NC). The comparisons were made between control and treated animals within the same period. Data are presented as means ± SEM.

3 Results

3.1 Corporal, testicular and epididymal weights

In Balb/C mice, the busulfan treated animals presented lower body weight 90 days after the administration in relation to control animals ($p<0,05$; Fig. 1A). The testicular and epididymal weights decreased ($p<0,0001$) in Balb/C busulfan treated animals in both analyzed periods related to respective controls (Fig. 1C and E). We also could observe that in Swiss lineage, the testicular and epididymal weights decreased in busulfan treated animals analyzed after 30 days ($p<0,0001$ and $p<0,01$ respectively), but no differences were observed 90 days after busulfan ($P>0,05$; Fig. 1D and F).

3.2 Spermatozoa motility, vigor and morphology

The spermatozoa motility was significantly lower, about 70% in BUS30 group compared to C30 ($p<0,0001$), and approximately 87% in BUS90 compared to C90 for Balb/C

animals ($p<0,0001$; Fig. 2A). Surprisingly, in the animals of Swiss lineage, the spermatozoa motility was approximately 53% lower in BUS30 than in C30 ($p<0,01$), but no differences were found between the groups at 90 days ($p>0,05$; Fig. 2B). In relation to vigor, the values found in Balb/C lineage animals were significantly lower in BUS30 (1,67 ± 0,21) than in C30 (4,37 ± 0,18; $p<0,0001$), and in BUS90 (0,07) than in C90 (4,17 ± 0,24; $p<0,0001$). Only one animal of the BUS90 group from Balb/C lineage presented a vigor classified as 1 (one), and all the other animals of this group presented a null vigor. The vigor found on Swiss lineage was reduced in BUS30 (1,67 ± 0,56) than in C30 (4,17 ± 0,40; $p<0,01$), but none difference was observed between BUS90 (3,17 ± 1,01; $p>0,05$) and C90 (4,4 ± 0,24; $p>0,05$).

The number of normal sperm in epididymis showed significant difference ($p<0,01$) among the experimental groups independent of the analyzed period for Balb/C animals (Fig. 2C). On the other hand, in Swiss animals, the amount of normal sperm in epididymis showed significant difference ($p<0,01$) among the experimental groups 30 days after treatment (C30 and BUS30), but it was not different 60 days later (C90 and BUS90) (Fig. 2D). For the two analyzed lineages, the most frequent abnormality found on spermatozoa of treated busulfan mice was in the middle piece and in control group was head defects ($p<0,01$) (Table 2). It was possible to observe, in Balb/C animals, that the number of distal cytoplasmic droplets was elevated in animals of the control group ($p<0,0001$), on the other hand, the number of proximal cytoplasmic droplets was superior in busulfan treated mice at 30 days ($p<0,0001$; Table 2). In some animals of Balb/C BUS90 group, it was not possible to count two hundred spermatozoa for morphological analysis.

3.3 Histopathology

The seminiferous tubules in control groups (C30 and C90) showed germinal epithelium and interstice morphologically normal in the two mice lineages (Fig. 3A and B),

for this reason, only one control for each lineage is shown. It was observed diffuse vacuolization (degeneration) in germinal epithelium, as well as decrease in the number of germ line cells and maintenance of Sertoli cells for Balb/C animals 30 and 90 days after busulfan treatment (BUS30 and BUS90) and in Swiss animals 30 days after busulfan injection (BUS30) (Fig. 3C - E). Nevertheless, for Swiss animals 90 days after the treatment it was possible to observe some degenerated germinal epithelium cells, but the spermatogenic lineage was present (Fig. 3F).

3.4 mRNA expression

For the animals of Balb/C lineage, we observed a statistically decrease in *Nanos2* mRNA in busulfan treated mice, independent of the analyzed period ($p<0,05$; Fig.4A). The gene *Nanos3* presented a decrease in mRNA expression only in mice analyzed 30 days after the drug administration ($p<0,001$; Fig. 4B). The mRNA expression pattern for the genes *Gdnf* and *Plzf* was similar, with the higher expression in busulfan treated groups ($p<0,05$; Fig. 4C and D). In Swiss mice the genes *Nanos2*, *Nanos3* and *Plzf*, reveal no statistic differences between the experimental groups independent of the analyzed period ($p>0,05$; Fig. 4E,F and H). For the gene *Gdnf* was observed a higher expression in busulfan treated animals analyzed at 30 days ($p<0,05$; Fig. 4G).

3.5 Fertility recovery

All the busulfan treated Swiss mice that were allocated in cage with females had litters around 34 days after the allocation, while the busulfan treated Balb/C mice did not recovery fertility through at least 90 days.

4 Discussion

This study revealed that busulfan treatment results in germ cell damage in both Balb/C and Swiss mice lineages. The main difference between them is that Balb/C remained with germ cell depletion during the analyzed period, while Swiss show signs of spontaneously regeneration 90 days after treatment.

According to Bucci et al. [16], the depletion of spermatogonia after the busulfan administration, occurs in a dose-dependent manner. Male mice that received a single dose of busulfan at 40 mg kg^{-1} remained sterile for at least 44 weeks [16]. However, Zohni et al. [20] related that some mice that received a single dose of busulfan at 45 mg kg^{-1} recovered fertility after 36 weeks. In this study, the Swiss mice recovered fertility 17 weeks after busulfan treatment, while the Balb/C did not recovery fertility until the end of analyzed period.

The testicular and epididymis weights decreased 30 days after drug administration in relation to control groups for both lineages. However, differently for Balb/C, in Swiss 90 days after treatment, it was not observed differences between the treatment and control. These data, related to testicular and epididymis weight decrease, are similar to the data observed by many authors after busulfan administration, due to loss of a majority of the germ cells [16, 19, 20, 23]. The increase in testicular and epididymal weight observed in Swiss at 90 days after busulfan, refers probably to the reestablishment of germ cells in this epithelium.

To our knowledge this is the first study that analyzed spermatozoa motility and vigor after busulfan treatment. Here we show that there are differences between the two lineages 90 days after treatment. Busulfan causes decrease in spermatozoa motility in Balb/C mice after 30 and 90 days and in Swiss only after 30 days. These results show that in Swiss mice have a higher number of live spermatozoa 90 days after the treatment than in Balb/C. The number of normal spermatozoa was higher in control groups than in busulfan groups, similar to those found by Bucci et al. [16], except in Swiss BUS90, where we did not find differences between the groups. Busulfan treated mice show a reduction in the number of distal cytoplasmic

droplets in comparison to normal animals; however, the number of proximal cytoplasmic droplets is higher in busulfan treated mice than in control mice. These data suggest that busulfan lead a delay in the sperm maturation and defects caused by testicular degeneration. The number of spermatozoa was reduced in Balb/C BUS90, where we could not count 200 spermatozoa in some animals. Bucci et al. [16] reported azoospermia in treated animals, where 60% of mice had no sperm in the cauda epididymis 44 weeks after 40 mg kg^{-1} treatment. The morphology of germinal epithelium shows that in Balb/C lineage, the utilized dose of busulfan is able to leave the epithelium degenerate for at least 90 days, unlike the Swiss strain, in which after 90 days, the germinal epithelium morphology of busulfan treated animals was similar to control. These findings complement the data obtained for fertility, which show that Swiss mice were fertile after 90 days of drug application and Balb/C not.

The A_s cells are called the true “actual stem cells”, which in favorable conditions are indeed the self-renewing cells. The A_{pr} cells comprise a second population of stem cells, the “potential stem cells”, which are capable of self-renewing but do not self-renew in the normal situation, only in stressful situations [24]. Considering the undifferentiated spermatogonial population, the *Nanos2* gene is expressed in A_s and A_{pr} cells and is fundamental to maintenance of stem cells during spermatogenesis [6, 25]. The *Nanos3* gene is mainly expressed in A_{al} until the transition to A_1 cells (differentiated) [9, 24]. The gene expression data obtained in this study suggests that the most affected cells by the treatment with busulfan in Balb/C are mainly the A_s spermatogonia, since the mRNA expression of *Nanos2* is severely decreased after the treatment. In Swiss busulfan treated animals, is possible to suggest that the same cell population is affect by this drug, once we observed a slight reduction expression of *Nanos2* mRNA after 30 days. However, the observed reduction did not differ from the control at any analyzed moment. In Balb/C the *Nanos3* expression decreased after 30 days of the treatment, but elevated at 90 days in relation to control, returning to physiological levels,

indicating that A_{al} and A_1 spermatogonia have recovered. These cells probably were not so affected by busulfan in Swiss lineage. The high levels of *Gdnf* expression, in both lineages, coincided with the period of rapid loss of SSC after administration of busulfan. This result was expected, once this factor regulates the testicular stem cell niche and is expressed by Sertoli cells [8], which are not affected after the busulfan use [16]. The *Gdnf* can induce or maintain the *Nanos2* expression, maintaining and renewing the SSC population [26]. The presented data corroborate which these findings since the expression of this factor in treated animals was superior to control animals. It suggests a rapid loss of SSC due to cytotoxic effect of the drug followed by an increase in *Gdnf* expression to provide a repopulation of A_s spermatogonia. In Swiss, the group BUS90 presented a decreased in *Gdnf* expression in relation to control, probably due to reestablishment of the SSC in the epithelium. For the *Plzf* gene, which is expressed by A_s, A_{pr} and A_{al} spermatogonia, and it is a transcriptional factor specific of spermatogonias, promoting the signaling necessary for SSC self-renewal and maintenance preventing differentiation into differentiated spermatogonia [7], we observed an increase in mRNA for BalB/C BUS30 and BUS90 probably indicating that the remaining undifferentiated spermatogonia of the testes, treated with busulfan, are multiplying and repopulating the testicular environment besides inhibiting the differentiation. All the mRNA gene expression data suggest us that the depletion of the SSC pool by busulfan is responsible by the decrease of *Nanos2* gene expression and consequently increase of *Gdnf* and *Plzf* gene expression.

The differences between the two analyzed lineages observed in our study are due to genetic variations, once the Balb/C lineage is based in crossing inbred, and Swiss in heterogamic crosses. This factor may be the reason that Swiss mice are more resistant to this drug than Balb/C mice.

Our data show for the first time the mRNA pattern expression profile of SSC markers after busulfan treatment aiming a better molecular explanation on which testicular cells are the busulfan targets. In conclusion the results of the present study show that the two studied lineages react differently to busulfan treatment revealing the needs of attention before the use of busulfan as testicular degeneration model in different lineages and species, therefore, the Swiss lineage is not a good model for prolonged testicular degeneration, probably, due to genetic variations between the lineages.

Conflict of interest

The authors declare that they have no competing financial interests.

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List of Tables

Fig. 1

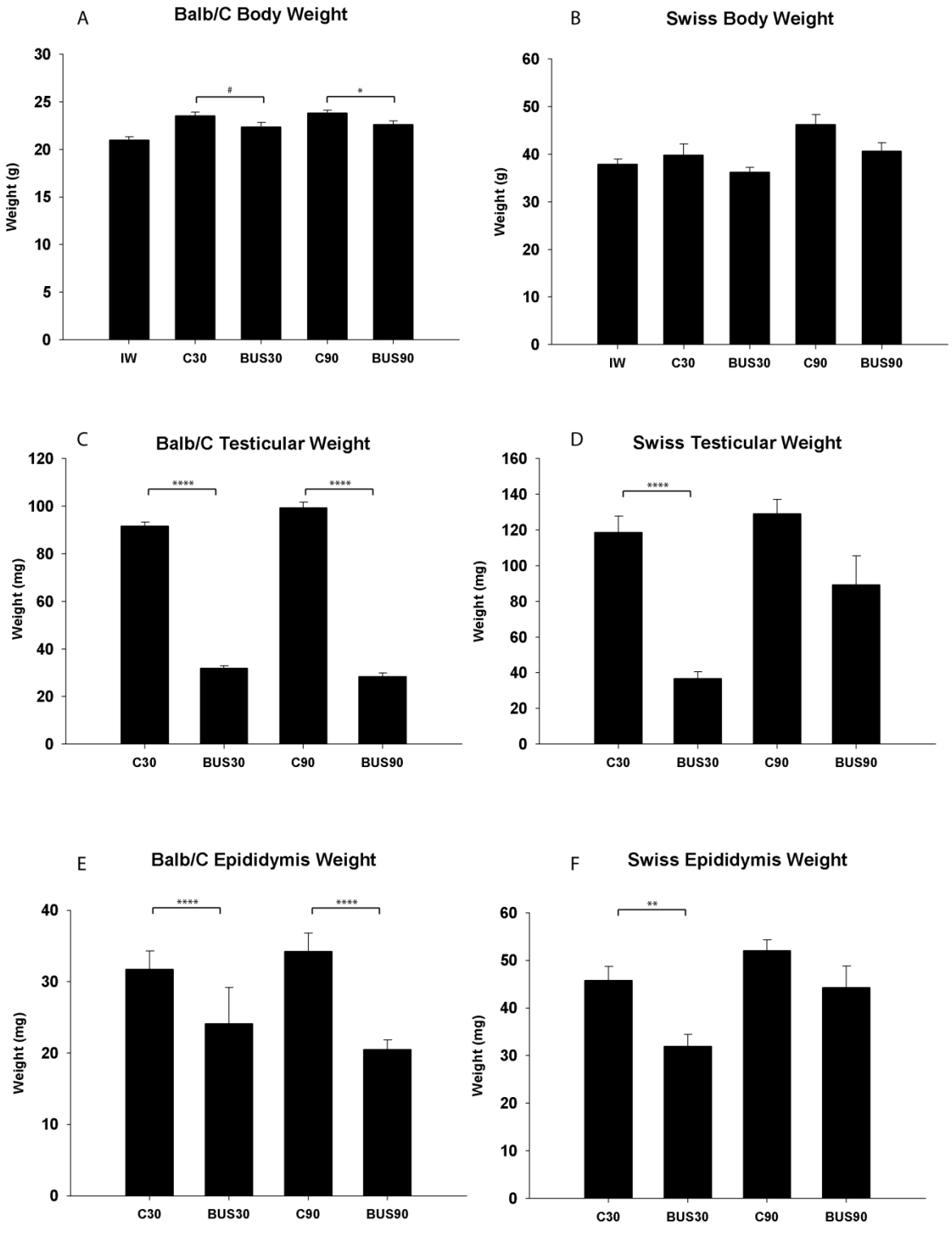
A
Balb/C Body Weight
Weight (g)
IW
C30
BUS30
C90
BUS90
B
Swiss Body Weight
Weight (g)
C
Balb/C Testicular Weight
Weight (mg)
D
Swiss Testicular Weight
Weight (mg)
E
Balb/C Epididymis Weight
Weight (mg)
F
Swiss Epididymis Weight
Weight (mg)

Fig. 2

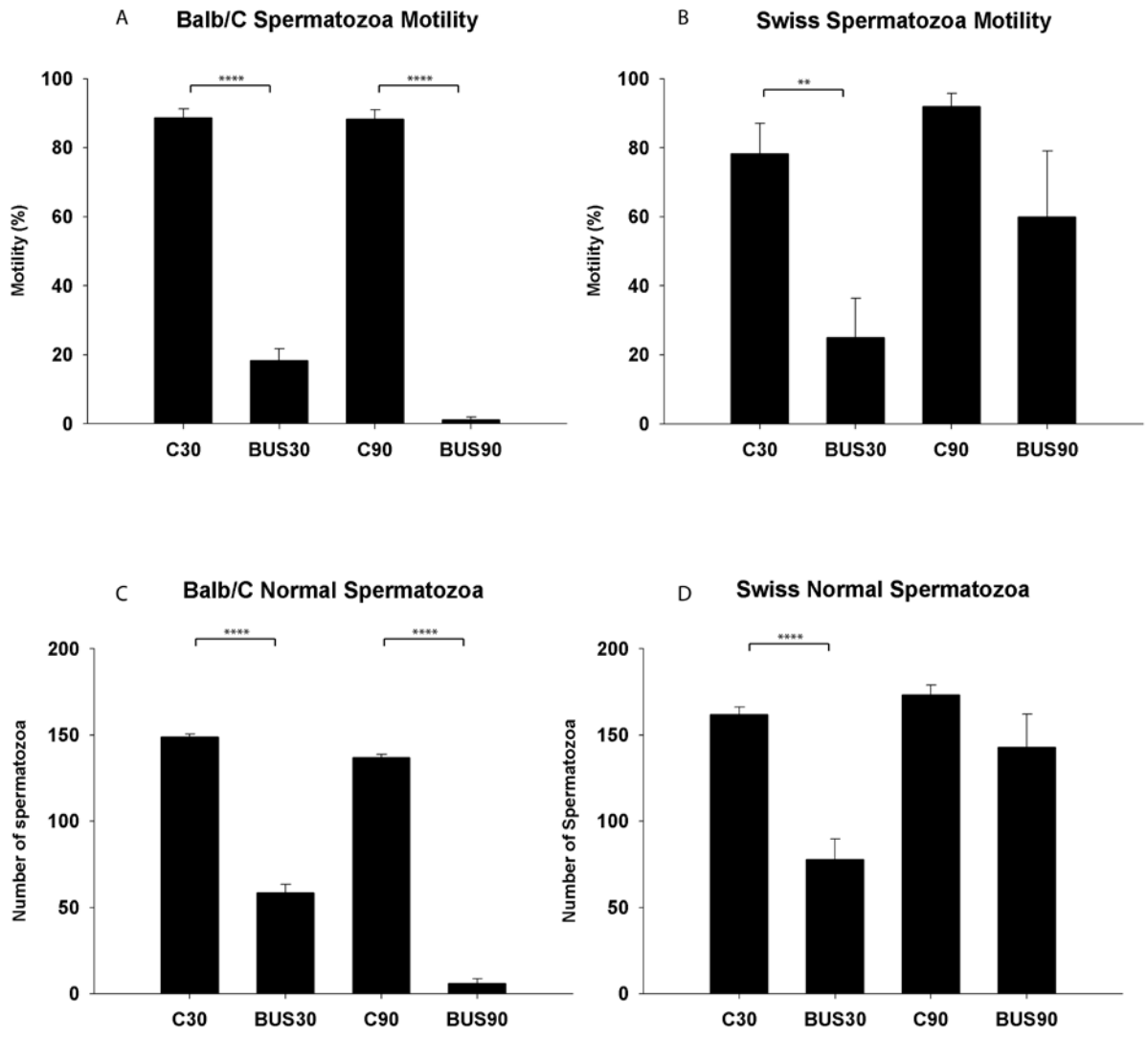
A
Balb/C Spermatozoa Motility
Motility (%)
100
80
60
40
20
0
****
****
C30
BUS30
C90
BUS90
B
Swiss Spermatozoa Motility
Motility (%)
100
80
60
40
20
0
**
C30
BUS30
C90
BUS90
C
Balb/C Normal Spermatozoa
Number of spermatozoa
200
150
100
50
0
****
****
C30
BUS30
C90
BUS90
D
Swiss Normal Spermatozoa
Number of Spermatozoa
200
150
100
50
0
****
C30
BUS30
C90
BUS90

Fig. 3

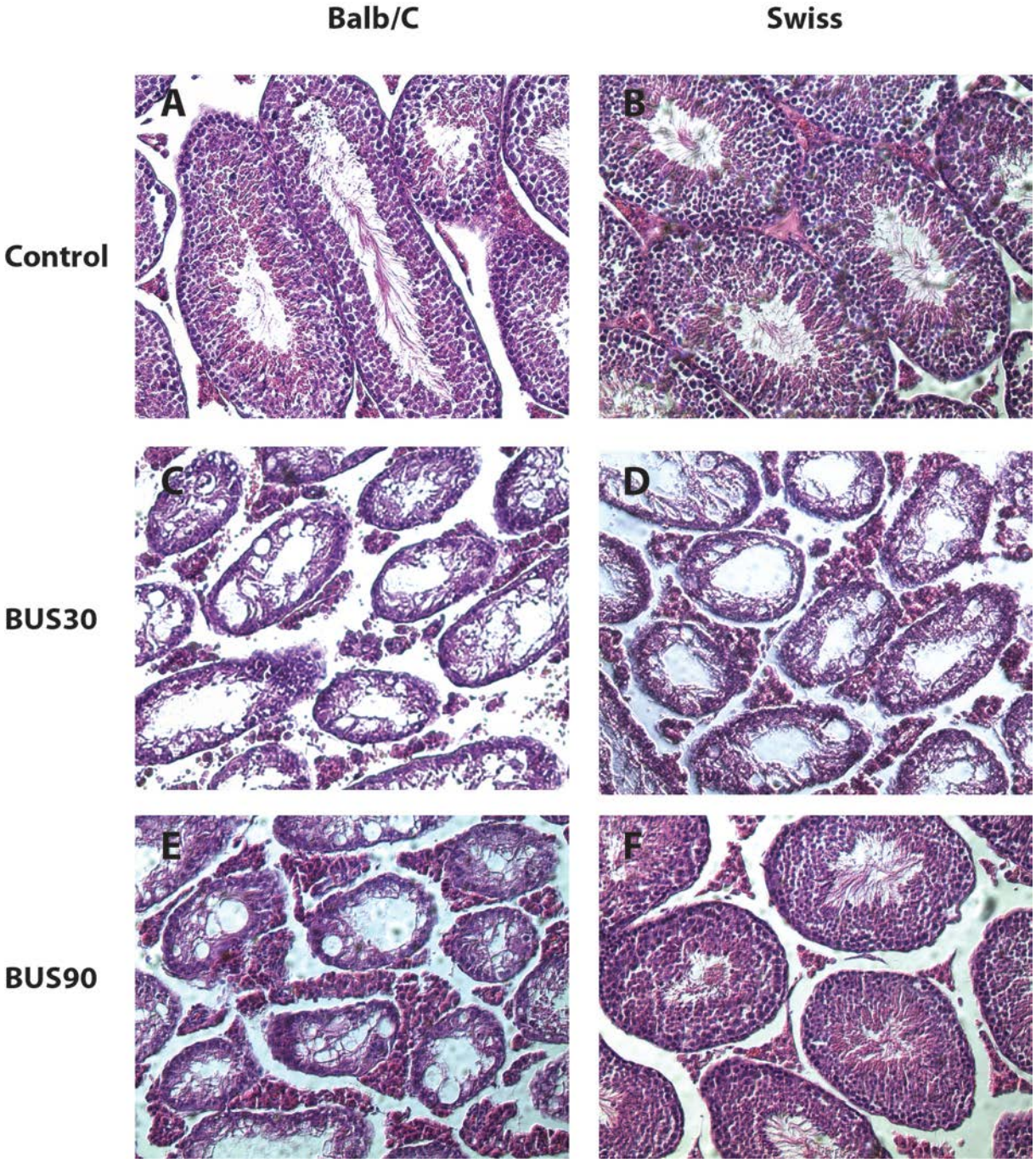

Fig.4

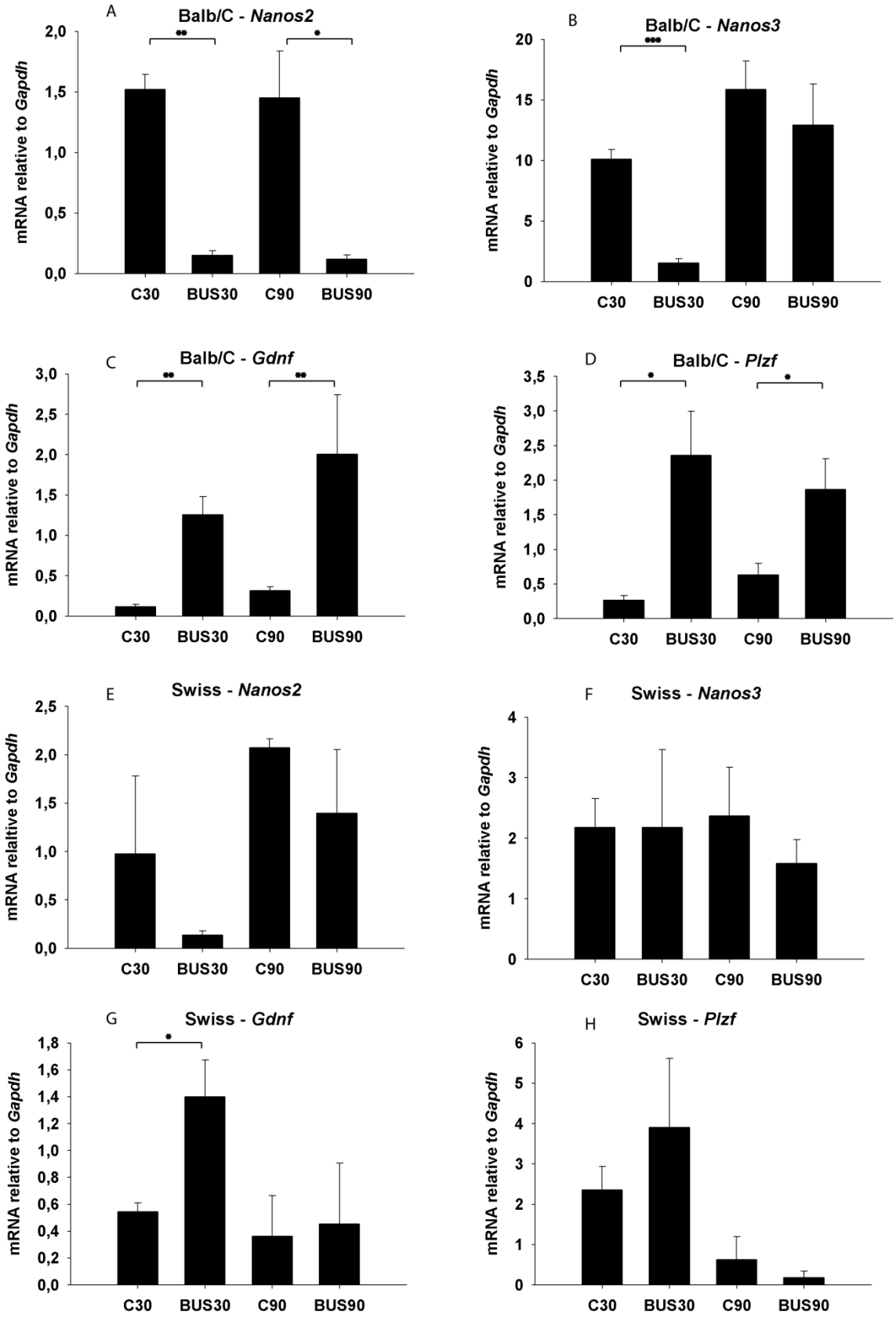
A
Balb/C - Nanos2
mRNA relative to Gapdh
2,0
1,5
1,0
0,5
0,0
**
*
C30
BUS30
C90
BUS90
B
Balb/C - Nanos3
mRNA relative to Gapdh
20
15
10
5
0
***
C30
BUS30
C90
BUS90
C
Balb/C - Gdnf
mRNA relative to Gapdh
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
**
C30
BUS30
C90
BUS90
D
Balb/C - Plzf
mRNA relative to Gapdh
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
C30
BUS30
C90
BUS90
E
Swiss - Nanos2
mRNA relaltive to Gapdh
2,5
2,0
1,5
1,0
0,5
0,0
C30
BUS30
C90
BUS90
F
Swiss - Nanos3
mRNA relative to Gapdh
4
3
2
1
0
C30
BUS30
C90
BUS90
G
Swiss - Gdnf
mRNA relative to Gapdh
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
C30
BUS30
C90
BUS90
H
Swiss - Plzf
mRNA relative to Gapdh
6
5
4
3
2
1
0
C30
BUS30
C90
BUS90

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

Gene	Primer forward	Primer reverse	Accession number
Nanos2	AGGTAGCTGAGGAGCC CAACTC	TGCTTGCAGAAGTTGCA TATGG	NM_194064.2
Nanos3	CTACTGCTACACCACCC GGAAT	AGACACCTGCTGCTGCT TCTC	NM_194059.2
Gdnf	GATTCGGGCCACTTGGA GTT	GACAGCCACGACATCC CATAA	NM_010275.2
Plfz	CGAGCTTCCGGACAAC GA	AAATGCATTCTCAGTCG CAAAC	NM_001033324.2
Gapdh	CAGCCTCGTCCCGTAGA CAA	GTAGACCATGTAGTTGA GGTCAATGAA	NM_008084.2

Table 2. Analysis of defective spermatozoa in mice treated or not with busulfan.

Lineage	Days	Treatment	Head Defect	Defective Middle Piece	Tail Defect	Proximal Cytoplasmic Droplets	Normals	Distal Cytoplasmic Droplets
Balb/C	30	Control	35,03 ± 1,61	9,10 ±0,70	2,44 ± 0,30	4,48 ± 0,54	148,99 ± 1,50	107,61 ± 4,37
		Busulfan	41,91 ± 3,17	65,67 ± 2,33 ***	8,05 ± 1,15 ***	19,80 ± 2,16 ***	58,43 ± 4,97 ***	35,46 ± 2,59 ***
	90	Control	40,01 ± 1,95	12,30 ± 0,91	4,24 ± 0,60	6,45 ± 0,71	137,00 ± 1,77	93,59 ± 4,24
		Busulfan	67,60 ± 11,81 *	90,80 ± 12,70 ***	19,00 ± 5,00 **	8,13 ± 3,39	5,78 ± 2,76 ***	1,57 ± 1,22 ***
Swiss	30	Control	20,50 ± 3,15	9,17 ± 1,55	7,83 ± 1,47	0,50 ± 0,34	162,00 ± 10,45	64,67 ± 11,53
		Busulfan	28,80 ± 6,03	79,83 ± 14,12 **	9,21 ± 0,85	4,46 ± 1,87	77,70 ± 11,88 ***	41,64 ± 9,94
	90	Control	16,59 ± 4,80	4,99 ± 0,77	3,99 ± 0,31	1,20 ± 0,37	173,20 ± 12,99	86,34 ± 25,77
		Busulfan	22,98 ± 5,92	22,18 ± 10,26	7,33 ± 3,85	4,75 ± 3,81	142,80 ± 19,37	82,39 ± 13,90

Asterisks indicate significant statistical differences between treated and control animals for each periods (* p<0,01; ** p<0,001; *** p<0,0001).

4. CONCLUSÃO

Com base nos resultados obtidos, é possível concluir que o busulfan atua sobre as células germinativas masculinas de camundongos das duas linhagens estudadas, sendo que o efeito em camundongos Balb/C é mais severo e mais duradouro do que na linhagem Swiss. Com relação ao perfil de expressão gênica, é possível observar que o busulfan atua nas células-tronco espermatogoniais, afetando a espermatogênese dos animais, porém, na linhagem Swiss, o período de 90 dias após a aplicação do fármaco é suficiente para reestabelecer a espermatogênese, a ponto dos animais gerarem prole. Essa diferença entre as linhagens se deve provavelmente a variações genéticas entre as linhagens, sendo que os animais provenientes de cruzamentos heterogâmicos aparentam ser mais resistentes aos efeitos do fármaco. Estes dados chamam atenção para o cuidado que se deve ter ao utilizar este fármaco em modelos de degeneração testicular relacionados com estudos de infertilidade masculina nas diferentes espécies e linhagens animais.

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