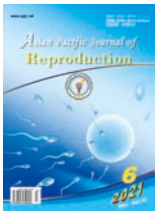

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Protective effects of honey compound syrup on busulfan-induced azoospermia in male rats

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ABSTRACT

Objective: To evaluate the protective effects of honey compound syrup on sperm count and testis tissue in rats.

Methods: Thirty rats were randomly assigned to five groups. The control group received 1 mL normal saline with dimethyl sulfoxide intraperitoneally; the busulfan group received busulfan 10 mg/kg body weight at the first and twenty-first days of the experiment *via* intraperitoneal injection; the last three groups received busulfan 10 mg/kg body weight to induce azoospermia, and then received 1.0, 1.5, or 2.0 mg/kg honey compound syrup, respectively, after induction of azoospermia. After administration, the testis and epididymis of all rats were removed. Then, reproductive organ weight and sperm parameters (sperm concentration, epididymal sperm reserve and daily sperm production) were measured. After hematoxylin-eosin staining, seminiferous tubule cells and diameters were assessed.

Results: Busulfan damaged the testis tissue and impaired spermatogenesis. Administration of honey compound syrup in three doses improved testis tissue and spermatogenesis. The protective effects of honey compound syrup may relate to the antioxidant properties of honey and other compounds in this syrup.

Conclusions: Administration of honey compound syrup could be an ameliorative agent for the side effects of chemotherapy drugs such as busulfan on the male reproductive system.

KEYWORDS: Busulfan; Azoospermia; Honey compound syrup; Sperm; Rat; Iranian traditional medicine

1. Introduction

Infertility is an important health problem that involved male disorders in almost 40% of infertile couples; the inability of the males to conceive fertile women is the fundamental cause. There are many and various reasons for male infertility occurrences, such as physical, structural, hormonal, sexual, environmental, and dietary factors[1-3]. No existence of sperm in the ejaculation is azoospermia. There are two types of obstructive and non-obstructive azoospermia; 10%-15% of all infertile men and 1% of all men are suffering from these types of azoospermia[4]. In the obstructive type, due to obstruction of the ejaculatory duct, sperm is not in the ejaculate, but maybe it can be found in the testes. Non-obstructive azoospermia is another type which can occur due to different causes

Significance

In many studies, azoospermia and testis tissue-damage have been reported and confirmed as side effects of busulfan and also infertility is an important health problem that involved 40% of infertile couples. In this study, we present Iranian traditional medicine syrup, with beneficial effects in the treatment of sexual disorders. Honey compound syrup is an herbal and almost safe drug that can be used as a tonic for male sexual problems as azoospermia and infertility.

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such as congenital defects, genetic abnormalities, infectious diseases, endocrine disorders, exposure to gonadotoxins, traumas, varicocele, and medications such as chemotherapy drugs[5].

Busulfan is a chemotherapy drug, which can be used for leukemia before and after bone marrow transplantation. Busulfan administration in men with cancers can cause permanent or complete infertility[6]. In different studies, azoospermia and testis tissue-damage have been reported and confirmed as side effects of this drug[7,8]. Although azoospermia can be treated by hormonal and surgical methods, it is essential to find new ways that have a lower risk than surgery and have satisfactory results. Moreover, efforts to compensate for the harmful effects of cancer therapy cytotoxic drugs on spermatogenesis are required[5].

To date, various treatments that involved traditional medicine are conventional beside modern therapy. Iranian traditional medicine is one of the oldest types of complementary and alternative medicine that have ancient annals with 10 000 years old (The texts of, *The Canon of Medicine* by Avicenna, *The Continens* by Rhazes, *The Treasure of Kharazmshah* by Sorsanus, and *Liber Regius* by Haly-Abbas were central to western medical science)[9]. Nowadays, Iranian traditional medicine, known as a therapeutic plan, is conventional among significant numbers of patients[10]. In Iranian traditional medicine, compound-honey syrup is used as conventional treatment in different diseases. Honey and extract of seven medicinal plants are components of honey compound syrup. These components are *Zingiber officinale*, *Cinnamomum verum*, *Crocus sativus*, *Elettaria cardamomum*, *Alpinia officinarum*, *Pistacia lentiscus*, *Myristica fragrans* and the common names of these plants are ginger, cinnamon, saffron, cardamom, galangal, mastic, and nutmeg, respectively. Each of the components of the honey compound syrup has beneficial effects in the treatment of various and different diseases. In a study, Kaveh *et al* examined the effect of honey compound on children's asthma before and after orally using the drug, which showed promising results as a perfect anti-inflammatory and anti-asthma agent[11]. In the present study, the protective effects of honey compound syrup against busulfan-induced azoospermia in rats were assessed.

2. Methods and materials

2.1. Animals

In this study, thirty male Wistar rats weighing 200–250 g and aged 6-8 weeks that were purchased from the Pasteur Institute of Iran were used. Rats were kept in the animal laboratory in a controlled environment, a cycle of 12 h of darkness–light and temperature (22±2) ℃, with free access to food and water.

2.2. Honey compound syrup preparation

Honey compound syrup is a standard syrup in Iranian traditional medicine that has been used for sexual treatments for many years[12,13]. In this study, honey compound was prepared according to Iranian traditional medicine pharmaceutical manuscripts, with slight modifications[12–14]. Honey compound is an Iranian traditional medicine produced by Niak Pharmaceutical Company and registered in the Food and Drug Organization affiliated with the Ministry of Health of Iran (license number: S-94-0425). Plants used in honey compound syrup are considered as well-known medical plants and were prepared by Niak Company and controlled with standard methods at quality control laboratory. As a syrup formulation, honey compound syrup is a mixture of fine honey, water, and extract of herbs of *Cinnamomum verum*, *Crocus sativus*, *Alpinia officinarum*, *Zingiber officinale*, *Myristica faragrans*, *Elettaria cardamomum*, and *Pistacia lenticus*.

2.3. Experimental groups and treatment

Rats were randomly assigned into five groups of six in each. The control group (group 1) received 1 mL normal saline with dimethyl sulfoxide (DMSO) intraperitoneally. The busulfan group (group 2) received 10 mg/kg busulfan (Sigma, St.Louis, MO, USA); the method of this study was derived from a study by Panahi *et al* with slight modifications[15] with 10 mg/kg DMSO (Sigma, USA) diluted with the same dose of normal saline to achieve 5 mg/mL density. Rats received one dose of this combination in the first and twenty-first days of the experiment *via* intraperitoneal injection[15]. Groups 3, 4 and 5 were treated with the combination of busulfan and honey compound. Rats in these three groups were given 1.0, 1.5 and 2.0 mL/kg honey compound orally, respectively, for 14 days (every other day from 42th to 56th days of the experiment). This treatment period was chosen based on the duration of the spermatogenesis in rats. The mentioned doses of the honey compound syrup were selected according to the doses that were used in the other clinical trials (1 mL/kg of body weight)[11].

2.4. Preparation of samples

At the 57th day of treatment, intraperitoneal injection of 100 mg/kg ketamine hydrochloride [100 mg/1 mL-10%-Alfasan (Netherlands)] and 10 mg/kg xylazine [20 mg/1 mL-2%-Alfasan (Netherlands)] was performed to anesthetize rats[16]. Then, rats' testis and epididymis were removed from the body and washed in saline, and the weight of reproductive organs was assessed. For hematoxylin-eosin staining, the testis tissues were fixed in 10% formalin. The caudal part of epididymis was used for the evaluation of sperm count[16].

2.5. Collection of sperm

For sperm collection, the caudal part of epididymis was torn apart into fine pieces in pre-warmed 37 ℃ normal saline[16].

2.6. Assessment of the body and reproductive organ weights

Final total body mass, testis, and epididymis weights were determined with a digital scale and recorded. Gonadosomatic index (GSI) was also assessed[16].

2.7. Assessment of sperm concentration, epididymal sperm reserve and daily sperm production

For sperm concentration, sperm suspension was diluted with 4 mL of formaldehyde. Then, a specific volume of the diluted sample was placed on hemocytometer chambers. Light microscopy (Olympus) at 400× magnifications was used to count sperm heads in 5 microscopic fields. The sperm count was expressed as 10^6 sperm per milliliter[16].

For epididymal sperm reserve, 10 μL of diluted sample was placed on hemocytometer chambers. Sperm heads were counted in 5 microscopic fields using light microscopy at 400× magnifications. Then, the epididymal sperm reserve was assessed based on this formula[17]:

Number of sperms in 400 small square × dilution rate ×10 000

For daily sperm production, 10 μL of diluted sample was placed on hemocytometer chambers. Sperm heads were counted in 5 microscopic fields using light microscopy at 400× magnifications. Then, the above formula was used to determine total sperm in each testis. After that, for sperm count in each gram of the testis, data were divided into testis weights[18]. The duration of spermatogenesis is 6.3 days in rats; therefore, the data obtained were divided to 6.3 to assess the daily production of sperm[17].

2.8. Counting different cell populations in seminiferous tubules

After the preparation of tissue sections, fifty seminiferous tubules were randomly chosen to estimate the number of Sertoli and Leydig cells, spermatogonia, primary spermatocytes and spermatids. Evaluation of the tissue sections was carried out by using light microscopy (Olympus) 200× and the results were reported by two independent observers.

2.9. Morphometric assessment of seminiferous tubules

For assessment of seminiferous tubules diameters, round contour seminiferous tubules were chosen randomly. Measurement of 10 sections of the seminiferous per animal was performed. The analysis of images was performed by Motic Image Plus 2.0 Ml[19].

2.10. Histological procedure

Testicular tissue samples for histological assessment were fixed in 10% formalin buffer and then embedded in paraffin. Afterwards, tissue section preparations were done. Subsequently, the changes in the seminiferous tubules were examined. The spermatogenesis and the statue of seminiferous tubules such as the depletion of Sertoli and Lydig cells were assessed.

2.11. Statistical analysis

The data analysis was made by Spss 22 (IBM, California). Data were expressed as mean±standard deviation (mean±SD). One-way analysis of variance followed by Tukey's *post hoc* was used. $P<0.05$ was considered statistically significant.

2.12. Ethics statement

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1395.899).

3. Results

3.1. Effect of honey compound syrup on the body and reproductive organ weights

Body weight decreased in the busulfan group compared to the control group, but it was not significant. It was significantly increased in the busulfan+honey compound syrup (1.0 and 1.5 mg/kg) groups compared to the bulsulfan group ($P<0.001$), but it did not significantly alter in the busulfan+honey compound (2.0 mg/kg) group compared to the busulfan group (Table 1) .

Busulfan caused a significant decrease in the testis weight compared to the control group ($P<0.001$). Administration of honey compound syrup (1.0, 1.5 and 2.0 mg/kg) increased testis weight compared to the busulfan group ($P<0.001$) (Table 1).

Busulfan decreased epididymis weight compared to the control group, but it was not significant. Epididymis weight increased significantly in the busulfan+honey compound syrup (1.0, 1.5 and 2.0 mg/kg) groups compared to the busulfan group ($P<0.001$) (Table 1) .

GSI decreased in the busulfan group compared to the control group, but it was not significant. Administration of the honey compound syrup at dose of 1.0 mg/kg decreased GSI compared to the busulfan group, but it was not significant. GSI increased in the busulfan+honey compound syrup (1.5 and 2.0 mg/kg) groups compared to the busulfan group (P<0.001) (Table 1).

3.2. Effect of honey compound syrup on sperm count, epididymal sperm reserve and daily sperm production

Busulfan resulted in a significant decrease in sperm count compared to the control group (P<0.001). Administration of the honey compound (1.0, 1.5, and 2.0 mg/kg) increased sperm count compared to the busulfan group (P<0.001) (Table 2).

Busulfan caused a significant decrease in epididymal sperm reserve compared to the control group (P<0.001). Epididymal sperm reserve significantly increased in the busulfan+honey compound (1.0, 1.5 and 2.0 mg/kg) groups compared to the busulfan group (P<0.001) (Table 2).

Busulfan significantly decreased daily sperm production compared to the control group (P<0.001). Daily sperm production significantly increased in the busulfan+honey compound (1.0, 1.5 and 2.0 mg/kg) groups compared to the busulfan group (P<0.001) (Table 2).

3.3. Effect of honey compound syrup on the number of different cell populations and seminiferous tubule diameter

Rats exposed to busulfan alone significantly decreased the number of Sertoli and Leydig cells, spermatogonia, spermatids and primary spermatocytes and seminiferous tubule diameter compared to the control group (P<0.001). Administration of the honey compound syrup (1.0, 1.5 and 2.0 mg/kg) increased the mentioned cells and seminiferous tubule diameter in comparison to the busulfan group (P<0.001) (Table 3).

3.4. Effect of honey compound syrup on testicular histopathology

In the control group, seminiferous tubules had a normal structure and an active spermatogenesis process was detected in the tubules (Figure 1A). In the busulfan group, most of the spermatogonia, primary spermatocytes, Sertoli cells, spermatids and Leydig cells were destroyed. Also, this busulfan considerably depleted the seminiferous tubules in comparison to the control group (Figure 1B). However, rats treated with honey compound syrup (1.0, 1.5 and 2.0 mg/kg) improved the structure of seminiferous tubules, with the extent that was comparable to the control group (Figure 1C, D, E).

Table 1. Body and reproductive organ weights.

Parameters	Control	Busulfan	Busulfan+honey compound syrup (1.0 mg/kg)	Busulfan+honey compound syrup (1.5 mg/kg)	Busulfan+honey compound syrup (2.0 mg/kg)
Body weight, g	347.33±9.30	276.33±6.60	371.5 0±19.3$^{###}$	348.60±16.30$^{###}$	341.40±19.10
Testis weight, g	1.53±0.10	0.98±0.06***	1.59±0.07$^{###}$	1.66±0.03$^{###}$	1.60±0.07$^{###}$
Epididymis weight, g	0.28±0.04	0.16±0.01	0.34±0.01$^{###}$	0.33±0.01$^{###}$	0.34±0.04$^{###}$
GSI	0.44±0.03	0.35±0.02	0.04±0.02	0.05±0.02$^{###}$	0.05±0.01$^{###}$

Data are presented as mean±SD with one-way analysis of variance followed by Tukey's *post hoc* test. ***: *versus* the control group, P<0.001; $^{###}$: *versus* the busulfan group, P<0.001. GSI: Gonadosomatic index.

Table 2. Sperm parameters.

Parameters	Control	Busulfan	Busulfan+honey compound syrup (1.0 mg/kg)	Busulfan+honey compound syrup (1.5 mg/kg)	Busulfan+honey compound syrup (2.0 mg/kg)
Sperm count, $\times10^6$	14.70±1.90	0.00***	25.40±1.05$^{###}$	28.10±0.30$^{###}$	23.40±1.30$^{###}$
Epididymal sperm reserve, $\times10^6$	3 683.30±483.70	0.00***	6 370.30±264.60$^{###}$	7 040.00±966.30$^{###}$	5 860.00±342.80$^{###}$
Daily sperm production, $\times10^6$	5 846.08±766.30	0.00***	1 011.30±420.50$^{###}$	1 117.30±153.40$^{###}$	9 301.70±543.20$^{###}$

Data are presented as mean±SD with one-way analysis of variance followed by Tukey's *post hoc* test. ***: *versus* the control group, P<0.001; $^{###}$: *versus* the busulfan group, P<0.001.

Table 3. Seminiferous tubule cells and diameter.

Parameters	Control	Busulfan	Busulfan+honey compound syrup (1.0 mg/kg)	Busulfan+honey compound syrup (1.5 mg/kg)	Busulfan+honey compound syrup (2.0 mg/kg)
Sertoli cell, *n*	28.00±0.30	18.00±0.40***	28.00±0.80$^{###}$	23.00±0.50$^{###}$	29.00±0.90$^{###}$
Lydig cell, *n*	25.00±0.30	15.00±0.50***	25.00±0.40$^{###}$	23.00±0.10$^{###}$	24.00±0.10$^{###}$
Spermatogonia, *n*	60.00±1.10	54.00±0.50***	60.00±1.50$^{###}$	63.00±0.60$^{###}$	60.00±0.30$^{###}$
Spermatid, *n*	215.00±0.90	100.00±2.00***	214.00±1.20$^{###}$	205.00±1.00$^{###}$	210.00±0.70$^{###}$
Primary spermatocyte, *n*	85.00±0.90	50.00±0.70***	85.00±0.60$^{###}$	82.00±0.70$^{###}$	80.00±1.50$^{###}$
Seminiferous tubule diameter, μm	350.01±0.50	310.00±1.60***	350.10±0.30$^{###}$	350.20±0.50$^{###}$	350.30±0.50$^{###}$

Data are presented as mean±SD with one-way analysis of variance followed by Tukey's *post hoc* test. ***: *versus* the control group, P<0.001; $^{###}$: *versus* the busulfan group, P<0.001.

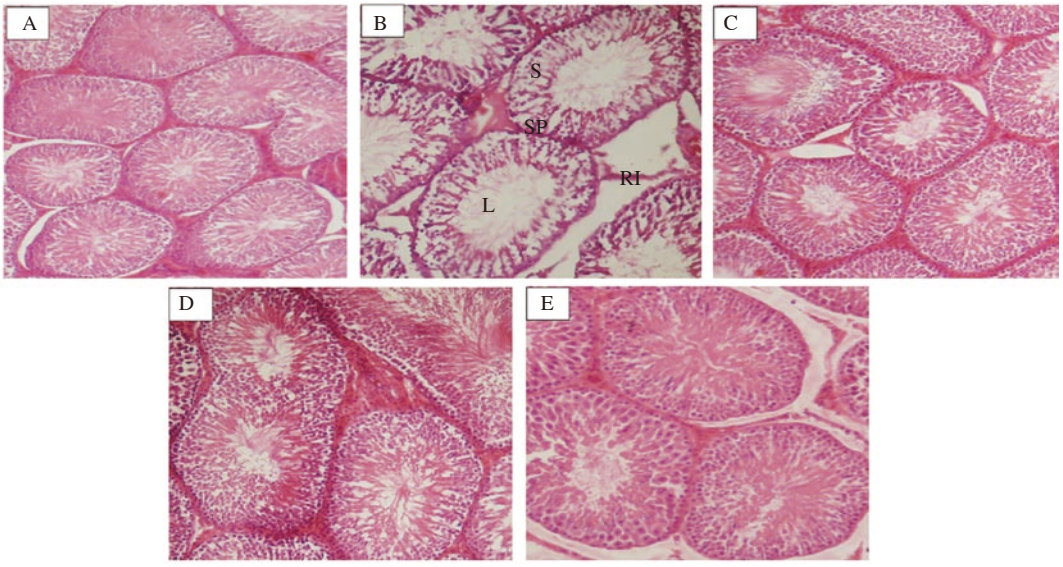

Figure 1. Changes in testicular histopathology in different groups (hematoxylin-eosin staining, magnification ×10). A: In the control group, seminiferous tubules are intact. B: In the busulfan group, seminiferous tubules are depleted of spermatogonia (S), primary spermatocytes (SP), Sertoli cells, spermatids and Leydig cells (L). C, D, and E: Honey compound syrup (1.0, 1.5 or 2.0 mg/kg) improves the structure of seminiferous tubules. RI: rupture of interstitum.

4. Discussion

In this study, the effect of honey compound syrup was investigated on busulfan-induced azoospermia in male rats. According to the results of the present study, busulfan caused destructive effects on sperm parameters and testis tissue functionally and structurally. Administration of three different doses of honey compound syrup (as a natural and potent treatment) ameliorates reproductive organ weight, sperm count, daily sperm production, epididymal sperm reserve cells of seminiferous tubules, and testicular histopathology.

Busulfan is one of the anti-cancer drugs which are used to treat lymphoma, chronic leukemia, and ovarian cancer. The male reproductive system is one of the systems that are vulnerable to the side effects of busulfan[20]. This drug can decrease testis weight[21], increase abnormal sperm parameters[22], induce azoospermia, oligospermia, destroy almost all testicular germ cells[23], and finally cause temporary or permanent sterility. Busulfan is an alkylating agent that can lead to stem cell death by attaching to double strand DNA that could prevent DNA replication and RNA transcription. Spermatogenesis was inhibited in this study that could be explained by these[24]. In the present study, sperm count and sperm production significantly decreased in busulfan-exposed rats. Reactive oxgen speciese can damage bio-molecules such as DNA and lipids. Sperms are highly susceptible to oxidative stress because sperm plasma membrane has a high amount of polyunsaturated fatty acids. Oxidative stress, which can be caused by busulfan administration, could affect fatty acid and decrease sperm count and motility[25,26]. In agreement with our data, Anjamrooz *et al*[22] and Dehghani *et al*[27] reported that sperm count markedly declines after exposure to busulfan. With the increased occurrence of cancer all over the world today, the use of anti-cancer drugs is increasing. Therefore, many studies as well as this study today focus on the increasing fertility potential after treating with drugs such as busulfan.

Nowadays, plant extracts and herbal plants as sources of antioxidants and phenolic compounds have attracted considerable attention. Several studies reported the improving effect of various plant extracts and herbal plants on busulfan-induced testis toxicity[20]. In this study, for the first time, we examined the effects of different doses of honey compound syrup in busulfan-treated rats. There are two perspectives and strategies for investigating the mechanism of the honey compound syrup effect. First, from the point of view of traditional medicine, this potent syrup improves sperm and has also been used in various sources to enhance libidio, increase semen, reinforce the body and increase sperm count[13]. Second, this syrup is composed of components that affect spermatogenesis by various mechanisms, such as antioxidant, anti-inflammatory, strengthening of Leydig cells, increasing spermatogenesis, and increasing testosterone.

Antioxidants have beneficial effects in busulfan-induced testis injury. Flavonoids are the main group of antioxidants that are found in honey. Additionally, various compounds could be found in honey, such as antioxidants, amino acids, vitamins (B2, B4, B5, B6, B11, and vitamin C), carbohydrates, proteins, minerals, and 18 free amino acids. Acid phosphorylase, catalase, invertase, and glucose oxidase are the enzymes that can be found in honey[28]. Several studies reported many medicinal effects of honey. These effects are antibacterial, hepatoprotective, hypoglycaemic, reproductive, and anti-hypertensive. It may act *via* amelioration of oxidative stress in tissues[29,30].

In this study, honey compound syrup improved sperm-count, testis

and epididymis weight, depletion of cells in seminiferous tubules, and diameter of seminiferous tubules, which were destructed by busulfan administration *via* its antioxidant effects. It was reported that honey improved spermatogenesis in ischemia–reperfusion-induced injury in rats the same as the present study[31]. In addition to honey, honey compound syrup also contains other medicinal plants. Ginger is one of them. Phenolic compounds of *Zingiber* were found to scavenge superoxide anion and to inhibit lipid peroxidation induced by active oxygen species such as superoxide anion or hydroxyl radical, respectively[32]. This component improved sperm parameters in adult male rats treated with cyclophosphamide[33]. It was reported that other components in honey compound syrup, such as cinnamon and saffron, showed protective effects on the reproductive system. For instance, cinnamon ameliorated spermatogenesis and testis tissues in busulfan-induced infertile rats[7]. Cardamom, galangal, mastic, and nutmeg are other components of honey compound syrup that have various effects, such as antioxidant and anti-inflammatory effects[11]. These findings show that all improvement that occurred in sperm parameters and testis tissue was appeared by antioxidant effects of all the components of the honey compound syrup.

In conclusion, this study shows that honey compound syrup (honey and seven other medicinal plants) could repair the damaged testis tissues and improve the spermatogenesis process in busulfan-induced rats. It seems that these plant compounds can be useful to reduce chemotherapy side effects such as azoospermia in the future.

Conflict of interest statement

There is no conflict of interest in this study.

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Authors' contributions

Rasool Choopani and Haniye Kashafroodi conceived of the presented idea. Rasool Choopani developed the theory, performed the computation, and supervised the findings of this work. Seyyed Shamsadin Athari carried out the experiment. Keivan Lorian and Haniye Kashafroodi wrote the manuscript with support from Rasool Choopani. Saadat Ghafarzadeh aided in interpreting the results of pathology samples. All authors discussed the results and contributed to the final manuscript.

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