Progress on the study of the mechanism of busulfan cytotoxicity

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Abstract The preparation of spermatogonial stem cell (SSC) transplant recipients laid the technical foundation for SSC transplant technology and the understanding of spermatogenesis mechanisms. Busulfan is commonly used to prepare recipients for mouse SSC transplantation; however, its safety and efficiency have been questioned. This review summarizes the relationship between SSCs and Sertoli cells (SCs), and the mechanism of busulfan toxicity against sperm cells. We concluded that the proliferation, differentiation, and apoptosis of SSCs are regulated by SCs. The endogenous spermatogenic cells are depleted by busulfan treatment via alkylation of DNA, destruction of vimentin filament distribution, disruption of SSC differentiation, promotion of SSC dormancy, and generation of oxidative stress. However, the mechanisms require further exploration. The recent establishment of a model in vitro culture system has provided a good technical foundation to further explore these mechanisms, which will help us to find more efficient methods of recipient preparation and optimal transplantation times.

Keywords Mice · Busulfan · Spermatogenic cells · Spermatogonial stem cells · Sertoli cells

Introduction

The preparation of spermatogonial stem cell (SSC) transplantation recipients is an important step in the exploration of the mechanisms of spermatogenesis. Busulfan was used previously for SSC transplantation recipient preparation, because it damages the testicular niche, leading to disrupted spermatogenesis and infertility. Intraperitoneal (i.p.) injection of busulfan was first established to prepare recipients for the transplantation of SSCs, which was widely used afterward (Kanatsu-Shinohara et al. 2003), but the high mortality rate caused by busulfan was not resolved (Wang et al. 2010). Therefore, local testicular irradiation, heat shock, and other receptor preparation techniques were established, obviously improving mortality. However, transplant efficiency was inadequate with these methods, and the application of these technologies was not reported. Qin et al. (2016a) established a method of intratesticular injection of busulfan in mice for the preparation of recipients, and the high mortality rate was effectively overcome. Moreover, studies have shown that this intratesticular injection method has a large potential to improve recipient transplantation success in both pigs and mice (Ganguli et al. 2016; Lin et al. 2017). However, under

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physiological conditions, tight junctions and reactions between Sertoli cells (SCs) and SSCs help to stabilize the microenvironment inside the testicles, which ensures normal physiological functions of SSCs, such as proliferation, differentiation, and apoptosis. It was documented that infertility was automatically restored in recipients treated with busulfan (Kanatsu-Shinohara et al. 2016; Qin et al. 2016b; Zohni et al. 2012), which influenced the success of recipient preparation. With respect to recipient preparation, the mechanism of busulfan toxicity on SSCs and spermatogenic cells is not yet clear, which hampers further exploration and optimization of this recipient preparation method. This review paper summarizes the pathways and mechanisms of busulfan toxicity against spermatogenic cells. These findings will help to determine a mode of action and to optimize dosing regimens during recipient preparation with busulfan. This will serve as an important guide to determine the optimal timing of transplantation to study the treatment of human infertility.

The relationship between SCs and SSCs

SCs and SSCs are two functional cells in testicular somniferous tubules. The former cells are larger and have an irregular, pyramidal shape; they are distributed throughout the testis spermatogenic seminiferous epithelium, with one end on the basement membrane and the other end extending into the lumen. In contrast, the latter cells are smaller and cling to the inside of the epithelial basement membrane; they are connected with the SCs and are completely surrounded by them to form the chimera (Franca et al. 2016; Oatley and Brinster 2012). In the stable niche environment and dependent on the morphological structure of the seminiferous tubule, SSCs and SCs interact with each other to promote self-renewal, proliferation, and differentiation, which constitutes the normal ordered cycle of spermatogenesis that occurs throughout the life of male animals (Hofmann 2008). The tight junctions between SCs and myoid cells around the seminiferous tubules form the blood-testis barrier (BTB) that provides a suitable microenvironment for SSC self-renewal and normal spermatogenesis by protecting spermatogenesis from interference and damage by harmful substances. In vitro culture of mouse SSCs and SCs showed that

SSC self-renewal and proliferation could be promoted via direct interaction between these two cell types and paracrine secretion by SCs (Xiong et al. 2010). SCs are the “feeder cells” of the spermatogenic cells, and are tightly bound to SSCs in vivo to ensure secretion of various cytokines; this promotes self-renewal, proliferation, differentiation, and apoptosis of SSCs and maintains the proper number of SSCs to stabilize the niche for continuous and orderly spermatogenesis (de Rooij 2009; Hai et al. 2014).

Toxicity mechanism of busulfan against spermatogenic cells

Busulfan destroys DNA structure, prevents proliferation and differentiation of SSCs, and initiates apoptosis

Busulfan contains two sulfonated methane functional groups and acts in the G0/G1 phase in cells. In principal, through bimolecular nucleophilic substitution, busulfan can alkylate DNA on N7 and O6 of guanine, and N3 of adenine, forming intrastrand crosslinks at 5'-GA-3' and, to a lesser extent, at 5'-GG-3'. Consequently, the emergence of DNA crosslinking, DNA–protein crosslinking, and single-strand breaks (SSBs) results in the blockage of DNA replication and transcription, and inhibition of cell proliferation and differentiation (Iwamoto et al. 2004).

The expression of p53 was shown to be increased in response to busulfan-induced DNA damage, causing apoptosis (Furukawa et al. 2007). In the nucleus, p53 upregulates the adaptor protein, ASK (activator of S-phase kinase), which promotes the activation of BAX and its interaction with mitochondria, and the expression of apoptosis target genes, such as Bax, Bid, and PUMA (p53 upregulated modulator of apoptosis), are induced (Zhivotovsky and Kroemer 2004); these genes are also located in the mitochondrial membrane, causing increased permeability, release of cytochrome C, and induction of apoptosis. Interestingly, when busulfan-induced DNA damage is detected by the presence of p53 in the cytoplasm, Bax-Bcl-xl and Bax-Bcl-2 heterodimers depolymerize to release Bax (or directly activate Bak) to induce apoptosis. The Bax/Bak heterodimer is a checkpoint in the intracellular apoptotic pathway (Lindsten et al. 2000; Wei et al. 2001). Fas, induced by busulfan in spermatogenic

cells, specifically binds to Fas ligand (FasL) of SCs and the new death domain protein, FADD (Fas-associated protein with death domain). This combines with procaspase-8 to form the death-inducing signaling complex (DISC), which activates caspase-8 (Muzio et al. 1998). Caspase 3 and caspase-7 are subsequently activated (Gupta et al. 2006), cleaving the DNA repair enzyme, PARP (poly (ADP-ribose) polymerase) and increasing $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease activity; this results in DNA cleavage and cell apoptosis. The apoptotic induction factor, Bid/Bax, can also be activated, promoting the release of mitochondrial cytochrome C to initiate the intracellular apoptosis pathway (Choi et al. 2004).

Busulfan destroys vimentin filaments (VFs) and intercellular cell adhesion molecule-1 (ICAM-1)

VFs ensure that SCs timely regulate germ cells by maintaining tight connections between SCs and spermatogenic cells (Amlani and Vogl 1988). Upon continuous treatment with busulfan in adult rats, the apical VFs in the SCs gradually collapse towards the nuclei, becoming disorganized in the basal region of the SCs. After the effect of busulfan effect subsides, spermatogenesis begins to be restored and the VFs reorganize in the basal and perinuclear regions of the SCs among the spermatogonia and spermatocytes (ElGhamrawy et al. 2014; Kopecky et al. 2005). ICAM-1 is expressed by several cell types (such as: SCs and germ cells), and it regulates the movement of preleptotene/leptotene spermatocytes across the BTB and the release of elongated spermatids into the tubule lumen (Mruk and Cheng 2004; Xiao et al. 2012). After i.p. injection of busulfan in the mouse, testicular ICAM-1 significantly decreased at both the mRNA and protein levels and then recovered gradually. The BTB is damaged by the reduction in ICAM-1 expression, which affects the connection between SSCs and SCs. However, the disordered VF distribution in SCs could destroy cellular morphology by separating the close link between SSCs and SCs, which affects the stability of the niche microenvironment and the SSCs (Amann 2008; Cai et al. 2016). In particular, we found that the damage and disappearance of SSCs, spermatogenic cells, and SCs occurred synchronously, and SCs recovered along with the SSCs (Qin et al. 2016b). We speculate that VFs and ICAM-1 play an important

regulatory role in the degradation and recovery of SSCs and SCs.

Busulfan disrupts differentiation factors and hinders differentiation of SSCs

Feeding pregnant rats a single oral dose of busulfan caused reduced expression of bone morphogenetic protein 4 (BMP4) in embryonic apical ectodermal ridge (AER) and anterior mesenchyme cells. BMP4 is a member of the bone morphogenetic protein family, which is found in early embryonic development in the ventral marginal zone, for example. This increased cell death in the AER and mesenchyme cells (Otsuji et al. 2005), and approximately 80% of embryo hind limbs exhibited malformations, such as polydactyly and syndactyly (Naruse et al. 2007). Therefore, we suggest that the reduction in Bmp4 expression by busulfan reduces cell proliferation, resulting in embryo hypoplasia. One week after i.p. injection of busulfan in mice, the expression of c-kit became undetectable. Notably, this decrease in c-kit expression occurred earlier than did the increase in p53/Bax/Bcl-2 in TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive cells, resulting in apoptosis of most spermatogonia and some spermatocytes. Two to three weeks after the treatment, a marked cell-specific increase in apoptosis was observed in meiotic spermatocytes. At 4 weeks, testes from busulfan-treated mice showed a complete loss of differentiating germ cells, with the exception of some undifferentiated spermatogonial stem cells. However, c-kit expression was detectable, and it was restored to control levels by 5 weeks. Similarly, Stem cell factor (SCF) mRNA levels and differentiated spermatogenic cells were detected again in the sixth week. These results indicate that the c-kit/SCF pathway is critical for male germ cell survival and that busulfan treatment results in transient, rather than permanent, infertility due to the downregulation of c-kit (Choi et al. 2004). In mice with c-kit mutations, only a few micro spermatogenic cells can be detected and spermatogenesis is blocked (Kissel et al. 2000). BMP4 may be downregulated by busulfan and may inhibit the expression of SSCs differentiation marker genes through the BMP4/Bmpr1a/Smads/Sohlh2/c-kit pathway, thereby inhibiting spermatogonia differentiation to spermatocytes (Carlomagno et al. 2010; Feng et al. 2000; Li et al. 2014).

Busulfan promotes protective proliferation and dormancy of SSCs by regulating expression of glial cell line-derived neurotrophic factor (GDNF) and retinoblastoma (RB) phosphorylation

It was reported that 5 days after i.p. injection of busulfan, GDNF mRNA levels in seminiferous tubules increased to their highest level, then gradually decreased, with no significant difference detected after 4 weeks compared with the pretreatment level (Zohni et al. 2012). Regardless of busulfan dose, more than 95% of SSCs were lost within 3 days, and fertility was lost within 4 weeks after treatment. Fertility and SSC numbers gradually recovered, but slower recovery was observed at higher busulfan doses (Zohni et al. 2012). Similar results were obtained after continuous injection in mice, although the maximal level of testicular GDNF lasted for approximately 3 weeks (Luo et al. 2010). It has been speculated that busulfan briefly exhibits protective effects and can promote proliferation, inhibit differentiation, and regulate spermatogenesis by regulating high SC secretion of GDNF and by both Akt and Src family kinase (SFK) signaling in SSCs (Ebata et al. 2011; Oatley et al. 2007; Wu et al. 2011). Busulfan-treated mouse testes exhibit strong pRB (the hypophosphorylated form of Rb, primarily found in resting SSCs) and weak proliferating cell nuclear antigen (PCNA) signals, while control adult testes exhibit strong ppRB (hyperphosphorylated form, found in proliferating cells) and PCNA signals. PCNA is very strongly expressed in the testis at postnatal day 5, while pRB and ppRB are expressed at equal levels (Choi et al. 2004). Busulfan treatment may inhibit Rb phosphorylation in the testis via inhibition of cyclin E; pRB then remains bound to E2F, which in the complexed form is inactive, inhibiting PCNA gene synthesis and hindering SSC transition from G0 to G1 phase. At the same time, the expression of cyclin E gradually decreases and recovers progressively after 4 weeks (Choi et al. 2004). This indicates that busulfan may inhibit pRB phosphorylation to cause SSC dormancy by disrupting the activity of cyclin E.

Redox equilibrium is disrupted by busulfan, which results in oxidative damage in a series of spermatogenic cells

Redox balance normally occurs in cells. Both feeding in vivo and culturing liver cells in vitro showed that busulfan is conjugated with glutathione. It was documented that free glutathione decreased 50–60%, glutathione was rapidly depleted, and reactive oxygen species (ROS) increased, leading to oxidative stress and liver cell injury (DeLeve and Wang 2000; Hassan et al. 2002). However, ROS were reduced by the addition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitors after busulfan treatment. It was suggested that a continuous increase in ROS production via NADPH was induced by busulfan and that the NADPH oxidase inhibitor could effectively alleviate this oxidative stress (Probin et al. 2007). Many studies have shown that busulfan-treated testes contain significantly increased lipid peroxidation levels during week 1 and 2 (Cai et al. 2016). Cell membrane fluidity and permeability were altered by busulfan treatment, as indicated by ROS-induced malondialdehyde (MDA) production, resulting in damaged cell structure and function. Forty-five days after a single i.p. injection of busulfan, mice showed significant degenerative changes in their germ cells and loss of spermatogenesis was observed. In contrast, mice that received melatonin injection followed by busulfan treatment showed improved spermatogenesis (Mirhoseini et al. 2014). Research on the related mechanism indicated that melatonin increased the expression of manganese superoxide dismutase (MnSOD), which successfully ameliorated the busulfan-induced SSC apoptosis caused by high concentrations of busulfan-induced ROS (Li et al. 2017). In addition, resveratrol might be an approach for therapeutic intervention to promote SSC proliferation and cease SSC loss in an azoospermia mice model induced by busulfan (Wu et al. 2016). Antioxidants could successfully ameliorate busulfan-induced SSC apoptosis caused by high concentrations of ROS, and induce reproductive cell proliferation.

Conclusion and outlook

Busulfan alters the expression of proliferation, differentiation, and apoptosis factors in spermatogenic cells

by changing their redox state and by DNA alkylation modification, which affects the interrelationship between SCs and SSCs. As a result, the proliferation and differentiation of SSCs is inhibited, SSC dormancy is induced, and apoptosis of a series of spermatogenic cells is promoted. This leads to depletion of endogenous spermatogenic cells and provides an environment for the migration and colonization of exogenous SSCs. However, previous studies only focused on the changes of SSCs and a series of spermatogenic cells with busulfan toxicity, but they did not analyze the interrelationships between SSCs and SCs. Therefore, it is not sufficient to study only the mechanism of toxicity of busulfan on SSCs. At present, an in vitro culture system of SSCs and SCs has been established and gradually improved (Liu et al. 2011). This system provides a convenient model for studying the regulatory relationship between SCs and SSCs in vitro to reveal the mechanisms involved. Related studies will establish efficient, low toxicity SSC recipient preparation methods and the best time for transplantation to lay a solid theoretical foundation. This should promote exploration of the mechanism of mammalian sperm formation and methods for the treatment of human infertility.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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