Original Article
Male reproductive health and infertility

pISSN: 2287-4208 / eISSN: 2287-4690
World J Mens Health 2023 Oct 41(4): 882-891
https://doi.org/10.5534/wjmh.220100

The World Journal of MEN's HEALTH

Effect of N-acetyl-L-cysteine on Testicular Tissue in Busulfan-Induced Dysfunction in the Male Reproductive System

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Purpose: This study aimed to evaluate the protective effect of N-acetyl-L-cysteine (NAC) as an antioxidant on busulfan-induced testicular dysfunction in mice and elucidate its possible mechanism of action.

Materials and Methods: Thirty-two C57BL/6 male mice were randomly divided into four groups (n=8/group) as follows: (1) control group (oral administration of saline [0.1 mL daily] for 35 days); (2) NAC group (oral administration of NAC [10 mg/kg daily] for 35 days); (3) busulfan group (double intraperitoneal injections of 20 mg/kg; total dose of 40 mg/kg); and (4) busulfan+NAC group (after double intraperitoneal injections of 20 mg/kg; total dose of 40 mg/kg, NAC administration [10 mg/kg daily] for 35 days). The testes were removed, weighed, and subjected to sperm parameter analysis and morphology assessment. Reproductive hormone, serum/testicular reactive oxygen species (ROS) level, oxidative stress and antioxidant markers were evaluated. The testicular expression of *Nrf2* and *HO-1* was examined using RT-qPCR.

Results: Busulfan treatment significantly decreased testicular weight, sperm count, and serum testosterone levels. Atrophy and degeneration of germinal epithelium were observed in the busulfan group. NAC administration after busulfan treatment partially attenuated the deterioration of testis weight, sperm quality, serum hormones, histomorphometric changes, and oxidative and antioxidative status. NAC treatment resulted in a considerable improvement in *Nrf2* and *HO-1* mRNA expression levels.

Conclusions: This study provides compelling evidence that NAC as a potent antioxidant has significant protective effects against busulfan-induced male reproductive impairment possibly through modification of the Nrf2/HO-1 signaling pathway.

Keywords: Acetylcysteine; Antioxidant effect; Busulfan; Mouse; Reproductive system; Testis

INTRODUCTION

Male factor infertility is responsible for approximately 50% of infertile couples and plays a crucial role in reproductive diseases [1,2]. Major causes of male infertility are associated with spermatogenetic failures due to endocrine-disrupting environmental chemicals, obesity, smoking, and oxidative stress [3,4]. Recent stud-

Received: May 21, 2022 **Revised:** Jun 20, 2022 **Accepted:** Jul 24, 2022 **Published online** Jan 1, 2023
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ies reported declining both sperm count and quality in men, thereby resulting in reduced fertility [3].

Abnormal sperms with transformation and fragmented DNA, and decreased sperm count are correlated with free radical-induced oxidative stress in the testes [4-6]. The testes are vulnerable to oxidative stress because they contain low antioxidant enzyme content compared with other organs, such as the liver and kidneys [6,7]. Studies have shown that testicular function affecting sperm parameters could be improved by antioxidant therapies, which positively influence interaction between free radicals and antioxidants in testicular cells [5,8].

Busulfan (1,4-butanediol dimethanesulfonate) is an alkylating agent often used to treat chronic myelogenous leukemia [9]. Additionally, it has been used to induce azoospermia in animal models [10-12]. Imbalance between reactive oxygen species (ROS) levels and antioxidant defense results in reduced sperm motility, damaged sperm DNA, and subsequent impairment of embryonic genome [13,14]. During oxidative stress, nuclear factor erythroid 2-related factor 2 (Nrf2) combines with the antioxidant response elements and upregulates various antioxidant genes [15]. Heme oxygenase-1 (HO-1), one of the major oxidative stress markers that is induced by Nrf2 activation [15,16], engages in the testicular response to stress [17].

N-acetyl-L-cysteine (NAC), a glutathione (GSH) precursor, is a potent free radical scavenger. Recently, there has been a growing interest in the therapeutic potential of NAC, *via* oral and intravenous administration, against testicular damage in testicular torsion–detorsion model [14]. This model demonstrates the protective effect of NAC in attenuating negative biochemical changes related to excessive ROS generation and antioxidant enzyme depletion. However, to our knowledge, there are no studies demonstrating the therapeutic potential of NAC on busulfan-induced dysfunction if the male reproductive system.

This study aimed to investigate the therapeutic potential of NAC on body and testis weight, sperm parameters, histological changes, reproductive hormone levels, and antioxidant activity in a busulfan-induced infertility mouse model. We also assessed the expression of key regulatory factors, such as *Nrf2* and *HO-1*, associated with busulfan-induced spermatogenic disorders.

MATERIALS AND METHODS

1. Animals

In this study, 32 two-month-old C57BL/6 male mice (20–22 g) were included. The animals were housed in plastic cages under 12 hours light/dark cycle. Mice were allowed free access to water and food prior to and during the experiment.

2. Experimental protocol

The C57BL/6 mice were randomly divided into four groups (n=8/group). The control group received saline (0.1 mL daily oral administration). The NAC group received oral NAC administration (10 mg/kg daily) for 35 days. The busulfan group received saline (0.1 mL daily oral administration) after busulfan (Sigma, St. Louis, MO, USA) treatment (double intraperitoneal injections of 20 mg/kg [total dose of 40 mg/kg] at an interval of 3 h). The busulfan+NAC group received oral NAC administration (10 mg/kg daily) for 35 days after busulfan treatment as described previously. All groups received NAC or saline for 35 days. The animals were weighed before the experiment and one day prior to sacrifice. Blood samples were obtained *via* heart puncture, immediately prior to organ collection, and the serum was stored at -80°C. The testes were weighed immediately after removal at the end of treatment. One of the two testes was fixed in Bouin's solution to analyze histopathological parameters, while the other was snap-frozen in liquid nitrogen and stored at -80°C.

3. Ethics statement

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Pusan National University Hospital (IACUC approval No. PNUH-2020-168) and were performed according to the Guide for the Care and Use of Laboratory Animals.

4. Sperm analysis

The caudal epididymis was carefully separated from the testis to evaluate sperm parameters. After mincing the cauda with a 30-gauge needle, the suspension was placed in a Petri dish containing phosphate-buffered saline (PBS; Sigma) supplemented with 0.5% bovine serum albumin at 37°C in 5% CO_2 for 10 minutes. Sperm counts were measured using a Neubauer hemocytometer (HBG Company, Giessen, Germany). The total number of sperms and the number of motile sperms

were determined. On movement detection, sperms were scored for motility. The percentage of motile sperms in each treatment group was calculated.

5. Histopathological evaluation

The testis tissue was fixed in 4% paraformaldehyde (Sigma), dehydrated and paraffin-embedded. We stained 5-μm thick sections with hematoxylin and eosin stain (H&E) and visualized under a light microscope (Nikon E100; Nikon, Tokyo, Japan). The photomicrographs were measured for germinal epithelium depth and seminiferous tubule diameter using ImageScope analysis software (Aperio Technologies, Vista, CA, USA).

6. RNA preparation and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated from frozen testes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further treated with DNase (RNase-free) (TaKaRa, Dalian, China) to remove genomic DNA. The total RNA concentration and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, 1 μg of total RNA was reverse transcribed to cDNA with M-MLV reverse transcriptase (TaKaRa) using random hexamers (Takara Bio, Inc., Otsu, Japan) for 1 hour at 42°C, followed by enzyme inactivation at 95°C for 5 minutes. Subsequently, these cDNA libraries were quantified using qPCR, carried out with LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). The gene-specific primers were designed based on the corresponding mRNA sequences (Table 1). PCR cycling conditions consisted of initial denaturation at 95°C for 30 seconds, followed by 42 amplification cycles of denaturation at 95°C for 5 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. The expression of target genes was determined relative to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as an internal control, and the relative mRNA expression was calculated using $2^{-\Delta\Delta CT}$ method. Each experiment was performed in duplicate using different cell batches at least three times.

7. Estimation of serum testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), and ROS

Serum testosterone, FSH, and LH levels were measured by enzyme-linked immunosorbent assay kits (Cusabio China Inc., Wuhan, China) according to manufacturer's protocol. The ROS levels were evaluated using OxiSelect™ *in vitro* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's instructions. The absorbance was measured at 450 nm.

8. Evaluation of total antioxidant capacity (TAC) and associated parameters

The testes were homogenized in ice-cold PBS (50 mM) containing sucrose (0.25 M). TAC of the testes was measured using an antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI, USA)and calculated using a Trolox standard curve. Total intracellular GSH levels were measured using the commercial ApoGSH Glutathione Detection Kit (BioVision, Mountain View, CA, USA). Briefly, after filtering through 10 kDa pore-size filters (R&D System, Inc., Minneapolis, MN, USA), 40 L of the filtrate was diluted using lysis buffer (final dilution 100:1). In a fluorometric plate, GSH standards (100:1) were mixed with 2 L of monochlorobimane dye and incubated for 30 minutes at 37°C. The fluorescence was determined using a microplate reader at excitation/emission wavelength of 380/460 nm. Total GSH concentration was calculated using the GSH standard

Table 1. Primers sequences used for real time PCR amplification

Gene	Sequence (5′→3′)	
	Forward	Reverse
Nfr2	GCTATTTTCCATTCCCGAGTTAC	ATTGCTGTCCATCTCTGTCAG
HO-1	CCAGCAACAAAGTGCAAGAATC	CCACCAGAAAGCTGAGTGTAAG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGT

PCR: polymerase chain reaction, *Nrf2*: nuclear factor erythroid 2-related factor 2, *HO-1*: heme oxygenase-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase.

curve.

9. Statistical analysis

All data are presented as mean±standard deviation. Data analyses were performed with SPSS software version 22.0 (IBM Corp., Armonk, NY, USA) and evaluated using one-way analysis of variance (ANOVA) and Student's t-test. p-values <0.05 were considered statistically significant.

RESULTS

1. Weight and sperm parameters

No remarkable difference was observed in the total body weight of mice among the four groups (Fig. 1A). The busulfan group showed significant decrease in the weight and size of the testes ($p<0.05$). Interestingly, oral administration of NAC slightly restored the testes weight in the busulfan+NAC group as compared with the busulfan group (Fig. 1B, 1C). The busulfan group showed a significant decrease in sperm counts and motility ($p<0.05$). Moreover, oral administration of NAC significantly restored sperm counts and motility (Fig. 1D, 1E; $p<0.05$).

2. Histological changes in seminiferous tubules

The effect of NAC on testes histoarchitecture in different groups is shown in Fig. 2. The control and NAC groups exhibited a typical testicular architecture with normal development of spermatozoa at all stages and Leydig cells, which were packed closely in the seminiferous tubule. The testes of the busulfan group displayed degenerated and irregular histopathologi-

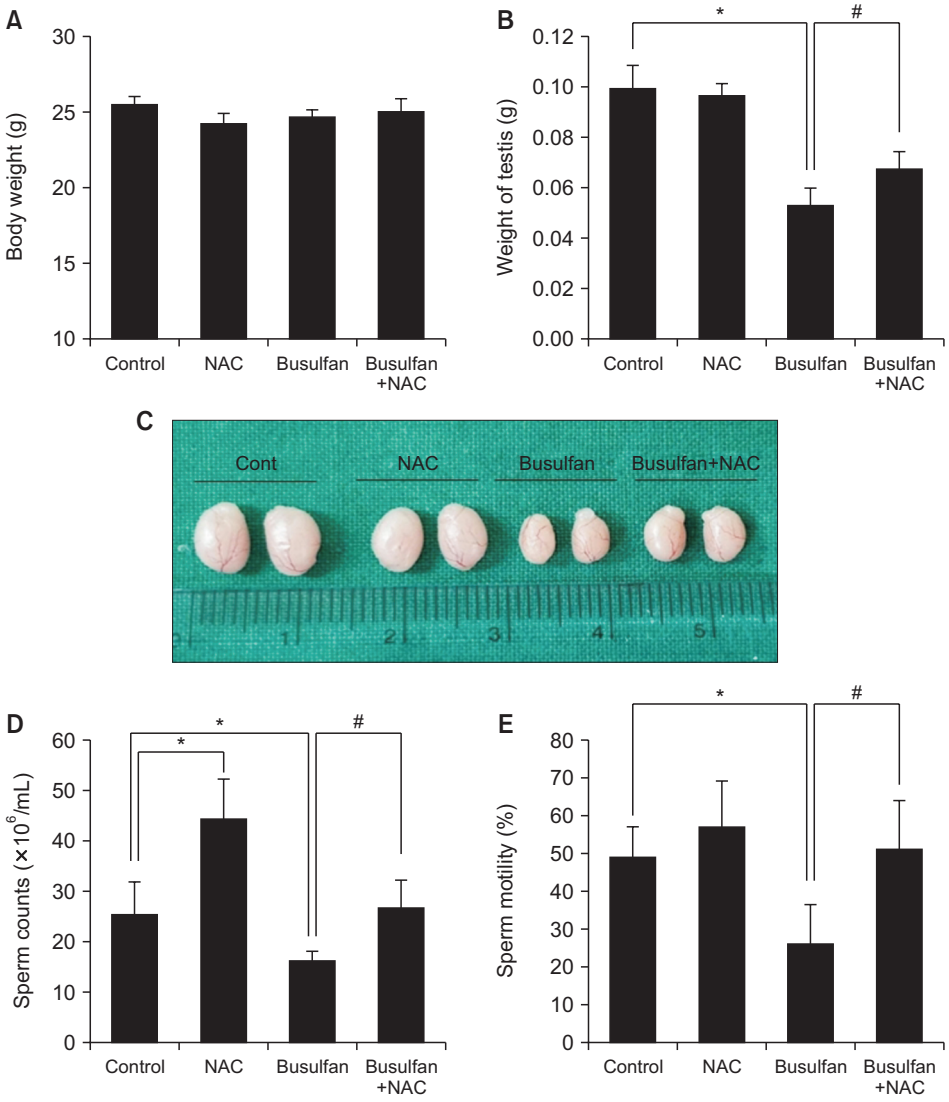

Fig. 1. Effects of busulfan and NAC on testicular weight, size, and semen parameters. Comparison of the (A) body weight (g), (B) weight of testis (g), (C) size of testis (mm), (D) sperm counts ($\times 10^6$/mL), and (E) sperm motility (%) among the four treatment groups. *$p<0.05$ *versus* the Cont group, #$p<0.05$ *versus* the busulfan group. Busulfan: 1,4-butanediol dimethanesulfonate, Cont: control, NAC: N-acetyl-L-cysteine.

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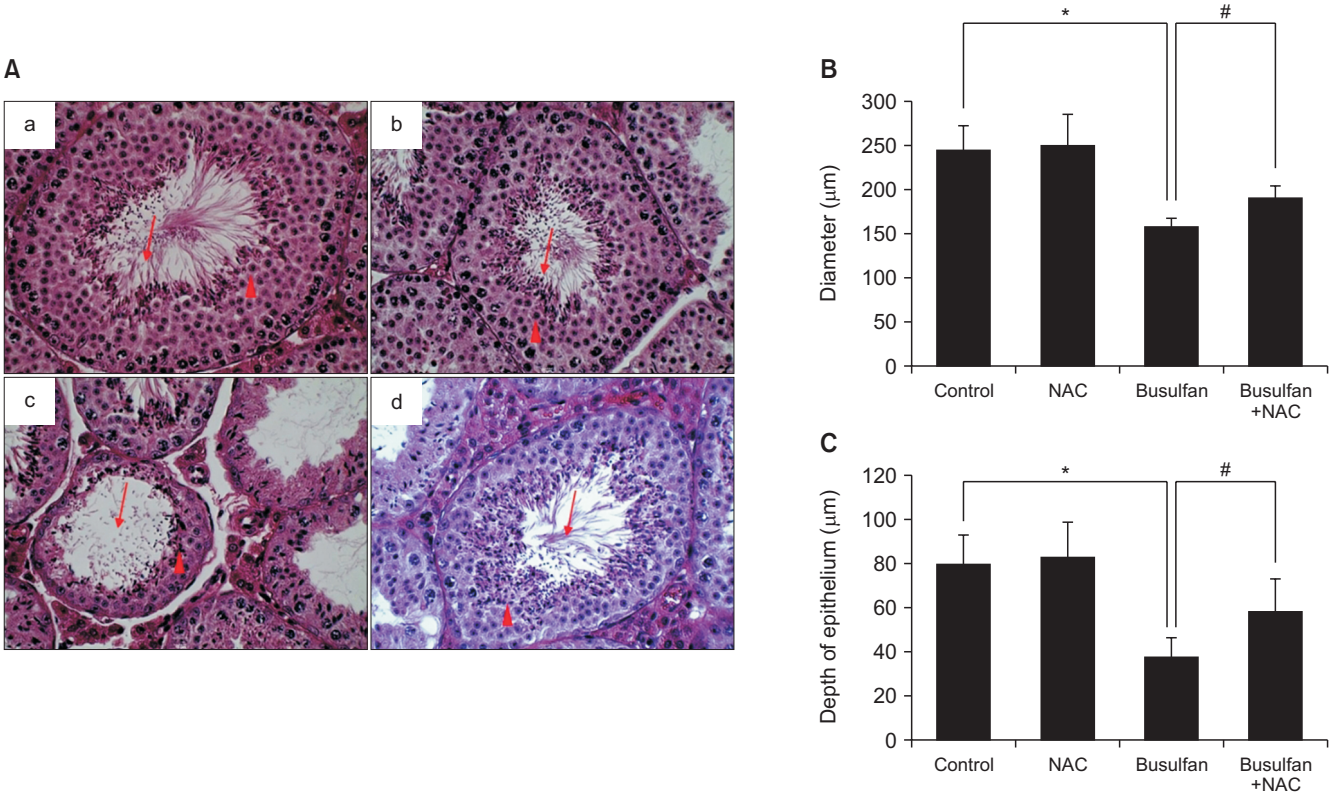

Fig. 2. Effects of busulfan and NAC on seminiferous tubules. Comparison among the four groups in terms of (A) histopathological features. (a) Cont: normal spermatogenesis. (b) NAC: normal spermatogenesis. (c) Busulfan: disorganized germinal cell layers in the testis of mouse treated with busulfan. (d) Busulfan+NAC: restored germinal cell layers in the testis of mouse after NAC administration following busulfan treatment. Hematoxylin and eosin stained (magnification ×400). Spermatozoa and germinal epithelium are indicated with red arrow and arrowhead, respectively. (B) Diameter of seminiferous tubule (μm). (C) Depth of germinal epithelium (μm). *p<0.05 *versus* the Cont group, #p<0.05 *versus* the busulfan group. Busulfan: 1,4-butanediol dimethanesulfonate, Cont: control, NAC: N-acetyl-L-cysteine.

cal arrangements of spermatogenic cells. Detachment of the spermatogenic epithelium from the basement membrane and vacuolated features of the seminiferous tubules were observed. However, NAC treatment significantly alleviated these abnormalities by inducing partially complete spermatogenesis with increased number of epithelial layers and diameter of the seminiferous tubule in the busulfan+NAC group (p<0.05).

3. Reproductive hormone levels

The levels of testosterone and LH were significantly decreased in the busulfan group compared to those in the control group (p<0.05). In contrast, NAC administration in busulfan-treated mice restored testosterone and LH levels, although the levels were lower than those observed in the control group. Moreover, NAC administration without busulfan treatment significantly improved testosterone and LH levels compared to those in the control group (Fig. 3A, 3B; p<0.05). FSH levels were not significantly different among the four groups (Fig. 3C).

4. Oxidative stress and *Nrf2*/*HO-1* expression

ROS levels in serum and testicular homogenate were significantly increased in the busulfan group (Fig. 4A, 4B; p<0.05). This increase was markedly alleviated in the busulfan+NAC group. The NAC group showed a non-significant change in the testicular ROS levels compared to that in the control group. Busulfan significantly reduced the intratesticular TAC and GSH levels (Fig. 4C, 4D; p<0.05). NAC administration restored TAC levels in the testes of busulfan-treated mice. Compared with the busulfan group, GSH levels after NAC administration did not increase significantly in busulfan+NAC group (Fig. 4D).

Expression levels of *Nrf2* and *HO-1*, were analyzed using RT-qPCR to understand the molecular antioxidant properties of NAC (Fig. 5). Busulfan significantly decreased *Nrf2* and *HO-1* mRNA expression levels in the testes of mice (p<0.05). Conversely,

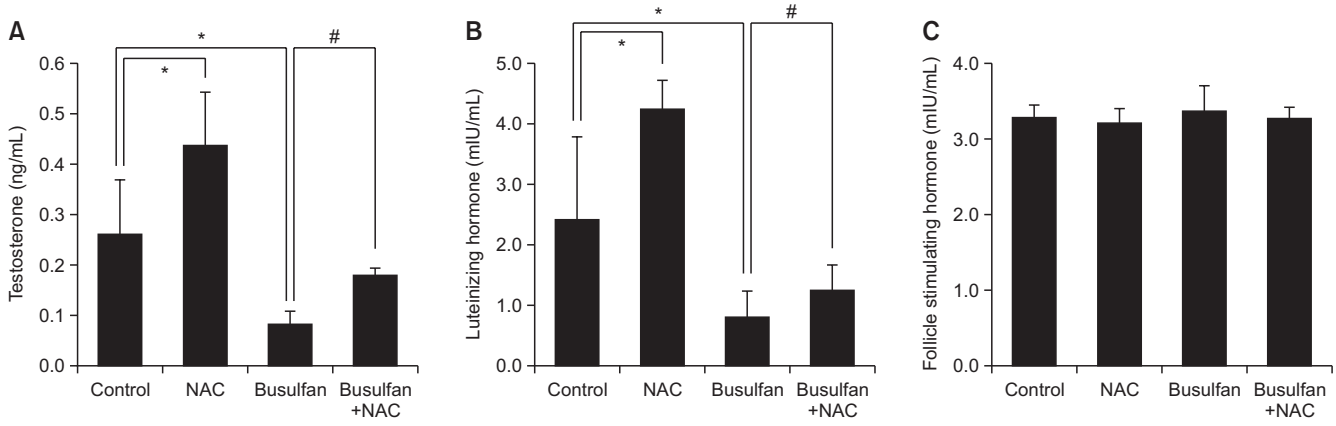

Fig. 3. Effects of busulfan and NAC on reproductive hormone levels. Comparison between the levels of (A) testosterone (ng/mL), (B) luteinizing hormone (mIU/mL), (C) follicle stimulating hormone (mIU/mL) among the four groups. *p<0.05 *versus* the Cont group, #p<0.05 *versus* the busulfan group. Busulfan: 1,4-butanediol dimethanesulfonate, Cont: control, NAC: N-acetyl-L-cysteine.

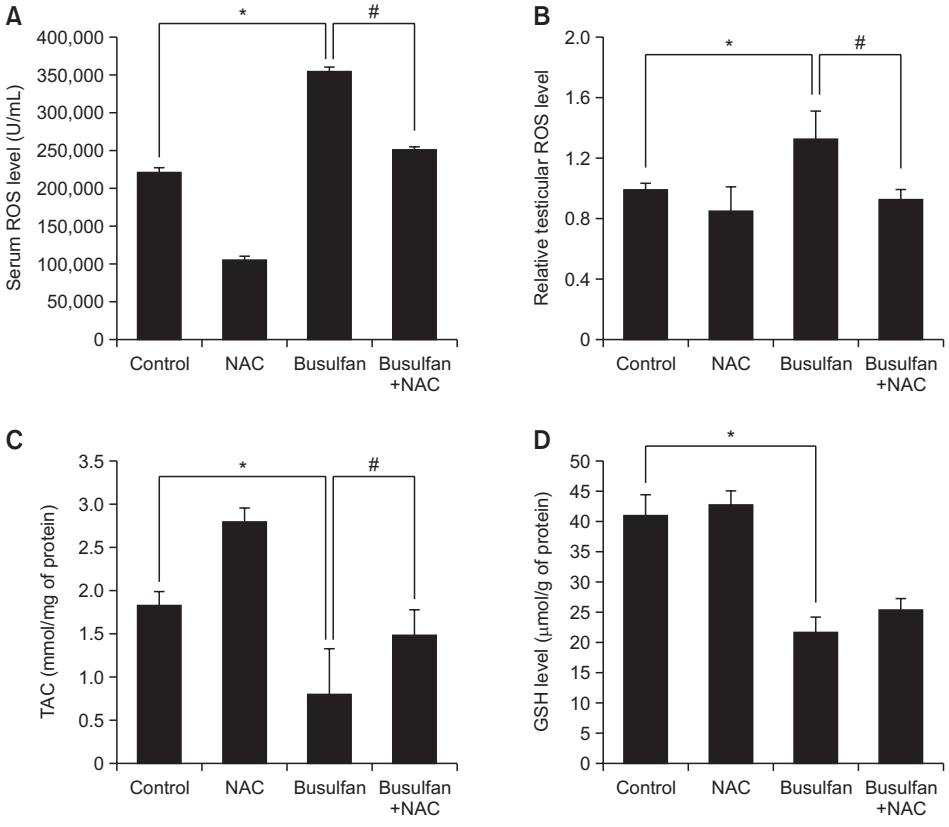

Fig. 4. Effects of busulfan and NAC on serum and testicular ROS, TAC, and GSH levels. Comparison between (A) semen ROS level (U/mL), (B) relative testicular ROS level, (C) TAC (mmol/mg of protein), (D) GSH content (μmol/g of protein) among the four groups. *p<0.05 *versus* the Cont group, #p<0.05 *versus* the busulfan group. Busulfan: 1,4-butanediol dimethanesulfonate, Cont: control, GSH: glutathione, NAC: N-acetyl-L-cysteine, ROS: reactive oxygen species, TAC: total antioxidant capacity.

NAC supplementation restored their expression in the busulfan+NAC group, thus indicating its potent antioxidant activity against busulfan-induced oxidative stress in the testes.

DISCUSSION

In the present study, the germinal epithelium of male mice was damaged by administering intraperitoneal busulfan injections to investigate the protective effect of NAC on impaired spermatogenesis. The damage was evident by decreased testicular weight, reduced sperm count and motility, disorganized morphological structure of the seminiferous tubule, and decreased testosterone levels, which might be due to elevated oxidative stress in the testes. This damage appeared to be attenuated by NAC administration. Interestingly, NAC upregulated *Nrf2*/*HO-1* mRNA expression, thereby at-

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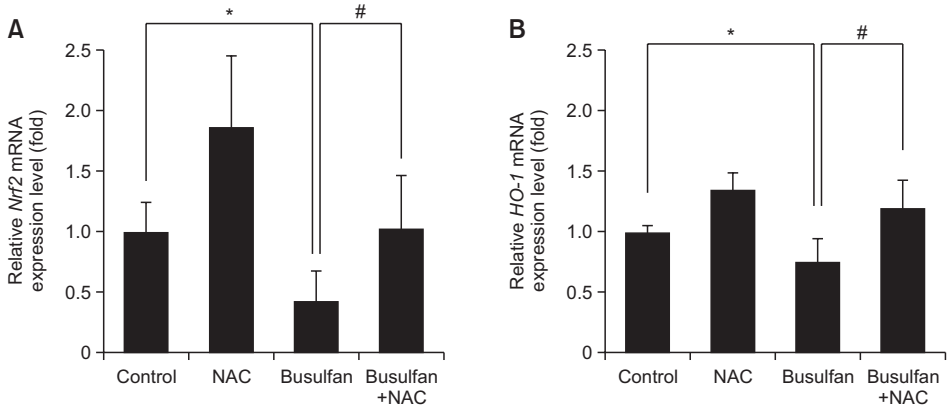

Fig. 5. Effects of busulfan and NAC on mRNA levels of *Nrf2* and *HO-1*. (A) Relative *Nrf2* mRNA expression and (B) Relative *HO-1* mRNA expression among the four groups. The fold-changes in mRNA expression levels of *Nrf2* and *HO-1* were quantified relative to the *GAPDH* mRNA levels. *p<0.05 *versus* the Cont group, #p<0.05 *versus* the busulfan group. Busulfan: 1,4-butanediol dimethanesulfonate, *Nrf2*: nuclear factor erythroid 2-related factor 2, *HO-1*: heme oxygenase-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, Cont: control, NAC: N-acetyl-L-cysteine.

tenuating oxidative stress during spermatogenesis.

Several chemotherapeutic agents pose a high risk of male infertility. These agents may alter the reproductive process and cause side effects, such as the disruption of not only spermatogonial stem cells (SSCs) but also niche cells (Sertoli, Leydig, and peritubular cells) [18]. Busulfan is widely used as a chemotherapeutic agent in chronic diseases, such as leukemia, before bone marrow transplantation, and induces temporary or permanent sterility in male patients. Recent reports have shown various adverse effects of busulfan injection on the male reproductive system, including oligospermia, azoospermia, testicular atrophy, altered serum levels of male reproductive hormones, increased apoptotic sperm, oxidative stress induction, and cytotoxicity [19]. Intraperitoneal administration of busulfan exerts toxic effects on the male reproductive system by preferentially destroying dividing cells in the testes [9,20]. Zohni et al [9] reported reduced SSCs after a single intraperitoneal injection of busulfan (15, 30, and 45 mg/kg). Further, the testicular weight decreased by 30% to 50% depending on the dosage, and SSC recovery was delayed at higher doses of busulfan. Bordbar et al [21] induced infertility in a mouse model by injecting a single dose of 5 mg/kg busulfan, resulting in reduced seminiferous tubule volume and decreased testosterone levels, sperm motility, and sperm count. Moreover, Wang et al [22] reported that a single intraperitoneal injection of 30 mg/kg busulfan is optimal to prepare recipient mice for spermatogonial transplantation. Jung et al [23] reported that a single intraperitoneal injection of 40 mg/kg busulfan caused testicular injury to aggravate the sperm parameters, testosterone levels, and eventually, the number of offspring. According to Dobrinski et al [24], an intraperitoneal injection of 50 mg/kg busulfan was lethal, probably because of its cytotoxic effects on the hematopoietic system. Although the optimal dose of busulfan for depleting testicular germ cells in animal models has not been defined, doses of 20 to 40 mg/kg of busulfan could deplete germ cells in the seminiferous tubules while maintaining the lowest possible death rate.

In our preliminary experiment, we examined the dose-dependent response of single doses of busulfan (10, 20, and 40 mg/kg). A single intraperitoneal injection of 10 mg/kg did not show considerable histological changes in the testes when compared with the control group. However, the mortality rate was 10% to 15% after a single intraperitoneal injection of 40 mg/kg busulfan in C57BL/6 mice and resulted in a significant decrease in sperm count and motility by day 35. To balance mortality and spermatogenesis dysfunction, we administered two intraperitoneal injections of 20 mg/kg busulfan at 3-h intervals making a total dose of 40 mg/kg. Our results are consistent with those of previous studies wherein mice received two injections of busulfan with rare spontaneous restoration of germ cells [9,11,23]. As spermatogenesis in mice requires approximately 35 days [25], we administered exogenous NAC in busulfan-treated mice for 35 days.

In this study, busulfan administration resulted in an ideal mouse model of azoospermia with no unexpected deaths. Busulfan treatment decreased testosterone levels and increased morphological abnormalities in

sperms. The serum testosterone and LH levels declined, whereas FSH levels were similar among all groups. This can be explained by the fact that the busulfan-induced decline in serum testosterone is associated with fewer LH receptors on Leydig cells, leading to the loss of functional Leydig cells, as validated by histological analysis (Fig. 2).

To elucidate the molecular mechanism underlying the antioxidant properties of NAC, we assessed ROS levels in the serum and testes. TAC and GSH levels represent a balance between free radicals and antioxidants [26]. GSH is sensitive to intracellular ROS; as one of the major endogenous antioxidants, GSH is readily neutralized by ROS [17]. In this study, we demonstrated that busulfan reduced TAC and GSH levels in the testes, suggesting that increased ROS levels decreased TAC and GSH in the busulfan-induced reproductive system dysfunction mouse model. The protective mechanism of NAC in testicular tissue could be explained by the following two mechanisms: First, NAC acts as a free radical scavenger in presence of cellular oxidative stress, and second, NAC attenuates GSH depletion during oxidative stress [27,28]. The protective effect of NAC against busulfan-induced reproductive system dysfunction was not appropriately reflected by GSH concentration, since detection of changes in testicular endogenous GSH concentrations after NAC oral administration needs time. Another possible explanation is that intraperitoneal busulfan injection and oral NAC administration have different absorption routes that involve GSH synthesis. In this study, NAC administration by oral gavage was not correlated with increased GSH levels, suggesting that NAC-mediated protection against busulfan-induced testis damage is most attributed to its potent ROS-scavenging effect.

Nrf2 has been reported as a key component in the antioxidant system that modulates the expression of genes related to endogenous antioxidant mechanisms [29]. Moreover, *Nrf2* downregulation is associated with testicular injury in animal models [17]. *Nrf2* attenuates oxidative stress by promoting the expression of the downstream anti-oxidative gene, *HO-1*. Several studies have reported NAC as a potent regulator of the Nrf/HO-1 pathway [29,30]. A recent study reported that NAC pretreatment restored H_2O_2-induced cell damage by activating the Nrf2/HO-1 pathway [16]. In the present study, we demonstrated that *Nrf2* and *HO-1* were significantly downregulated in the busulfan-treated groups compared with the control group, due to oxidative stress. NAC administration significantly increased *Nrf2* and *HO-1* mRNA expression, suggesting that NAC activates the Nrf2 pathway in normal testes tissue. Whether HO-1 is directly regulated by NAC or is dependent on Nrf2 remains unclear. To the best of our knowledge, this is the first report to demonstrate that NAC supplementation ameliorates abnormal spermatogenesis and testicular injury caused by the busulfan-induced impairment of spermatogenesis, potentially due to its antioxidative activity and ability to activate *via* Nrf2/HO-1 pathway.

Unexpectedly, NAC administration elevated the LH level in NAC group. Considering of the homeostatic mechanism of the hypothalamus–pituitary–gonadal (HPG) axis, decreased LH level was expected in contrast to the increased testosterone level. We hypothesized that NAC may affect upstream of the HPG hierarchy. This hypothesis, however, needs to be verified. FSH levels did not significantly vary, irrespective of the sperm counts, among the four groups. Thus, the sperm counts might not have decreased enough to affect FSH secretion from the hypothalamus. Alternatively, more time may be required to induce changes in FSH level. Further studies are needed to explore this issue.

Our study has several limitations. A more accurate infertility animal model, such as one demonstrating the oxidative stress-induced impairment of Nrf2 and related antioxidative enzymes, is needed. Moreover, the present study did not adopt different concentration gradients or time periods of NAC treatment. It is possible that employing a different dose or period of gavage would reinforce the effects of NAC due to its ability to enhance other antioxidant states.

CONCLUSIONS

NAC mitigates busulfan-induced spermatogenic damage by reducing oxidative stress in the testes. The beneficial effects of NAC potentially rely on the regulation of the *Nrf2*/*HO-1* signaling pathway. The present study provides new insights into busulfan-mediated impairment of spermatogenesis and provides evidence for the protective role of NAC in male fertility. Our findings suggests that NAC administration may be a promising strategy to ameliorate male reproductive dysfunction caused by chemotherapeutic agents, such

as busulfan.

Conflict of Interest

The authors have nothing to disclose.

Funding

This study was supported by Biomedical Research Institute Grant (20200007), Pusan National University Hospital.

Author Contribution

Conceptualization: HJP. Data curation: KHK, MJP. Formal analysis: KHK, MJP. Funding acquisition: KHK. Investigation: KHK, MJP, HJP. Methodology: HJP. Project administration: KHK, MJP. Resources: NCP, MJP. Software: KHK, HJP. Supervision: NCP, HJP. Validation: KHK, MJP. Visualization: KHK, MJP, HJP. Writing – original draft: KHK, MJP. Writing – review & editing: NCP, HJP.

Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

REFERENCES

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol 2015;13:37.
2. Luján S, Caroppo E, Niederberger C, Arce JC, Sadler-Riggleman I, Beck D, et al. Sperm DNA methylation epimutation biomarkers for male infertility and FSH therapeutic responsiveness. Sci Rep 2019;9:16786.
3. Ravitsky V, Kimmins S. The forgotten men: rising rates of male infertility urgently require new approaches for its prevention, diagnosis and treatment. Biol Reprod 2019;101:872-4.
4. Finelli R, Leisegang K, Kandil H, Agarwal A. Oxidative stress: a comprehensive review of biochemical, molecular, and genetic aspects in the pathogenesis and management of varicocele. World J Mens Health 2022;40:87-103.
5. Agarwal A, Leisegang K, Majzoub A, Henkel R, Finelli R, Panner Selvam MK, et al. Utility of antioxidants in the treatment of male infertility: clinical guidelines based on a systematic review and analysis of evidence. World J Mens Health 2021;39:233-90.
6. Jana K, Dutta A, Chakraborty P, Manna I, Firdaus SB, Bandyopadhyay D, et al. Alpha-lipoic acid and N-acetylcysteine protects intensive swimming exercise-mediated germ-cell depletion, pro-oxidant generation, and alteration of steroidogenesis in rat testis. Mol Reprod Dev 2014;81:833-50.
7. Payabvash S, Salmasi AH, Kiumehr S, Tavangar SM, Nourbakhsh B, Faghihi SH, et al. Salutary effects of N-acetylcysteine on apoptotic damage in a rat model of testicular torsion. Urol Int 2007;79:248-54.
8. Agarwal A, Finelli R, Selvam MKP, Leisegang K, Majzoub A, Tadros N, et al. A global survey of reproductive specialists to determine the clinical utility of oxidative stress testing and antioxidant use in male infertility. World J Mens Health 2021;39:470-88.
9. Zohni K, Zhang X, Tan SL, Chan P, Nagano MC. The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice. Hum Reprod 2012;27:44-53.
10. Qin Y, Liu L, He Y, Wang C, Liang M, Chen X, et al. Testicular busulfan injection in mice to prepare recipients for spermatogonial stem cell transplantation is safe and non-toxic. PLoS One 2016;11:e0148388.
11. Mirhoseini M, Saki G, Hemadi M, Khodadadi A, Mohammadi Asl J. Melatonin and testicular damage in busulfan treated mice. Iran Red Crescent Med J 2014;16:e14463.
12. Pérez-Crespo M, Pericuesta E, Pérez-Cerezales S, Arenas MI, Lobo MV, Díaz-Gil JJ, et al. Effect of liver growth factor on both testicular regeneration and recovery of spermatogenesis in busulfan-treated mice. Reprod Biol Endocrinol 2011;9:21.
13. Barekat F, Tavalaee M, Deemeh MR, Bahreinian M, Azadi L, Abbasi H, et al. A preliminary study: N-acetyl-L-cysteine improves semen quality following varicocelectomy. Int J Fertil Steril 2016;10:120-6.
14. Bodur A, Alver A, Kahraman C, Altay DU, İnce İ. Investigation of N-acetylcysteine on contralateral testis tissue injury by experimental testicular torsion: long-term effect. Am J Emerg Med 2016;34:1069-74.
15. Luo Q, Li Y, Huang C, Cheng D, Ma W, Xia Y, et al. Soy isoflavones improve the spermatogenic defects in diet-induced obesity rats through Nrf2/HO-1 pathway. Molecules 2019;24:2966.
16. Lee D, Kook SH, Ji H, Lee SA, Choi KC, Lee KY, et al. N-acetyl cysteine inhibits H2O2-mediated reduction in the mineralization of MC3T3-E1 cells by down-regulating Nrf2/HO-1 pathway. BMB Rep 2015;48:636-41.
17. Guo Y, Sun J, Li T, Zhang Q, Bu S, Wang Q, et al. Melatonin ameliorates restraint stress-induced oxidative stress and

apoptosis in testicular cells via NF-κB/iNOS and Nrf2/ HO-1 signaling pathway. Sci Rep 2017;7:9599.
18. Kadam P, Ntemou E, Baert Y, Van Laere S, Van Saen D, Goossens E. Co-transplantation of mesenchymal stem cells improves spermatogonial stem cell transplantation efficiency in mice. Stem Cell Res Ther 2018;9:317.
19. Nasimi P, Tabandeh MR, Roohi S. Busulfan-mediated oxidative stress and genotoxicity decrease in sperm of Satureja Khuzestanica essential oil-administered mice. Syst Biol Reprod Med 2018;64:348-57.
20. Xie Y, Deng CC, Ouyang B, Lv LY, Yao JH, Zhang C, et al. Establishing a nonlethal and efficient mouse model of male gonadotoxicity by intraperitoneal busulfan injection. Asian J Androl 2020;22:184-91.
21. Bordbar H, Esmaeilpour T, Dehghani F, Panjehshahin MR. Stereological study of the effect of ginger's alcoholic extract on the testis in busulfan-induced infertility in rats. Iran J Reprod Med 2013;11:467-72.
22. Wang DZ, Zhou XH, Yuan YL, Zheng XM. Optimal dose of busulfan for depleting testicular germ cells of recipient mice before spermatogonial transplantation. Asian J Androl 2010;12:263-70.
23. Jung SW, Kim HJ, Lee BH, Choi SH, Kim HS, Choi YK, et al. Effects of Korean red ginseng extract on busulfan-induced dysfunction of the male reproductive system. J Ginseng Res 2015;39:243-9.
24. Dobrinski I, Avarbock MR, Brinster RL. Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 2000;57:270-9.
25. Kanatsu-Shinohara M, Naoki H, Shinohara T. Nonrandom germline transmission of mouse spermatogonial stem cells. Dev Cell 2016;38:248-61.
26. Mirzaei Khorramabadi K, Reza Talebi A, Abbasi Sarcheshmeh A, Mirjalili A. Protective effect of vitamin E on oxidative stress and sperm apoptosis in diabetic Mice. Int J Reprod Biomed 2019;17:127-34.
27. Wang AL, Wang JP, Wang H, Chen YH, Zhao L, Wang LS, et al. A dual effect of N-acetylcysteine on acute ethanol-induced liver damage in mice. Hepatol Res 2006;34:199-206.
28. Arfsten D, Johnson E, Thitoff A, Jung A, Wilfong E, Lohrke S, et al. Impact of 30-day oral dosing with N-acetyl-L-cysteine on Sprague-Dawley rat physiology. Int J Toxicol 2004;23:239-47.
29. Cai Z, Lou Q, Wang F, Li E, Sun J, Fang H, et al. N-acetylcysteine protects against liver injure induced by carbon tetrachloride via activation of the Nrf2/HO-1 pathway. Int J Clin Exp Pathol 2015;8:8655-62.
30. Bae WJ, Ha US, Choi JB, Kim KS, Kim SJ, Cho HJ, et al. Protective effect of decursin extracted from Angelica gigas in male infertility via Nrf2/HO-1 signaling pathway. Oxid Med Cell Longev 2016;2016:5901098.