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# Royal Jelly Administration Recover Spermatogenesis and Sexual Hormone Levels in a Busulfan-injured Rat Model

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**ABSTRACT**

The role of the busulfan-mediated pathway in its anti-estrogenic effects and resulting changes of spermatogenesis is still unknown, while royal jelly may improve spermatogenesis. Our objective was to determine the effects of royal jelly on the recovery of spermatogenesis, histology testis and sexual hormones and in of a busulfan-injured rat model. Sixty adult rats were randomly assigned to three treatment groups, consisting of four replicates with five rats in each replicate. Animals received a tow intraperitoneal vehicle (control) or 10 mg/kg busulfan injection (at 0 and 21 day), and 35 days after, the animals received orally 100 mg/kg royal jelly for 14, 28 and 56 days. One day after, blood plasma was obtained for hormone analysis, sperm was recovered from epididymis, and testes were processed for histology. The weight of the body and testis were recorded. Body and testis weights were significantly lower in the busulfan- vehicle group comparing to the other treatments ($P<0.05$). Whereas, this decrease was recovered in the royal jelly+ busulfan rat. Control groups did not show significant changes in most parameters, but busulfan decreased sperm counts, motility and normal morphology, induced seminiferous tubule depletion, intertubular space and decreased blood testosterone, FSH and LH. Royal jelly treatments partially recovered spermatogenesis, decreasing tubular atrophy. The royal jelly treatments in the busulfan-treated animals significantly increased sex hormones levels and sperm parameters, but, except for sperm motility and normal morphology at 56 d, they did not recover up to the values in the no-busulfan group ($p<0.05$). Royal jelly was the most efficient treatment, also increasing the concentration of sexual hormones close to no-busulfan levels. The royal jelly treatments reverted spermatogenesis, hormonal levels and histology similar to controls, however not attaining the same sperm quality than controls busulfan.

**Keywords:** Busulfan, Sex hormones, Rat, Spermatogenesis, Royal jelly.

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## INTRODUCTION

The fertility science has faced major challenges in the management of sperm disorders. Nowadays, many chemotherapeutic drugs affect the male reproductive system, and common therapeutic methods often result in unsatisfactory results. There is an increasing interest in this topic (reproductive toxicities), due to the overall fertility decrease in industrialized countries, but there is still much to know concerning the effect of chemotherapeutic drugs on the reproductive system.

Further understanding of chemotherapy drugs and their effects on the male reproductive system could help improve infertility treatments in patients. Busulfan (chemically known as 1, 4-butanediol dimethanesulfonate) has commonly been used to treat breast cancer after surgery and radiation therapy. Several studies have confirmed the negative effects of busulfan on spermatogenesis and can suppress testicular activity in human and animal models (4, 7, 13, 16). Although the process is still unclear, busulfan seems to exert its effects in the testicle primarily by its toxicity to spermatogonial stem cells (DNA alkylation, cytoskeletal disruption, oxidative stress, etc.), but it affects Sertoli, Leydig, and peritubular cells too (2, 13).

Most of the current published data have focused on royal jelly as a chemopreventive agent, but there is increasing interest in its use for the treatment of infertility. Royal jelly has several biological activities and pharmacological properties, including antioxidant capacity, antitumor, and a protective effect against testicular atrophy and the reproductive system (5, 9). Amirshahi *et al*. (2013) and Delkhoshe-Kasmaie *et al*. (2014) observed that Taxol (chemotherapeutic agent) and Bleomycin (chemotherapic antibody) have acute reproductive toxicity in rats, respectively, but the administration of royal jelly remarkably reduced the chemotherapy drugs-induced histopathological injuries (such as cellular shrinkage and seminiferous tubule depletion), and could help to improve sex hormone levels, the maintenance of germinal epithelium, and reverse spermatogenesis (1, 5).

This study, using rats as a model, tests the hypothesis that exposure to busulfan alters testicular histology and function, and this may influence sex hormones, altogether suppressing spermatogenesis, whereas royal jelly treatment could reverse these effects. Therefore, we have evaluated the effect of royal jelly on the testicular structure, sex hormone concentration, and sperm characteristics in busulfan-injured rats.

## MATERIALS AND METHODS

### *Reagents and equipment*

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). The ELISA kits (Cosmo Bio Co., Tokyo, Japan) were prepared to evaluate some reproductive hormones. Royal jelly was collected from beehives no 28 and 74, Pars Asal Co., Fars province, Iran, during 2018 and kept at -20 °C until use. It was dissolved in distilled water and given orally.

### *Animals*

All the experimental protocols were approved by the Ethics Review Committee (No. February/2018 on 31s July 2018) of Shiraz University of Medical Sciences (Permit Number: A: 21-5035). Healthy adult male Wistar rats (200±20 g; 6-week-old) were purchased from the Pasteur Research Center (Karaj, Iran). Rats were kept in an air-conditioned animal room under a 12 h light: 12 h dark cycle under standard environmental

conditions (23-24 °C, humidity 50-55%) with free access to tap water and commercial dry pellet diet. During the experiment, rats were housed in polypropylene cages lined with pine wood husk and changed daily.

***Experimental design***

Sixty adult rats were randomly assigned to three treatment groups, consisting of four replicates with five rats in each replicate. Animals received a tow intraperitoneal vehicle (control) or 10 mg/kg busulfan injection (at 0 and 21 day), and 35 days after, the animals received orally 100 mg/kg royal jelly for 14, 28 and 56 days. On completion of treatment, the rats were anesthetised with 0.64 mg/kg xylazine and 20 mg/kg ketamine (Alfasan, Woerden, the Netherlands) and then euthanized by exsanguination. Blood plasma was obtained for hormone analysis, sperm were recovered from the epididymis, and testes were processed for histology. The weight of the body and testis were recorded.

***Hormonal assays***

Blood samples were obtained via the tail vein in order to evaluate the serum hormones. Samples were collected in vacuum tubes early in the morning before treatments. In order to evaluate the testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) concentration, the serum was separated by centrifugation (×4000g for 10 min) and readily frozen at -20 °C. Samples were assessed in groups by radioimmunoassay (kits of Monobind Inc). Standard commercial kits were used for analysis, and the procedures were adopted as recommended by the manufacturer.

***Histological analysis***

The left testes were removed for histology parameters. After macroscopic observation, testicular tissue samples were fixed in 10% buffered neutral formaldehyde for at least 72 hours. Tissue samples were directly dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Thin sections (5-7 μm), perpendicular to the longest axis of the testis, were cut using a rotary microtome, stained with hematoxylin and eosin (H&E), and examined under a light microscope for histological studies (Olympus, Tokyo, Japan, BX60).

***Epididymal sperm analysis***

In order to assess the epididymal sperm count, the cauda epididymides were placed in 1 ml of Ham's F10 medium. The epididymis was cut into 2-3 pieces and incubated at 37 °C for a few minutes (5% CO2) in order to allow sperm to swim out of the epididymal tubules. An aliquot of sperm suspension was diluted 1:20 with Ham's F10 medium and transferred into a Neubauer's hemocytometer. Spermatozoa were counted under a light microscope at ×400 and expressed as million/ml of suspension (World Health Organization, Department of Reproductive Health and Research, 2010).

Sperm motility was determined by placing a drop of 10 μl of the sperm suspension in a 37 °C pre-warmed slide and covered with a coverslip. At least 10 fields were assessed for each sample using a bright-field microscope with a closed diaphragm, and the percentage of motile spermatozoa was estimated subjectively (11).

Sperm morphology was analyzed by eosin-nigrosin staining in 500 spermatozoa. A drop of stained sperm suspension was put on a clean slide and a thin smear was made and allowed for drying.

This slide was examined under a light microscope at ×1000, and spermatozoa with white and pink heads were considered alive or dead, respectively (8).

### *Statistical analysis*

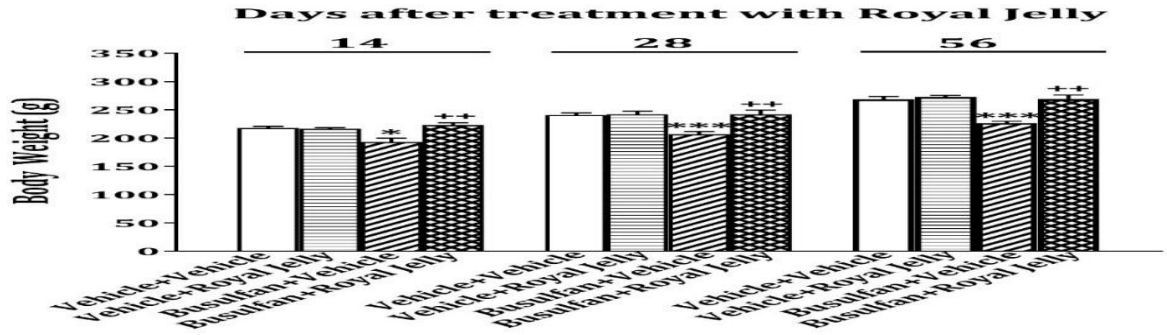
Our data, except histological parameters, were analysed by SPSS 17.0 software. In order to compare the treatments, one-way ANOVA, t-test, and Duncan test were used for multiple comparisons between groups ($P<0.05$). The results were expressed as the mean ± standard deviation (SD).

## RESULTS

### *Weight*

Figure 1 and Table 1 show body and testis (average) weights in each group (mean±SD). Body and testis weights were significantly lower in the busulfan-vehicle group compared to the other treatments (P<0.05). Whereas, this decrease was recovered in the royal jelly+ busulfan rat.

**Figure Legend**




| Days | 14 | | | | 28 | | | | 56 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | V+V | V+R | V+B | B+R | V+V | V+R | V+B | B+R | V+V | V+R | V+B | B+R |
| Testis (g) | 2.61±0.09[a] | 2.83±0.13[a] | 1.62±0.15[b] | 2.18±0.54[a] | 2.77±0.11[a] | 2.91±0.08[a] | 1.81±0.7[b] | 2.32±0.17[a] | 2.81±0.14[a] | 2.93±0.2[a] | 1.85±0.11[b] | 2.4±0.12[a] |

V: Vehicle, R: Royal jelly, B: Busulfan; groups with different letters differ, P<0.05.

**Fig. 1** Body weight index after vehicle or busulfan injections (at 0 and 21 day), and 35 days after, the animals received orally 100 mg/kg royal jelly for 14, 28 and 56 days. *Significant difference with control group (p<0.05). **Significant difference with royal jelly group (p<0.05). ***Significant difference with busulfan+royal jelly group (p<0.01).

### *Sperm and hormone*

The administration of busulfan (10 mg/kg busulfan injection at 0 and 21 day) clearly decreased spermatogenesis and altered testicular and hormonal results. The sperm production and quality (Figure 2), blood serum levels of testosterone, FSH, and LH (Figure 3) significantly decreased in comparison to the animals in the control groups (p<0.05). The royal jelly treatments in the busulfan-treated animals significantly increased sex hormone levels and sperm parameters, but, except for sperm motility and normal morphology at 56 days, they did not recover to the values in the no-busulfan group (p<0.05).

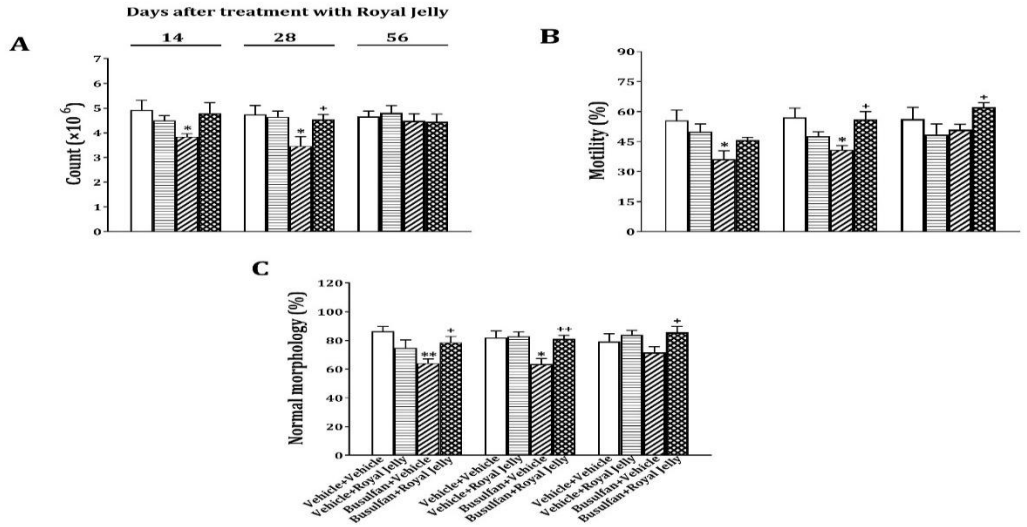



**Fig. 2** Sperm variables after vehicle or busulfan injections (at 0 and 21 day), and 35 days after, the animals received orally 100 mg/kg royal jelly for 14, 28 and 56 days. *Significant difference with control group ($p<0.05$). **Significant difference with royal jelly group ($p<0.05$).

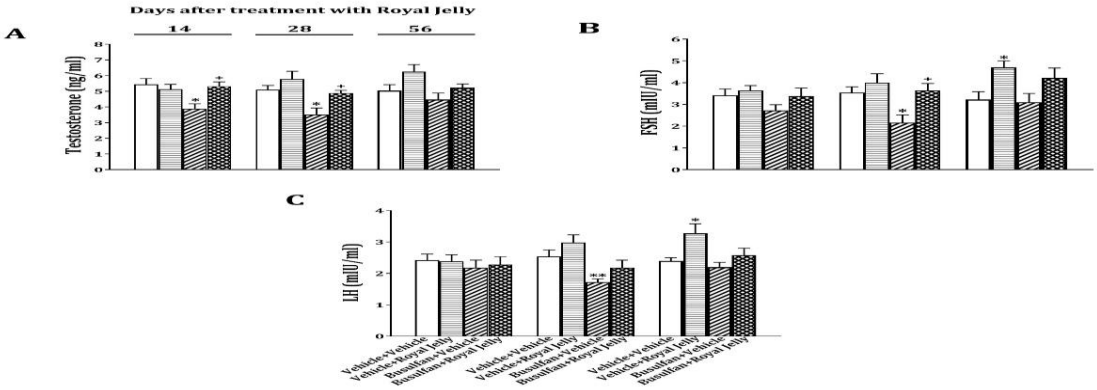



**Fig. 3** Sex hormones levels after vehicle or busulfan injections (at 0 and 21 day), and 35 days after, the animals received orally 100 mg/kg royal jelly for 14, 28 and 56 days. *Significant difference with control group ($p<0.05$). **Significant difference with royal jelly group ($p<0.05$).

***Histology***

The testicular histological structure and the seminiferous tubules architecture of no-busulfan rats were normal, irrespective of the hormonal treatment (Figure 4a), whereas the busulfan treatment caused extensive disruption of the histology (Figure 4b). We observed atrophy of the seminiferous tubules with damage to the germinal epithelium and shrinkage of the basal lamina. It was accompanied by and an increase of the interstitial space apoptosis rate, which presented congested blood vessels and vascular hyperemia. The administration of royal jelly recovered the histological structure (Figure 4d). Royal jelly still showed increased interstitial space and some damage in the seminiferous tubules. In the royal jelly-treated groups, we observed Leydig cells adjacent to the basal lamina, as well as elongating and round spermatids in the epithelium.

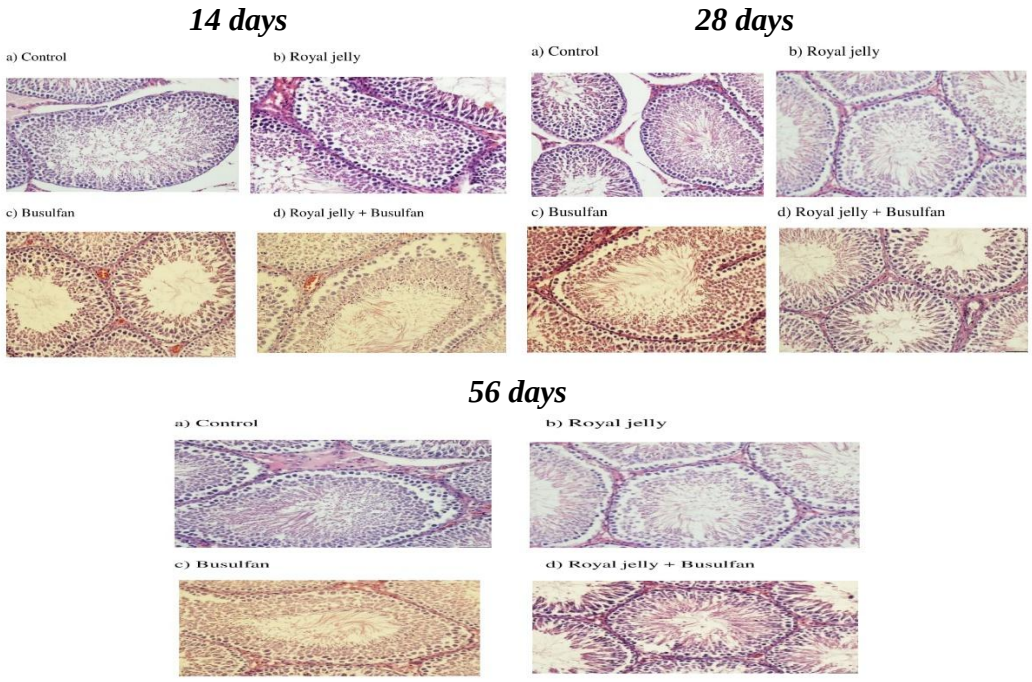



**Fig. 4** Testicular histological structure of busulfan rat after treatment for 14, 28, and 56 days with: (a) Vehicle (control); (b) Royal jelly; (c) Busulfan; (d) Royal jelly+Busulfan. (hematoxylin and eosin, ×100). The histological structure and seminiferous tubule architecture were normal in control (a) and royal jelly (b) groups for 14, 28, and 56 days. Sections of testis tissues from rats treated with busulfan for 14, 28, and 56 days (c) showed extensive atrophy in the seminiferous tubules, destruction of the germinal epithelium, increased interstitial space and apoptosis rate, congested blood vessels and vascular hyperemia, and shrinkage of the basal lamina. After royal jelly administration for 14, 28, and 56 days, the royal jelly groups (d) showed an organized germinal epithelium and a proper spermatogenesis rate.

## DISCUSSION

Nowadays, assessment of sperm parameters and testicular histological structure in rodents is a significant issue owing to the increase in reproductive toxicities data, looking at the impact of chemotherapeutic drugs on the male reproductive system. In recent years, several investigations have been done in order to evaluate the possible toxic effects of various chemotherapeutic drugs on the reproductive system (1, 2, 4, 5, 7, 13, 16). In this regard, the role of the busulfan-mediated pathway in its anti-estrogenic effects and the resulting changes of spermatogenesis is still unknown.

During the 8-week experiment (before royal jelly treatment), clinical signs of chemotherapy, such as body weight (Figure 1) and hair loss, were observed, which may be related to the busulfan-induced anorexia and low food consumption. Delkhoshe-Kasmaie *et al*. (2014) reported that anorexia might be due to either the chemotherapy drugs (Taxol)-related central effect or chemotherapy drugs-related pathologic effects on gastrointestinal movements and secretion (5).

Few studies have focused on the effects of busulfan on the rat testis, its histological changes, and how to reverse them (5). Together with the results present study in rats, Jafarian *et al*. (2014) in mice and Olfati *et al*. (2018) in rats were found that busulfan play an essential role in male infertility (7, 16). Our findings are well

coordinated by the current literature on busulfan toxicity, indicating that these chemotherapeutic drugs can cause testicular dysfunction. In all rats exposed to busulfan, spermatogenesis rate and spermatogenic cells production (count and motility) were significantly ($p<0.05$) lower than in the control group. Morphological changes in the testis were paralleled by functional disorders such as lower sperm count and motility (5).

Our study shows new information for the rat as an animal model for royal jelly effects on the male reproductive system. One of our more relevant findings was the effects of the royal jelly treatments (100 mg/kg bw for 14, 28, and 56 days) on spermatogenesis recovery in the busulfan-injured rats by impacting the normal structure of the testis, secretion of testosterone, and spermatogenesis. In agreement, Delkhoshe-Kasmaie *et al*. (2014) showed a protective effect of royal jelly, in particular at a 100 mg/kg (bw) dose level, on the Taxol-induced structural damage and also on functional activities of the testis (3). Taking royal jelly has several pharmacological properties, including remarkable antioxidant capacity (5, 13) and a protective effect against testicular atrophy and the reproductive system (9, 10), and the physiological functions of its proteins, on the one hand, may explain its beneficial and protective effects on sperm quality (10).

Maintenance of normal male fertility depends on spermatogenesis, which is under complex endocrine control by mechanisms involved in gonadotropin and steroid hormones (6). Our hormonal evaluation confirms that busulfan destroys the sexual hormone homeostasis, likely by acting on estradiol receptors in the testicle and in the hypothalamus-pituitary axis. Busulfan-injured rats showed reduced serum testosterone, FSH, and LH levels, which may be responsible for the inhibition of spermatogonial proliferation and differentiation in the testicles of these animals. However, we need to confirm if they directly affected the testicular tissues or if their effects were mediated by other systems. Also, busulfan disrupts estradiol signalling, which is critical for the maintenance of testicular function, and concomitantly alters hormonal balance and sperm production. It seems that supplementation with royal jelly can counteract busulfan effects and help recovering the hormonal balance. One of the effects seems to be a partial recovery of LH levels (Figure 3), which results in testosterone production, possibly contributing to the restoration of spermatogenesis/steroidogenesis.

Spermatogenesis is a fundamental process of proliferation and differentiation of germ cells into spermatozoa, which is dependent on FSH, produced by the adenohypophysis, and androgens, locally produced in response to LH. Testosterone, as the major androgen in the testis, is essential for regulating the development, growth, and metabolism of the male reproductive system. Zhou *et al*. (2011) reported that the decrease of circulating FSH, LH, and testosterone was also related to changes in gene expression in the testis (15). Moreover, the decrease in testosterone secretion in busulfan-treated rats could worsen the effects of decreasing estrogen synthesis, since testosterone regulates some genes supporting the complex development of germ cells (12). The treatment of busulfan-injured male rats with royal jelly induced the recovery of testosterone and FSH levels (steroidogenesis), causing testicular restoration and qualitatively normal spermatogenesis.

Earlier reports have demonstrated that busulfan treatment in mice (45 mg/kg daily for 35 d) (7) and rats (45 mg/kg daily for 42 d; 16) caused several histopathological changes in the testis and epididymis, such as thicking of the tunica albuginea, distortion and deforming some

seminiferous tubules, degenerating Leydig cells, and increasing interstitial space. Whereas we would need mechanistically experiments to confirm it, the antiestrogenic effect of busulfan seems to be the culprit here. The subsequent hormonal disruption could worsen its effects, leading to spermatogenic arrest. A line with the results of the present study (Figure 4c, d), a previous study on male rats (5) has also confirmed the potential of royal jelly to help reverse spermatogenesis.

In conclusion, the administration of royal jelly in busulfan-induced rats recovered the histological structure. Also, our study also confirmed that the histological disruption in the testis of busulfan-treated rats was related to widespread apoptosis.

## CONCLUSION

In conclusion, the administration of busulfan can impair spermatogenesis and cause infertility, seriously altering the histological structure of the testicle and the hormonal balance.

These effects can be caused either directly by acting on the testis or indirectly by negative effects on the regulation of gonadotropin secretion. These questions should be addressed in future mechanistic studies.

Royal jelly helped recovering spermatogenesis and histological testicular structure in the testis of busulfan-injured rats.

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**Transparency declaration**

There is no conflict of interests.

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