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Effects of Aflatoxin on AgNOR Activity of Cells in Different Hepatic Zones of Liver, and Protective Effectiveness of Esterified Glucomannan in Ram

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Summary
In this study, the effects of total aflatoxin (AF) given orally on silver-staining nucleolus organizer regions (AgNORs) activity were studied on epithelial cells (hepatocytes) in the 3 functionally different hepatic zones of liver. 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. 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 liver. Liver tissue samples were evaluated in terms of the AgNOR areas, nuclear areas and the ratio to the nuclear area of the AgNOR area in the hepatocytes. In conclusion, the adverse effects causing by aflatoxicosis on the liver AgNOR activity could be ameliorated by adding EG to the ration.
Key Words*:* Aflatoxin, AgNOR, glucomannan, liver, ram.

Koçlarda Karaciğerin Farklı Bölgelerindeki Hücrelerin AgNOR Aktivitesi Üzerine Aflatoksinin Etkileri ve Esterifiye Glukomannan'ın Koruyucu Etkinliği

Sunulan bu çalışma, ağızdan verilen total aflatoksinin (AF) karaciğerin 3 fonksiyonel olarak farklı bölgesindeki hepatositlerin AgNOR aktivitesi üzerine olan etkilerinin ve esterifiye glukomannanın (EG) aflatoksikoza karşı koruyucu etkinliğinin belirlenmesi açısından yapıldı. Materyal olarak 32 adet 1 yaşlı Merinos ırkı koç kullanıldı. Kontrol (K) grubuna ticari yem, AF grubuna ticari yem ile günlük 250 µg AF, EG grubuna ticari yem ile günlük 2 gr EG, AF+EG grubuna ise ticari yemle birlikte günlük 250 µg AF ve 2 gr EG verildi. 92 günlük besleme periyodunu takiben hayvanlar kesildi ve doku örnekleri alındı. Karaciğer hücrelerinde AgNOR alanları, çekirdek alanları ile AgNOR alanının çekirdek alanına oranları değerlendirildi. Sonuç olarak, karaciğer AgNOR aktivitesi üzerine aflatoksikozun sebep olduğu istenmeyen etkilerin rasyona EG ilavesiyle iyileştirilebildiği bulundu.
Anahtar kelimeler: Aflatoksin, AgNOR, glukomannan, karaciğer, koç.

1. Introduction
The aflatoxins (AFs) are a group of closely related mycotoxin metabolites (1). The main biological effects of AFs are carcinogenicity, immunosuppression, mutagenicity and teratogenicity (2). Contamination of AF in feed causes aflatoxicosis in poultry production characterised by listlessness, anorexia, decreased weight gain, and increased mortality (3). AFs are well known for its hepatotoxic and hepatocarcinogenic effects (4). The liver is the target organ following the ingestion of the toxin. High doses of AFs cause severe hepatocellular necrosis (5). In utero exposure of AF through mother's blood has also been reported in human beings (6).

Removing AF from contaminated food and foodstuffs remains a major problem and there is a great demand for effective decontamination technology (3). 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

(7). Recent years, researchers suggested that the best approach for decontamination would be biological degradation (8). One of these methods is the use of organic adsorbents like modified yeast cell wall preparation, containing glucomannan (9). Esterified glucomannan (EG) showed considerably high binding ability (80-97%) with AF (10), and it has been preferred for detoxification of AF in poultry animals. Several studies (11) showed that EG partially and/or completely reserved the adverse effect of AF on performance, biochemistry-haematology and immune responses of birds.

Nucleolar-organizer regions (NORs) are loops of DNA containing ribosomal RNA genes (12). 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 (13). 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 (14).

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

2. Material and Methods

2.1. 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, 1ml/50 kg) and oksifendazol (oxa-F, 1 tablet/50 kg) were performed. In addition, enterotoxaemia (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*. AF and EG that were mixed of 250 g commercial feed were given to animals before morning feeding and then morning feeding was continued.

Table 1. Composition of the commercial feed

Crude matter	%88	Na	%0.1-0.4
Crude protein	%12	NaCl	%1.0
Crude Celluluos	%12	Metabolic energy	2750 kcal/kg
Crude ash	%9	Vit A	7000 IU-kg
Insoluble ash in HCL	%1.0	Vit D3	700 IU-kg
Ca	%0.6-1.6	Vit E	25 mg/kg
P	%0.4		

2.2. Aflatoxin:

The AF was produced from *Aspergillus parasiticus* NRLL 2999 culture (USDA, Agricultural Research Servise, Peoria, IL) via fermentation of rice by the method of Shotwell et al. (15) with minor modifications by Demet et al. (16). Fermented rice was sterilized in autoclave, dried at 70º C, and ground to a fine powder. According to the method reported by Vicam (17) 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. (18). The amount of total AF in the fermented rice was found 73.96 ppm. The AF within the rice consisted of 84.15% AFB_1, 6.29% AFB_2, 9.13% AFG_1 and 4.25% AFG_2. (rate of return method 97.4%; sensitivity 0.4 ppb).

2.3. Experimental Design:

The experimental design consisted of four dietary treatments. Control group (C) fed with the commercial feed (Table 1). 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. AF+EG group fed with commercial feed added 250 µg/day of total AF and 2 g/day of EG. AF and EG doses which were given to animals throughout the study were calculated by pharmacologists.

2.4. Tissue Samples:

At the end of the 92nd day, after sacrificed all of the animals tissue samples were taken from the liver in 10% neutral-buffered formalin solution. The tissues were processed and paraffin sections (6 µm) were stained with a solution containing one volume of 2% gelatine in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck). The staining was performed at 37º C in the dark for 20-30 minutes (19). The

histological preparations were examined with a light microscope (Leica DM-2500 attached to a DFC-320 digital camera). In different hepatic zones of the liver (zone 1, zone 2, and zone 3) of each animal, 25 hepatocytes having nuclei were evaluated. The nuclear area and the AgNOR area were analysed with an image analysis programme (IM-50). Also, the percentage of the AgNOR area relative to the nuclear area was calculated.

2.5. Statistical Analysis :

The obtained results were statistically analysed using Duncan test in SPSS software [version 17.00; SPSS Inc., Chicago, IL, USA]. The level of significance was $P<0.05$.

3. Results

We obtained the nuclear area, the AgNOR area (Figure 1, Figure 2), and the ratio of the AgNOR area to whole nuclear the of hepatocytes in different hepatic zones of the liver (Table 2).

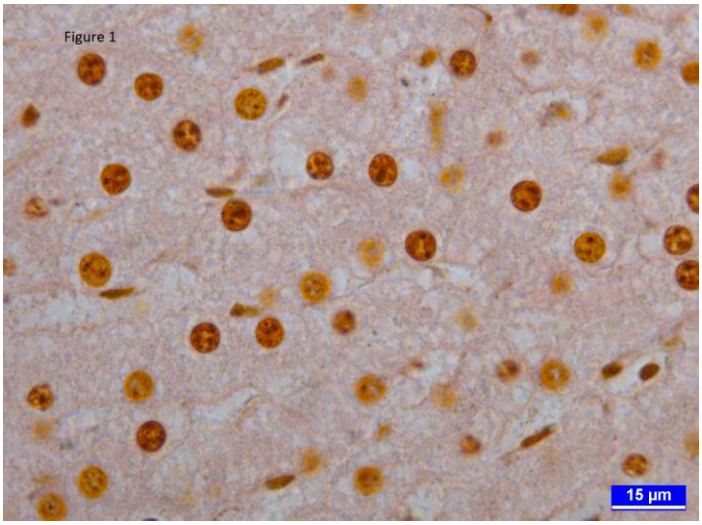

Figure 1

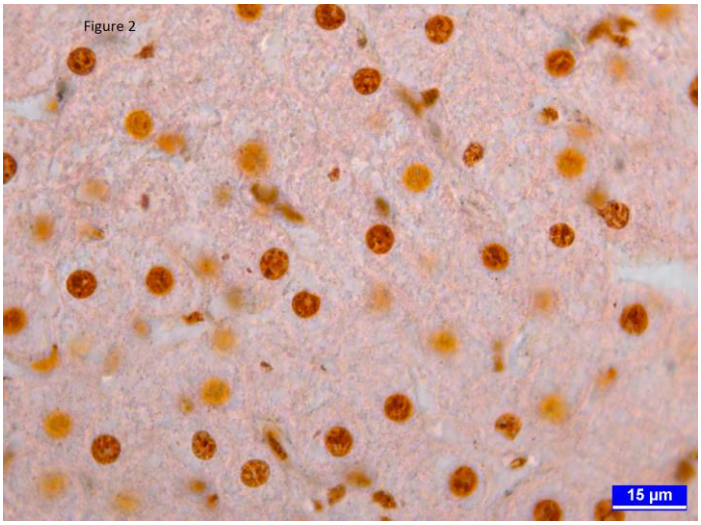

Figure 2

Table 2. AgNOR parameters of hepatocytes in different hepatic zones of liver (µm²)±SE

Groups (n=8)	Nuclear areas of hepatocytes in different hepatic zones of liver (µm²)±SE			AgNOR areas of hepatocytes in different hepatic zones of liver (µm²)±SE			The ratio of the AgNOR area to whole nuclear area of hepatocytes in different hepatic zones of liver (%) ±SE		
	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3
C	33,55±0,42^{a}	33,35±0,51^{a}	33,04±0,60^{a}	1,34±0,04^{a}	1,38±0,04^{a}	1,24±0,03^{a}	4,06±0,21^{a}	4,01±0,22^{a}	4,38±0,18^{a}
AF	28,74±0,26^{c}	28,60±0,27^{c}	27,47±0,28^{c}	0,71±0,02^{c}	0,72±0,02^{c}	0,65±0,02^{d}	2,60±0,11^{c}	2,66±0,09^{c}	2,55±0,09^{c}
EG	32,62±0,38^{ab}	32,42±0,26^{a}	32,53±0,32^{ab}	1,21±0,03^{ab}	1,29±0,03^{b}	1,18±0,04^{b}	3,81±0,15^{ab}	3,79±0,14^{ab}	3,92±0,12^{a}
AF+EG	29,82±0,31^{b}	30,77±0,39^{b}	28,96±0,42^{b}	0,75±0,02^{c}	0,77±0,02^{c}	0,79±0,02^{c}	3,57±0,09^{b}	3,62±0,11^{b}	3,45±0,09^{b}

C: Control, AF: Aflatoxin, EG: Glucomannan, AF+EG: Aflatoxin+glucomannan
a—d Values within a column with no common superscripts are significantly (P<0.05) different.

4. Discussion

It is reported that liver, kidney and immune system organs are considered to be target organs for AF and these are primarily affected in aflatoxicosis cases by Ortatatli et al. (20). Lakkawar et al. (5) declared that liver and kidney were the most affected organs in rabbits which fed an AFB_1 contaminated diet. The effects of AFs on histopathological changes are directly correlated with the concentration of AF and the duration of the exposure (21). A study of Colakoglu and Donmez (22) has found the adverse effects causing by experimental aflatoxicosis could be ameliorated by adding esterified glucomannan (EG) to the ration. EG showed considerably high binding ability (80-97%) with AF (10), and it has been preferred for detoxification of AF in poultry. These results clearly indicated that EG addition effectively diminished the adverse effects of AF on the investigated values.

Classical histological lobule of the liver can be functionally divided into three zones based upon oxygen supply. These zones called zone 1, 2, and 3 from perifery of the lobule to the central vein, respectively (23). Nuclear areas of hepatocytes were significantly ($P<0.05$) decreased in all hepatic zones of AF group compared to the C group. While measurements in zone 1 of EG and AF+EG groups were decreased compared with the C group, it was found statistically higher ($P<0.05$) than that of AF group. Measurements in zone 2 in EG group were similar to C group. It was known that AFs have toxic effect on p53 gene which is a protective effect against DNA damage in cells (24). Some researchers have declared that AFs lead to reducing the number of ribosome in hepatocytes, and ultimately that this situation caused to decreased in protein synthesis (25, 26). 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 hepatocytes of liver decreased significantly ($P<0.05$) in all hepatic zones of AF group compared to the other groups. This situation showed that AFs have an inhibitory effect on proliferation activity and synthesis in hepatocytes. AgNOR area of AF+EG group was found statistically decreased ($P<0.05$) than that of C and EG group. But, it was found statistically higher than AF group. These data were in agreement with findings of the researches who reported that AFs caused genetic disorders in cells (27, 28). In this study, these data also showed that EG ameliorated suppressive effects of AFs on protein synthesis.

The ratio to nuclear area of the AgNOR area of hepatocytes of liver decreased significantly ($P<0.05$) in the AF group compared to the other groups. This result reveals that AFs which are caused DNA damage (29) reduce activity of protein synthesis (25, 26). Zaczek et al. (14) have reported that AgNOR parameters associated with the proliferation activity of the epithelium.

In this study, histopathological findings obtained from AF+EG group is close to the C and EG groups. This results have shown EG is an important adsorbent in decreasing the detrimental effects of AFs. This finding was in agreement with a study of Colakoglu and Donmez (22).

5. Conclusion

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 ameliorative agent which can be used successfully to prevent aflatoxicosis. Obtained data showed that there are important changes in the AgNOR parameters during aflatoxicosis. Therefore, we think that the AgNOR parameters will also utilize taking into account besides other histopathological assessments for future similar studies.

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