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RESEARCH ARTICLE

Protective Role of Lycopene on Aflatoxin B_1 Induced Changes Sperm Characteristics and Testicular Damages in Rats

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Summary

The aim of this study was to investigate if lycopene could diminish the adverse effects of aflatoxin B_1 (AFB_1) on sperm characteristics, the testes, epididymis and oxidative stress in rats. A total of 28 adult male Wistar-Albino rats (8 weeks old weighing 180-220 g) were divided into four groups; Controls, lycopene treated rats (10 mg/kg BW, daily by gavage), AFB_1 treated rats (2.5 mg/kg BW, single dose intra peritonal) and lycopene (10 mg/kg BW, daily by gavage) + AFB_1 (2.5 mg/kg BW, single dose intra peritonal) treated rats. Traits of reproductive organs (testes and sperm characteristics, testicular histological findings and the testicular tissue oxidative status) were determined after 15 days of treatment. The sperm motility was significantly decreased while the rate of total abnormal sperms was significantly increased in rats treated with AFB_1 alone compared to their levels in controls ($P<0.001$). The level of testes malondialdehyde (MDA) in rats treated with AFB_1 was significantly higher than its level in controls ($P<0.001$). Treatment with AFB_1 significantly decreased testes diameter and seminifer epithelium thickness ($P<0.0001$) when compared with the others. Similarly, the thickness of the germinative cell layer at seminifer tubul (ST) in rats treated with AFB_1 was significantly smaller than controls. Treatment with lycopene significantly increased sperm motility ($P<0.001$) and alleviated the many negative effects of AFB_1 on sperm characteristic and testicular damage in rats. In conclusion our results showed that consumption of 10 mg of lycopene/ BW given by gavage protected the rats to the toxicity of AFB_1 when it is administrated as a single dose intraperitoneally.

Keywords: *Alfatoxin B_1, Lycopene, Sperm quality, Testes, Epididymis, Rat*

Ratlarda Aflatoksin B_1'in Spermatozoa Özellikleri ve Testislerde Yarattığı Hasar Üzerine Likopenin Koruyucu Etkisi

Özet

Bu çalışma, likopen uygulamasının aflatoksin B_1 (AFB_1) uygulanmış ratların üreme sistemi, sperm kalitesi ve oksidatif stres üzerine olan önleyici etkilerini saptamak amacıyla yürütülmüştür. Çalışmada, toplam 8 haftalık yaşta ve ortalama 180-220 g canlı ağırlığa sahip toplam 28 adet erkek Wistar Albino ratlar kullanıldı. Ratlar rastgele 4 eşit gruba ayrıldı. Gruplar; Kontrol, Likopen grubu (10 mg/kg CA/gün), AFB_1 grubu (2.5 mg/kg CA, tek doz ip.) ve Likopen(10 mg/kg CA/gün) + AFB_1 (2.5 mg/kg CA, tek doz ip.) şeklinde oluşturulmuştur. Toplam 15 gün süren araştırma sonunda, testis ve spermatozoa özellikleri ile testiküler histoloji ve oksidasyon özellikleri incelenmiştir. AFB_1 ve Kontrol grubları, motilite ve toplam anormal spermatozoon sayısı özellikleri bakımından karşılaştırıldığında, AFB_1 uygulanan hayvanların motilitesinde önemli düzeyde azalma ve toplam anormal spermatozoon sayısında ise artış saptanmıştır ($P<0.001$). AFB_1 uygulanan hayvanların testis malondialdehyde (MDA) seviyesi Kontrol grubu hayvanlarınkinden daha yüksek bulunmuştur ($P<0.001$). Testis çapı ve seminifer epitelyum kalınlığı AFB_1 uygulanan grupta diğer deneme gruplarına göre daha düşük tespit edilmiştir ($P<0.0001$). Benzer şekilde, AFB_1 uygulanan hayvanların germinatif hücrelerin kalınlığı Kontrol grubu hayvanlarınınkinden daha küçük tespit edilmiştir. Çalışmada Likopen uygulamasının spermatozoa motilitesini önemli düzeyde artırdığı ($P<0.001$) ve AFB_1'in spermatozoa özellikleri ve testis üzerindeki olumsuz etkilerini giderdiği saptanmıştır. Sonuç olarak, 10 mg/ kg CA düzeyinde likopen uygulamasının, ratlara intraperitoneal olarak uygulanmış tek doz AFB_1 toksisitesine karşı koruyabileceği tespit edilmiştir.

Anahtar sözcükler: *Alfatoksin B_1, Likopen, Sperm kalitesi, Testis, Epididymis, Rat*

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INTRODUCTION

Aflatoxins are extremely toxic metabolites which are produced by certain species of fungi, e.g. *Aspergillus flavus* and *A. parasiticus.* They can occur as natural contaminants of foods and feeds. Among the aflatoxins (B_1, B_2, G_1 and G_2), aflatoxin B_1 (AFB_1) is known the most toxic compound and it has generated much concern due to its carcinogenicity, mutagenicity and teratogenicity for human and a wide range of animals [1,2]. AFB_1 is classified as natural human carcinogen by The International Agency for Research in Cancer [3]. Aflatoxins may affect energy, nucleic acid and protein metabolism [4]. AFB_1 concentration in the organs and tissues were categorized in the order from high to low concentrations as follows: the gonads, the parenchymatous organs (liver and kidney), the lymphopoietic organs (spleen, Bursa cloacalis and thymus), the endocrine glands, the muscles and the lungs, while the brain had the lowest concentration [5].

In mice, scattered testes weight and disorganized cell population in the seminiferous tubules were diminished significantly after orally administration of aflatoxin at level of 25 and 50 µg/animal/day [6]. Similarly, sperm count and motility were significantly decreased and also large number of nonviable spermatozoa has been observed in mice treated wit aflatoxin [7]. Pathogenesis of kidney, liver, testicular damage following AFB_1 exposure is generally ascribed to oxidative damage. AFB_1 may cause lipid peroxidation and decreases the activity of enzymes that protect against oxidative damage in these tissues. The administration of antioxidants such as vitamin E, selenium, vitamin C, carotenoids and others may protect against xenobiotic-induced damage [8-11].

Carotenoids are the principal pigments responsible for the colors of vegetables and fruits. They are known as antioxidants that have been shown to inhibit various types of cancers. Lycopene, a naturally present carotenoid in tomatoes and other fruits, has attracted considerable attention due to its potent antioxidant properties [12] and free radical scavenging capacity [13]. Epidemiological studies indicated that dietary intake of lycopene is correlated with decreased the risk for gastric cancer [14] and has antiproliferative effects on prostate [15] and breast cancer cell lines [16].

Due to these properties, lycopene was selected for use in this experiment. The objective of this experiment was to investigate the potential protective effects of lycopene against the toxicity of AFB_1 in rats by observing their effects on sperm characteristics, testicular system (testes-epididymis) and oxidative stress.

MATERIAL and METHODS

Chemicals

Lycopene 10% FS (Redivivo, Roche, code 7791) was purchased from DSM Nutritional Products (Istanbul, Turkey). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Animals and Treatments

The experiment was performed at the Animal Experimental Research Center of the Dicle University and it was conducted according to the guidelines for animal experimentation of Dicle University and approved by the Ethical Committee.

A total of 28 male Wistar-Albino rats (8 weeks old, weighing 180-220 g) were divided into 4 treatment group (7 rats per treatment). Rats were housed in wire cages with filter tops at 24°C of temperature, 55-60% of humidity and a 12 h light/12 h dark cycle. Throughout the studies rats were fed the Standard commercial rat diets (Elazığ Yem Inc. Elazığ, Turkey) and water ad libitum. The rats in control and group 3 were treated by gavage daily with corn oil for 15 days. Rats in both groups 2 and 4 (lycopene alone and lycopene + AFB_1) were treated daily with 10 mg/kg BW lycopene dissolved in corn oil for 15 days. On day of 12, groups 3 and 4 were administrated by intraperitonal (IP) with single dose of AFB_1 (2.5 mg/kg BW) dissolved in PBS (pH 7.4).

Experimental Procedures

Rats were weighed at the beginning and the end of the experiment. At the end of the experiment all rats were sacrificed after the last exposure by anaesthesia with ketamine (50 mg/kg IM). Then, rats were laid to death by an overload of sodic pentobarbital. Testes and epididymes were dissected, weighed and cleared of adhering connective tissue and assayed immediately. One of the testes from all rats was fixed in 10% formalin and stored for histopathological examinations. Other testis samples were kept -20°C for biochemical analyses.

Determination of Epididymal Sperm Concentration and Motility

Spermatozoa in the epididymis were counted by a modified method of Yoki [17]. Briefly, the epididymis was minced with anatomical scissors in 5 ml of physiological saline, placed in a rocker for 10 min, and incubated at room temperature for 2 min. Sperm progressive motility was evaluated by an earlier method described by Sonmez [18]. For this purpose, fluid was obtained from the caudal epididymis with a pipette and diluted to 2 ml

with Tris buffer solution. The system was pre-warmed (35°C) and percentage of motility was evaluated visually at 400× magnification. Motility estimations were performed from three different fields in each sample. The mean was used as the final motility score. The method described by Evans and Maxwell [19] was used for determination of the percentage of morphologically abnormal spermatozoa after adapting the method for use in rats. According to this method, slides were prepared with India ink. A total of 300 sperm cells were counted on each slide under light microscope at 400× magnification.

Biochemical Assays

The levels of malondialdehyde (MDA) in the testicular tissue was measured using the colorimetric method described by Yoshioka [20], based upon the reaction of thiobarbituric acid (TBA) with MDA. The absorbance of the MDA-TBA adduct was measured at 535 nm wavelength using a spectrophotometer (Shimadzu 1601 UV/Visible). The concentration was expressed as µmol/l.

Histopathological Examinations

Testes and epididymis tissues were fixed in solutions of 10% formalin for 24 h. Sections of testes and epididiymis were processed by standard histological techniques, and stained with Crossman's Triple for light microscopy. Measurements of testes and epididiymis weight, length, thickness were recorded. The diameter and thickness of tubulus seminiferus and ductus epididymis from 5 rats per treatment were measured by using an ocular micrometer in a light microscope (Nikon Eclipse-400, Coolpix-4500), and the average size of ST and germinal cell layer thickness were calculated. Seminiferous tubule diameter (µm) was measured by ocular micrometer [12] and results were given as average.

Statistical Analyses

Data were analyzed using the General Linear models (GLM) procedure of SPSS 9.0 [21]. If appropriate, post-hoc analyses were carried out using the Duncan's test for multiple comparisons. Statements of statistical significance are based on $P<0.05$.

RESULTS

Testes Characteristics

Testes weights (right and left), length, thickness, diameters and seminifer epithelium thickness in all groups are shown in *Table 1*. Right and left testes weights and length were not affected by treatments. Treatment with AFB_1 significantly decreased testes diameter and seminifer epithelium thickness ($P<0.0001$) when compared with the others. Treatment with lycopene alleviated the negative effects of AFB_1 on testes diameter and seminifer epithelium thickness.

Epididymis Characteristics

As shown in *Table 1*, epididymis weights (right and left) were not affected by treatments. However, the

Table 1. *Effects of lycopene treatment on testes, epididymis and sperm characteristics in aflatoxin B1 treated rats* [1]
Tablo 1. *Aflatoksin uygulanmış ratlar da likopenin testis, epididimis ve spermatozoa karekterleri üzerine etkileri* [1]

Measurements	Treatments					
	Control	Lycopene	AFB_1	AFB_1+ Lycopene	SEM	P
Testes Characteristics						
Diameter, (µm)	258.4 ^{b}	360.8 ^{a}	219.4 ^{c}	241.7 ^{c}	10.66	0.0001
Weight (right), (mg)	324.8	327.3	284.2	352.9	22.31	0.770
Weight (left),(mg)	340.5	342.6	267.6	316.5	18.04	0.444
Lenght, (cm)	1.95	1.94	1.86	1.93	0.03	0.802
Seminifer epithelium thickness, (µm)	32.4 ^{b}	42.0 ^{a}	15.0 ^{d}	22.7 ^{c}	2.00	0.0001
Epididymis Characteristics						
Diameter (µm)	291.0 ^{a}	276.4 ^{ab}	256.0 ^{b}	275.7 ^{ab}	4.48	0.041
Weight (right), (mg)	62.2	47.4	49.4	56.9	3.18	0.338
Weight (left), (mg)	61.1	58.6	64.9	45.4	4.59	0.487
Thickness, (µm)	16.0 ^{b}	18.3 ^{a}	16.3 ^{b}	16.8 ^{b}	0.21	0.0001
Sperm Characteristics						
Motility	72.1 ^{a}	70.0 ^{a}	47.1 ^{b}	65.0 ^{a}	3.14	0.012
Head defects	5.3 ^{b}	6.6 ^{b}	9.1 ^{a}	7.0 ^{b}	0.39	0.002
Tail defects	7.2 ^{c}	9.1 ^{bc}	15.6 ^{a}	10.8 ^{b}	0.75	0.0001
Total defects	12.6 ^{c}	15.7 ^{bc}	24.7 ^{a}	17.8 ^{b}	1.00	0.0001

^{a,b,c,d} *Means within line with different superscripts differ significantly ($P<0.05$),* [1] *Each value represents the least square mean from 7 rats per each treatment,* ***SEM:*** *Standard error of the mean*

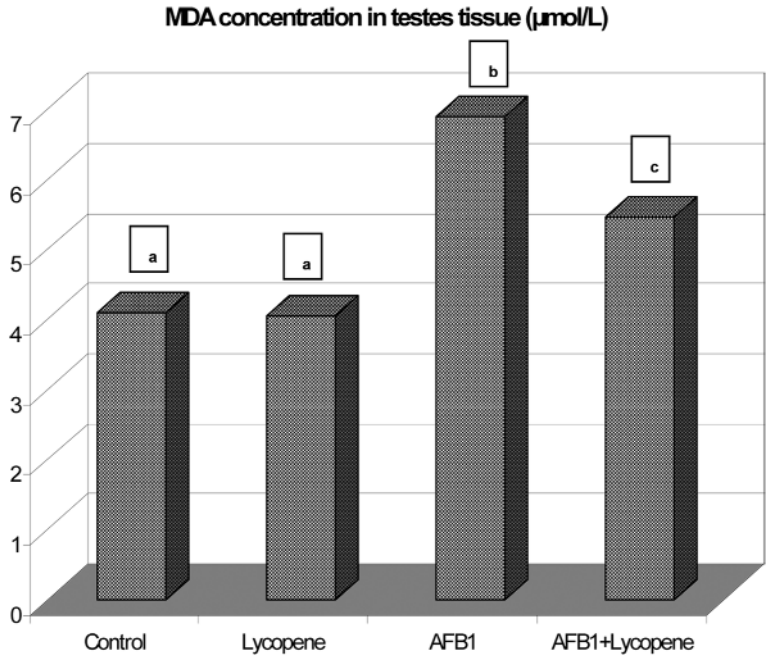

Fig 1. The concentration of MDA in testes tissue of rats treated with AFB_1 and lycopene

[a,b,c] Means within colon with different superscripts differ significantly (P<0.001)

Şekil 1. Aflatoksin ve Likopen uygulanmış ratların testis dokularındaki MDA konsantrasyonu

diameter of epididymis were decreased in rats received the single dose of AFB_1 as compared to controls. In addition, epithelium thickness was significantly increased (P<0.0001) in rats treated with lycopene alone compared to other groups. There was not difference between AFB_1 plus lycopene group and control for the epididymis diameter.

Sperm Quality Characteristics

Sperm motility was significantly decreased while the rate of the defects of head, tail and total defects were significantly increased in rats administrated with single dose of AFB_1 alone compared to their levels in controls *(Table 1)*. Treatment with lycopene significantly increased sperm motility and prevented sperm abnormalities induced by AFB_1.

Lipid Peroxidation in Testes Tissue

The levels of MDA in the testicular tissue in rats treated with AFB_1 were significantly higher than their levels in controls (P<0.001) *(Fig. 1)*. Treatment with lycopene prevented elevation of MDA levels significantly in AFB_1 + lycopene group (P< 0.001).

Histopatholigical Examinations

The testes tissue of the control groups showed normal structure under light microscopy *(Fig. 2)*. The spermatogenic cells in the seminiferous tubules (ST) and

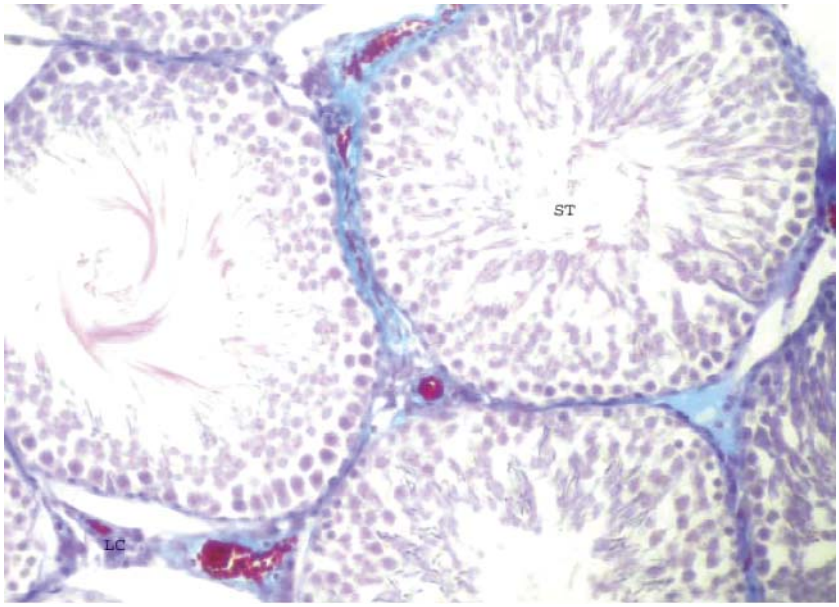

Fig 2. The picture of the seminiferous epitelhium and leyding cell of the control rat, **ST:** Seminifer tubul, **LC:** Leyding cell, TriplleX2

Şekil 2. Kontrol ratlarda seminifer epitel ve leyding hücrelerinin görünümü, **ST:** Seminifer tubul, **LC:** Leyding hücresi, TriplleX20

leyding cells (LC) are seen in the inertial areas *(Fig. 3)*. The ST of rats treated with AFB_1 was disorganized, with decreased diameter as compared to controls. Similarly, the thickness of the germintive cell layer at ST in rats treated with a single dose of AFB_1 was significantly smaller than controls group *(Table 1)*. After aflatoxin application, the germinal and spermatogenic epithelium of the seminiferous tubules were detachment. Desquamative germinal cells were seen in ST lumen *(Fig. 3)*. The extent of the histological changes testifies to a significant damage caused to the spermiogenic epithelium of seminiferous tubulus after single dose AFB_1 administration. The AFB_1 induced changes in histopathological findings were partially reversed by treatment with lycopene. The spermatogenic cells in seminiferous epithelium and leyding cells in interstitial areas were markedly prominent *(Fig. 4)*. The epididymis tissue of the control groups showed that the epithelium is pseudostratified columnar-the free surface of the cell possesses stereocilia and the duct, which is surround by smooth muscle, is embedded in connective tissue. *(Fig. 5)*. In the AFB_1 treated rat, vacuoles were always present. In some segments, vacuoles were large but few and in some segment, vacuoles were small but profuse *(Fig. 6)*. The vacuolated epididymal epithelial cells were not observed in the lycopene group *(Fig. 7)*.

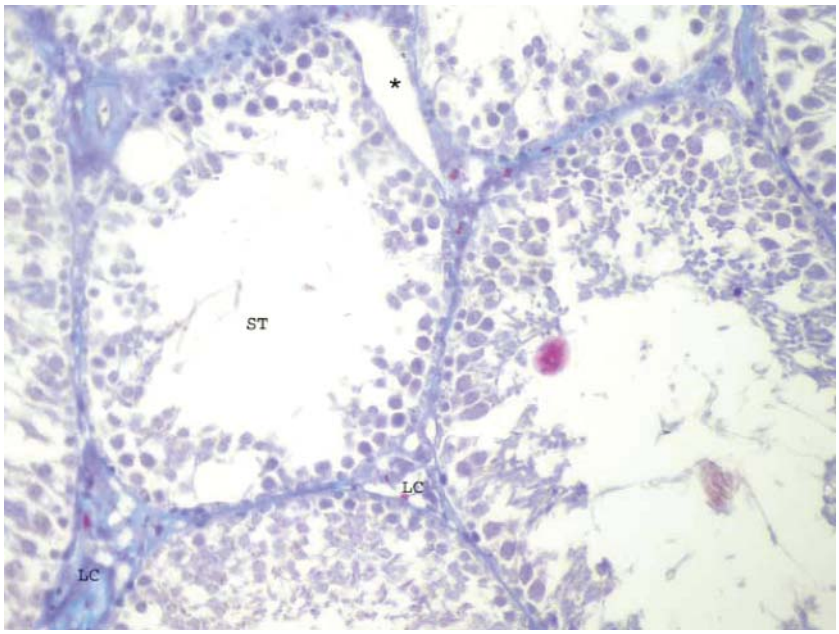

Fig 3. The picture of the testes degeneration and desquamation of germinal epitelhium AFB_1 treated rat, **ST:** Seminifer tubul, ***** Detachment and denudation of the germinal epitelhium) TriplleX20

Şekil 3. Aflatoksin uygulanmış ratlarda germinal epitelde ki dökülme ve dejenerasyonun görünümü, **ST:** Seminifer tubul, ***** Germinal epitelde ayrılma ve aşınma, TriplleX20

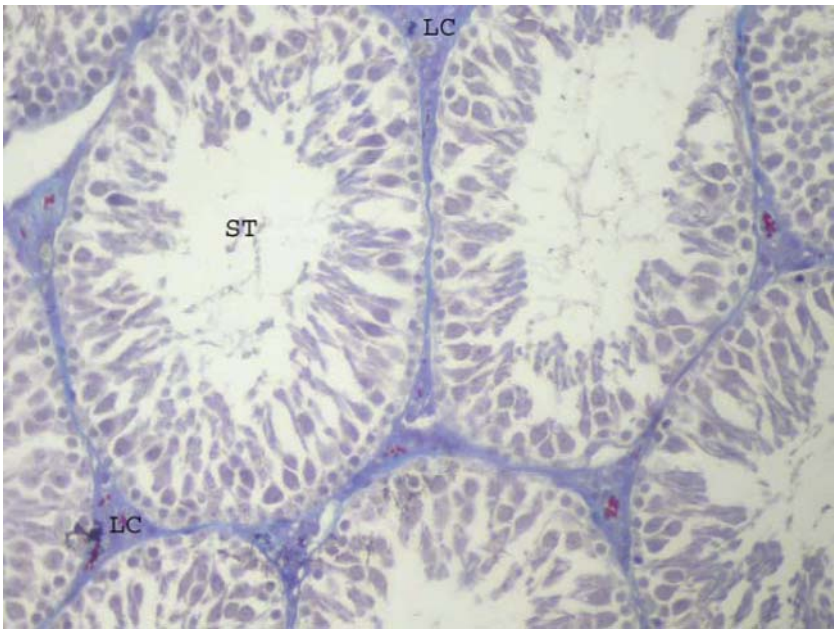

Fig 4. The picture of the seminiferous epitelhium and leyding cells of AFB_1 plus lycopene treated rat. **ST:** Seminifer tubul, **LC:** Leyding cell, TriplleX20

Şekil 4. Aflatoksin ve likopen uygulanmış ratlarda seminifer epitel ve leyding hücrelerinin görünümü, **ST:** Seminifer tubul, **LC:** Leyding hücresi, TriplleX20

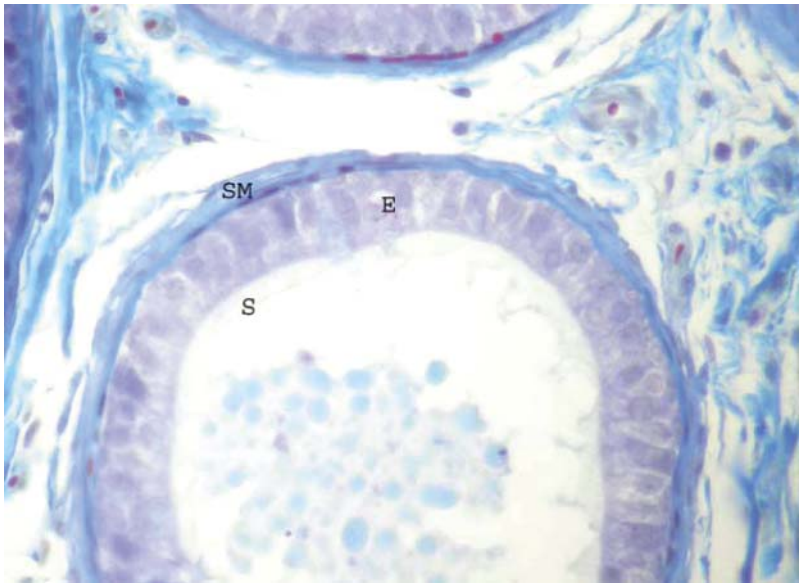

Fig 5. The picture of the epididymis tissue of the control rat. **E:** Epitelhium, **S:** Sterocilia, **SM:** Sirculer muscle, TriplleX20

Şekil 5. Kontrol ratlarda epididimis görünümü, **E:** Epitel, **S:** Sterosilya, **SM:** Sirküler kas, TriplleX20

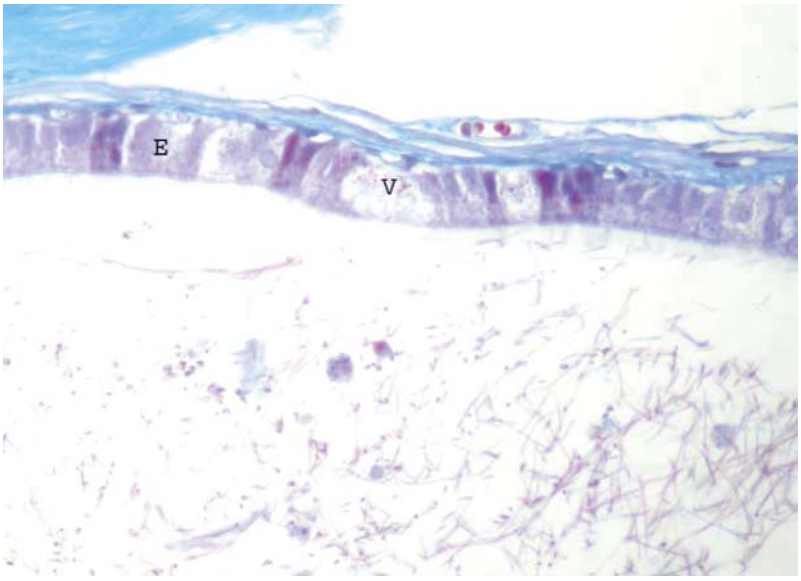

Fig 6. The picture of the epididymis tissue of AFB_1 treated rat. **E:** Epitelhium, **V:** Vacuole TriplleX40

Şekil 6. Aflatoksin uygulanmış ratlarda epididimis görünümü. **E:** Epitel, **V:** Vakuol TriplleX40

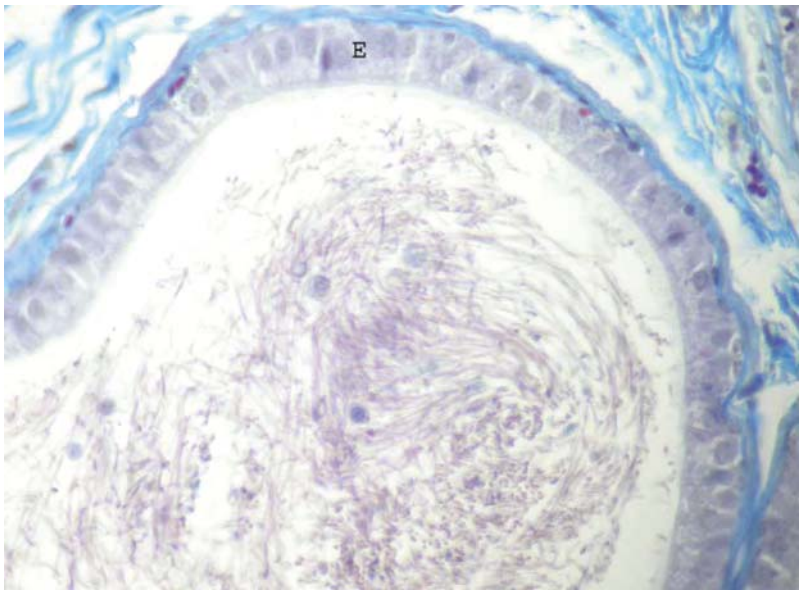

Fig 7. The picture of the epididymis tissue of AFB_1 plus lycopene treated rat, **E:** Epitelhium, TriplleX20

Şekil 7. Aflatoksin ve likopen uygulanmış ratlarda epididimis görünümü, **E:** Epitel, TriplleX20

DISCUSSION

Effects of AFB1

Food contamination with aflatoxins is increasing and exposure of these aflatoxins at levels that do not cause clinical effects may threats to animal and human health by suppress immune functions and decrease resistance to disease. Rats from the AFB_1 group presented clinical signs of intoxication. Differences were observed between rats treated with AFB_1 and the control group in various parameters including testes diameter, seminifer epithelium thickness, and epididymis diameter and thickness and the concentration of MDA in testes. Pro-oxidant and antioxidant balance is vital for normal biological functioning of the cells and tissues [13]. Increases of the lipid peroxidation products (MDA) can be used as sensitive indicator of the toxicity of AFB_1 [19]. In our study, significant increases were observed in the level of MDA in testes tissue of rats treated with a single dose of AFB_1. Similar observations have been reported by different researchers [22,23]. The increases of MDA concentration during aflatoxicosis may confirm the impaired immuno-modulation in resulting in underlining mechanisms for AFB_1 induced cell injury and DNA damage. The effects of AFB_1 on testes have been studied previously [7]. The testicular changes were observed in the present study are in agreement with earlier reports [24-26]. AFB_1 induced many regressive changes of different intensity in the germinal epithelium of the seminiferous tubules resulting in a severe dystrophic alteration of the spermatogenic epithelium along with edematous changes in the interstitial tissue of adult male rats [27]. Similarly, in our study we observed the decrease of seminifer epithelium thickness. Salem [28] reported that decreases of relative weight of testes and increase of the number of abnormal dead sperms following a 9 week administration of sub-lethal doses of AFB_1 to mature male rabbits. Sperm motility is one of the indicators of the fertility status in the male [7]. The results of the present study indicated that AFB_1 administration at the acute dose resulted in both a significantly decrease in sperm motility and increase the number of abnormal spermatozoon in the rats. Our findings, especially impairment in sperm characteristics and in histopathological findings are compatible with report of some workers confirm the spermiotoxic effects of AFB_1 in rat testes [29]. Similarly, prolonged consumption of aflatoxins by rats and pigs caused histological alterations of male reproductive organs [30]. In our study the administration of single dose of AFB_1 induced reduction in sperm motility and increased the rate of abnormal spermatozoon in the rats. Agnes and Akbarsha [7], reported that aflatoxins induced damages in the histology of testis and cause anomalies in sperm morphology. Epididymal toxicity gains importance in view of the role of the epididymis in post-testicular maturation of spermatozoa [31,32].

Effects of Lycopene

Lycopene has been shown to have the highest anti-oxidant activity among the carotenoids in cell protection against hydrogen peroxide and nitrogen dioxide radicals. Thus, lycopene has received a particular attention because it is a strong antioxidant and has been shown to reduce the amount of oxidative DNA damage and also reduce lipid peroxidation in cell culture and in rats *in vivo* [13]. Besides, it has been reported that lycopene has high efficient antioxidant and free radical scavenging capacity [33]. In our study, administration of lycopene alleviated the negative effects of AFB_1. This agree with the study showing the administration of lycopene to cyclosporine A treated rats improved nearly all the cyclosporine A-induced damages in the structure of testis [34]. AFB_1-induced histopathological alterations were effectively reverted by pre-treatment with lycopene. This study clearly indicates that, AFB_1 treatment markedly impaired testicular function and that pre-treatment with lycopene might prevent this toxicity. In addition, it has been reported that lycopene attenuates oxidative stress and exert anticancer effects both *in vitro* and *in vivo* [31,32,35]. In the present study, we found that lycopene decreased the damages of the AFB_1 in the epididymal epithelial cells. These observations might also indicate that lycopene has protective and therapeutic effects on aflatoxin induced oxidative stress.

In conclusion, our study showed that AFB_1 treatment markedly impaired testicular function and administration of lycopene reduced oxidative stress and alleviated the negative effects of AFB_1 on parameters tested.

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