The effects of ellagic acid on testicular tissue changes, sexual hormones, antioxidant system and Gene Expression of Caspase-9 and Bcl-2 in the relative sterility rat model following administration of busulfan: A stereological study

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Abstract

Background: Busulfan is an antineoplastic medication that is broadly utilized for cancer treatment. On the other hand, prescription of busulfan may cause sterility in male patients. Therefore, the decrease of this side effect is important. The aim of the present study was to evaluate the effects of ellagic acid on testicular tissue changes, sexual hormones, antioxidant defense system, and caspase-9 and Bcl2 gene expression in the relative sterility rat model following administration of busulfan.

Methods: Rats were randomly assigned to five groups of 13 animals per group. Sterility was induced by a single injection of busulfan (10 mg/kg) in groups 3, 4 and 5. The control group was not treated. The healthy group received 50mg/kg ellagic acid. Groups 4 and 5 (treatment group) received 10mg/kg and 50mg/kg ellagic acid, respectively for 48 days. Then, the serum levels of antioxidant enzymes, Malondialdehyde, sexual hormones and the testicular damage were evaluated.

Results: The significant increment of total antioxidant capacity and catalase was seen in both treatment groups (p<0.001). Also, both treatment groups significantly increased spermatogonia, round spermatids and long spermatids. Treatment with 50mg/kg ellagic acid significantly increased the testis weight, testis volume, seminiferous tubule volume, germinal epithelium volume, interstitial tissue volume, spermatocyte, Sertoli cells, and Leydig cells in the busulfan group(P<0.05). Additionally, 50mg/kg ellagic acid significantly increased the gene expression of Bcl2 and decreased caspase 9 in the busulfan group (P<0.05).

Conclusions: The consumption of ellagic acid may have beneficial effects on antioxidant defense system, sexual hormones abnormality and testicular tissue damage.

Keywords: Ellagic acid, Testicular tissue, Sterility, Rat
Background

Chemotherapy are associated with many changes in the reproductive system and among them, alkylating agents cause the most adverse effects on the gonad[1, 2]. Busulfan is one of the drugs that has alkylating properties[3, 4], and leads to enhanced oxidative stress, apoptosis, necrosis and finally decreases the activity of the gonads and endocrine abnormality[5, 6, 1].

According to studies, the fetus or neonate of rats that were born from pregnant mothers who have been exposed to this drug during pregnancy had gonadal dysfunction and reduced testicular germ cells and somatic cells[7, 8].

Administration of busulfan as a single dose in high doses (40-55 mg/kg body weight) in adult mice induces azoospermia [9, 10]. It has been shown that treatment with busulfan combined with cyclophosphamide leads to enhanced oxidative stress, apoptosis, necrosis and finally decreases the activity of the gonads and endocrine abnormality[5, 6, 1].

Biological compounds with antioxidant properties such as ellagic acid with antioxidant properties are able to protect the tissues against reactive oxygen species[11, 12]. increases the activity of the three antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase, which are altered in diseases caused by free radicals[13]. Studies have shown that ellagic acid has beneficial neuroprotective effects against ischemic brain injury[12]. Therefore, according to these findings, the aim of the present study was to investigate the effect of ellagic acid on the testicular tissue changes, sexual hormones (testosterone, LH, and FSH), antioxidant defense system, and caspase-9 and Bcl2 gene expression in the relative sterility rat model following administration of busulfan.
Methods

Experimental animals

Sixty-five healthy male Sprague-Dawley rats (2-3-month-old, 200-250 g) were purchased from the animal laboratory of Shiraz University of Medical Sciences. They were housed in standard cages, five per cage, with 12:12 hours light-dark cycles at temperature of 23±2°C.

Induction of relative sterility

The relative sterility rat model was induced by intraperitoneal administration of a single dose of 10 mg/kg busulfan (Pierre Fabre, France).

Experimental design

The rats were divided randomly into five groups of 13 rats per group.

Group 1, the control group, did not undergo any treatment and received only regular water and food.

Group 2, the healthy group (E.A 50), received 50 mg/kg b.w ellagic acid once per day for 48 days.

Group 3, the busulfan group (BUS), received single injection of 10 mg/kg busulfan.

Group 4, the treatment group (BUS+ E.A 10), received single dose of busulfan (10 mg/kg) + 10 mg/kg b.w ellagic acid once per day for 48 days.

Group 5, the treatment group (BUS+ E.A 50), received single dose of busulfan (10 mg/kg) + 50 mg/kg b.w ellagic acid once per day for 48 days.

Determination of biochemical parameters
At the end of the study, after 12 hr fasting and under anesthesia with ketamine (10%)/xylazine (2%) mixture (80/5 mg/kg) (Alfasan, Netherland), 5 ml blood was collected by cardiac puncture. Afterward, the animals were sacrificed by sodium thiopental intraperitoneally (100 mg/kg). The blood samples were centrifuged at 3500 rpm for 10 min to separate the serums and stored at -80°C prior to biochemical measurements. The sex hormones including testosterone, LH and FSH were assessed by specific hormone kits (Bioassay Technology laboratory, China) and ELISA. Catalase activity and total antioxidant levels (Zellbio Co, German) were measured using spectrophotometry and glutathione peroxidase (GPX) enzyme activity by the Biorex kit. Serum malondialdehyde (MDA) concentrations were determined by a calorimetric method [14].

**Stereological study**

At the end of the assay, the left testicle tissue was separated from all the surrounding tissues; then, the weight of the testicles was calculated by scales, and the primary volume was determined using the immersion technique. In this study, “Orientator method” was used to acquire Isotropic uniform random. In the next step, we put the slice testes in paraffin molds, so that the trocar fragment is placed in the middle of the other parts. Five and 20 μm thickness sections were then prepared. Tissue sections were dyed with Hematoxylin-Eosin (H&E) and Trichrome Masson [15]. After preparing the slides, the stereology software was used for analysis of the results of the present study [15].

The degree of shrinkage was assessed by the following formula based on the volume of the tissue [15]:

\[
\text{Volume Shrinkage}=1-(\text{Area after}/\text{Area before})^{1.5}
\]

Then, the following formula was used to calculate the germinal epithelium, the tubules, and the interstitial space volume ratio [15, 16].
\[ Vv(\text{structure}) = \frac{\sum_{i=1}^{n} p(\text{structure})}{\sum_{i=1}^{n} (\text{reference})} \]

Where the “\( \Sigma P_{\text{structure}} \)” was the number of points hitting the profiles of the germinal epithelium or tubules or interstitial tissue and “\( \Sigma P_{\text{references}} \)” was the number of points hitting the testis.

The method of calculation of numerical density and absolute number of cells [15-17] was as follows:

\[ Nv = \frac{\sum_{i=1}^{n} Q}{\sum_{i=1}^{n} P \times h \times \left( \frac{a}{f} \right) \times \frac{t}{BA}} \]

Where \( \Sigma Q \) was the number of the whole cells counted in all the disectors, \( h \) was the height of the optical dissector, \( a/f \) was the area of the counting frame, \( \Sigma p \) was the total number of the counted frames, \( BA \) was the microtome block advance to cut the block, and \( t \) was the mean of the final section thickness.

**RNA isolation and quantitative RT-PCR Gene expression levels**

The total RNA from the testicular tissue was isolated using the TRIzol reagent (Invitrogen), and the cDNA was synthesized following the manufacturer’s protocol, using 1 μg RNA (Prime Script™ RT reagent Kit, Takara). RT-PCR was done using a standard SYBR-green PCR kit (SYBR Premix EX Taq™ II, Takara), and the gene-specific PCR amplification was conducted using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The qRT-PCR reactions, including the no-template controls, were done in triplicate. Each PCR reaction was performed in a 20 μL solution containing 0.8 μL (10 μM) each of forward and reverse primers, 10 μL of Premix Ex Taq DNA polymerase, 0.4 μL of ROX reference dye, 6 μL of dH2O, and 2 μL of reverse transcription reaction products. The qRT-PCR primers used in the experiment are listed in Table 1. All experiments were performed in quadruplicate. Relative expression was determined by
the $2^{-\Delta \Delta Ct}$ method using the housekeeping gene, GAPDH, as an internal control, and the fold change was calculated by comparing with the corresponding control group. The PCR efficiency was 98% for each gene approximately. Primer sequences are demonstrated in Table 1.

Table 1. Gene specific-forward and reverse primer sequences

| Primer   | GC% | Length (bp) | TM  | Sequences (5'-3')             | PCR Product length |
|----------|-----|-------------|-----|------------------|-------------------|
| Cas9:F   | 55  | 20          | 60.39 | ACATCTTCAATGGGACCGGC | 85bp              |
| Cas9:R   | 52.38 | 21        | 60.20 | TCTTTCTGCTCACCACCACAG |                  |
| GAPDH:F  | 50  | 20          | 59.96 | AAAGAGATGCTGAACGGGCA  | 100bp             |
| GAPDH:R  | 47.62 | 21       | 59.79 | ACAAGGGAAAATTTGTCCACGA |                  |
| Bcl-2:F  | 50  | 20          | 57.78 | GGAGGATTGTGGCCTTTCTTT | 100bp             |
| Bcl-2:R  | 50  | 20          | 57.98 | GTCATCCACAGAGCGATGT |                  |

Statistical analysis

Statistical analysis was done using SPSS software, version 23 (SPSS Inc, Chicago IL). Data were expressed as mean ± SD. Normally distributed data were compared between the groups by one-way ANOVA test (and Tukey test as post hoc) and abnormal data were compared by the Kruskal-Wallis test (and Mann–Whitney U-test as post hoc) and related histograms were plotted using graphpad software. A P-value of <0.05 was considered statistically significant.

Result

Sexual hormones

Concentration of LH and FSH significantly increased in the BUS group compared to the healthy group (P<0.001). BUS also significantly decreased he testosterone level compared to the healthy group (P<0.001). In addition, LH and FSH concentration significantly decreased in the BUS+
E.A.50 group compared to the BUS group (P<0.001). The testosterone level significantly increased in the BUS+ E.A.50 group compared to the BUS group (P<0.001) (Table 2).

Table 2. Evaluation of LH, FSH, and testosterone concentrations in experimental groups

| Group        | LH(mIU/ml)    | FSH(mIU/ml)   | TES (nmol/L) |
|--------------|---------------|---------------|--------------|
| Con          | 21.68±2.93<sup>a</sup> | 24.34±2.21<sup>a</sup> | 96.32±14.78<sup>a</sup> |
| E.A.50       | 20.19±1.09<sup>a</sup> | 23.60±3.20<sup>a</sup> | 96.60±11.00<sup>a</sup> |
| BUS          | 36.98±1.28<sup>b</sup> | 40.58±4.68<sup>b</sup> | 42.73±9.30<sup>b</sup> |
| BUS+ E.A.10  | 29.04±5.89<sup>c</sup> | 33.08±3.89<sup>b</sup> | 60.69±3.92<sup>bc</sup> |
| BUS+ E.A.50  | 23.80±1.78<sup>ac</sup> | 25.46±3.79<sup>a</sup> | 76.99±11.16<sup>ac</sup> |

The results are presented as mean ± SD. There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (p < 0.05).

Antioxidant parameters

A significant decrease in TAC, catalase and GPX level was observed in the BUS group compared to the control group (P<0.001). BUS also significantly increased the MDA level compared to the healthy group (P<0.001) (Figure 1. A-D). BUS+ E.A.10 and BUS+ E.A.50 consumption significantly increased TAC and catalase (P<0.01) and significantly decreased MDA (P<0.001) compared to the BUS group. BUS+E.A.50 consumption also significantly increased the GPX (P=0.009) (Figure 1. A-D).

The mRNA expression levels of Bcl-2 and Caspase-9

BUS significantly decreased the gene expression of Bcl-2 level than the control group (P=0.004). Bcl-2 significantly increased in the BUS+ E.A.50 group compared to the BUS group (P=0.006).
Moreover, BUS significantly increased the gene expression of Caspase-9 level than the control group (P=0.002). Also, the BUS+ E.A.50 group significantly decreased the gene expression of caspase-9 level compared to the BUS group (P=0.002) (Figure 2. A-B).

*Stereological parameters*

Bus significantly decreased the body weight more than the control group, but it was prevented from reducing the body weight average in the group receiving salicylic acid compared to the busulfan group. Thus, BUS+ E.A.50 treatment significantly increased the body weight as compared to the BUS group. (Figure 3. A-L)

These parameters significantly decreased in the BUS group more than the control group (P<0.05). The testis weight, testis volume, seminiferous tubule volume, germinal epithelium volume, interstitial tissue volume, spermatocyte, sertoli cells, leydig cells of BUS+ E.A.50 and spermatogonia, round spermatids and long spermatids for the BUS+ E.A.10, and BUS+ E.A.50 were significantly increased compared to the BUS group (P<0.05) (Figure 3. A-L), (Figure 4. A1-E3).

**Discussion**

The present study evaluated the protective effects of ellagic acid on the testicular tissue changes and related complications in the relative sterility rat. The main findings of this study were that 50 mg/kg b.w ellagic acid improved the sexual hormones abnormality, antioxidant parameters, stereological and apoptotic gene expression changes in rats with the relative sterility. These beneficial effects of ellagic acid can be attributed to its potential anti-oxidative and anti-apoptotic properties.
In this study, administration of a single dose of 10mg/kg busulfan led to lower spermatogenesis maturation and major testicular parameters. It has been shown that busulfan destroys all the testicular germ cells, which is due to the alkylating property of busulfan [18]. Busulfan also stopped the spermatogonia division or their death and which could be related to decreased spermatozoon maturation [19], [1].

Additionally, busulfan induced ultrastructural and morphological changes not only in the germ cells, but also in the testicular somatic cells including Leydig cells and sertoli that could result in many changes in the testis and spermatogenesis. Spermatogenesis results from the effect of germ cells and somatic cells on each other [20]. In other words, it causes a reduction in spermatogenesis maturation, number of germ cells, and quantitative parameters of seminiferous. The current study also showed a decrease in the number of Leydig and sertoli cells. It was confirmed that chemotherapy had an indirect effect on the function of the Leydig cells, thereby causing functional disorders [21].

Moreover, busulfan has a potential role in lowering synthetic function of the Leydig cells. It has been shown that there is a direct link between the volume of Leydig cells, amount of endoplasmic reticulum, and secretory capacity of the Leydig cell; in other words, the more active Leydig cells had higher volumes [22]. Thus, the amount of androgen produced by Leydig cells is likely to be reduced. In addition, the study by Chatterjee demonstrated that serum testosterone level notably decreased in patients with congenital lymphoma and chemotherapy [23], which is consistent with our findings.

In the present study, the effect of ellagic acid administration alone and in combination with busulfan on spermatogenesis was investigated. Administration of 50 10mg / kg of ellagic acid for 48 days, along with Busulfan, reduced the effects of busulfan on spermatogenesis. Therefore, it
seems that the improvement in spermatogenesis is due to the antioxidant activity of Ellagic acid. The study carried out by Motlag et al. showed that ellagic acid could prevent the reduction of spermatogonia, Leydig and sertoli cells as well as diameter of spermatozoa tubules in the testicular tissue of the rats exposed to cadmium chloride [24], which is similar to our results.

Our major findings showed that ellagic acid potentially augmented the defense antioxidant enzymes such as catalase and GPx along with ameliorate MDA level. Ellagic acid is a natural phenol compound with a polyphenolic structure that has a DPPH-free radical scavenging activity and inhibited lipid peroxide production. It has been shown that ellagic acid enhanced the activity of three antioxidant enzymes, SOD, CAT and GPx, which are altered in various diseases involving free radical attack [25]. The cryprotective and antioxidative properties of ellagic acid have been previously reported in a reduction of the LPO and increment of the total glutathione (tGSH) and GPx levels in rats [26]. Other studies also reported anti-oxidative properties of ellagic acid against oxidative stress [25].

In the present study, ellagic acid also declined the serum FSH and LH levels. Because of the anti-proliferative properties of ellagic acid [27], it may inhibit the proliferation of spermatogonia cells and then cease their differentiation to spermatocyte. In the same line, Glode et al. demonstrated that any agent that could reduce the FSH and LH secretion and inhibit the pituitary-hypothalamic-gonadal axis had a role in inhibition of spermatogonia cells during chemotherapy. Glode et al. also showed that treatment with GnRHa gonadotropin releasing hormone analogues had a role in maintenance of spermatogenesis in rats [28].

Moreover, the study by Hosseini Ahar et al. showed that the use of busulfan can reduce the body weight and testicular weight in male rats [29], which is consistent with our results. Zheng Wei et al. also showed a direct relationship between the testis weight and germinal cells number [30].
Additionally, Bucci et al. demonstrated that busulfan led to chromosomal disorders and mutations in the sperm [18].

Chemotherapy drugs can induce apoptosis in the germ cells of the testicular tissue [31]. In the present study, single doses of 10 mg/kg busulfan induce apoptosis in the spermatogonia and primary spermatocytes [32] and may induce ultrastructural forms of apoptosis in the male reproductive system. Changes such as nucleation of the germ cells, especially spermatogonia, separation of the germ cells, presence of large spaces between the adjacent cells, cellular shrinkage, presence of vacuoles in the germ cells, and apoptotic bodies in the sertoli cells were often observed several days after injection of busulfan. This effect is associated with genotoxic and apoptotic roles of busulfan on healthy cells of patients who have undergone chemotherapy [31]. In this study, administration of ellagic acid could ameliorate the apoptotic condition which is induced by busulfan. EA has potential anti-apoptosis and anti-inflammatory effects [33]. These results are in accordance with those of Çeribasi et al., who reported the effects of ellagic acid on the ameliorating adriamycin-induced high LPO levels and apoptosis in rats [26]. It seems that ellagic acid with its phenolic structure may enhance the anti-oxidative capacity by protecting against the detrimental effects of free radicals [26].

It has been shown that Bcl-2 is a key factor in the inhibition of apoptosis; it is assumed that its over-expression can effectively prevent the apoptosis induced by hydrogen peroxide, free radicals and microbial contamination [33]. Thus, these findings suggest that ellagic acid exhibits anti-oxidant activity, through the down-regulation of caspase-9 and activation of Bcl-2. In line with these findings, our results demonstrated that ellagic acid improved the abnormal gene expression level of Bcl-2 and caspase-9.

**Conclusion**
The results demonstrated that the consumption of ellagic acid may have beneficial effects on antioxidant defense system, sexual hormones abnormality and testicular tissue damage. Therefore, ellagic acid therapy may be effective in the treatment of reproductive defects caused by chemotherapy.

**Abbreviation**

LH: Luteinizing Hormone; FSH: Follicle Stimulating Hormone; E.A: Ellagic Acid; BUS: Busulfan; GPX: Glutathione Peroxidase; MDA: Malondialdehyde; TAC: Total Antioxidant Capacity; H&E: Hematoxylin-Eosin; BA: Block Advance; GnRHa: Gonadotropin Releasing Hormone Analogues; SOD: Super Oxide Dismutase; ELISA: Enzyme-linked immunosorbent assays; PCR: Polymerase chain reaction; b.w: Body Weight; GPx: Glutathione Peroxidase; CAT: Catalase; DPPH: 2,2-diphenyl-1-picrylhydrazyl; tGSH: Total glutathione

**Declarations**

**Acknowledgment**

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**Authors’ contributions**

SN, SV, FK, FS, and MM contributed to the conception and design of the study. KRJ, AM, NJ, and MJK collected and analyzed the data. SN, FS, FK, SV and MM drafted the manuscript. FS, SN and MM critically revised the manuscript. All authors read and approved the final manuscript.

**Consent for publication**

Not applicable.

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**Availability of data and materials**
All data generated and analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

This study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences and performed in accordance with the Ethical Standards laid down in the 1964 Declaration of Helsinki and its later amendments.

**Competing interests**

The authors declare that they have no competing interests.

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Figure legend:

Figure 1. Comparison of TAC, MDA, catalase, and GPX levels in experimental groups
The results are presented as mean ± SD. There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (p < 0.05).

**Figure 2.** The effect of treatment with ellagic acid on mRNA expression. Levels of Bcl-2, and Caspase-9. Data are presented as mean ± SD. There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (p < 0.05).

**Figure 3.** Evaluation of the body weight and testis stereological parameters after 48 days of treatment. The column graph of the body weight(A), testis weight(B), the volumes of the testicle (C), seminiferous tubules (D), Germinal epithelium (E), and interstitial tissue (F), and the number of spermatogonia (G), spermatocytes (H), round spermatids (I), long spermatids (J), Sertoli (K), and Leydig (L) in the experimental groups. Data have been presented as mean ± SD. There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (p < 0.05).

**Figure 4.** Photomicrograph of the testicles’ histology in different groups (A1, A2, A3): the control rats with normal structure seminiferous tubules, interstitial tissue, and the number of sexual lineage cells. (B1, B2, B3): the healthy group (E.A 50), received 50 mg/kg ellagic acid with normal testis histopathological features. (C1, C2, C3): the busulfan group: the seminiferous tubules appeared atrophic, the germinal epithelium height was destroyed, and many testicular cells were lost. (D1, D2, D3): azoospermia rats treated with ellagic acid 10 mg/kg showed fewer pathological changes and improved testis architecture. (E1, E2, E3): the sexual cell population significantly ameliorated in the rats treated with ellagic acid 50 mg/kg compared to those that received busulfan. A-E: Trichrome Masson staining with magnification at ×40, ×100, ×400.