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*Original article*

# Activity of resveratrol on the influence of aflatoxin B1 on the testes of Sprague dawley rats

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## Abstract

Twenty eight male Sprague Dawley rats (aged 3 months) were used in the study. The animals were given feed and water as ad libitum. Sprague dawley rats were randomly divided into 4 groups as 7 rats in each group. Except for the control one, aflatoxin B1 (7.5 µg / 200 g), resveratrol (60 mg / kg) was administered to rats of 3 other groups. At the end of the 16th day, blood, semen and tissue specimens were taken by decapitation under ether anesthesia. When we evaluate the spermatological parameters, it is understood that resveratrol has a statistically significant difference in terms of sperm motility and viability (membrane integrity) compared to the control group and aflatoxin B1 administration groups, indicating a protective effect on spermatological parameters. In terms of pathological parameters - histopathological examination - in the control and resveratrol groups, seminiferous tubules were observed to be in normal structure. In the group treated with aflatoxin, the regular structure of the spermatogenic cells deteriorated and the seminiferous tubules became necrotic and degenerative. In the group treated with Afb1 + res, the decreasing of necrotic and degenerative changes were determined compared with in the group treated with aflatoxin. As immunohistochemical examination, cleaved caspase 3 expression was found to be very low in the control and resveratrol groups. Cleaved caspase 3 expression was severely exacerbated in seminiferous tubules in aflatoxin group but cleaved caspase 3 expression level decreased in Afb1 + res. In the biochemical direction, resveratrol has been shown to inhibit the adverse effects of aflatoxin on antioxidant levels and to show a protective effect. For this purpose, the use of resveratrol with antioxidant activity was investigated in preventing or ameliorating damage to aflatoxin B1. It has been concluded that resveratrol effectively prevent the aflatoxin-induced testicular damage and lipid peroxidation. It has also been shown that resveratrol has protective effects on sperm motility and viability.

**Key words:** aflatoxin B1, rat, resveratrol, sperm

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## Introduction

The most widely known mycotoxins, aflatoxins, are the metabolites of Aspergillus flavus, Aspergillus paraciticus, Aspergillus nomius and some Penicillium and Rhizopus species that cause acute and chronic poisoning in humans and animals. They consist mainly of six compounds; aflatoxins B1, B2, G1, G2, M1 and M2 (Marth 1979, Steyn 1995, Mello and Macdonald 1997, Sibanda et al. 1999). Aflatoxins are carcinogenic, mutagenic and teratogenic, as well as being important for health because they are resistant to heat treatments (Veldman 1992, Govaris et al. 2001, Srivastava et al. 2001).

Firstly, resveratrol was described in 1940 as a structure at the roots of white herlebore (Veratrum grandiflorum O. Loes), and then in Polygonum cuspidatum roots called Kojo-kon in Japan (Aggarwal and Shishodia 2006). The strong antioxidant properties of resveratrol are 50 times more potent than vitamin E and 30 times more potent than vitamin C (Celotti et al. 1996, Cheong et al. 1999). It has anti-aging effectiveness; it is thought that has slowing effect the aging (Falchetti et al. 2001). It has also been shown in several studies in which resveratrol increases sperm quality in rats (Jiang et al. 2008, Collodel et al. 2011, Giovana et al. 2013, Abdelali et al. 2016).

The aim of this study was to determine the effects of aflatoxin B1 and resveratrol on spermatological, pathological and biochemical parameters were in Sprague dawley rats.

## Materials and Methods

The approval of Atatürk University Animal Experimentations Local Ethics Committee (Approval number: 2015/161) was taken before starting the study.

### Animals and experimental procedure

Twenty eight male Sprague Dawley rats (aged 3 months) were used in the study. The animals were given feed and water as ad libitum. Sprague dawley rats were randomly divided into 4 groups as 7 rats in each group. I. group (n=7) referred as control group and physiological saline was given as oral gavage. II. group (n=7) referred as Aflatoxin B1 and 7.5 µg/200 g Aflatoxin B1 was given as oral gavage to animals. III. Group (n=6) referred as resveratrol and 60 mg/kg resveratrol was given as oral gavage to animals. IV. Group (n=7) referred as Aflatoxin B1 + Resveratrol and 7.5 µg/200 g Aflatoxin B1 and 60 mg/kg resveratrol were given as oral gavage to animals. At the end of the 16th day, blood, semen and tissue specimens were taken by decapitation under ether anesthesia.

### Collection of samples

Following decapitation procedure, the testes and cauda epididymidis of the rats were removed from the body and cleaned from adipose or connective tissues with anatomical scissors and tweezers. Cauda epididymal semen samples and testes tissues were collected. Routine semen examinations were performed.

### Semen evaluation

One of cauda epididymidis was used to obtain semen sample for each animal. For this purpose, randomly selected cauda epididymidis was minced in Petri dish including 5 mL of physiological saline. To provide the migrations of spermatozoa from cauda epididymidis to fluid, the solution-tissue mixture was incubated in a warmed stage at 35°C for 5 min. Following the incubation period, cauda epididymitis residue was removed by using anatomical tweezers from the Petri dish. The fluid remaining in the Petri dish was used as semen sample. Evaluation of semen was conducted using routine spermatological parameters including motility and alive sperm rate of spermatozoa. To evaluate the percentage of sperm motility, light microscope (Primo Star; Carl Zeiss, Oberkochen, Germany) equipped with the heated stage was used. Briefly, a slide was placed on a heated stage warmed up to 35°C placed on a conventional light microscope. Approximately 20 µL of semen sample was dropped on the slide. The percentage of sperm motility was detected by visual investigation of the sample. To estimate the sperm motility, randomly selected three different fields from each sample were evaluated. The average of three field estimations was calculated as the final motility score of the sample (Turk et al. 2008, Aksu et al. 2016). Sperm viability was evaluated with light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan) after eosin nigrosin staining (Akyol et al. 2015). The smear was prepared for counting. A total of 200 cells were counted and the results are presented as percentages.

### Biochemical evaluations of testicular tissues

The obtained testis tissue samples were washed with cold (+4°C) 0.15 M potassium chloride (KCl) and dried with drying paper for biochemical analyzes. The tissues were homogenized for 3 minutes at 16000 rpm in 0.15 M KCI solution with a homogenizer (Ultra Turrax Type T25-B, IKA Labortechnic, Germany). Homogenization was carried out in an ice cube. The homogenate was centrifuged at 5000 g for 1 hour (at + 4°C) and the levels of GSH, MDA, CAT, GPX and SOD from the supernatant were measured with a Biotek ELISA Reader.

Table 1.

| Testicular biopsy scores (MTBS) | |
|---|---|
| Score | Description |
| 1. | No cells |
| 2. | Sertoli cells without germ cells |
| 3. | Only spermatogonia |
| 4. | Only a few spermatocytes |
| 5. | Many spermatocytes |
| 6. | Only a few early spermatids |
| 7. | Many early spermatids without differentiation |
| 8. | Few late spermatids |
| 9. | Many late spermatids |
| 10. | Full spermatogenesis |

### 1. CAT measurement

Testis CAT activity was measured according to the method of Goth (1991).

### 2. MDA measurement

MDA activity of testis tissue was measured according to the method of Placer et al. (1966).

### 3. SOD measurement

SOD activity was measured according to the method of Sun et al. (1988).

### 4. GPx measurement

Testis GPx activity was measured according to the method of Matkovics et al. (1988).

### 5. GSH measurement

Tissue extraction and analysis of GSH was performed according to the method of Ball (1966), Fernandez and Videla (1981).

Blood samples were centrifuged at 3000 rpm for 10 min at + 4°C, plasma samples were separated and stored at -20°C deep freeze until biochemical analyzes were performed. Plasma MDA, GSH, CAT, SOD and GPx levels were measured respectively according to the method of Yoshioka et al. (1979), Tietze (1969), Goth (1991), Sun et al. (1988) and Matkovics et al. (1988) using the Biotek ELISA Reader.

### Histopathological examination of testicular tissues

The testes were removed immediately, fixed in 10% neutral formalin for 24 -48 hours, and then processed to obtain paraffin blocks. Paraffin-embedded blocks were routinely processed; 5-µm thick sections were stained with hematoxylin-eosin and examined under a microscope, and 10 randomly selected tubules were examined under 20× magnification. It was assessed histopathologically using Johnsen's mean testicular biopsy score (MTBS) criteria (31). A score of 0–10 was given to each tubule according to epithelial maturation (Table 1).

### Immunohistochemical examination of testicular tissues

After deparaffinization, the slides were immersed in antigen retrieval solution (pH 6.0) and heated in microwave for 15 minute to unmasked antigens. The sections were then dipped in 3% H2O2 for 10 min to block endogenous peroxidase. Sections were incubated at room temperature with polyclonal rabbit active/cleaved caspase 3 antibody (cat no. NB600-1235, dilution 1/200; Novus Biological, USA) for apoptosis. Expose mouse and rabbit specific HRP/DAB detection IHC kit was used as follows: sections were incubated with goat anti- mouse antibody, then with streptavidin peroxidase, and finally with 3,3′ diaminobenzidine + chromogen. Slides were counterstained with hematoxylin. Then 10 randomly selected tubules were examined under 20× magnification. İmmunreactivity in the sections were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe).

### Statistical analysis

Statistical comparisons of data were analysed using General Linear Model/Repeated Measures (SPSS, Version IBM 20.0 Microsoft, Chicago, IL, USA) in-group comparisons. Data were expressed as mean±standard error of the mean (SEM). Differences were considered significant when $p<0.05$.

In terms of immunohistochemical parameters statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 20.0). All data were presented in mean (±) standard error (S.E.). Dif-

Table 2. Testicular biochemical parameters.

| Groups | GSH (mmol/g) | MDA (nmol/g) | CAT (kU/g) | GPx U/mg | SOD (EU/mg) |
|---|---|---|---|---|---|
| Control | 0.49±0.03$^{c}$ | 36.49±1.26$^{ab}$ | 230.73±3.24$^{ab}$ | 0.05±0.00$^{bc}$ | 5.32±0.09$^{b}$ |
| Aflatoxin B1 | 0.24±0.01$^{a}$ | 66.46±0.66$^{c}$ | 213.89±0.84$^{a}$ | 0.04±0.00$^{a}$ | 3.97±0.36$^{a}$ |
| Resveratrol | 0.65±0.03$^{d}$ | 32.41±1.34$^{a}$ | 257.88±10.73$^{b}$ | 0.06±0.00$^{d}$ | 8.60±0.36$^{d}$ |
| Aflatoxin B1 + Resveratrol | 0.35±0.01$^{b}$ | 40.09±1.90$^{b}$ | 224.17±4.60$^{ab}$ | 0.05±0.00$^{bc}$ | 7.48±0.12$^{cd}$ |
| p | *** | *** | * | *** | *** |

Table 3. Plasma biochemical parameters.

| Groups | GSH (mmol/L) | MDA (mmol/l) | CAT (kU/L) | GPx U/mL | SOD (EU/mL) |
|---|---|---|---|---|---|
| Control | 2,86±0,24$^{ab}$ | 13,30±0,39$^{b}$ | 225,63±37,44$^{ab}$ | 0,22±0,01$^{ab}$ | 12,25±0,28$^{b}$ |
| Aflatoxin B1 | 2,27±0,10$^{a}$ | 16,67±0,52$^{c}$ | 166,85±13,26$^{a}$ | 0,20±0,00$^{a}$ | 9,77±0,18$^{a}$ |
| Resveratrol | 3,02±0,06$^{b}$ | 11,19±0,25$^{a}$ | 287,50±29,84$^{b}$ | 0,24±0,01$^{b}$ | 12,37±0,18$^{b}$ |
| Aflatoxin B1 + Resveratrol | 2,41±0,21$^{ab}$ | 12,11±0,49$^{ab}$ | 222,25±9,90$^{ab}$ | 0,21±0,01$^{ab}$ | 11,74±0,25$^{b}$ |
| p | * | *** | ** | * | * |

Table 4. The values (Mean ±SEM) of spermatological parameters in male rats used.

| Groups | Motility (%) | Viability (%) |
|---|---|---|
| Control | 71.42±0.52$^{b}$ | 63.85±1.33$^{b}$ |
| Aflatoxin B1 | 60.71 ± 1.30$^{a}$ | 55.00±1.54$^{a}$ |
| Resveratrol | 72.85±1.48$^{c}$ | 70.42±2.61$^{c}$ |
| Aflatoxin B1 + Resveratrol | 57.14±2.40$^{a}$ | 56.57±0.89$^{a}$ |
| p | *** | *** |

ferences in measured parameters among the four groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test ($p<0.05$).

## Results

In the biochemical direction, resveratrol has been shown to inhibit the adverse effects of aflatoxin B1 on antioxidant levels (GSH - mmol/L, CAT - kU/L, GPx -U/mL, SOD - EU/mL) and to show a protective effect in Table 2,3.

In table 4, when we evaluate the spermatological parameters, it is understood that resveratrol has a statistically significant difference in terms of sperm motility and viability (membrane integrity) compared to the control group and aflatoxin B1 administration groups, indicating a protective effect on spermatological parameters (groups: control, resveratrol, aflatoxin B1 and Afb1 + res; respectively, values of motility: 71.42±0.52, 72.85±1.48, 60.71± 1.30, 57.14 ± 2. 40; values of viability: 63.85±1.33, 70.42±2.61, 55.00±1.54, 56.57±0.89.

### Histopathological examination

In the control and Resveratrol groups, seminiferous tubules were observed as normal (Figs. 1-2). It was observed that regular structure of spermatogenic cells deteriorated in aflatoxin-treated group and seminiferous tubules became necrotic and degenerative (Fig 3). In the group treated Aflatoxin + Resveratrol, necrotic and degenerative changes were decreased in comparison with group treated with aflatoxin (Fig. 4, $p<0.05$).

### Immunohistochemical examination

Cleaved Caspase 3 expression was found to be very low in the control and resveratrol groups. Cleaved Caspase 3 was expressed severely in seminiferous tubules in aflatoxin group but Cleaved Caspase 3 expression level decreased in aflatoxin+resveratrol treated group (Figs. 4-8, $p<0.05$).

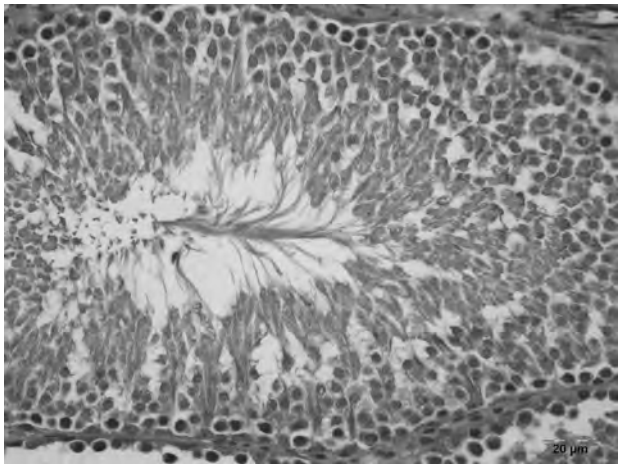


Fig 1. Control group. In normal structure. Hematoxylin-eosm (H-E).

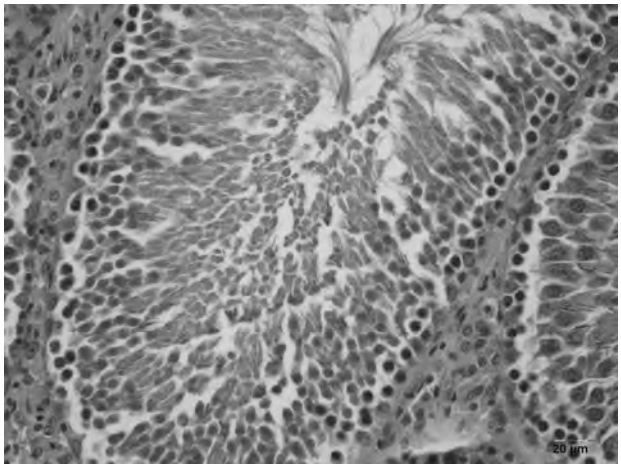


Fig 2. Resveratrol group. In normal structure (H-E).

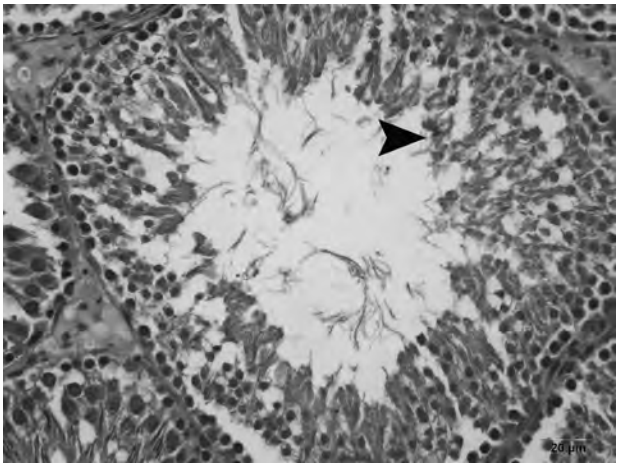


Fig 3. Aflatoxin group. Severe necrotic and degenerative changes in seminiferous tubules (arrowhead).

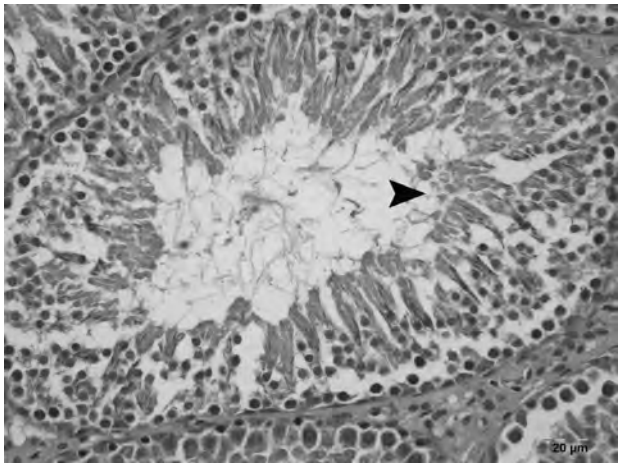


Fig 4. Aflatoxin + resveratrol group. Mild necrotic and degenerative changes in seminiferous tubules (arrowhead).

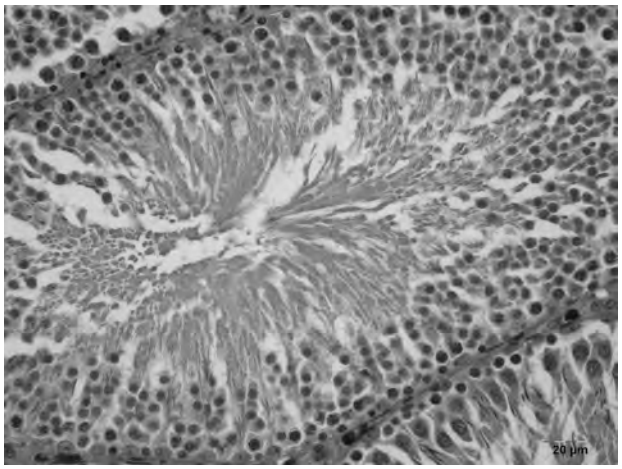


Fig 5. Control group. Immunohistochemistry (IHC).

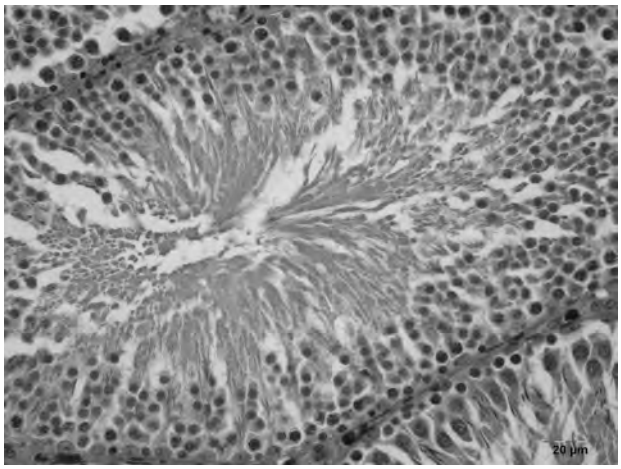


Fig 6. Resveratrol group (IHC).

## Discussion

It is a fact that environmental factors influence spermatological parameters (Agnes and Akbarsha 2003, Mathuria and Verma 2008). Aflatoxins are also important in terms of public health, which can be found in animal products such as milk and eggs (Fan et al. 2015, Giovati et al. 2015, Yuan et al. 2016). It has been shown the harmful effects of aflatoxin on various animals and tissues in previous studies (Verma and Nair 2001, Verma and Mathuria 2009, Wang et al. 2013, Nazz et al. 2014, Shi et al. 2015). Therefore, it is important to identify

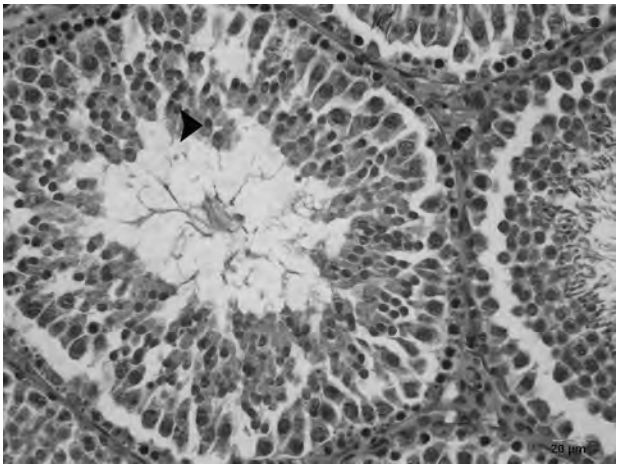

Fig 7. Aflatoxin group. Severely cleaved Caspase 3 expression in seminiferous tubules (arrowhead). (IHC)

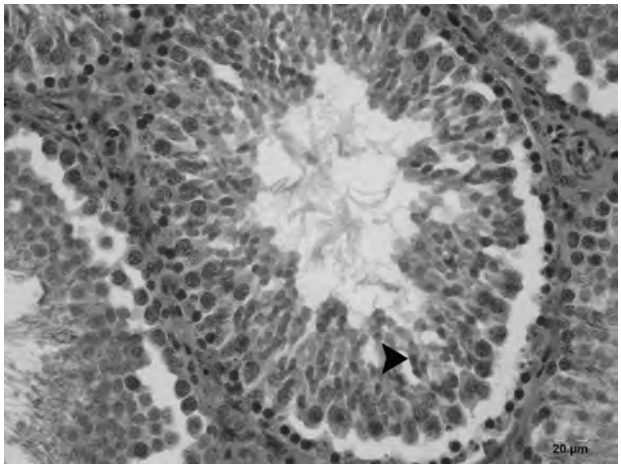

Fig 8. Moderate Expression of Cleaved Caspase 3 in seminiferous tubules in the aflatoxin + Resveratrol group (arrowhead). (IHC).

protective strategies in aflatoxin-induced experimental studies. In this sense, resveratrol, a potent antioxidant, was used in our study to determine its protective effect as in other studies (Jiang et al. 2008, Collodel et al. 2011, Giovana et al. 2013, Abdelali et al. 2016).

Spermatogenesis occurs in seminiferous tubules. If they are damaged, spermatological parameters (viability and motility) are affected. And it is well known that sperm viability and motility are so important in fertilization. If there is a decrease in the viability of the sperm cells, there is a reduction in plasma membrane integrity and motility (Roca et al. 2016).

Malondialdehyde (MDA) is one of the final products of lipid peroxidation in the cells. MDA level is a marker of oxidative stress (Gaweł et al. 2004). Besides, GSH wards off the radical species such as superoxide radicals and hydrogen peroxide and protects the membrane protein thiols (Mitchell et al. 1973). CAT convert the harmful hydrogen peroxide to water and oxygen and protects tissues from highly reactive hydroxyl radical (Chance et al. 1952). GPx is one of the metalloenzymes (glutathione peroxidase) that capable of removing hydrogen peroxide by converting the oxidized glutathione to reduced glutathione and containing selenium and partly availability in cell membrane. GPx also can restrict the chain reaction of lipid peroxidation by removing the lipid hydroperoxides from cell membrane (Sising and Pathak 1990, Jung and Henke 1996). As the prime antioxidant enzymes, SOD, can prevent oxidative stress and preventing free radical-induced cellular damage though catalyzing the dismutation reaction of reactive oxygen species (ROS) into oxygen (O2) and $H_2O_2$ in biological systems (Vaughan 1997, Umasuthan et al. 2012, Zelko et al. 2002).

There are a number of studies showing the negative effects of stress-inducing applications and toxic agents on testicular apoptosis and spermatological parameters resulting in lipid peroxidation (Ceribasi et al. 2012, Siervo et al. 2015, Turk et al. 2015, Allai et al. 2016, Korkmaz et al. 2017).

Similarly, our findings have shown that aflatoxin B1 (7.5 µg / 200 g; oral gavage) causes the lipid peroxidation in the testis tissue and the increasing in MDA level, negatively affects the spermatological parameters (motility, viability) and destroys the seminiferous tubules. On the other hand, the protective effect of resveratrol (60 mg/kg; oral gavage) was determined in all parameters [(antioxidant levels; GSH - mmol/g, CAT - kU/g, GPx -U/mg, SOD - EU/mg), (spermatological parameters; motility, viability), (testicular tissue; cleaved caspase 3 expression level in seminiferous tubules)] which aflatoxin had negative effect.

In conclusion, the use of resveratrol with antioxidant activity was investigated in preventing or ameliorating damage to aflatoxin B1. It has been concluded that resveratrol effectively prevent the aflatoxin B1-induced testicular damage and lipid peroxidation. It has also been shown that resveratrol has protective effects on sperm motility and viability.

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