

Effects of Aflatoxin on *AgNOR* Activity of Cells in Different Regions of Kidney, and Protective Effectiveness of Esterified Glucomannan in Ram ^[1]^[2]

Fatma COLAKOGLU ¹ Hasan Huseyin DONMEZ ¹ 

[1] This study forms part of the PhD Thesis of Fatma KAYIKCI COLAKOGLU, for which we thank the "Selcuk University Scientific Research Projects (BAP) Coordinating Office", Project no: 09202023, and TUBITAK, Project No: 1070866, for financial support

[2] We are grateful to Prof. Dr. Mustafa ORTATATLI for contributions

¹ Department of Histology and Embryology, Faculty of Veterinary Medicine, University of Selcuk, TR-42075 Konya - TURKEY

Makale Kodu (Article Code): KVFD-2012-8176

Summary

In this study, the effects of total aflatoxin (AF) given orally on silver-staining nucleolus organizer regions (*AgNORs*) activity were studied in glomerulus and tubular epithelial cells of kidney in Merino rams. In addition, this study was conducted in order to evaluate the efficacy of an esterified glucomannan (EG) for protection against to aflatoxicosis. As materials, 1 year-old 32 Merino rams were used. Rams were fed through the 92 days. Control group (C) fed with the commercial feed. AF group fed with commercial feed added 250 µg/day of total AF. EG group fed with commercial feed added 2 g/day of EG daily. AF+EG group fed with commercial feed added 250 µg/day of total AF and 2 g/day of EG. At the end of the 92nd day the animals were sacrificed, and tissue samples were taken from the kidneys. Whereas ratio of nuclear area of the *AgNOR* area of cells in examined regions of kidney was found decreased significantly ($P<0.05$) in the AF group compared to the control group, AF+EG group was found similar to control group ($P>0.05$). In conclusion, the adverse effects causing by aflatoxicosis on the kidney *AgNOR* activity could be ameliorated by adding EG to the ration.

Keywords: Aflatoxin, *AgNOR*, Glucomannan, Kidney, Ram

Koçlarda Aflatoksinin Böbreğin Farklı Bölgelerindeki Hücrelerin *AgNOR* Aktivileri Üzerine Etkileri ve Glukomannanın Koruyucu Etkinliği

Özet

Bu çalışmada Merinos ırkı koçlara oral yolla verilen total aflatoksinin (AF) glomerulus ve böbrek tubuler epitel hücrelerindeki gümüşleme metoduyla çekirdekçik organizatör bölgelerinin (*AgNORs*) aktiviteleri üzerine olan etkileri ve AF ile birlikte verilen glukomannanın (EG) aflatoksikozun neden olduğu etkileri azaltıcı/engelleyici etkinliğinin belirlenmesi amaçlandı. Hayvan materyali olarak, 32 adet 1 yaşlı Merinos ırkı koç kullanıldı. Beslemeye 92 gün süreyle devam edildi. Kontrol (K) grubuna ticari yem, AF grubuna ticari yem ile günlük 250 µg AF, EG grubuna ticari yem ile günlük 2 g EG, AF+EG grubuna ise ticari yemle birlikte günlük 250 µg AF ve 2 g EG verildi. 92 günlük besleme periyodunu takiben hayvanlar kesildi. Böbreğin incelenen bölgelerindeki hücrelerin *AgNOR* alanının çekirdek alanına oranları AF grubunda kontrol grubuna göre önemli oranda düşük ($P<0.05$); AF+EG grubunda ise kontrol grubuna benzer ($P>0.05$) bulunmuştur. Sonuç olarak; elde edilen veriler, yemle birlikte alınan AF'nin böbrek *AgNOR* aktiviteleri üzerinde olumsuz etki oluşturabileceğini, bu etkilerin yeme EG ilave edilerek önemli oranda önlenilebileceğini ortaya koymuştur.

Anahtar sözcükler: Aflatoksin, *AgNOR*, Böbrek, Glukomannan, Koç

INTRODUCTION

One of the most important problems in human and animal nutrition is contamination of human foods and

animal feeds with molds and mycotoxins ¹. Aflatoxins are a group of mycotoxins produced by the strains of *Aspergillus*

İletişim (Correspondence)

 +90 332 2233619

 hdonmez@selcuk.edu.tr

flavus and *A. parasiticus* ². They are produced on cereal grains during growth, harvest, storage, or transportation ³. It is well known AFs are teratogenic ⁴, immunosuppression ⁵, mutagenic, and carcinogenic ⁶. There is evidence that it has harmful effects on the kidneys, though not as much as on the liver ⁷.

Prevention of feed and feedstuffs from possible mold growth and AF contamination is very important. Practical and cost-effective methods for detoxification of AF containing feed and feedstuff are in great demand ⁸. Since the early 1990s, the adsorbent-based several studies have been performed to detoxify AF in contaminated food and foodstuffs and to minimize the deleterious effects of AF ⁹. An approach to the problem has been the usage of non-nutritive and inert adsorbents in the diet to bind AF and reduce the absorption of AF from the gastrointestinal tract ¹⁰. The non-nutritive clays such as aluminosilicates, zeolites, bentonites, and clinoptilolite were preferred by the researchers ^{5,11}. Recent years, researchers suggested that the best approach for decontamination would be biological degradation ¹². Live yeast (*Saccharomyces cerevisiae*) initially used as a performance promoter in the early 1990s, was found to have beneficial effects on aflatoxicosis ¹³. Esterified glucomannan (EG) showed considerably high binding ability (80-97%) with AF ¹⁴, and it has been preferred for detoxification of AF in poultry animals ⁶.

Nucleolar-organizer regions (NORs) are loops of DNA containing ribosomal RNA genes ¹⁵. These regions can be easily stained with silver methods to appear as black dots (AgNORs) in the cell nucleus since they are argyrophilic. NORs are used by cytogeneticists for studying chromosomal disorders. This staining technique is very simple and does not require any special instruments or costly reagents ¹⁶. Additionally, the size, number and dispersion of the silver deposits on the NOR reflect the degree of transcriptional, nucleolar and proliferative activity of the cells ^{17,18}.

The aim of the study was to determine the effects of total AF given orally on AgNOR activity of cells in different regions of kidney in Merino rams. In addition, this study was conducted in order to evaluate the efficacy of EG for protection against to aflatoxicosis.

MATERIAL and METHODS

Animals and Diet

Approval for the present study was obtained from the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Selcuk University (2008/061). Thirty-two Merino rams were approximately purchased 1-year-old (12-14 months old). Animals were examined for general health. Antiparasitic ivermectin injection (Avromec-F, 1 ml/50 kg) and oksifendazol (oxa-F, 1 tablet/50 kg) were performed. In addition, enterotoxemia (Pluritoxiven-8, 1 ml) and smallpox

vaccines were performed. For adaptation to the environment and the implementation of a new 15-day training program was applied to feeding. Individually weighted rams were divided into four equal groups. Experimental feeding was continued throughout ninety-two days. The rams were fed a commercial food (Table 1). Water and alfalfa were given *ad libitum*.

Experimental Design

The experimental design consisted of four dietary treatments. Control group (C) fed with the commercial feed. AF group fed with commercial feed added 250 µg/day of total AF. EG group fed with commercial feed added 2 g/day of EG (Alltech, Turkey). AF+EG group fed with commercial feed added 250 µg/day of total AF and 2 g/day of EG. AF dose was determined by the views of the researches ¹⁹⁻²¹ who were articles on the effects of AF and the pharmacologists who were studies on AF in our faculty. EG dose was also determined according to the dose in the prospectus (Alltech, Turkey). To make sure feed consumed, AF and EG that were mixed of 250 g commercial feed were given to animals before morning feeding and then morning feeding was continued.

Aflatoxin

The AF was produced from *Aspergillus parasiticus* NRLL 2999 culture (USA, Agricultural Research Service, Peoria, IL) via fermentation of rice by the method of Shotwell et al.²² with minor modifications by Demet et al.²³. Fermented rice was sterilized in autoclave, dried at 70°C, and ground to a fine powder. According to the method reported by Vicam ²⁴ extraction and cleaning of AF in fermented rice was used immunoaffinity column (Down Test®; Vicam). The amount of AF carried out by high performance liquid chromatography (HPLC) according to the method reported by Stroka et al.²⁵. The amount of total AF in the fermented rice was found 73.96 ppm. The AF within the rice consisted of 84.15% AFB₁, 6.29% AFB₂, 9.13% AFG₁ and 4.25% AFG₂ (rate of return method 97.4%; sensitivity 0.4 ppb).

Collection and Processing of Tissue Samples

At the end of the 92nd day, after sacrificed of the all animals

Table 1. Composition of the commercial feed

Tablo 1. Ticari yemin içeriği

Chemical Composition	Percentage Rate	Chemical Composition	Percentage Rate
Dry matter	%88	Na	%0.1-0.4
Crude protein	%12	NaCl	%1.0
Crude Cellulos	%12	Metabolic energy	2750 kcal/kg
Crude ash	%9	Vit A	7000 IU/kg
İnsoluble ash in HCL	%1.0	Vit D3	700 IU/kg
Ca	%0.6-1.6	Vit E	25 mg/kg
P	%0.4		

tissue samples were taken from the kidney in 10% neutral-buffered formalin solution. The tissues were processed and paraffin sections (6 μ m) were stained with a solution containing one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck). The staining was performed at 37°C in the dark for 20-30 min²⁶. The histological preparations were examined with a light microscope (Leica DM-2500 attached to a DFC-320 digital camera). In different regions of the kidney (glomerulus, proximal tubules, distal tubules and collecting tubules) of each animal, 25 cells having nuclei were evaluated. The nuclear area and the AgNOR area (Fig. 1 and 2) were analyzed with an image analysis program (IM-50). Also, the percentage of the AgNOR area relative to the nuclear area was calculated.

Statistical Analysis

The obtained results were statistically analyzed using Duncan test in SPSS software [version 17; SPSS Inc., Chicago, IL, USA]. The level of significance was $P < 0.05$.

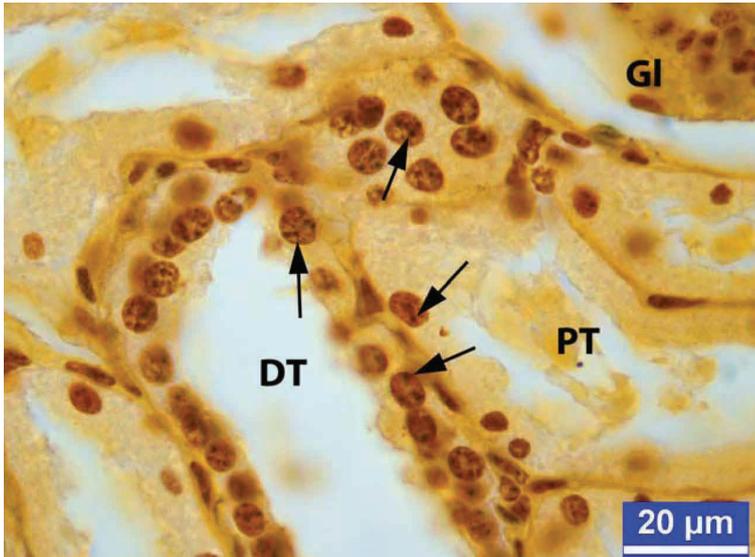
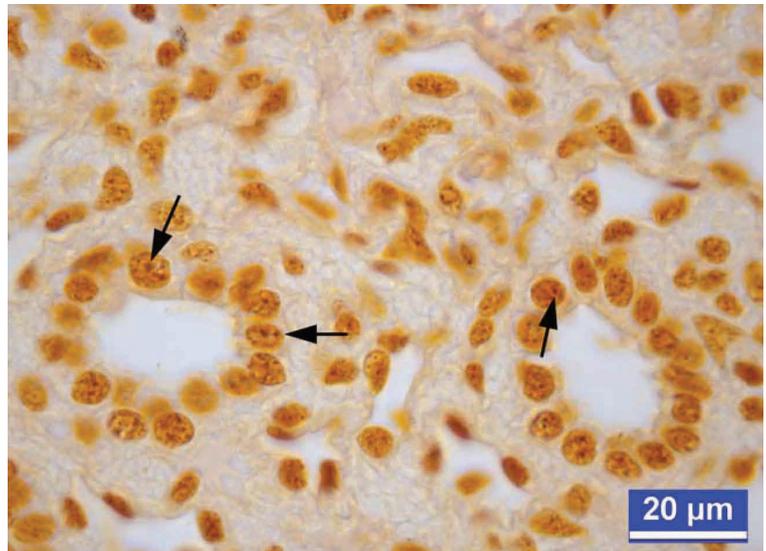


Fig 1. A section from cortex of kidney. DT: Distal tubule, GL: Glomerulus, PT: Proximal tubule, Arrows: AgNOR areas, AgNOR staining

Şekil 1. Böbreğin korteks bölgesinden bir kesit. DT: Distal tubul, GL: Glomerulus, PT: Proksimal tubul, Oklar: AgNOR alanları, AgNOR boyama

Fig 2. Collecting tubules in medulla of kidney. Arrows: AgNOR areas, AgNOR staining

Şekil 2. Böbreğin medula bölgesindeki toplayıcı borucuklar. Oklar: AgNOR alanları, AgNOR boyama



RESULTS

We obtained the nuclear area (Table 2), the AgNOR area (Table 3), and the ratio of the nuclear of the AgNOR area (Table 4) of cells in different regions of the kidney. Nuclear areas and ratio of nuclear area of the AgNOR area of glomerulus and tubular epithelial cells of kidney decreased significantly ($P < 0.05$) in the AF group compared to the other groups. Furthermore, AF group was lowest ($P < 0.05$) AgNOR area.

DISCUSSION

The liver and kidney are considered the main target organ for aflatoxicosis^{7,27-29}. Lakkawar et al.³⁰ reported that liver and kidney were the most affected organs in rabbits which fed an AFB₁ contaminated diet. The effects of AFs on histopathological changes are directly correlated with the concentration of AF and the duration of the exposure³¹.

Table 2. Nuclear area of cells in different regions of kidney**Tablo 2.** Böbreğin farklı bölgelerindeki hücrelerin çekirdek alanı

Groups (n=8)	Glomerulus (μm^2) \pm SE	Proximal Tubules (μm^2) \pm SE	Distal Tubules (μm^2) \pm SE	Collecting Tubules (μm^2) \pm SE
C	15.75 \pm 0.34 ^a	26.02 \pm 0.46 ^a	23.11 \pm 0.32 ^a	28.95 \pm 0.48 ^a
AF	13.13 \pm 0.28 ^c	22.20 \pm 0.29 ^b	20.32 \pm 0.32 ^c	25.33 \pm 0.30 ^c
EG	15.17 \pm 0.32 ^{ab}	25.30 \pm 0.39 ^{ab}	22.29 \pm 0.33 ^{ab}	28.24 \pm 0.35 ^{abc}
AF+EG	14.33 \pm 0.31 ^b	22.33 \pm 0.36 ^b	21.65 \pm 0.30 ^b	27.00 \pm 0.29 ^b

C: Control, AF: Aflatoxin, EG: Glucomannan, AF+EG: Aflatoxin+glucomannan; a-c Values within a column with no common superscripts are significantly ($P<0.05$) different

Table 3. AgNOR area of cells in different regions of kidney**Tablo 3.** Böbreğin farklı bölgelerindeki hücrelerin AgNOR alanı

Groups (n=8)	Glomerulus (μm^2) \pm SE	Proximal Tubules (μm^2) \pm SE	Distal Tubules (μm^2) \pm SE	Collecting Tubules (μm^2) \pm SE
C	0.23 \pm 0.06 ^a	0.43 \pm 0.01 ^a	0.41 \pm 0.01 ^a	0.51 \pm 0.01 ^a
AF	0.17 \pm 0.00 ^c	0.27 \pm 0.01 ^c	0.26 \pm 0.01 ^c	0.38 \pm 0.01 ^b
EG	0.22 \pm 0.01 ^a	0.41 \pm 0.01 ^a	0.38 \pm 0.01 ^a	0.49 \pm 0.01 ^a
AF+EG	0.19 \pm 0.01 ^b	0.33 \pm 0.01 ^b	0.29 \pm 0.01 ^b	0.39 \pm 0.01 ^b

C: Control, AF: Aflatoxin, EG: Glucomannan, AF+EG: Aflatoxin+glucomannan; a-c Values within a column with no common superscripts are significantly ($P<0.05$) different

Table 4. The ratio of the nuclear of the AgNOR area of cells in different regions of kidney**Tablo 4.** Böbreğin farklı bölgelerindeki hücrelerin AgNOR alanının çekirdek alanına oranı

Groups (n=8)	Glomerulus (%) \pm SE	Proximal Tubules (%) \pm SE	Distal Tubules (%) \pm SE	Collecting Tubules (%) \pm SE
C	1.80 \pm 0.09 ^a	1.53 \pm 0.07 ^a	1.50 \pm 0.06 ^b	1.76 \pm 0.07 ^a
AF	1.23 \pm 0.05 ^c	1.18 \pm 0.05 ^b	1.27 \pm 0.05 ^c	1.44 \pm 0.06 ^b
EG	1.60 \pm 0.09 ^a	1.79 \pm 0.09 ^a	2.08 \pm 0.09 ^a	1.82 \pm 0.06 ^a
AF+EG	1.59 \pm 0.07 ^a	1.49 \pm 0.07 ^a	1.49 \pm 0.06 ^b	1.68 \pm 0.08 ^a

C: Control, AF: Aflatoxin, EG: Glucomannan, AF+EG: Aflatoxin+glucomannan; a-c Values within a column with no common superscripts are significantly ($P<0.05$) different

Nuclear areas of glomerulus and tubular epithelial cells were significantly ($P<0.05$) decreased in all regions of AF group compared to the C group. Measurements in glomerulus, distal and collecting tubule regions of AF+EG group were similar compared with the C group. But, it was observed that measurements in proximal tubule regions in AF+EG group were significantly ($P<0.05$) different compared with the C group. It was known that AFs have toxic effect on p53 gene which is a protective effect against DNA damage in cells ^{32,33}. The findings of the study were showed that AF was significantly decreased nuclear areas of cells and formed negative impact on metabolic activity of cells.

AgNOR areas of glomerulus and tubular epithelial cells of kidney were similar with C and EG groups. We were observed AF group was lowest ($P<0.05$) AgNOR area. AgNOR area of AF+EG group was found statistically higher ($P<0.05$) than that of AF group. These data were in agreement with findings of the researches ^{34,35} who reported that AFs caused genetic disorders in cells. In this study, we found the lowest AgNOR area of the AF group since AF caused to modification ³⁶ and reduce number of ribosome. Ultimately,

this situation causes to reduce synthesis of proteins ^{37,38}. On the other hand, AgNOR area of AF+EG group to be higher than those of AF group, it shows aflatoxin is relatively eliminated pressure on protein synthesis by EG.

The ratio of nuclear area of the AgNOR area of glomerulus and tubular epithelial cells of kidney decreased significantly ($P<0.05$) in the AF group compared to the other groups. This result reveals that AFs which are caused DNA damage ^{28,33,39} reduce activity of cell's protein synthesis ^{37,38}. Zaczek et al. ¹⁷ have reported that AgNOR parameters associated with the proliferation activity of the epithelium.

As a conclusion, reason of decline in protein synthesis activity of the cells, we can be said AF is partly eliminated the negative impact on the cells by used EG. We were concluded EG is an agent which can be used successfully to prevent aflatoxicosis. Obtained data are showed that there are important changes in the AgNOR parameters. Therefore, we think that AgNOR parameters will also utilize taking into account besides other histopathological assessments for future similar studies.

REFERENCES

1. **Devegowda G, Raju MVLN, Afzali N, Swamy HVLN:** Mycotoxins picture: worldwide, novel solutions for their counteraction. In, Lyons TP, Jacques KA (Eds): *Biotechnology in the Feed Industry. Proceedings of Alltech's 14th Annual Symposium*. Nottingham, 24 February 1998, UK, pp 241-255, 1998.
2. **Bennett JW, Klich M:** Mycotoxins. *Clinical Microbiology Reviews*, 16, 497-516, 2003.
3. **CAST:** Council for Agricultural Science and Technology Task Force, Mycotoxins: Economic and Health Risks. Ames, IA, US, Rep No 116, 1989.
4. **Sur E, Celik I:** Effects of AFB1 on the development of the bursa of Fabricius and blood lymphocyte acid phosphatase of the chicken. *Brit Poult Sci*, 44, 558-566, 2003.
5. **Oguz H, Hadimli HH, Kurtoglu V, Erganis O:** Evaluation of humoral immunity of broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. *Rev Med Vet*, 154, 483-486, 2003.
6. **Basmacıoğlu H, Oguz H, Ergul M, Col R, Birdane YO:** Effect of dietary esterified glucomannan on performance, serum biochemistry and haematology in broilers exposed to aflatoxin. *Czech J Anim Sci*, 50 (1): 31-39, 2005.
7. **Eraslan G, Karaoz E, Bilgili A, Akdogan M, Oncu M, Essiz D:** The effects of aflatoxin on kidney function in broiler chicks. *Turk J Vet Anim Sci*, 27, 741-749, 2003.
8. **Peraica M, Domijan A, Jurjevic Z, Cvjetkovic B:** Prevention of exposure to mycotoxins from food and feed. *Arch Hig Rada Toksikol*, 53 (3): 229-237, 2002.
9. **Ibrahim IK, Shareef AM, Al-Joubry KMT:** Ameliorative effects of SB on phagocytosis and Newcastle disease antibody formation in broiler chickens during aflatoxicosis. *Res Vet Sci*, 69, 119-122, 2000.
10. **Rosa CAR, Miazzo R, Magnoli C, Salvano M, Chiacchiera SM, Ferrero S, Saenz M, Carvalho EC, Dalcerro A:** Evaluation of the efficacy of bentonite from the south of Argentina to ameliorate the toxic effects of AF in broilers. *Poult Sci*, 80, 139-144, 2001.
11. **Kececi T, Oguz H, Kurtoglu V, Demet O:** Effects of polyvinylpyrrolidone, synthetic zeolite and bentonite on serum biochemical and haematological characters of broiler chickens during aflatoxicosis. *Br Poult Sci*, 39 (3): 452-458, 1998.
12. **Bata A, Laszity R:** Detoxification of mycotoxin contaminated food and feed by microorganisms. *Trends Food Sci Technol*, 10, 223-228, 1999.
13. **Stanley VG, Ojo R, Woldensenbet S, Hutchinson DH:** The use of *Saccharomyces cerevisiae*, to suppress the effect of aflatoxicosis in broiler chicks. *Poult Sci*, 72, 1867-1872, 1993.
14. **Diaz DE, Hagler WM, Hopkins BA, Whitlow LW:** Aflatoxin binders I: *in vitro* binding assay for aflatoxin B₁ by several potential sequestering agents. *Mycopathologia*, 156, 223-226, 2002.
15. **Robert-Fortel I, Junera HR, Geraud G, Hernandez-Verdun D:** Three-dimensional organization of the ribosomal genes and Ag-NOR proteins during interphase and mitosis in PtK1 cells studied by confocal microscopy. *Chromosoma*, 102, 146-157, 1993.
16. **Khanna AK, Yadav SK, Dixit VK, Kumar M:** AgNOR count and subjective AgNOR pattern assessment (SAPA) score in carcinoma of the pancreatic head including tumors. *J Pancreas*, 6 (6): 575-580, 2005.
17. **Zaczek M, Maciejowski J, Gil K, Szot W, Chlap Z:** Silver-binding nucleolar organiser regions (AgNORs) in the normal epithelium of different parts of the digestive tract in rats. *Acta Pathol Jpn*, 42 (8): 573-578, 1992.
18. **Bukhari MH, Niazi S, Khan SA, Hashmi I, Perveen S, Qureshi SS:** Modified method of AgNOR staining for tissue and interpretation in histopathology. *Int J Exp Path*, 88, 47-53, 2007.
19. **Abrams L:** Mycotoxicosis. *J S Afr Vet Med Assoc*, 36, 5-13, 1965.
20. **Lewis G, Markson LM, Allcroft R:** The effect of feeding toxic groundnut meal to sheep over a period of five years. *Vet Rec*, 80, 312-314, 1967.
21. **Edrington TS, Harvey RB, Kubena LF:** Effect of aflatoxin in growing lambs fed ruminally degraded or escape protein sources. *J Anim Sci*, 72, 1274-1281, 1994.
22. **Shotwell OL, Hasseltine CW, Stubblefield RD, Sorenson WG:** Production of aflatoxin on rice. *Appl Microbiol*, 5, 425-429, 1966.
23. **Demet O, Oguz H, Celik I, Nizamlioglu F:** Pirinçte aflatoksin üretilmesi. *Vet Bil Derg*, 11 (1): 19-23, 1995.
24. **Vicam LP:** Fluorometer USDA-FGIS procedure for corn, corn meal, corn/soy blend, milled rice, popcorn, sorghum and soybeans. In, Vicam LP (Ed): *Afla Test Instruction Manuel*. pp 36-38, Vicam, Watertown, USA, 1999.
25. **Stroka J, Anklam E, Otterdijk R:** Immunoaffinity column clean-up prior to thin layer chromatography for determination of aflatoxins in various food matrices. *J Chromat A*, 904, 251-256, 2000.
26. **Aydın MF, Celik I:** Determination of the distribution of silver-stained nucleolar organizing regions (AgNORs) in the livers broilers and laying hens. *Vet Bil Derg*, 21 (1-2): 91-99, 2005.
27. **Yildirim E, Yalcinkaya I, Kanbur M, Cinar M, Oruc E:** Effects of yeast glucomannan on performance, some biochemical parameters and pathological changes in experimental aflatoxicosis in broilers chickens. *Revue Med Vet*, 162 (8-9): 413-420, 2011.
28. **Ozen H, Karaman M, Cigremis Y, Tuzcu M, Ozcan K, Erdag D:** Effectiveness of melatonin on aflatoxicosis in chicks. *Res Vet Sci*, 86, 485-489, 2009.
29. **O'Brien E, Dietrich DR:** Mycotoxins Affecting the Kidney. In, Hook JB, Tarloff JB, Lash LH (Eds): *Toxicology of Kidney*. First ed., pp. 895-936, Boca Raton, CRC Pr, FL, 2004.
30. **Lakkawar AW, Chattopadhyay SK, Johri TS:** Experimental aflatoxin B₁ toxicosis in young rabbits-a clinical and patho-anatomical study. *Slov Vet Res*, 41 (2): 73-81, 2004.
31. **Boonyaratpalin M, Supamattaya K, Verakunpiriya V, Suprasert D:** Effects of aflatoxin B₁ on growth performance, blood components, immune function and histopathological changes in black tiger shrimp (*Penaeus monodon fabricius*). *Aquacult Res*, 32, 388-398, 2001.
32. **Gerbes AL, Caselmann WH:** Point mutations of the p53 gene, human hepatocellular carcinoma and aflatoxins. *J Hepatol*, 19 (2): 312-315, 1993.
33. **Ozturk M:** P53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet*, 338, 1356-1359, 1991.
34. **Shi CY, Phang TW, Lin Y, Wee A, Li B, Lee HP, Ong CN:** Codon 249 mutation of the p53 gene is a rare event in hepatocellular carcinomas from ethnic Chinese in Singapore. *Br J Cancer*, 72, 146-149, 1995.
35. **Sengstag C:** The molecular mechanism of aflatoxin B₁- induced liver cancer: is mitotic recombination involved. *Mol Carcinog*, 19, 147-152, 1997.
36. **Lyman BA, Erki L, Biedrzycka DW, Devlin TM, Ch'ih JJ:** Modification of protein synthetic components by aflatoxin B₁. *Biochem Pharmacol*, 37 (8): 1481-1486, 1988.
37. **Ergun E, Ergun L, Essiz D:** Light and electron microscopic studies on liver histology in chicks fed aflatoxin. *Dtsch Tierarztl Wschr*, 49, 243-247, 2006.
38. **Sehu S, Ergun L, Cakir S, Ergun E, Cantekin Z, Sahin T, Essiz D, Sareyyupoglu B, Gurel Y, Yigit Y:** Hydrated sodium calcium aluminosilicate for reduction of aflatoxin in quails (*Coturnix coturnix japonica*). *Dtsch Tierarztl Wschr*, 114, 252-259, 2007.
39. **Salem MH, Kamel KI, Yousef MI, Hassan GA, EL-Nouty FD:** Protective role of ascorbic acid to enhance semen quality of rabbits treated with sublethal doses of aflatoxin B₁. *Toxicology*, 162, 209-218, 2001.