Effect of aflatoxin on performance, hematology, and clinical immunology in lambs

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Abstract

Twenty-four female lambs were intoxicated with a diet contaminated with 2 ppm aflatoxin for a period of 37 d. Twelve lambs were maintained as the control group. After this period, the lambs were left for 35 d without aflatoxin in their feed. Performance, hematology and clinical immunology were examined in the intoxicated lambs. A non-significant decrease in body weight was observed in the intoxicated lambs during the intoxication period, whereas a significant decrease ($P < 0.001$) in average daily gain was noted on the last day of intoxication and during the clearance period. No significant differences were observed in erythrocyte count, white blood cell count or differential leukocyte count between the groups. Bacteriostatic activity of the serum was lower in the intoxicated lambs, however, there was no effect on serum opsonic activity. Phagocytosis by the neutrophils was higher during the intoxication period and the levels of IgG were elevated in the intoxicated lambs. In vivo cellular immunity was assessed by intradermal injection of phytohemagglutinin; the response was lower during intoxication period. These results indicate that a lowering in the average daily gain was the most sensitive indicator of aflatoxicosis in lambs, and that the immune response was altered, which could render the animals more susceptible to infectious diseases.

Résumé

Une intoxication à l'aflatoxine fut induite chez vingt-quatre agnelles en les alimentant pendant 37 j avec une diète contenant 2 ppm de toxine. Un groupe témoin de douze agnelles fut également constitué. Suite à cette période, les animaux furent laissés pendant 35 j sans aflatoxine dans leur alimentation. Les performances zootechniques, de même que des critères hématologiques et immunologiques furent déterminés chez les animaux ayant reçu la toxine. Quoique non-significative, une perte de poids corporel fut notée chez les animaux intoxiqués durant la période de 37 j, alors qu'une diminution significative (P < 0,001) du gain moyen journalier fut enregistrée au dernier jour de l'intoxication de même que durant la période d'élimination de la toxine. Aucune différence significative ne fut observée entre les deux groupes pour ce qui est du comptage érythrocytaire, du comptage leucocytaire de la numération différentielle des leucocytes. L'activité bactériostatique du sérum était plus faible chez les animaux intoxiqués, mais aucune différence ne fut notée dans le pouvoir d'opsonisation du sérum. La phagocytose par les neutrophiles était plus élevée durant la période d'intoxication et les niveaux d'IgG étaient élevés chez les animaux intoxiqués. L'immunité cellulaire, telle qu'évaluée par injection intradermique de phytohémagglutinine, était diminuée durant la période d'intoxication. Les résultats démontrent qu'une diminution du gain moyen journalier était l'indicateur le plus sensible d'aflatoxicose chez des agnelles, et que la réponse immunitaire était modifiée, ce qui pourrait rendre les animaux plus susceptibles à une maladie infectieuse.

(Traduit par docteur Serge Messier)

Introduction

Aflatoxins are mycotoxins produced by the fungi *Aspergillus flavus* and *A. parasiticus*, which grow on numerous feedstuffs (mainly cereals) when environmental conditions are favorable. It is in this manner that they can affect livestock (1). Aflatoxins are one of the most dangerous mycotoxins known due to their high toxicity to both animals and humans. The effects of aflatoxins have been studied thoroughly in domestic animals, mainly in the monogastrics (2,3), which feed on high quantities of cereal grains.

Aflatoxicosis has a wide variety of effects on animals, including weight loss and poor performance, changes in the clinical biochemistry panel, and an increase in susceptibility to infectious diseases (4). There are recent papers (5–7) showing that a decrease in body weight, growth rate, and an alteration in mineral metabolism, coagulation profile, and clinical biochemistry occur in young lambs. In birds, clinical and analytical recovery of the animals after withdrawal from the toxic diet is usually quick (8). On the other hand, 8 d was not a sufficient period of time for lambs to return to the basal levels in the studied parameters (6).

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Increased susceptibility to infectious disease and impairment of cellular and humoral immune functions have been observed in animals. The animals' responses to vaccines have been variable; a decrease have all been documented, an increase, or no effect at all on antibody levels (3,9,10). The effects of the aflatoxin on the phagocytic function (11), immunoglobulin concentrations, and cellular immune function (12) have been described for pigs and other animals. Little is known about the effect of aflatoxins on the immune function in sheep livestock.

The aim of this experiment was to study the effects on performance, hematology and clinical immunology that aflatoxins had on female lambs treated with aflatoxin — contaminated feed for 37 d. The second objective was to monitor the recovery of these lambs over a 35-d period after the end of the treatment.

Material and methods

Animals

The experimental protocol was approved by the Servicio de Apoyo a la Experimentación Animal of the University of Zaragoza. Thirty-six 5-month-old female lambs (Fleischschaff × Romanov) were obtained from a flock belonging to the Servicio de Investigación Agroalimentaria in Zaragoza, and were housed under drylot conditions in pens, with straw and water available ad libitum.

The health of all animals was closely monitored before and during the study. The lambs were randomly allotted into 2 groups (average body weight 27 ± 2.9 kg). The treatment group consisted of 24 lambs that received the concentrate feed containing 2 ppm aflatoxins. The control group consisted of 12 lambs receiving an aflatoxin-free diet (0 ppm). Lambs consumed the toxic feed for 37 d (intoxication period), then the aflatoxins were removed from the diet and the lambs were monitored for 35 d (clearance period). The weight and feed intake of the lambs were measured weekly.

Aflatoxin production

The aflatoxin used in this study was produced by *A. parasiticus* NRRL 2999, via fermentation on sterile, polished rice as described by Shotwell et al (13). The aflatoxins were isolated on thin-layer chromatographic (TLC) plates (14) and were analyzed spectrophotometrically for aflatoxin content by the Nabney and Nesbitt method (15). The aflatoxin content was determined to be 0.5374 mg/g rice powder. The percentage of aflatoxin in the rice powder was 83.4% aflatoxin (AF) AFB1, 3.5% AFB2, 12.3% AFG1 and 0.8% AFG2. It was incorporated into the basal diet to provide the desired level of 2 mg of aflatoxins/kg of diet (2 ppm).

Blood samples

Three different tubes of blood were obtained from each lamb by jugular venipuncture on Days 0, 7, 14, 23, 30 and 37 of the intoxication period and then on Days 7, 14, 21, 28 and 35 of the clearance period. The anticoagulant EDTA-K^+ was used in one tube for the hematological study, heparin was used for the phagocytic study, and a tube without anticoagulant for serum. Hemoglobin, hematocrit, erythrocyte count (RBC), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined with a cell counter (Sysmex Microcell counter F-800, Kobe, Japan) following the manufacturer's instructions. The differential leukocyte counts were made using the method and the differential criteria of Jain (16). Blood smears were stained with Panóptico Rápido (QCA, Tarragona, Spain) and observed under a microscope.

Table I. Body weight and average daily gain (ADG) in lambs intoxicated with 2 ppm of aflatoxin and in the control group (0 ppm)

	Body weight (kg)		Average daily gain (g/day)[a]	
Day	0 ppm	2 ppm	0 ppm	2 ppm
Intoxication period				
0	27.1 ± 2.7	27 ± 3	—	—
7	28.8 ± 2.9	28 ± 3	232 ± 101	157 ± 115
14	29.5 ± 3.1	29 ± 3.1	172 ± 50	148 ± 63.7
21	29.5 ± 2.8	28.4 ± 3.2	113 ± 36.7	68 ± 57*
28	30 ± 3.1	28.9 ± 3.1	102 ± 35	70 ± 39*
35	31.5 ± 3.3	29.7 ± 3.2	125 ± 35.3	79 ± 39***
Clearance period				
7	32.1 ± 3.1	30.9 ± 3.2	116 ± 34	91 ± 40.6
14	33.4 ± 3.2	32.1 ± 3.6	129 ± 33.8	104 ± 36.6*
21	33.4 ± 3.5	33.2 ± 3.7	113 ± 29.5	112 ± 37
28	35.6 ± 3.5	34.1 ± 3.7	136 ± 28.8	114 ± 32.6*
35	36.4 ± 3.8	34.9 ± 3.8	130.8 ± 32	111 ± 31.7

Data are expressed as mean ± standard deviation

[a] Each value was calculated by the difference of weights between the first day of the experiment and the day of the test

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Bacteriostatic activity of the serum

The bacteriostatic activity of the serum was analyzed as described by Thurston et al (17). A single strain of *Escherichia coli* (*E. coli*) was used. The stock culture was added to 10 mL of trypticase soy broth (TSB) (Difco, Detroit, Michigan, USA). After a 2-hour incubation at 37°C, the culture was adjusted with calcium-magnesium-veronal buffer at pH 7.2 (buffer composed of 8.500 g sodium chloride, 0.575 g diethylmalonylurea, 0.185 g sodium diethylmalonylurea, 0.168 g magnesium chloride-6$H_2$0, 0.028 g calcium chloride, 1000 mL distilled water) (bioMérieux, l'Étoile, France) for a 50% of transmittance at 540 nm (Perkin Elmer Lambda 5, UV-Vis, Barcelona, Spain) against a blank of distilled water at 100% of transmittance. The *E. coli* in the 10 mL of adjusted culture was washed 3 times and then resuspended in 10 mL of veronal buffer for use in the test procedure. The test was conducted by mixing 0.2 mL of the resuspended *E. coli* with 0.2 mL of the suspension of lamb serum previously diluted to 1:16 with veronal buffer. The mixture was incubated at 37°C for 30 min. A control tube was prepared with veronal buffer as a substitute for the serum. At the end of the 30 min, 5 mL of TSB were added to the tubes and the tubes were incubated at 37°C. The percentage of transmittance at 540 nm against a blank of TSB at the end of 6 h was recorded. Growth was indicated by a decrease in the percent light transmittance.

Table II. Bacteriostatic activity of the serum (BAS), phagocytosis of neutrophils (PN) and serum opsonic activity (SOA) in lambs intoxicated with 2 ppm of aflatoxin and in the control group

	Control group			Intoxicated group		
Day	BAS (%T[a])	PN (RLU/10000 neutrophils)	SOA (RLU/10000 neutrophils)	BAS (%T)	PN (RLU/10000 neutrophils)	SOA (RLU/10000 neutrophils)
Intoxicated period						
0	100 ± 2.1	0.84 ± 0.53	1.23 ± 0.06	96.7 ± 6.2	0.89 ± 0.4	1.21 ± 0.14
7	77.8 ± 9.1	0.81 ± 0.4	1.25 ± 0.07	74.5 ± 15.8	0.9 ± 0.36	1.24 ± 0.07
14	81.8 ± 8.3	0.63 ± 0.41	1.34 ± 0.14	81.4 ± 1.2	0.51 ± 0.18	1.3 ± 0.09
23	71.6 ± 6.4	0.41 ± 0.18	1.2 ± 0.13	63.8 ± 10.3*	0.46 ± 0.21	1.22 ± 0.13
28	64.3 ± 12	0.93 ± 0.36	1.46 ± 0.11	58.7 ± 14.6	1.18 ± 0.4	1.43 ± 0.13
37	46.1 ± 20.1	0.28 ± 0.12	1.49 ± 0.1	26.5 ± 17***	0.46 ± 0.26*	1.47 ± 0.07
Clearance period						
7	39.9 ± 18	0.86 ± 0.4	1.85 ± 0.11	37.3 ± 21	0.67 ± 0.27	1.7 ± 0.22*
14	37.7 ± 8.7	0.75 ± 0.26	1.94 ± 0.11	44.8 ± 14	0.87 ± 0.48	1.9 ± 0.14
21	46.1 ± 21	0.92 ± 0.15	1.51 ± 0.15	47.2 ± 22	0.87 ± 0.45	1.54 ± 0.28
28	46.8 ± 23	1.1 ± 0.27	1.25 ± 0.32	51.1 ± 26.4	0.83 ± 0.41	1.26 ± 0.26
35	54.2 ± 26.9	0.63 ± 0.23	1.1 ± 0.06	59.3 ± 22.7	0.61 ± 0.27	1.01 ± 0.09

Data are expressed as mean ± standard deviation. PN and SOA values are in RLU/10000 neutrophils

[a] Percentage of transmittance

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Phagocytic assay

Respiratory burst activity of the neutrophils phagocytosis was determined using a chemiluminescence (CL) assay method (18). Heparinized blood (10 mL) was hemolized by flash hypotonic lysis and centrifuged at 600 × *g* to obtain the leukocyte layer. This fraction was washed twice in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} ions. The pellet was resuspended in 1 mL of Hanks with Ca^{2+} and Mg^{2+} ions at a aproximate concentration of 10^7 cells/mL. Zymosan (Sigma Chemical Co., St. Louis, Missouri, USA) was hydrated with NaCl at 0.9%, boiled for 30 min and then resuspended to achieve a concentration of 10 mg/mL. Serum, from a healthy donor sheep, and zymosan were preincubated at 37°C for 30 min to opsonize the zymosan. The final serum concentration was 10%. The chemiluminescent assay was performed in a luminometer (Luminoskan RS 1.0, Labsystem, Finland). For the test, a 96-well opaque and flat-bottomed microtitre plate was used. The following reactants were added: 100 µL of resuspended leukocyte, 100 µL of 10^{-4} M luminol (Sigma Chemical Co.) in phosphate-buffered saline and 100 µL of opsonized zymosan. The plate was placed in the measuring chamber of the machine with continous agitation at 37°C. Thirty readings of the chemiluminescence from each sample were recorded and a chemiluminescence curve and absolute peak response were read. Each sample was tested in duplicate and the results were recorded as peak chemiluminescence in relative light units (RLU)/10 000 neutrophils.

Serum opsonic activity

Serum opsonic activity was also performed using a CL assay. Serum from each lamb was used to opsonize zymosan particles at a 10% serum concentration. In a microtitre plate, 100 µL of opsonized zymosan, 100 µL of 10^{-4} M luminol, and 100 µL of a leukocyte suspension at 10^7 cells/mL from a donor sheep, were suspended. The chemiluminescence assay was performed in a luminometer and the results were obtained as above.

IgG levels

The level of immunoglobulin G (IgG) in serum was determined using a radial immunodiffusion test. The plates were purchased from The Binding Site (United Kingdom) with antibodies for sheep. The plates were incubated at room temperature for 72 h, after which the ring of precipitation was measured. The concentration of IgG in the serum was calculated by linear regression of a standard curve from the size of the ring.

Delayed type hypersensitivity test

A delayed type hypersensitivity (DTH) test was performed to assess the in vivo cellular immunity. Each lamb was injected on Day 30 of the intoxication period and on Day 30 of the clearance period with 0.1 mL of 2.5 mg/mL phytohemagglutinin (PHA, Sigma Chemical Co.), in isotonic saline solution). The injection was intradermal on the right inside flank in a region without wool. Approximately 10 cm from this site, 0.1 mL of saline solution was injected, serving as control. Skin thickness was measured using a Hauptner caliper 0, 12, 24, and 48 h after injection. Data was expressed as ratio between skin thicknesses of PHA-injected, and of saline solution. Biopsies were taken for the histopathological studies.

Statistics

The data was analysed using a Student's *t*-test and a value of $P < 0.05$ was considered to be significant.

Table III. Levels of IgG (mg/dl) in lambs intoxicated with 2 ppm of aflatoxin and in the control group

Day	Control group	Intoxicated group
Intoxicated period		
0	1758 ± 246	2038 ± 297
23	1554 ± 302	1938 ± 570*
37	1761 ± 232	2025 ± 733
Clearance period		
7	1830 ± 278	1999 ± 705
35	1718 ± 330	1923 ± 446

Data are expressed as mean ± standard deviation
*$P < 0.05$

Table IV. Delayed hypersensitivity test in lambs intoxicated with 2 ppm of aflatoxin and in the control group. Data shows ratio between thickness of the skin with PHA and isotonic saline solution

	Day 30 of intoxication		Day 30 of clearance	
Hour	Control	Intoxicated	Control	Intoxicated
0	1.31 ± 0.2	1.21 ± 0.24	1.06 ± 0.14	1.19 ± 0.21
12	3.51 ± 1.05	2.78 ± 0.97*	2.39 ± 0.23	2.31 ± 0.62
24	2.93 ± 0.67	2.47 ± 0.52*	2.62 ± 1.08	2.32 ± 0.88
48	1.74 ± 0.71	1.98 ± 0.7	2.18 ± 0.65	2.23 ± 0.67

Data are expressed as mean ± standard deviation
*$P < 0.05$

Results

Lambs in both groups appeared to be clinically normal and no deaths resulted from the intoxication. The intoxication caused alterations in the lambs' performance, which is shown in Table I. The body weight of the intoxicated lambs was always lower than those in the control group but this difference was not statistically significant ($P > 0.05$). The average daily gain (ADG) was lower in the intoxicated lambs. The difference was statistically significant beginning from Day 21 of intoxication, and was more significant ($P < 0.001$) on the last day of intoxication. Differences between both groups during the clearance period were observed for ADG.

Feed intake during the intoxication period in the control group was of 900 g/lamb/day (feed:gain = 7.2) and in the intoxicated group was of 625 g/lamb/day (feed:gain = 7.9). In the clearance period, the daily intake was 925 g/lamb/day (feed:gain = 7) and 850 g/lamb/day (feed:gain = 7.7), in the control and intoxicated group, respectively.

No effects due to aflatoxins were observed in the total WBC in the intoxicated or clearance periods. No significant differences ($P > 0.05$) were noted in the differential leukocyte count (data not shown) and also no effects observed on RBC, HCT, HG, MCHC, MCH and MCV values (data not shown).

Data on the bacteriostatic activity, phagocytosis of neutrophils and serum opsonic activity are shown on Table II. Lambs receiving 2 ppm of aflatoxins in the feed had a lower bacteriostatic activity than the control group, being the lowest on Day 37 of intoxication period ($P < 0.001$), whereas during the clearance period, the levels quickly recovered to those of the control group. Phagocytic activity of the neutrophils was higher during the intoxication period ($P < 0.05$), but were variable in the clearance period. No effect due to the aflatoxins was observed on serum opsonic activity in the intoxicated lambs and only on Day 7 of the clearance period there was a statistically significant difference ($P < 0.05$).

The levels of IgG in serum were higher in the intoxicated group during the experiment (Table III), and were statistically significant ($P < 0.05$) on Day 23 of intoxication period. The response to PHA in intoxicated lambs was lower on Day 30 of intoxication ($P < 0.05$), but on Day 30 of the clearance period there were no significant statistical differences ($P > 0.05$) (Table IV). Histologically, there were polymorphonuclear neutrophils within the dermal blood vessels, with some lymphocytes, histiocytes and fibroblasts, and superficial edema and fibrin.

Discussion

No mortality or clinical signs of decrease were seen in the treated lambs. Abdelsalam et al (19) reported depression, inappetence, anorexia, ataxia, and dyspnea in sheep intoxicated with extremely high doses (200 ppm) of aflatoxin, 100 times higher than that used in this study. The toxic properties of aflatoxins differ with dose and duration of exposure. Animal susceptibility to aflatoxins also depends on other important factors such as species, breed, age, sex, and nutritional status (4). The results of our experiment demonstrate that 2 ppm of aflatoxins may be a subclinical dose for sheep, since only a slower increase in body weight and ADG were noted. This is in agreement with previous results found in studies done in young male lambs (6). Poor performance is a recognized effect in those farm animals intoxicated by aflatoxins, and, in this experiment, it was associated with a lower food intake by the intoxicated animals. The clearance time used here was not sufficient to achieve the complete recuperation of the lambs, and a longer period would be necessary in order to rehabilitate an animal adequately for fattening.

Aflatoxin effects on hematological parameters are variable, since both a decrease and increase in the hemogram of animals have been reported. We did not find any change in the WBC or differential leukocyte levels, as the results observed in pigs (20), goats (21) and lambs (22) have shown. Hematological values were variable and no clear effect was observed. An increase in RBC and hemoglobin values was seen in lambs intoxicated with 2.5 ppm of aflatoxins (23,6), yet other investigators observed no variation (22). The clinical effects of aflatoxins can be influenced by the dose, sex, age or breed of animal used in the intoxication experiments.

Aflatoxicosis altered the bactericidal activity of serum. This bactericidal property, which depends on the complement immune system, causes loss of viability in Gram-negative bacteria (24,25). A decrease in this activity has been reported in steers administered different doses of aflatoxins (17). It has been observed in guinea pigs that there was a decrease in the C4 fraction of the complement (25,26) system, which is an essential component to the activation of the membrane attack complex (24). In this study, no residual effects were noted, as the intoxicated animals eventually regained proper immune function after the clearance period, and the clearance period reached levels similar to those in the control group.

A decrease in monocyte phagocytosis was observed in pigs ingesting milk from dams intoxicated with aflatoxins. Neutrophil function was highly affected in those animals treated with high doses

of AFG1 (11). In our experiment, the percentage of AFG1 in the feed was 12.3%, probably too low to significantly change the function of the neutrophils. Ovine neutrophils have fewer granules that produce superoxide anions, which are necessary for the respiratory burst (26,27), and, therefore, have a lower microbicide activity. Thus, this function was not altered by intoxication with aflatoxins. These findings, together with the number and percentage of leukocytes, indicate that the effect of aflatoxins on the cellular fraction in lambs was negligible.

No effect of the aflatoxins on opsonic serum capacity was observed by a chemiluminescence test using the opsonization of zymosan. Opsonins are antibody molecules and C3 fractions of the complement, which join to Fc receptors and to the complement of the phagocyte (25,27). Therefore, no effects of this component could be found in the intoxicated lambs.

Aflatoxicosis increased IgG levels over both periods of the study. It has been reported that aflatoxins decreased IgG concentrations in poultry (18,29); whereas, in porcine livestock, an increase has been described both in IgG and IgM (3), or, no variations were found at all (12), depending of the dose of aflatoxin used. This observation corroborates findings of an increase in γ-globulin concentrations observed in lambs (10), characteristic of aflatoxin intoxication, whose target organ is the liver, and other hepatic disease.

The decrease in cellular immune responses in lambs with aflatoxicosis was demonstrated with the intradermal test using PHA. It has been reported that the main effect of the aflatoxins is on cellular immunity in birds (28), in lactating rats (30) and in pigs and this effect has been observed as a low response to intradermal tests (12). This response is due to the toxic effects of the aflatoxins on the thymus and T lymphocytes. The thymus of intoxicated birds was smaller than in those of the control birds, and was to related to the toxin dose (29). In this present study, no effect was observed during the clearance period, indicating a recovery of the toxic effects on cellular immunity after the withdrawal of the toxic feed.

The results of these experiments, namely a lower feed intake and lower ADG in the intoxicated animals indicate the susceptibility of sheep to intoxication by aflatoxin. The immunotoxic effects of the aflatoxins were variable, and the bactericidal activity and intradermal tests were affected but the leukocyte function was unaffected. The findings of the study imply that the lambs consuming aflatoxins may be susceptible to disease due to suppression of some humoral and cellular immune responses; however, further studies are necessary in order to understand the immunological status of sheep intoxicated with aflatoxin.

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