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Abstract

Objectives: Methotrexate (MTX) is an anticancer drug used in chemotherapy. MTX was known for its toxic effects involving most of the organs including testis. Bee pollen is healthy food for human and has antioxidant effect. We intended to determine protective effect of bee pollen against testicular injury caused by MTX in rats.

Methods: Thirty-two adult Sprague Dawley male rats were used, and 4 groups were formed: control, MTX, pollen, and MTX + pollen. Rats were given pollen at a dose of 400 mg/kg with intragastric gavage for 10 days. On day 7, MTX was administered a single dose of 30 mg/kg ip. Serum testosterone and LH, tissue MDA level, and SOD and CAT enzyme activities were examined. In addition, spermatological parameters were evaluated.

Results: MDA level and SOD activity increased while testosterone level decreased significantly in the MTX group compared to the control group. In the MTX + pollen group, MDA level and SOD activity decreased while testosterone level increased. There was no significant change in CAT activity and LH values. Abnormal sperm ratio decreased in the MTX + pollen group compared to the MTX group.

Conclusions: Our results suggest that bee pollen has a healing effect on reproductive parameters in testicular damage caused by MTX.

Keywords: methotrexate; oxidative stress; pollen; testicular damage; testosterone.

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Introduction

Methotrexate (MTX) is a chemotherapeutic agent that shows its effects by inhibiting folic acid metabolism [1] and it is used in the treatment of many diseases such as rheumatoid arthritis and psoriasis [2]. MTX is reported to be toxic not only in cancer cells but also leading to the formation of oxidative damage in many organs such as testis, kidney, and liver [3, 4]. The testis produces testosterone, a hormone necessary for as well as auxiliary reproductive gland functions, and spermatozoa, that are essential for reproduction. This organ is easily affected by toxic substances given or received externally [5]. Oxidative stress is important factor causing dysfunctions in the reproductive system in males [6]. Oxidative stress develops due to disturbed balance between oxidants and antioxidants, against antioxidants or in favor of oxidants [7]. Free radicals are constantly formed as intermediate products in the active sites of enzymes during the cellular enzymatic events. Sometimes free radicals leak from the active sites of enzymes, which accidentally come into contact with oxygen to generate reactive oxygen species (ROS). The ROS produced in the cell are removed by antioxidants. In some cases, the amount of ROS produced can be over the removing capacity of antioxidants. As a result of this, oxidative tissue damage occurs [8]. Free radicals affect cell membranes first and lipids in the membranes react with free radicals to form peroxidation products [9]. Malondialdehyde (MDA) is a marker of lipid damage caused by oxidative stress. The resulting MDA polymerizes the membrane lipids by cross-linking. As a result of polymerization, some changes occur that affect membrane properties such as ion transport and degradation of enzyme

activities [10]. Cells have antioxidant systems that prevent oxidative damages of free radicals, control the level of products formed, and capture and stabilize free radicals. Antioxidants prevent cell damage by reducing free radicals and lipid peroxidation [11]. Superoxide dismutase (SOD) is an antioxidant enzyme and dismutates superoxide to molecular oxygen and hydrogen peroxide (H_2O_2). Another important antioxidant enzyme is catalase (CAT) decomposes H_2O_2 by converting it into water and molecular oxygen [12]. All these antioxidant enzymes prevent tissue and organ damage caused by oxidative stress. It is known that free radicals generated as a result of destruction of the balance between oxidant and antioxidant systems in the testis can cause tissue damage, spermatozoa dysfunction, and abnormal spermatozoa production and infertility [13]. Studies demonstrated that MTX caused oxidative damage in germ cells and testicular tissue by reducing antioxidant activity of the cells, making them vulnerable to the deleterious effects of reactive oxygen species [14].

Bee pollen has been used as a healthy nutritional food for many years. Bee pollen contains carbohydrates, vitamins, minerals, and amino acids and also flavonoid and phenolic compounds [15]. Bee pollen possesses biological activities such as antibacterial, antifungal [16], and antioxidant [17].

As far as we know, there is no study on the reproductive effect of bee pollen in MTX-induced testicle damage. So the purpose of this study is to investigate whether bee pollen has a protective effect on testicular damage caused by MTX, a chemotherapeutic agent, at spermatological, endocrine and biochemical levels.

Materials and methods

Experimental animals and study groups

Thirty-two male Sprague Dawley rats (about 250–300 g) were used. Fırat University, Local Ethics Committee for Animal Experiments was applied at the planning stage and the study was planned in line with the ethics committee decision (protocol no. 2017/72). The rats were kept in cages at 22 ± 2.0 °C, under 12/12 h dark-light cycle. They were fed ad libitum and were randomly divided into 4 groups: control, MTX, pollen, and MTX + pollen (n=8). The control group was fed with standard rat diet and tap water for 10 days. MTX was administered by intraperitoneal injections at 30 mg/kg on day 7 [14] and pollen was given by intragastric gavage at 400 mg/kg/day [17] each day throughout the experiment, which lasted for 10 days.

Preparation of pollen extract

The pollen extract was flower pollen extract obtained from Macahel Apiculture Co. Ltd. It was weighed at a dose of 400 mg/kg and extracted with distilled water for 24 h and then filtered. The aqueous portion was stored at +4 °C until the experiment.

Sample collection

At the end of the study, animals were decapitated and blood samples were collected. Serum samples were separated for determination of LH and testosterone levels and frozen at −20 °C until analysis. The testis and epididymis were weighed after removing the fat tissue. Testis was removed and stored at −80 °C for analyses of MDA level, CAT and SOD activities. Of the accessory glands, vesicula seminalis and the ventral prostrate were removed and weighed.

Biochemical analyses

Determination of serum testosterone and LH levels

The serum testosterone and LH levels were determined by Enzyme-Linked Immuno Sorbent Assay (ELISA) for Rat kit (SunRed Biological Technology, Co., Ltd. Shanghai) according the manufacturer's instruction.

Determination of MDA, SOD and CAT levels

Testis tissues were homogenized at 1200 rev/min for 1–2 min in ice after adding 0.1 M phosphate buffer at pH 7.4. Homogenates were centrifuged at 4000 × g for 30 min to obtain supernatant. MDA levels, SOD, and CAT enzyme activities were measured in these supernatants.

The MDA level in the testis tissue was determined by using the spectrophotometric method of Mihara and Uchiyama [18]. SOD activity was detected according to the method of Sun et al. [19]. CAT activity was determined by using Aebi [20] method, which is based on decomposition and removal of hydrogen peroxide (H_2O_2).

The total protein levels in the testis was determined according to the Bradford [21] method, based on the Coomassie Brilliant Blue G-250 dye generating varying intensities of blue color in different concentrations of protein solutions.

Assessment of sperm characteristics

Sperm motility, sperm density, vesicula seminalis, prostate and right cauda values were evaluated by Turk et al. [22].

Statistical analysis

SPSS 23.0 (SPSS Inc., Chicago, IL, USA) program was used for the statistical analysis of the findings obtained in the study. Normality of the distribution of data was analyzed

by Kolmogorov-Smirnov test while variance analysis of the quantitative variables by one way ANOVA and post-hoc Tukey test. Non-normal distribution of data was assessed by nonparametric Kruskall–Wallis test.

Results

Serum testosterone and LH levels

As shown in Figure 1, there was a significant decrease in testosterone level of MTX group (563.99 ± 43.91 pg/mL) compared to the control group (861.53 ± 39.70 pg/mL). On the other hand, the testosterone level of the treatment group (MTX + pollen) (853.20 ± 38.60 pg/mL) was increased ($p<0.05$) compared to MTX. However, there was no difference in serum LH levels of the groups ($p>0.05$).

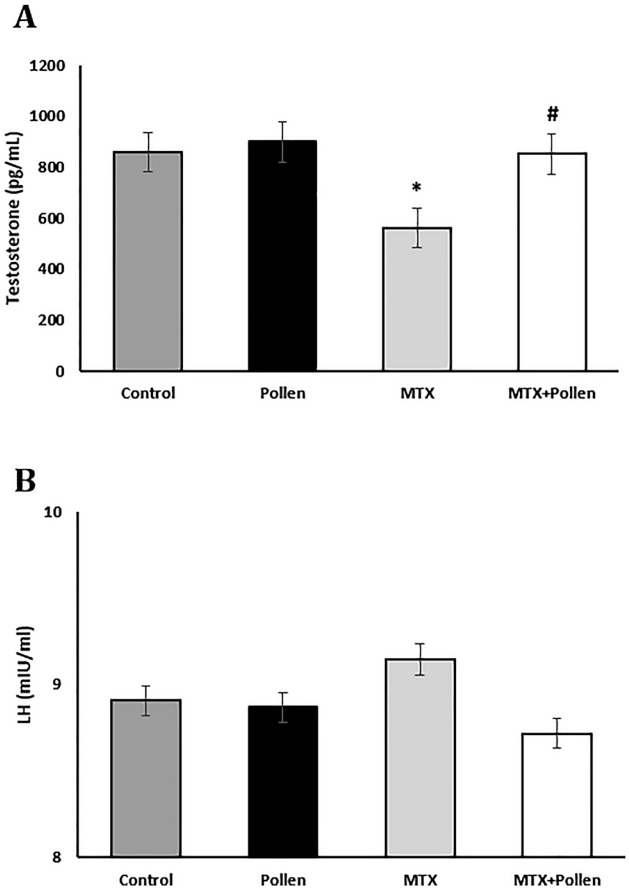

Figure 1: Serum (A) testosterone and (B) LH levels in rats. Data is presented as mean ± standard deviation. MTX, Methotrexate; LH, Luteinizian hormone. *Significantly different when compared with control group, ($p<0.05$). #Significantly different when compared with MTX group, ($p<0.05$).

Result of MDA level, SOD and CAT activities

Biochemical results of the experimental groups are presented in Figure 2. MTX appears to significantly increase MDA level and SOD enzyme activity compared to control and pollen groups ($p<0.05$). However, there was no statistically difference in CAT activities ($p>0.05$). In addition, tissue MDA and SOD levels of treatment group (MTX + pollen) decreased significantly compared to MTX group ($p<0.05$) There was no statistically difference in the serum CAT levels of the treatment group compared to the MTX group ($p>0.05$).

Spermatologic results

Sperm motility, sperm density, vesicula seminalis, prostate, and right cauda values are given in Table 1. There was no statistically difference in sperm motility of the study groups ($p>0.05$). Abnormal sperm percentage was found to be statistically higher in the MTX group than the control group while the values concerning vesicula seminalis, prostate, and right cauda were statistically significantly lower ($p<0.05$). In the MTX + pollen group, vesicula seminalis and prostate values showed statistically significant increase with respect to the MTX group, while abnormal sperm percentage decreased ($p<0.05$). There was no statistically difference in terms of sperm density and right cauda values in MTX + pollen group with respect to MTX group ($p>0.05$). In comparison to the control group, pollen group showed no statistically differences in motility, density, abnormal sperm percentage, vesicula seminalis, and right cauda values ($p>0.05$), but only prostate values were found higher than the control group ($p<0.05$).

Testicular and epididymal weights results

Testicular and epididymal weights of the groups are presented in Table 2. There was no statistically difference between the groups in right and left testis weights and epididymis weights ($p>0.05$).

Discussion

Methotrexate (MTX) is a frequently used for chemotherapy and produce toxic side effects in various organ systems. It also has harmful effect on testes [3]. MTX impairs fertility by causing defective oogenesis and spermatogenesis. Its significant side effects on men were reported as infertility and persistent azoospermia [23]. Since ancient times, mankind

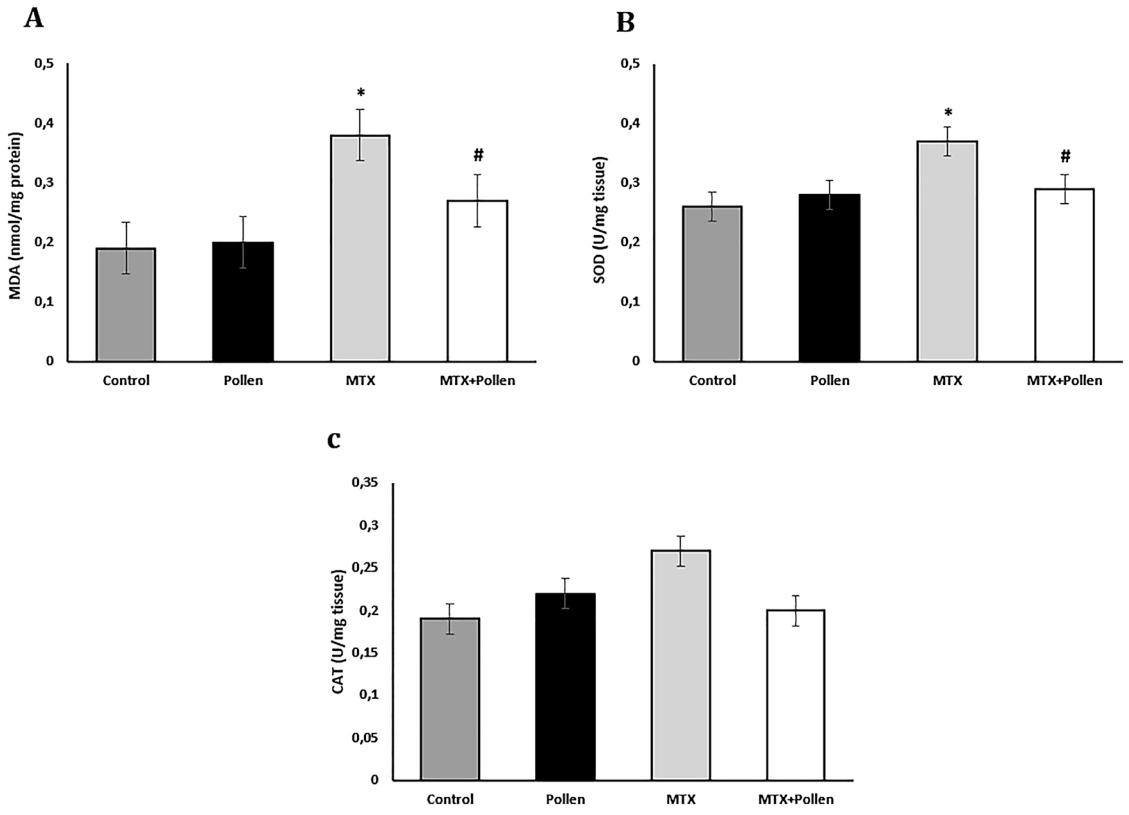

Figure 2: (A) MDA, (B) SOD and (C) CAT activities testis tissue data is presented as mean ± standard deviation. MTX, Methotrexate; CAT, Catalase; MDA, Malondialdehyde; SOD, Superoxide dismutase. *Significantly different when compared with control group, ($p<0.05$). #Significantly different when compared with MTX group, ($p<0.05$).

Table 1: Spermatological parameters of the groups.

Parameters	Control	Pollen	MTX	MTX + pollen
Density (million/cauda)	96.0 ± 4.60	113.97 ± 22.54[b]	75.28 ± 3.60	87.50 ± 24.95
Motility	76.80 ± 4.60	81.75 ± 6.90	72.00 ± 9.18	76.37 ± 9.25
Abnormal sperm rate	8.5 ± 1.87	6.71 ± 2.49[b]	12.00 ± 2.28[a]	8.12 ± 0.88[b]
Vesicula seminalis	40.80 ± 2.58	43.00 ± 7.77[b]	30.66 ± 4.36[a]	32.80 ± 2.77[b]
Prostate	26.40 ± 3.84	33.40 ± 5.85[a,b]	17.66 ± 8.73[a,c]	25.66 ± 2.65[b,c]
Right cauda	17.00 ± 2.12	17.00 ± 1.15[b]	12.75 ± 0.95[a]	13.37 ± 1.92[a,c]

Data is presented as mean ± standard deviation. MTX, Methotrexate.
[a]Significantly different when compared with control group, ($p<0.05$).
[b]Significantly different when compared with MTX group, ($p<0.05$).
[c]Significantly different when compared with Pollen group, ($p<0.05$).

has been used bee pollen in the treatment of many diseases such as burns, ulcers, and carcinogenesis. It has also been reported to protect cells from oxidative stress [17, 24]. To the best of our knowledge, there are no studies in the literature investigating the effects of bee pollen on MTX-induced testicular damage. That's why we have investigated in this present study, that whether dietary supplementation of bee pollen plays any role in preventing damage in MTX-induced testicular tissue damage or not.

Methotrexate (MTX) creates a distinct risk for organs having elevated mitotic activity, such as testicular tissue, and leads to impairment of new spermatozoon formation, and eventually infertility [25]. In present study, statistically decrease in serum testosterone levels was sighted with MTX administration but no statistically difference in serum LH levels. However, bee pollen was observed to increase testosterone levels significantly in the MTX + pollen group. Pınar et al. [26] shown that MTX caused significant

Table 2: Testis and epididymis weights of the groups.

Parameters	Control	Pollen	MTX	MTX + Pollen	p-Value
Right testis	1.69 ± 0.13	1.68 ± 0.19	1.54 ± 0.21	1.59 ± 0.11	ns
Left testis	1.70 ± 0.14	1.66 ± 0.19	1.53 ± 0.21	1.57 ± 0.12	ns
Right epididymis	41.20 ± 3.03	43.50 ± 5.04	43.25 ± 5.84	37.87 ± 3.84	ns
Left epididymis	35.80 ± 5.93	41.00 ± 4.37	39.50 ± 4.56	37.50 ± 3.73	ns

Data is presented as mean ± standard deviation. MTX, Methotrexatens; ns, not significance.

decrease in serum testosterone concentration. In another study, MTX has been shown to reduce serum testosterone levels in rats but not LH levels [27]. The effects of MTX in the literature on testosterone and LH levels are consistent with our results.

Malondialdehyde (MDA) actually shows the level of lipid peroxidation. Increased MDA levels are an important marker of cell membrane damage induced by oxygen radicals [28]. Dagguli et al. [29] administered a single dose of 20 mg MTX to rats and reported an increased MDA level. The fact that testis wall is rich in polyunsaturated fatty acids, had an accelerating effect on the process of oxygen-dependent lipid peroxidation, as they also pointed out in their study. In our study results consistent with the literature, MTX administration was observed to increase testicular tissue MDA levels statistically significantly. This increase was suppressed by bee pollen and this suggests that bee pollen has a protective effect against lipid peroxidation in systemic oxidative damage induced by MTX. This effect may be related to the polyphenolic compounds of bee pollen and their antioxidant properties.

In a healthy organism, deleterious effects of ROS are not observed through the delicate balance between ROS and antioxidant activity. Produced ROS are neutralized by CAT and SOD enzymes, thus providing the generation and removal of ROS to be balanced [8]. SOD plays great role in spermatogenesis and testicular development. Changes in enzyme activity may lead to impaired testicular functions and stopping of sperm development [30]. In previous study, demonstrated that MTX decreased the activity of the antioxidant enzyme system and sensitized the cells to reactive oxygen particles and caused testicular toxicity [3]. Yulug et al. [14] showed that MTX induced a decreasing of CAT and SOD activity in testicular tissue. They consider that decrease CAT and SOD enzyme activities may be related to the increase in consumption and unbalance of resynthesis mechanism. Our results were not consistent with the literature studies. In our study, it was observed that MTX increased SOD activities but did not change CAT activities significantly. SOD is an antioxidant enzyme that plays a role in clearing superoxide radicals formed by incomplete reduction of oxygen in metabolism.

Methotrexate (MTX) administration may cause oxidative damage in testicular tissue and increase in superoxide radicals. Accordingly, an increase in SOD activity may have been observed.

Since male fertility is related with continuous self-renewal of spermatogonia and their differentiation into spermatogenic cells, anticancer drugs can affect testicular tissue structurally and functionally and have adverse effects on fertility [30]. In a study, it is shown that MTX caused significant decrease in number, motility, and viability of spermatozoa [1]. Also in another study, MTX was shown to cause low sperm count and an increase in the number of sperm cells with head abnormalities [31]. Our results showed that, MTX increased abnormal sperm rate and decreased vesicle seminalis, prostate and right cauda but MTX didn't affect motility and density. Bee pollen decreased abnormal sperm rate and increased vesicle seminalis and prostate. However, no difference was found between the groups concerning the weights of testis and epididymis. Padmanabhan et al. reported that MTX administered at different doses showed no statistically difference in testis weights of mice when compared with healthy controls [32]. In another study, 20 mg/kg MTX was administered to rats and no difference was determined in testicular weights [33]. In a recent study, the effect of beta-glucan on MTX-induced testicular damage was investigated and there was no difference in testis weight between the groups [34].

Our study indicated that administration of MTX increased lipid peroxidation in testes and, so induced oxidative stress. In addition, it also indicated that MTX had caused spermatological damage. Bee pollen significantly reduced the toxicity of MTX on reproductive system. Considering all the results, we can say that bee pollen has a healing effect on sperm parameters and testicular tissue.

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