



Effects of ginsenoside as a phytoestrogen on DNA damage, and histopathological alteration in testicular tissue of mice exposed to busulfan

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Abstract

Introduction: Infertility is a global concern, with male factors playing a key role. Busulfan, a long-used alkylating chemotherapeutic agent, has notable reproductive toxicity despite its anticarcinogenic properties.

Objectives: This study investigates whether *Panax ginseng* extract rich in ginsenosides which can mitigate busulfan-induced testicular injury, DNA damage, and overall reproductive toxicity in mice.

Materials and Methods: In this experimental study, 50 mice were divided into five groups receiving varying regimens of busulfan and *P. ginseng* extract. Histopathologic changes and DNA damage (apoptosis) in the seminiferous tubules were evaluated by histomorphometric indices, and TUNEL assay, respectively.

Results: Significant pathological effects were observed in the busulfan-treated mice including collapsed seminiferous tubules, an increased number of damaged tubules (maturation arrest, eosinophilic degeneration and spermatid giant cells) and decreased tubule differentiated index (TDI) and spermiogenesis index (SI). Furthermore, *P. ginseng* administration improved all the mentioned conditions compares with busulfan alone group. We found, DNA damage and apoptotic cells were significantly decreased in *P. ginseng*-treated mouse testes compared to busulfan-received group. There were no significant difference between the control group with animals received dimethyl sulfoxide as a busulfan solvent.

Conclusion: Busulfan induced dramatic testicular injury, including impaired germ cell differentiation, histopathological alterations, reduced tubule differentiation and spermiogenesis indices, and increased apoptosis. Meanwhile, *P. ginseng* attenuated these effects, improving testicular histomorphometric indices and reducing TUNEL-positive apoptotic cells compared with the busulfan-only group. Further studies evaluating reproductive hormones, fertility, and the underlying mechanisms are needed to determine its potential reproductive benefits.



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Introduction

Infertility is a worldwide problem affecting approximately 15% of married couples. Although it is well known that male factor is the important cause of the infertility in 40-50% of the cases, the appropriate drugs for treating this condition have not yet been established effective treatments (1-3). The alkylating agent busulfan (myleran, 1, 4-butanediol methanesulfonate) has been used for more than 50 years as a chemotherapeutic agent. Despite its anticarcinogenic effects, the use of busulfan in clinical chemotherapy is limited because of a wide range of toxicities and gonadal dysfunction including azoospermia and oligospermia in human (2-5). It has been well understood that androgens and

gonadotropins play a pivotal role in spermatogenesis, function and development of male reproductive system (5). Estrogen is synthesized in male as well, suggesting a crucial role in development and function of male reproductive structures (4). The importance of estrogen in adult testis was highlighted by the phenotype of the aromatase knockout mouse (6,7).

Phytoestrogens are estrogenic plant-derived nonsteroidal substances that could be found in various herbal derivatives. In addition, herbal products contain a wide range of minerals, trace elements and phytochemicals, which are believed to protect cells from damage that can lead to chronic and acute disease (8-10).

Key point

Busulfan-induced testicular injury in mice has been attenuated by administration of *Panax ginseng*. These recoveries include increasing the number of spermatogonia, and restoring the tubule differentiation and spermiogenesis indices toward control levels. Ginseng treatment also markedly reduced busulfan-associated apoptosis in testicular tissue, supporting its potential protective role against chemotherapy-induced testicular injury.

Ginsenosides are phytoestrogens present in *Panax ginseng* and proposed to have estrogen-like effects akin to those of soy phytoestrogens. Steroid-like ginsenosides affect the estrogen receptor (11). Nevertheless, numerous studies demonstrated the principal pharmacological actions of *P. ginseng* on stress and nervous conditions (12), endocrine (13) and immune systems (14). In fact, *P. ginseng* assists in restoration of healthy condition either physically or mentally, through maintaining homeostasis and balance in the body.

Objectives

The aim of the present study was to investigate the *P. ginseng* extract and its main constituents (ginsenosides as a phytoestrogen) effect on busulfan-induced spermatogenesis disorder in mice.

Materials and Methods

In the present experimental animal study, 50 male BALB/c mice six-week-old were used, based on 10 per group accepted standard for this type of animal study and based on previous similar studies. One week before the test, the mice were accustomed to living in laboratory. The mice were kept under standard condition at 24 °C with 12 hours light and 12 hours dark and the mice were divided into 5 groups. First group defined as control (Ctrl), the second group the mice received only dimethyl sulfoxide (DMSO) (sigma D2650) as solvent of busulfan (B) (20 mg/kg). The third group mice received a single dose 20 mg/kg busulfan (Sigma USA) intraperitoneal (IP) (B); the fourth group after busulfan administration, the mice received 500 mg/kg *P.ginseng*, orally, every day for 40 days (G+B). Finally, in the fifth G/B group, *P. ginseng* was administered orally for two weeks before busulfan injection and continued daily

for 40 days thereafter (G/B), as pretreatment intervention (54 days total). Busulfan was initially dissolved in 50% DMSO and further was diluted with an equal volume of sterile distilled water to obtain a final concentration of 20 mg/kg. The busulfan solution was prepared immediately before use (Table 1).

The mice in all groups were sacrificed by cervical dislocation forty days after busulfan administration and testis was collected from mice for histopathological examination (tubule differentiated index [TDI] and spermiogenesis index [SI]) (15). The paraffin blocks of testicular tissue cut into 5 µm thick histological section, stained with hematoxylin and eosin (H&E) and TUNEL's method (detection of apoptotic cells) and examined under a light microscope. The staining kit did not color the nuclei of Sertoli cells (15).

Statistical analysis

Statistical analyses were performed using SPSS version 21.0. Data are expressed as mean ± standard deviation (SD). The normality of the residuals was assessed using the Shapiro–Wilk test, while Levene's test was conducted to assess the equality of variances. Differences among the five groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test when the assumptions for parametric analysis were satisfied. A two-sided *P* value < 0.05 was considered statistically significant.

Results

Histopathological and histomorphometric studies

In both G/B and G+B, spermatogonia numbers were significantly higher than group B. The most notable feature was the presence of occasional tubules lacking elongated spermatids (Figure 1A). Comparative analysis revealed significant reductions in TDI and SI in the B group relative to the control group. In contrast, TDI and SI in the G+B and G/B groups were not significantly different from controls (*P* < 0.0001; Figure 1B and C).

Histomorphometric evaluation the number of germ cells (spermatogonia) showed noticeable difference in testicular tissue in B group (31.66 ± 4.13) compared with control C group (44 ± 4.11) and DMSO administrated

Table 1. Experimental study groups and treatment regimens in BALB/c mice

Group	Treatment description	Route	Dose	Duration/Timing
Ctrl	Control group	-	-	-
DMSO	DMSO (solvent control)	-	-	-
B	Busulfan alone	Intraperitoneal (IP)	20 mg/kg	Single dose
G+B	Busulfan + <i>P. ginseng</i> (post-treatment)	Busulfan IP, then <i>P. ginseng</i> orally	Busulfan 20 mg/kg; <i>P. ginseng</i> 500 mg/kg	<i>P. ginseng</i> daily for 40 days after busulfan
G/B	<i>P. ginseng</i> pretreatment for 2 weeks, <i>P. ginseng</i> with busulfan concomitantly	<i>P. ginseng</i> was administered orally for 2 weeks before busulfan injection and continued daily for 40 days after busulfan administration.	Busulfan 20 mg/kg; <i>P. ginseng</i> 500 mg/kg	<i>P. ginseng</i> started two weeks after busulfan and continued daily for 40 days with busulfan

(sham) groups D (45.83 ± 3.29). However, some differences were apparent when comparing with busulfan and ginseng together administrated group in both synchronize G+B (38.36 ± 5.15) and protective administration of ginseng G/B (39.06 ± 4.29 ; [Figure 1A](#)).

Statistical analyses of the data showed that the number of germ cells in experimental group B was significantly lower than that in the control group ($P < 0.01$) but in the G+B and G/B group the number of spermatogonial cells was the approximately same of control group. The number of germ cells in the experimental groups G/B and G+B showed that the number of spermatogonia experiment significantly ($P < 0.01$) was higher than groups B. The most notable feature was the presence of occasional tubules lacking elongated spermatids.

To assess the diameter of seminiferous tubules, initially, 20 seminiferous tubules with small and large diameters in the cross section of each sample were measured and then the mean values were determined and the results in both groups was statistically evaluated and compared.

Our data showed that the mean diameters of seminiferous tubules in the control group were ($171.35 \pm 15.38 \mu\text{m}$), in sham group is ($173.81 \pm 17.78 \mu\text{m}$) and in the experimental group B is ($165.50 \pm 10.49 \mu\text{m}$). Our data also showed that the experimental groups G+B, with ($169.26 \pm 18.06 \mu\text{m}$) and group G/B, with ($172.14 \pm 20.09 \mu\text{m}$) both was similar

in terms of the numerical average with diameter of the control group. However, in a relative reduction in tubule diameter was observed in B group compared with the control group ([Figure 1B and D](#)).

The average size of the germinal epithelium was in the control group ($43.56 \pm 3.20 \mu\text{m}$), in the sham group ($43.38 \pm 4.42 \mu\text{m}$), in the experimental group B, ($40.36 \pm 6.19 \mu\text{m}$), in the experimental group G+B, ($42.21 \pm 4.86 \mu\text{m}$) and in the experimental group G/B, ($42.64 \pm 6.80 \mu\text{m}$). Our consideration revealed no statistically significant differences in germinal epithelium thickness among the five groups ($P > 0.05$). However, in the experimental group G+B, ($42.21 \pm 4.86 \mu\text{m}$) and the experimental group G/B, ($42.64 \pm 6.80 \mu\text{m}$), the size of the germinal epithelium were similar to the control group. In the B group, relative reduction in size of germinal epithelium was seen compared with the control group ([Figure 1](#)).

To determine the TDI, in $20\times$ microscopic fields from each sample, the tubules that contain three cell lines of differentiated spermatogenic cells more than spermatogonia type A, were compared with the remaining tubules. Data obtained for TDI in different groups is shown in ([Figure 1B and D](#)). The results showed that TDI were in the control group (80.25 ± 16.89), in sham group, (77.52 ± 17.78), in groups B, (27.28 ± 16.00), in

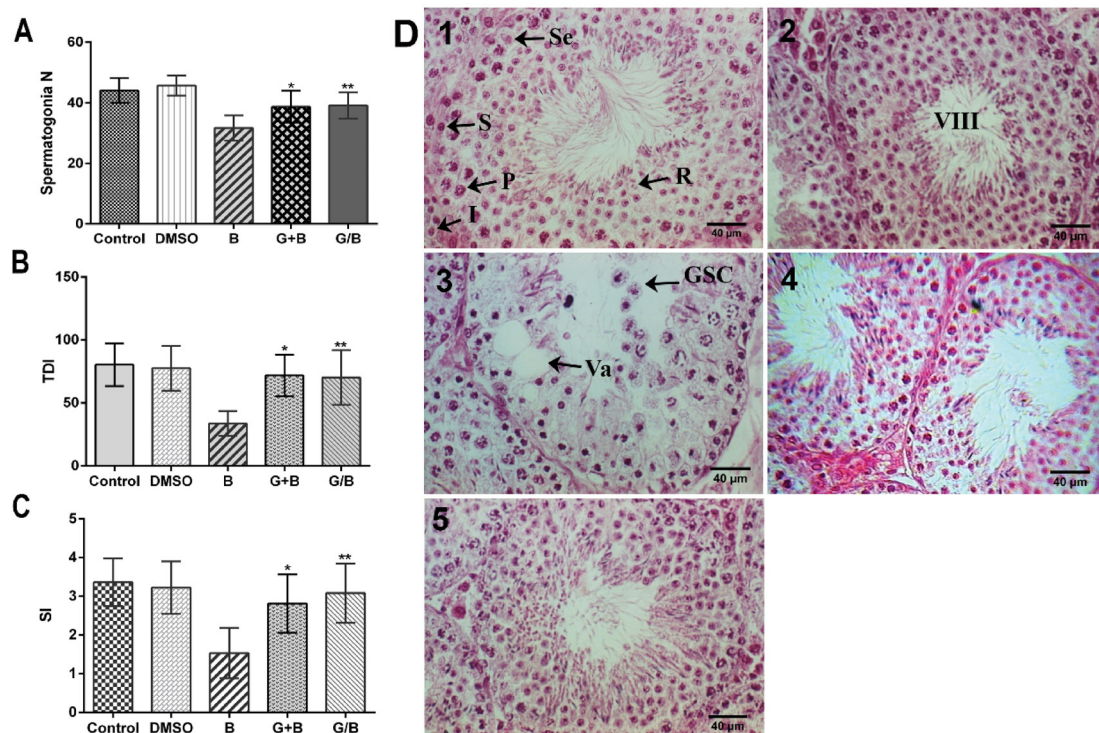


Figure 1. (A) Histomorphometric analysis of number of spermatogonia showed significant differences ($P < 0.0001$) between busulfan and *P. ginseng* receiving groups (G+B & G/B). (B & C) The SI and TDI evaluation indicated significant difference ($P < 0.0001$) between B and *P. ginseng* receiving groups. (D) the H&E staining showed Vacuoles (Va) and Giant spermatid cell (GSC) presence in the B group [3]. Note the germinal epithelium and its partial improvement, vacuoles, and various types of spermatogenic cells in (B+G) group, which received busulfan and *P. ginseng* simultaneously [4 & 5]. Rest of arrows show spermatogonia (S), Primary spermatocyte (P), Round spermatocyte (R), interstitial tissue (I), Sertoli (Se) and stage of Eight (VIII). Scaled bar = $40 \mu\text{m}$.

the experimental group G+B, (71.77 ± 16.52) and in the experimental group G/B, (70.23 ± 21.67). Comparison of these data showed that TDI in the experimental groups significantly decreased compared with the control group B ($P < 0.01$), but in the groups G+B and G/B, TDI was similar to the control group.

To determine the SI in $20\times$ magnification field for each sample, the number of seminiferous tubules containing sperm at stages 15 and 16 were determined in comparison to other seminiferous tubules. The mean SI are in the control group (3.3 ± 0.61), the sham group, (3.32 ± 0.78), the experimental group B, (1.19 ± 0.94), the test group G+B, (2.65 ± 0.95) and in the test group G/B, (2.86 ± 0.96). Comparison data showed that SI in the experimental group B with the other groups had significant differences ($P < 0.01$), the control group and the experimental G/B and G+B was similar to the control group and the experimental group D (Figure 1C and D).

Histochemical studies of apoptotic cells

Apoptotic cells were counted following TUNEL staining. Nuclei of apoptotic cells displayed brown staining, whereas nuclei of non-apoptotic cells were toluidine blue. Relative to the control, TUNEL-positive cells were significantly

elevated in the B group; however, apoptosis in the G+B group resembled that in the control group. The G/B group exhibited a significant reduction in TUNEL-positive cells compared with the B group ($P < 0.001$; Figure 2A-2E and F).

In detail, the number of apoptotic cells was counted after staining with TUNEL method. For this purpose, four-micron sections were utilized fixed by formaldehyde. The POD (peroxidase)-based diagnostic kit of cell death was conducted for the detection of apoptosis. The 3' end of DNA were labeled using these kits (Roche, Germany). The background staining was performed with toluidine blue. The nuclei of apoptotic cells using this kit came out brown, while the nucleus of normal cells was toluidine blue color (Figure 2A-2E and F).

The mean number of apoptotic cells (TUNEL positive) were in the control group 2.78 ± 0.44 , in the sham group (2.71 ± 0.75), the experimental group B, (5 ± 1.92), in the experimental group G+B, (3.83 ± 0.98) and in the test group G/B, (3.55 ± 1.36). Comparison data showed that TUNEL positive cells increased in the experimental group in comparison with control group significantly ($P < 0.01$) but apoptotic condition was similar in experimental group G+B cells to the control group. The statistical analysis of

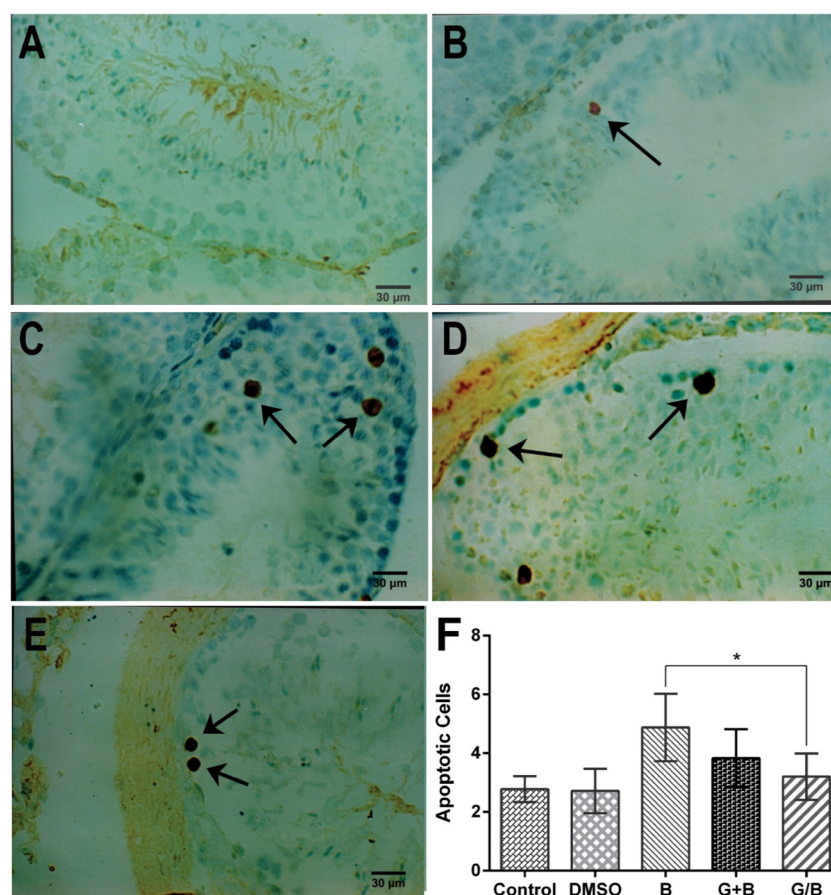


Fig. 2. TUNEL assay photomicrograph from seminiferous tubules; in control (A), sham (B), busulfan (C), G+B (D) and G/B (E) groups. Apoptotic cells are indicated by arrow. Histochemical analysis of number of apoptotic cells showed significant differences ($P < 0.0001$) between busulfan and *P. ginseng* receiving group (G/B). Scaled bar = 30 μ m.

the number of TUNEL positive cells in the experimental group G/B significantly ($P < 0.01$) lower than the experimental group B (Figure 2A-2E and F).

Discussion

Chemotherapeutic agents and related drugs can cause substantial side effects to multiple organs, including the reproductive system. Busulfan, as chemotherapeutic alkylating agent has toxic effects in sub-lethal doses especially in testicular tissue and germinal cells, which might lead to male infertility (16,17). Prolonged sterility has been observed in mice treated with 13 and 28 mg/kg busulfan, while recovery is limited at higher doses (35 mg/kg) (18,19). There is growing interest in reducing the side effects of chemotherapeutic drugs without diminishing anticancer efficacy. Spermatogonial cells are the most affected cells type by busulfan (20). Figure 1 shows busulfan toxicity effects were moderated aside which testicular tissue was improved by *P. ginseng* treatment. Saponins such as ginsenosides (Re, Rf, Rg1, Rg2 and Rh1) are involved in endocrine process and react at the hypothalamus-hypophyseal-gonadal axis. It seems damaged endocrine process by busulfan is reduced by *P. ginseng* and promote improvement in spermatogenesis in idiopathic sterility. A single dose of busulfan noticeably diminished TDI, SI and damaged germinal epithelium compared with control mice (Figure 1). In contrast, a recovery of the testicular tissue in all before mentioned indices was observed in busulfan group following the *P. ginseng* treatment. In addition, destruction and massive loss of spermatogonia were restored during spermatogenesis (Figure 1). Busulfan disrupts the germinal epithelium and vacuolized tissue between Sertoli cells, according to histomorphologic evaluations. In this regard, *P. ginseng* can affect Sertoli and spermatogenic cells and alter testis testosterone level. Previously Xie et al (17) reported that cytotoxicity of busulfan affected early germ cells, while *P. ginseng* treatment recovered its function. Several studies showed that *P. ginseng* administration reduced cytostatic side-effects of chemotherapy (21,22). Busulfan as an alkylating agent induces its effects through DNA strands alone or DNA and protein reaction (14,23). In this study, *P. ginseng* decreased apoptosis compared with busulfan groups (Figure 2) which implies that *P. ginseng* may play a role in DNA repair (24).

Spermatogenesis is essentially controlled by follicle stimulating hormone (FSH) and luteinizing hormone (LH). Remarkably, LH stimulates testosterone production by the testicular tissue, while FSH is involved in produce sperm of testicular tissue (25). In addition, *P. ginseng* increases LH, FSH levels and plasma testosterone and therefore stimulate sperm viability and motility (26,27). However, additional studies are required to elucidate the possible mechanism of *P. ginseng* on reproductive biology and sex hormone levels such as testosterone, FSH and LH. Regarding the numerous effects of Ginsenosides, long-

term use of standardized *P. ginseng* in a normal diet for cancer patients is suggested. Therefore, *P. ginseng* can be useful in recovery of azoospermia and oligospermia in chemotherapy candidates, precisely in which treated with busulfan or other alkylating agents.

Conclusion

In this mouse model, a single dose of busulfan caused clear testicular damage, including fewer spermatogonia, impaired spermatogenic differentiation, reduced tubule differentiation and spermiogenesis indices, abnormal tissue structure, and increased apoptosis within the seminiferous epithelium. Treatment with *P. ginseng*, whether given after busulfan exposure or started before busulfan administration and continued afterward, reduced these harmful effects. The ginseng-treated groups showed improved spermatogonial cell numbers and TDI and SI values that approached those of the control groups. They also had significantly fewer TUNEL-positive apoptotic cells than the busulfan-only group. Overall, these findings suggest that *P. ginseng* may help limit busulfan-induced structural and cellular damage in the testes and could have potential as a supportive approach for reducing chemotherapy-related testicular injury. It seems further research is needed to clarify the mechanisms of action and determine whether these protective effects translate into meaningful improvements in reproductive function.

Limitations of the study

Although histopathological and apoptotic changes were evaluated, assessment of FSH, LH, and testosterone levels, would have provided a more comprehensive evaluation of testicular function and fertility. These parameters will be considered in future studies.

Ethical issues

This study was extracted from MSc thesis of first author, in Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences. The ethical committee (Ethical code #86/2-2/4) approved the research protocol. All experimental protocols were conducted in compliance with the regulations of the research ethics committee of this university and the ethical guidelines of this university for the use of animals in research. Additionally, all animal procedures were conducted in accordance with the ARRIVE 2.0 guidelines for animal research. Moreover, all animal experiments were conducted in accordance with protocols approved by the United States National Institutes of Health (NIH, 1978). In addition, ethical issues (including plagiarism, data fabrication, and double publication) have been fully addressed by the authors.

Authors' contribution

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Supervision: Jafar Soleimanirad.

Validation: Abbas Ebrahimi-Kalan and Darush Mohammadnejad

Visualization: Abbas Ebrahimi-Kalan.

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Writing—review and editing: Abbas Ebrahimi-Kalan, Zeynab Aliyari-Serej and Jafar Soleimanirad.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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