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Protective effect of loboob (a Persian traditional remedy) on sexual hormones, antioxidant activities and stereological changes of testis tissue on busulfan induced oligospermia in rats

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Loboob as a traditional drug in Iranis known for its beneficial effects on busulfan-induced oligospermia. In this experimental study, protective effects of loboob (a Persian traditional remedy) on sexual hormones, antioxidant levels and stereological changes of testis tissue were evaluated in an oligospermia rat model induced by busulfan. Fifty male rats were randomly divided into five different groups: control, received no treatments; and the other groups administrated with a single dose of busulfan (10 mg/kg body weight). After 30 days, these groups were treated with 0, 35, 70 or 140 mg/kg/day of loboob for 60 days. Blood samples were collected for hormone and antioxidant enzyme assays. Unbiased stereology was performed on testis tissues to evaluate the volume of different parts of the testis and the number of various testis cells. Data indicated that FSH, LH and MDA were increased, and testosterone, catalase, SOD were decreased in the busulfan group, while treatment with loboob at 70 and 140 mg/kg significantly improved these parameters (P <0.05). Treatment with 70 and 140 mg/kg of loboob ameliorated the germinal epithelium volume, types A and B spermatogonia, spermatocytes, elongated and round spermatids, and Sertoli cells in the seminiferous tubules (P <0.05). High concentration of loboob also improved testis weight and volume, and leydig cell number (P <0.05). Thus, loboob is more effective for the recovery of seminiferous tubules and their cells than for the interstitial tissue. Loboob with various antioxidants, minerals and vitamins could overcome the side effects of busulfan.

Keywords: Catalase, Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Seminiferous tubules, Superoxide dismutase (SOD), Testosterone

Infertility has serious emotional and social consequences with a global prevalence of 10-15% in couples. Male infertility is related to many factors such as genital tract infection, genetic disorders, aging, environmental factors and medication[1]. Infertility is the most impressive effect of chemotherapy drugs such as Busulfan, 1,4-bis [methanesulfonyl-y] butane, in cancer patients[2]. Unbiased stereological principles and systematic sampling technique shows that most of testicular parameters like testis weight and volume, tubules volume density and interstitial tissue decreases after busulfan treatment in a dose-dependent manner[3]. Busulfan can also increase reactive oxygen species (ROS), which damages sperm function and spermatogenesis[4]. Furthermore, Busulfan's damaging effects on the testicular tissue have been proven to alter the levels of gonadotropin. In other words, it relates to the feedback effect that gonadal injury has on the release of gonadotropins, which raises Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) levels[5]. Thus, regarding busulfan electively destroying the spermatogonia differentiation in the testes, it is widely used for inducing the oligospermia in rats as an infertile animal model[6].

Nowadays, with respect of successful treatment of patients with malignant diseases, life expectancy has

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improved. But infertility after chemotherapy has received less attention. A potent strategy to diminish the side effects of chemotherapy drugs on the reproductive system and preventing infertility is herbal therapeutics. Because of lower side effects, there is an increasing trend towards the use of herbal remedies instead of chemical drugs[7,8]. Various research has been done to ameliorate the sperm parameters and childbearing potential using herbal remedies such as Safflower (*Carthamus tinctorius* L.) and Date palm (*Phoenix dactylifera* L.) pollen and gemmule extract [9-10].

Loboob is a multicompound Persian traditional herbal formulation for male infertility (locally called Laboob Saghir)[11]. However, there is no report in the literature about the effects of the combination of loboob ingredients on male infertility induced by busulfan, an alkylating antineoplastic agent. Hence, the present study, we have made an attempt to investigate the protective effect of loboob on sexual hormones, antioxidant enzymes and testes characteristics and spermatogenesis in oligospermia rats treated with busulfan. The possible impacts of loboob were assessed on the length and diameter of seminiferous tubules, testis volume and weight in rats by unbiased stereological methods.

Material and Methods

Animal and ethics

Fifty Wistar free feeding rats weighing 200-250 g, aged between 2 and 3 months, were kept under 12:12 h light: dark cycle, approximately 20-22°C with and relative humidity between 40 and 60%. All the animal experiment protocols were done based on the guidelines of the Animal Ethics Committee of the Shiraz University of Medical Sciences, Shiraz, Iran.

Experimental design

The rats were habituated to laboratory conditions for one week before the experiment started and were randomly divided into five different groups with ten animals each. Group I (Control): received no treatments; and the next four experimental groups (Gr. II-V) were treated with busulfan (Busilvex®; Pierre Fabre Medicament Boulogne, France) (intraperitoneally injected, 10 mg/kg body wt., once), 30 days after busulfan injection, and were given orally 0, 35, 70 or 140 mg/kg/day of loboob for 60 days. Thus, Gr. II (BUS) received 10 mg/kg of busulfan; Gr. III (BUS+Loboob35) received busulfan (10 mg/kg ip) + loboob (35 mg/kg/day, low dosage orally); Gr. IV (BUS+Loboob70) received busulfan (10 mg/kg ip) + loboob (70 mg/kg/day, moderate dosage orally); and Gr. V (BUS+Loboob140) received busulfan (10 mg/kg ip) + loboob (140 mg/kg/day, high dosage orally).

Preparation of loboob

A multicompound of loboob was chosen from traditional Persian medicine. Any ingredient with reported adverse effects on fertility was excluded from the original product; therefore, the formulation was prepared with slight changes. Table 1 presents scientific names of ingredients, their families, used parts, and their proportion in formulation.

All parts except sugar and honey were washed and dried completely. They were powdered using an electrical grinder, then sieved with a stainless-steel 30-mesh sieve. Powdered ingredients and sugar were added to warm dewaxed honey and homogenized with continues stirring for 30 min. The final product was ready to use after 40 days of storage in an air-tight glass container.

Hormone assays

The animals were sacrificed by anaesthetizing under 10% ketamine and 2% xylazine (Alfasan, Nederland). Blood samples were collected from cardiac puncture from each of the rats. Then, serum was provided by centrifugation at 700 g for 15 min.

Table 1 — Loboob ingredients, their families, and used parts

Ingredient	Family	Used part	%
Allium cepa L.	Amaryllidaceae	Seed	0.8
Alpinia officinarum Hance	Zingiberaceae	Root	0.8
Boswellia carterii Birdw.	Burseraceae	Oleogumresin	0.8
Brassica rapa L.	Brassicaceae	Seed	1.6
Cinnamomum zeylanicum Blume	Lauraceae	Bark	0.8
Cocos nucifera L.	Arecaceae	Fruit	1.6
Corylus avellana L.	Betulaceae	Kernel	1.6
Juglans regia L.	Juglandaceae	Kernel	1.6
Lepidium perfoliatum L.	Brassicaceae	Seed	1.6
Mentha piperita L.	Lamiaceae	Leaf	1.6
Piper cubeba L.F.	Piperaceae	Fruit	0.8
Piper nigrum L.	Piperaceae	Fruit	0.8
Pistacia vera L.	Anacardiaceae	Kernel	1.6
Prunus dulcis Mill.	Rosaceae	Kernel	1.6
Sesamun indicum L.	Pedaliaceae	Seed	1.6
Sugar	-	-	1.6
Syzygium aromaticum (L.) Merr.& L.M.Perry	Myrtaceae	Bud	0.8
Withania somnifera (L.) Dunal	Solanaceae	Root	0.8
Zingiber officinale Roscoe	Zingiberaceae	Rhizome	0.8
Honey	-	-	76.8

LH (Catalog No. E0179Ra), FSH (Catalog No. E0182Ra), and testosterone (Catalog No. E0930Ra) were measured by ELISA kits (Bioassay Technology laboratory, China).

Catalase assay

Catalase was determined spectrophotometrically (S1200, Unico, USA) based on the level of degraded H_2O_2. Briefly, 66.7 mM phosphate buffer (pH=7.0), 20 µL serum, and 355 µL distilled water were added to 75 µL of 120 mM H_2O_2. The consumption of H_2O_2 was followed at 240 nm at 25℃ for 3 min. Catalase activity was reported as µmol H_2O_2 consumed/min per mg serum protein using a molar extinction coefficient of 43.6 l/mol per cm for H_2O_2[12].

Glutathione peroxidase assay

The glutathione peroxidase (GPx) activity in serum was measured by continuous monitoring of the regeneration of reduced (GSH) from oxidized glutathione (GSSG) by activity of Glutathione Reductase (GR) and NADPH according to the method proposed by Fecondo and Augusteyn[13]. Firstly, 50 µL of serum and 100 µL of 2.5 mM GSH were added to a 750 µL reaction mixture containing 0.3 mM EDTA, 0.1 mM NADPH, 0.5 U GR, and 0.5 mM Na2N3 in 50 mM phosphate buffer (pH=7.2). Distilled water was used instead of GSH in control tubes. Then, 100 µL of 0.4 mM tert-butyl hydroperoxide was added to each experiment and control tube. Decrease in NADPH absorbance was measured at 340 nm at 37°C for 3 min. The decrease in absorbance reflecting the oxidation of NADPH, which is directly proportional to the GPX activity in the serum sample, was monitored at 340 nm (S1200, Unico, USA).

Malondialdehyde assay

The lipid peroxidation from malondialdehyde **(**MDA) formation was measured by the thiobarbituric acid assay (TBA). Briefly, blood serum was added to 2 mL TBA reagent consisting TBA (0.375%), trichloroacetic acid (15%) and 0.25 M HCl. Then, it was boiled for 15 min, cooled and centrifuged at 700 g for 15 min using a refrigerated centrifuge (4°C). The supernatant was collected and absorbance was measured spectrophotometrically at 532 nm (S1200, Unico, USA). TBARS concentration was measured by 1,1,3,3-tetramethoxypropane as a standard[14].

Superoxide dismutase assay

Superoxide dismutase (SOD) level was evaluated by Rat Superoxide Dismutase, Cu-Zn, SOD3 ELISA Kit (Cat.No E1185Ra) based on manufacture's instruction. Within 10 min of adding the last solution, the optical density was measured for each well using a microplate reader set to 450 nm (BioTek ELx800, USA).

Lactate dehydrogenase assay

The serum level of lactate dehydrogenase (LDH) was measured via diagnostic colorimetric kits (BioSystem, Spain) using the prestige instrument (Hitachi, Japan).

Stereological analysis

The animals were anesthetized by 10% ketamine and 2% xylazine (Alfasan, Netherland) on day 90 of the experiment and sacrificed. Then the testis was dissected and the weight was measured. The primary volume (V_1) of testis was determined by the Archimedes principle[3] and the testis tissue was fixed at 4% formalin. Isotropic Uniform Random sections (IUR) were required to achieve systematic random sampling to estimate the stereological parameters and shrinkages in testicular tissue during the tissue processing and staining. The isotropic uniform random sections were obtained by the orientator method. In brief, the testis was randomly put on the φ-clock with nine parts each equally. A random number was selected and cut along to the chosen number (Fig. 1 A-C). The cut surface of the first piece of testis derived from a φ-clock was placed along with the 0-0 axis of the θ clock and parallel cuts were randomly made along the chosen number. The cut surface of the second piece derived from the φ clock was vertically placed on the θ clock and was cut parallel based on a randomly selected number. Finally, 9-12 slabs were collected from each testis (Fig. 1 D and E). The slab of testis was punched circularly by a trocar (Fig. 1F).

The slabs were then embedded in paraffin and 5 and 25 µm sections were obtained and Hematoxylin-Eosin and Heidenhain's azan staining were done, respectively. The circular pieces' diameters were calculated and the degree of shrinkage was measured with the following formula:

$$\text{Degree of shrinkage} = 1 - \left(\frac{AA}{AB}\right)^{1.5}$$

where ''AA'' and ''AB'' are the area of the circular piece of tissue, post and prior to the processing and staining, respectively. The final volume of testis was measured using:

$$V_{final} = V_1 \times (1 - \text{Degree of shrinkage})$$

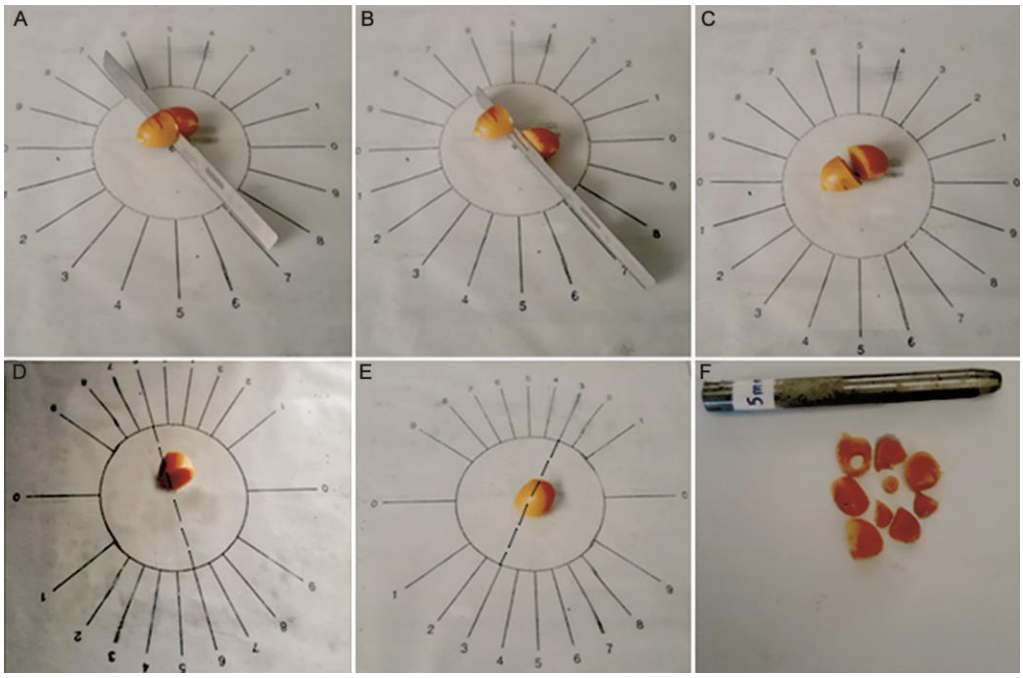

Fig. 1 — Orientator method was applied to obtain the isotropic uniform random sections. (A-C) φ-clock; (D & E) θ clock; and (F) a trocar were used in Orientator method.

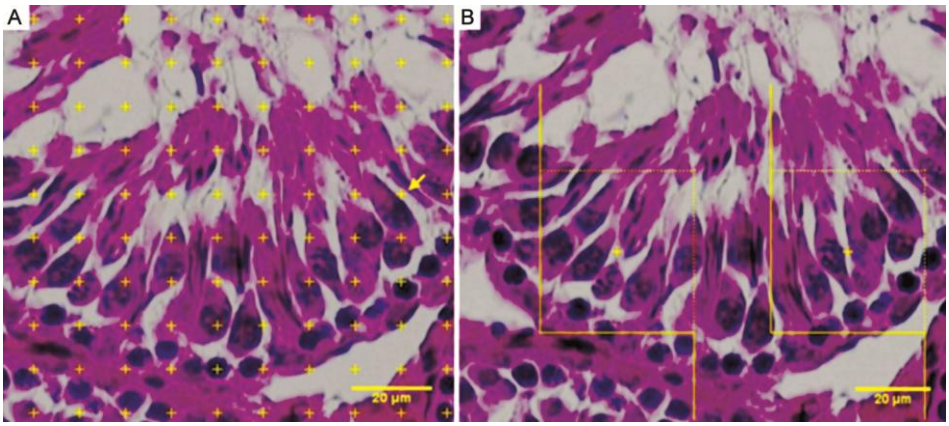

Fig. 2 — Estimation of (A) volume density; and (B) numerical density of cells using the point counting and optical dissector method, respectively.

The sections were analyzed with a video microscopy system (E-200, Nikon, Japan) connected to a video camera (TK-C1380E, JVC, Japan). The random microscopic field samples were designed with stereology software.

Estimation of germinal epithelium volume and seminiferous tubule

Randomly, five to seven fields per 5-µm section were estimated. The total points superimposed on the interstitial tissue were recorded (Fig. 2A).

$$Vv_{(structure/testis)} = \frac{\sum P_{structure}}{\sum P_{total}}$$

where the "$\Sigma P_{structure}$" was the number of points hitting the profiles of the tubules or interstitial tissue and "ΣP_{total}" was the number of points hitting the testis. The total volume of the parameters was calculated by multiplying the density by the final testis volume:

$$V_{structure} = Vv_{(structure/testis)} \times V_{final}$$

Estimation of the total number of spermatogonia, spermatids, and Sertoli cells

The total number of spermatogonia (type A and B), spermatocytes, and spermatids (round and long) and Sertoli cells was measured in a 25 µm thick section using the disector method (Fig. 2B). The number of

the germinal epithelium cells in the unit volume (or numerical density (Nv)) was estimated with the number of counted cells coming into focus, using the following formula:

$$Vv_{(structure/testis)} = \frac{\sum P_{structure}}{\sum P_{total}}$$

where "ΣQ" was the number of the entire cells counted in all the dissectors, "∑A" was the total area of the unbiased counting frame in all fields, and "h" was the height of the dissector, "t" was the mean of the final section thickness, and "BA" was the microtome block advance to cut the block.

Finally, the total number of the testis cells was estimated by the following formula:

$$N\ (testis\ cell) = Nv \times V_{final}$$

Statistical analysis

Sexual hormones, antioxidant activities and body weight (parametric data) were analyzed by one-way ANOVA followed by post-hoc Tukey's test. Stereological changes and weight and volume of the testis (non-parametric data) were analyzed by the Kruskal-Wallis test and then compared by Dunn's multiple comparison test using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Data were presented as mean± standard deviation (SD). Probability values less than 0.05 were considered statistically significant.

Results

Body weight

The results showed a significant decrease in the mean of body weight in the busulfan groups in comparison to the control group (P <0.01). However, no significant difference was observed among busulfan groups (Fig. 3).

Sexual hormones

The results indicated a significant increase in LH and FSH levels in the BUS group in comparison to the control group. In addition, the testosterone level was decreased significantly in the BUS group compared to the control group (P <0.05). However, the administration of 70 and 140 mg/kg loboob significantly decreased the LH level compared to the BUS group (P <0.05). The administration of 140 mg/kg loboob also significantly decreased the FSH level compared to the BUS group (P <0.05). Besides, the administration of 70 (Gr. IV) and 140 mg/kg (Gr. V) loboob significantly increased the testosterone level compared to the BUS group (P <0.05, Table 2).

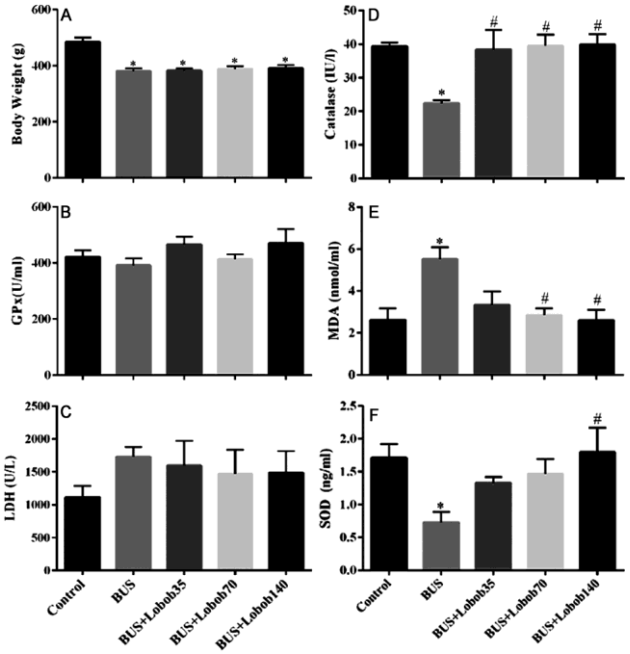

Fig. 3 — The effect of loboob treatment on body weight, and serum level of catalase, glutathione peroxidase (GPx), malondialdehyde (MDA), lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activities. * and # show significant difference with control and busulfan (BUS) groups at P <0.05, respectively.

Table 2 — Levels of LH, FSH and testosterone in treatment groups

Group	LH (IU/ml)	FSH (IU/mL)	Testosterone (nmol/L)
control	25.25±1.31†	27.76±1.42†	96.98±5.03†
BUS	34.27±1.85	34.83±1.03	64.65±2.24
BUS+Lobob35	30.62±1.02	31.55±1.70	85.18±7.43
BUS+Lobob70	28.46±1.07*	30.07±1.01	89.00±7.13*
BUS+Lobob140	25.28±1.25*	27.80±3.28*	92.58±6.37*

[†P <0.05, control *vs*. busulfan. *P <0.05, busulfan *vs*. busulfan + Lobob 35 or 70 or 140 groups]

Antioxidant enzymes

The results showed a significant decline in catalase in the BUS group (Gr. II) in comparison to the control group. However, a significant increase was observed in catalase in the BUS+Loboob 35 (Gr. III), 70 (Gr. IV) and 140 (Gr. V) compared to the Gr. II, and caused them to reach the level of the control group (Gr. I).

The mean level of MDA was significantly increased in the BUS group in comparison to the control group. Moreover, the MDA level was significantly decreased in the BUS+Loboob70 (Gr. IV) and 140 (Gr. V) compared to the BUS (Gr. II). Nevertheless, no significant difference was detected between the groups III, IV & V and Gr. I (control).

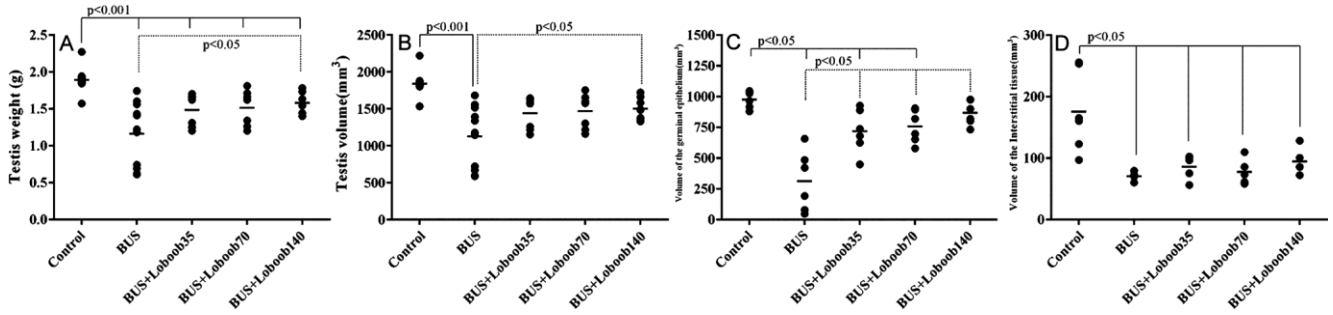

Fig. 4 — The effect of loboob treatment on (A & B) testis weight; and (C & D) volume of germinal epithelium and interstitial tissue by the stereological method in rats.

The mean level of SOD was significantly decreased in the BUS group (Gr. II) compared to the control group. Furthermore, the mean level of SOD was significantly increased in the BUS+Loboob140 (Gr. V) compared to the BUS (Gr. II). However, the results revealed no significant difference between the BUS+Loboob 35, 70 and 140 groups and the control group. The results also indicated no significant difference among the study groups in terms of the mean levels of GPX and LDH (Fig. 3).

Weight and volume of the testis, and volume of germinal epithelium and interstitial tissue

The results showed that the mean testis weight and volume were decreased in the BUS group compared to the control group (P <0.001). Also, the mean testis weight and volume of the BUS+Loboob140 was significantly increased in compared with the BUS group (P <0.05; Fig. 4 A & B). Also, the mean testis weight of loboob treatment groups was significantly decreased compared with the control group (P <0.001; Fig. 4B).

The results revealed that the germinal epithelium volume in the BUS group was significantly reduced in comparison with the control group (P <0.01). Also, a significant increase in the germinal epithelium volume was observed in loboob treatment groups in comparison with the BUS group (P <0.05). The germinal epithelium volume of BUS+Loboob35 and 70 groups was decreased in comparison with the control group (P <0.05; Fig. 4C). Data showed the interstitial tissue volume in all treatment groups was decreased in comparison with the control group (P <0.05; Fig. 4D).

Number of types A and B spermatogonia, spermatocytes, round and elongated spermatids, and Sertoli and leydig cells

The number of spermatogonia A and B in the BUS and BUS+Loboob35 groups was significantly decreased in comparison with the control groups,

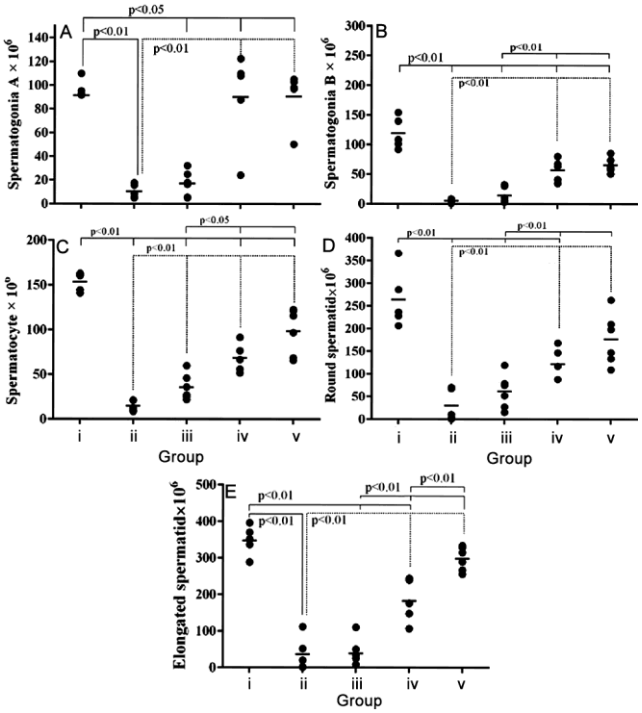

Fig. 5 — Effect of loboob treatment on the number of (A) a and b types; (B) spermatogonia; (C) spermatocyte; (D) round; and (E) elongated spermatid by the stereological method in rats.

BUS+Loboob70 and the 140 group (P <0.05). The mean number of spermatogonia A in BUS+Loboob 35 (Gr. III) and the mean number of spermatogonia B in BUS+Loboob35, 70 and 140 were significantly reduced when compared to the control group (P <0.01; Fig. 5 A & B).

The mean number of spermatocytes in the BUS significantly decreased in comparison with the control and loboob treatment groups (P <0.01). Also, the spermatocyte in all treatment groups was significantly decreased in comparison with the control group (P <0.01). The data showed BUS+Loboob70 and 140 significantly increased in comparison with the BUS+Loboob35 group (P <0.05; Fig. 5C).

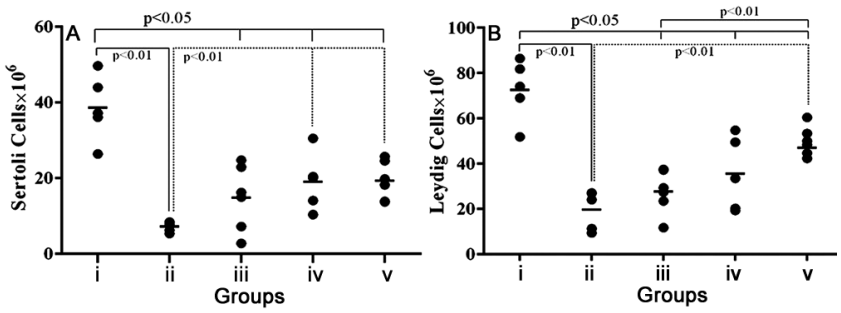

Fig. 6 —Effect of loboob treatment on the number of (A) Sertoli; and (B) leydig cells by the stereological method in rats.

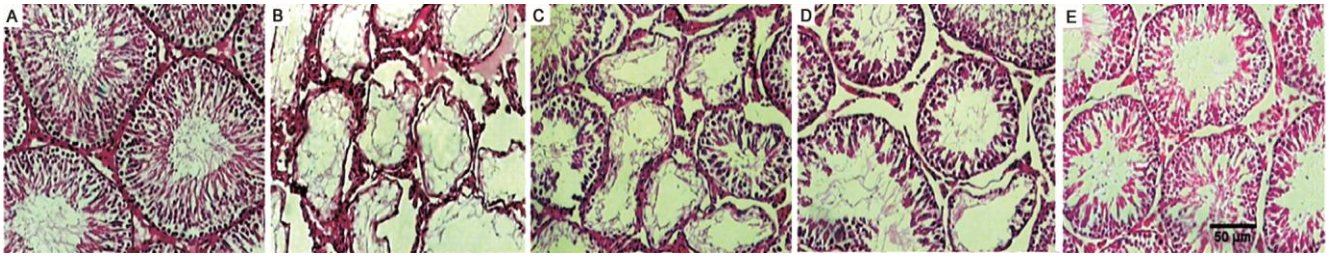

Fig. 7 — Seminiferous tubule cross-sections were evaluated with hematoxylin and eosin staining. (A) Control with normal tubules and germinal cells; (B) BUS with atrophic tubules, thin germinal epithelium, and abnormal lumen; (C) BUS+Loboob35 with slightly recover the destructive effects of busulfan; (D) BUS+Loboob70; and (E) BUS+Loboob140 groups with less atrophic changes, more germinal cells in the epithelium and more spermatozoa in the luminal space.

The number of round spermatids in the BUS and BUS+Loboob35 groups was significantly decreased in comparison with the control, BUS+Loboob70 and 140 groups (P <0.01). Also, the number of round spermatids in BUS+Loboob70 (Gr. IV) was significantly decreased in comparison with the control group (P <0.01; Fig. 5D).

The number of elongated spermatids in the BUS and BUS+Loboob35 was significantly decreased in comparison with the control, BUS+Loboob70, 140 groups (P <0.05). The mean number of elongated spermatids of BUS+Loboob70 (Gr. IV) was significantly lower than the control and BUS+Loboob140 groups (P <0.01; Fig. 5E).

The number of Sertoli cells in the BUS (Gr. II) and BUS+Loboob35 (Gr. III) was significantly depleted in comparison with the control (Gr. I), BUS+Loboob70 (Gr. IV) and 140 (Gr. V) (P <0.01). Also, significant reduction was observed in the number of Sertoli cells in BUS+Loboob35, 70 and 140 when compared to the control group (P <0.05; Fig. 6A).

The number of leydig cells in the BUS (Gr. II) and BUS+Loboob35 (Gr. III) significantly decreased in comparison with the control and BUS+Loboob140 groups (P <0.05). Also, leydig cell numbers in the BUS+Loboob70 and 140 groups were significantly decreased when compared to the control group (P <0.05; Fig. 6B).

Qualitative evaluation

The photomicrograph of the testis cells in the transverse section of seminiferous tubules is presented in Fig. 7. Seminiferous tubules and germinal cells appeared normal in the control group. While, with busulfan treatment, tubules showed the process of degeneration. 35 mg/kg/day of loboob could not improve the negative effects of busulfan treatment and a lot of seminiferous tubules were atrophied. The tubule lumina was abnormal and spermatozoa were rare in the lumina. The germinal epithelium was thinner in the BUS (Gr. II) and BUS+Loboob35 (Gr. III) than control, BUS+Loboob70 (Gr. IV) and BUS+Loboob140 (Gr. V), and overall cell mass appeared diminished. Administration of 70 or 140 mg/kg/day of loboob for 60 days ameliorated the destructive effects of busulfan, with less atrophic changes, more germinal cells in the epithelium and more spermatozoa in the luminal space.

Discussion

Cancer treatment by chemotherapy drug in patients with damage to gametes can induce infertility. It has been demonstrated that busulfan treatment with direct effect on the gonads results in germ cell damage by apoptosis, or loss of mediators of DNA repair[15]. Our

findings showed that administration of 10 mg/kg body wt. busulfan decreased the testosterone level and increased the LH and FSH levels. Furthermore, busulfan significantly decreased catalase and SOD, and increased the level of MDA. In the present study for first time was reported the ameliorative effects of loboob on LH, FSH, and testosterone levels, especially at 70 (except for the FSH level) and 140 mg/kg doses, in the oligospermia model. High dosage of loboob (140 mg/kg/day) improved the seminiferous epithelium and leydig cells, while mid dosage of loboob (70 mg/kg/day) only improved seminiferous epithelium in the busulfan treated rats.

Busulfan induces oxidative stress through GSH reduction and thioredoxin reductase inhibition[16]. In addition, busulfan decreases enzymatic antioxidant levels such as GPX and SOD, and increased the lipid peroxidation level in mice's testicular tissues. It has been demonstrated a relationship between high levels of lipid peroxidation and generation of ROS in the testes and epididymal sperms following busulfan treatment[17] which is consistent with our results. Also, our previous study showed that busulfan decreased all sperm parameters in rats[18].

The components of loboob possess antioxidant substances. Traditionally, some Iranian people consume herbal remedies for treatment of infertility. Loboob compound with easy preparation and inexpensiveness is a good choice for this purpose. According to Avicenna, consumption of loboob compound is a good choice for infertility treatment. About 25% of loboob contains different medicinal herbs of various parts (4 kernels, 4 seeds, 3 fruits, 2 roots, 1 oleogumresin, 1 bark, 1 leaf, 1 bud, and 1 rhizome). Numerous studies confirm positive effects of these ingredients on male reproductive system[19-22]. A previous study demonstrated that vitamins (C and E) and antioxidants strengthened the blood-testis barrier, repaired the sperm DNA, and improved male infertility through decreasing free radicals[23].

Some of ingredients of loboob play important role to improve steroidogenesis along with spermatogenesis. The onion *Allium cepa* L. increases testosterone levels by increasing antioxidant capacity to neutralize the negative effects of free radical production in the tests, improving insulin resistance, boosting nitric oxide generation, and changing the activity of adenosine 5′-monophosphate-activated protein kinase[24]. The common walnut *Juglans regia* L. significantly increased the levels of GSH, SOD and CAT, and decreased the level of MDA in testis of diabetic rats and increased the level of serum testosterone[25]. The clove *Syzygium aromaticum* L. also can increase testosterone production[26]. Previous study showed that ginger enhanced antioxidant enzymes activity and decreased MDA level in ethanol-induced testicular damage and increased the levels of testosterone[27]. It was reported that honey can increase serum testosterone levels by improving Leydig cell viability, diminishing oxidative damage in Leydig cells, and impairing aromatase activity in the testis[28]. Thus, these ingredients in the loboob with different ways could improve the level of testosterone in the serum of busulfan treated rats.

The pituitary gland is stimulated to release LH when blood testosterone levels are low, which may be the reason of high level of LH in busulfan group. In loboob groups, an increasing in testosterone may inhibit LH production by its aromatization into estradiol[29]. Furthermore, by increasing the number of Sertoli and leydig cells, FSH and LH receptors will respectively increase in these cells, which may lead to decrease the level of FSH and LH in the loboob treated rats.

In line with our results, previous studies reported that treatment of busulfan causes degenerative changes in germinal epithelium degeneration and atrophy in the epithelium of the seminiferous tubules testis[3]. Our results indicated that alterations in the volume of testis and seminiferous tubules had same patterns. Regarding the major portion of testis is seminiferous tubules, atrophy in this area could results in a depletion in testis volume and weight. Thus, it seems that the improvement in the volume of seminiferous tubules might be a part of reason for increasing the testis volume by loboob treatment. On the other hands, the volume of interstitial tissue did not affect by all concentrations of loboob, but in high dosage of loboob, the leydig cells number improved when compared to the control. Taken together, our observations may suggest that loboob is more effective on the recovery of seminiferous tubules and their cells than on the interstitial tissue. Oral administration of the aromatic resin *Boswellia thurifera* Roxb. increased fertility rate in male rats. Moreover, the organs reproductive weight, testes size and its secretory function showed a remarkable increase compared with control group[22]. The compounds of this herb could prevent DNA fragmentation and destruction of chromatin induced

by hydrogen peroxide[30]. One possible explanation is that the improvement of sexual hormones restored tubular activity by stimulating spermatogonia proliferation and Sertoli cell function. Other researchers have proposed that hormone treatments work together to boost spermatogonial stem cells and spermatogonia interactions with Sertoli cells, resulting in improved spermatogenesis and testicular histological restoration[31].

Our previous study revealed that 140 mg/kg loboob increased the motility of slow spermatozoa, improved progress motile sperm percentage and sperm viability[18]. *Allium cepa* seed[19], *Cinnamomum zeylanicum* bark[20], and *Cocos nucifera* oil[21] improved spermatogenesis and sperm parameters. These components contain different antioxidant and are effective in treating infertility. Therefore, these herbs may have beneficial effects on oligospermia. Almonds, pistachios, hazelnuts, walnuts, sesame and coconut are nutritional sources of arginine which has important role to inhibit the cell oxidation[32]. On the other hand, these ingredients usually contain variable concentrations of minerals and vitamins with preventing effects on the cells oxidation and positive influences on the sperm count and motility, like zinc, selenium, manganese, copper, arginine, L-carnitine, Q10, and vitamins B, C and E[33]. However, Pistachios, hazelnuts and walnuts are introduced as an excellent sources of zinc[34], Hazelnuts are also rich in monounsaturated fatty acids, vitamin E, L-arginine and folate[35].

Conclusion

Overall with the above results, it may be concluded that loboob ingredients with various antioxidants, minerals and vitamins in a dose-dependent manner could overcome the infertility induced by busulfan and improved the oxidative status, sexual hormones and structural of testis tissue, spermatogenesis and seminiferous tubules volume in the spermatogenesis process in a rat model. Therefore, loboob administration may have beneficial effects for patients treated chemotherapy with induced infertility. However, more investigations are needed to clarify the efficiency of loboob on male infertility.

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Ethical statement

All the animal experiments protocols were done based on the guidelines of the Animal Ethics Committee of the Shiraz University of Medical Sciences.

Conflict of interest

Authors declare no competing interests.

References

1 Niederberger C, WHO manual for the standardized investigation, diagnosis and management of the infertile male. *Urology*, 57 (2001) 208.

2 Dohle GR, Male infertility in cancer patients: review of the literature. *Int J Urol*, 17 (2010) 327.

3 Bahmanpour S, Jahromi BN, Koohpeyma F, Keshavarz M & Bakhtari A, Effects of Different Doses and Time-Dependency of Busulfan on Testes Parameters and Spermatogenesis in a Rat Model: A Quantitative Stereological Study. *J Adv Med Sci Appl Technol*, 3 (2017) 155.

4 Zhang X, Xia Q, Wei R, Song H, Mi J, Lin Z, Yang Y, Sun Z & Zou K, Melatonin protects spermatogonia from the stress of chemotherapy and oxidation via eliminating reactive oxidative species. *Free Radic Biol Med*, 137 (2019) 74.

5 Abarikwu S, Mgbudom-Okah C, Onuah C & Ogunlaja A, Fluted pumpkin seeds protect against busulfan-induced oxidative stress and testicular injuries in adult mice. *Drug Chem Toxicol*, 45 (2022) 22.

6 Galaup A & Paci A, Pharmacology of dimethanesulfonate alkylating agents: busulfan and treosulfan. *Expert Opin Drug Metab Toxicol*, 9 (2013) 333.

7 Kaur H, Singla N & Mahal AK, Antifertility effect of bait containing *Carica papaya* L. seed powder in male lesser bandicoot rat, *Bandicota bengalensis* (Gray and Hardwicke). *Indian J Exp Biol*, 59 (2021) 448.

8 Das B, Roy K, Talukdar AD, Dey A, Nongalleima K, Das S, Sharma P, Choudhury MD & Deb L, Antifertility activity of Oroxylum indicum (L.) Kurz: *In vitro* and *in vivo* study on human sperm and male wistar rats. *Indian J Exp Biol*, 61 (2023) 33.

9 Bahmanpour S, Vojdani Z, Panjehshahin MR, Hoballah H & Kassas H, Effects of *Carthamus tinctorius* on semen quality and gonadal hormone levels in partially sterile male rats. *Korean J Urol*, 53 (2012) 705.

10 Bahmanpour S, Talaei T, Vojdani Z, Panjehshahin M, Poostpasand A, Zareei S & Ghaeminia M, Effect of *Phoenix dactylifera* pollen on sperm parameters and reproductive system of adult male rats. *Iran J Med Sci*, 31 (2015) 208.

11 Ghaeni Heravi SM & Qarabadin Salehi, rewritten by Badr P, Mohagheghzadeh A, Shams MR. 1st edn. *Chogan Press*, (2013).

12 Gholizadeh F, Mokarram P, Dastgheib S & Rahpeima Z, The effect of the aquatic extract of *Stevia* on the MDA level and catalase activity in the testicular tissue of streptozotocin-nicotinamide-induced diabetic rats. *Siraz E-Med J*, 19 (2018) e61044.

13 Derhami A, Rajabi S, Rad GH & Jafari F, The Effect of Shilajit on Carboplatin-induced Thrombocytopenia and

Oxidative Stress in Rats. *J Pharm Negat Results*, 13 (SI7) (2022) 621. DOI: https://doi.org/10.47750/ pnr.2022. 13.S07.083.

14 Hagar HH, El Etter E & Arafa M, Taurine attenuates hypertension and renal dysfunction induced by cyclosporine A in rats. *Clin Exp Pharmacol Physiol*, 33 (2006) 189.

15 Choi YJ, Ok DW, Kwon DN, Chung Ji, Kim HC, Yeo SM, Kim T, Seo HG & Kim JH, Murine male germ cell apoptosis induced by busulfan treatment correlates with loss of c-kit-expression in a Fas/FasL-and p53-independent manner. *FEBS Lett*, 575 (2004) 41.

16 Abarikwu SO, Njoku R-CC, John IG, Amadi BA, Mgbudom-Okah CJ & Onuah CL, Antioxidant and anti-inflammatory protective effects of rutin and kolaviron against busulfan-induced testicular injuries in rats. *Syst Biol Reprod Med*, 68 (2022) 151.

17 Nasimi P, Tabandeh MR, Vahdati A & Khatamsaz S, Busulfan induces oxidative stress-and Bcl-2 family gene-related apoptosis in epididymal sperm and testis of adult male mice. *Physiol Pharmacol*, 19 (2015) 208.

18 Soghra B, Mojtaba K, Farhad K, Parmis B, Adel N, Dabbaghmanesh MH, Tahereh P, Najib FS, Najaf Z & Niloofar N, Preserving effect of Loboob (a traditional multi-herbal formulation) on sperm parameters of male rats with busulfan-induced subfertility. *JBRA Assist Reprod*, 26 (2022) 574.

19 Ige SF & Akhigbe RE, The role of Allium cepa on aluminum-induced reproductive dysfunction in experimental male rat models. *J Hum Reprod Sci*, 5 (2012) 200.

20 Yüce A, Türk G, Çeribaşi S, Sönmez M, Ciftci M & Güvenç M, Effects of cinnamon (C *innamomum zeylanicum*) bark oil on testicular antioxidant values, apoptotic germ cell and sperm quality. *Andrologia*, 45 (2013) 248.

21 Ogedengbe OO, Jegede AI, Onanuga IO, Offor U, Naidu EC, Peter AI & Azu OO, Coconut Oil Extract Mitigates Testicular Injury Following Adjuvant Treatment with Antiretroviral Drugs. *Toxicol Res*, 32 (2016) 317.

22 Nusier MK, Bataineh HN, Bataineh ZM & Daradka HM, Effect of Frankincense (*Boswellia thurifera*) on reproductive system in adult male rat. *J Health Sci*, 53 (2007) 365.

23 AlDean SLMS & Saleh WM, Evaluation of the antioxidant effect of vitamins C, E and Alpha lipoic acid (ALA) in improvement of Bull semen quality. *Ann Romanian Soc Cell Biol*, 25 (2021) 17510.

24 Banihani SA, Testosterone in males as enhanced by onion (*Allium cepa* L.). *Biomolecules*, 9 (2019) 75.

25 Nasiry D, Khalatbary AR, Ahmadvand H & Talebpour Amiri FB, Effects of *Juglans regia* L. leaf extract supplementation on testicular functions in diabetic rats. *Biotech Histochem*, 96 (2021) 41.

26 Mishra RK & Singh SK, Safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice. *Food Chem Toxicol*, 46 (2008) 3333.

27 Akbari A, Nasiri K, Heydari M, Mosavat SH & Iraji A, The protective effect of hydroalcoholic extract of *Zingiber officinale* Roscoe (Ginger) on ethanol-induced reproductive toxicity in male rats. *J Evid Based Complementary Altern Med*, 22 (2017) 609.

28 Banihani SA, Mechanisms of honey on testosterone levels. *Heliyon*, 5 (2019) e02029.

29 Pitteloud N, Dwyer AA, DeCruz S, Lee H, Boepple PA, Crowley WF, Jr. & Hayes FJ, Inhibition of luteinizing hormone secretion by testosterone in men requires aromatization for its pituitary but not its hypothalamic effects: evidence from the tandem study of normal and gonadotropin-releasing hormone-deficient men. *J Clin Endocrinol Metab*, 93 (2008) 784.

30 Miciński P, Pawlicki K, Wielgus E, Bochenek M, Gogol P & Ficek B, Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters. *Reprod Biol*, 11 (2011) 135.

31 Chen LY, Testosterone regulation of the spermatogonial stem cell niche in mice. Washington State University, 2008.

32 Shils ME & Shike M, Modern nutrition in health and disease. (Lippincott Williams & Wilkins), 2005.

33 El-Taieb MA, Herwig R, Nada EA, Greilberger J & Marberger M, Oxidative stress and epididymal sperm transport, motility and morphological defects. *Eur J Obstet Gynecol Reprod Biol*, 144 (2009) S199.

34 Schlegel PN, Supplements to Enhance Male Fertility. *Biennial Rev Infertil*, (2013) 3.

35 Abeer EE-K & Amr AR, Hypoglycemic effect of hazelnut and its effect on some sex hormones in alloxan induced diabetic in female rats. *Pak J Nutr*, 12 (2013) 229.