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To cite this version:
A Fernández, Jj Ramos, T Saez, Mc Sanz, Mt Verde. Changes in the coagulation profile of lambs intoxicated with aflatoxin in their feed. Veterinary Research, BioMed Central, 1995, 26 (3), pp.180-184. <hal-00902323>
Changes in the coagulation profile of lambs intoxicated with aflatoxin in their feed

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(Received 12 July 1994; accepted 7 February 1995)

Summary — Twenty-three male lambs were intoxicated with 2.5 ppm aflatoxins in their feed for a period of 3 weeks. Thirteen lambs were maintained as a control group (0 ppm aflatoxins in their feed). The coagulation profiles were determined from blood samples that were obtained at 0, 7, 14 and 21 d during the intoxication period and at 1, 2, 4 and 8 d of an 8-d clearance period. Aflatoxicosis in the animals was characterized by an increase in prothrombin time ($P < 0.01$) from d 14 of the intoxication period until the end of the experiment. An increase ($P < 0.05$) in fibrinogen concentration was detected beginning on d 21, instead of the expected decrease. This was probably due to the inflammation found in the lungs of the intoxicated animals. No difference in activated partial thromboplastin time was found between intoxicated and control animals. These results suggest that there was a significant change in some coagulation factors of the extrinsic pathway in the intoxicated lambs and that prothrombin time determination could be used as an indicator of aflatoxicosis in lambs.

aflatoxin / lamb / coagulation profile / hemostasis

Résumé — Modification du profil de la coagulation chez des agneaux intoxiqués par des aflatoxines dans la nourriture. Vingt-trois agneaux ont reçu un aliment contenant 2,5 ppm d’aflatoxines pendant 3 sem et un autre groupe constitué de 13 agneaux n’a rien reçu. Pour établir les profils de coagulation, les échantillons de sang ont été obtenus aux jours 0, 7, 14 et 21 après l’intoxication et aux jours 1, 2, 4 et 8 après une période de clairance de 8 j. L’aflatoxicose chez des agneaux a été caractérisée par un accroissement du temps de prothrombine ($P < 0.01$) 14 j après l’intoxication jusqu’à la fin de l’expérience. Une augmentation ($P < 0.05$) de la concentration de fibrinogène a été observée entre le j 21 de l’intoxication et la fin de l’expérience (au lieu d’une diminution attendue), ce qui pourrait être dû au processus inflammatoire présent dans les poumons d’animaux intoxiqués. Aucune différence du temps de céphaline activée entre les animaux intoxiqués et les témoins n’a été trouvée. Ces résultats suggèrent qu’il existe une modification significative des facteurs de coagulation de la voie extrinsèque pendant la période d’aflatoxicose et que la détermination du temps de prothrombine peut être un indicateur de l’aflatoxicose chez les agneaux.

aflatoxine / agneau / profil de coagulation / hémostase

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INTRODUCTION

Aflatoxins (AF) are a highly toxic group of mycotoxins, synthesized by some strains of Aspergillus flavus and A. parasiticus and occur naturally in several animal feeds (Wilson and Payne, 1994). The target organ in aflatoxicosis is the liver, where hepatocellular necrosis and fatty vacuolization of the cytoplasm is observed (Newberne, 1973; Pier, 1992). As a result of these lesions, hepatic metabolism is modified. Variations in serum biochemistry and of most coagulation factors have been described in poultry (Doerr et al, 1976; Fernández et al, 1994a), pigs (Harvey et al, 1989), cattle (Cook et al, 1986) and rabbits, (Clark et al, 1986; Baker and Green, 1987).

Spontaneous haemorrhage and bruises in animals fed aflatoxins together with an alteration of their coagulation profile have been observed (Tung et al, 1971; Clark et al, 1986; Baker and Green, 1987). Abdelsalam et al (1989) found petechiae on the gallbladder and focal haemorrhage on the renal cortex in sheep intoxicated with 200 ppm aflatoxin, an exceptionally high dose.

Coagulation factors have not yet been investigated in sheep intoxicated with aflatoxin and allowed to recuperate after removal of the toxin. Because there is limited data on this topic, our objective was to study the effect on the coagulation profile in lambs intoxicated with 2.5 ppm aflatoxins for a period of 3 weeks followed by an 8 d clearance period. The coagulation factors used for diagnosing the lambs’ aflatoxicosis are discussed.

MATERIALS AND METHODS

Animals

Thirty-six male lambs (Rasa Aragonesa) were obtained from a commercial farm and were housed under drylot conditions in the farms of the Servicio de Experimentación Animal (Zaragoza University). They were placed in pens with grass hay and water available for ad libitum consumption. The health of all animals was closely monitored before the beginning of the study, and was determined to be satisfactory. The lambs were between 40–45 d of age and had been weaned 15 d before the start of the experiment. The animals were given a 7 d period to adapt to the concentrate diet. The lambs were randomly allotted into 2 groups (average body weight (BW) = 15.3 ± 1.6 kg). The intoxicated group, containing 23 lambs, received a diet with 2.5 mg of aflatoxin/kg in their feed for a period of 21 d. The average quantity of aflatoxin intake for the intoxicated lambs was 80 μg/kg body weight and no refusal of the contaminated feed was noted. The control group of 13 lambs received a diet free of aflatoxins (0 mg/kg in the feed). On day 21, 12 intoxicated lambs and 6 of the control group were slaughtered by an iv injection containing a barbiturate overdose. The remaining lambs were fed 8 d without aflatoxins (clearance period) and were slaughtered as indicated above. The animals were searched during autopsy for macroscopic lesions such as bruises or petechiae, which would suggest some kind of coagulation disorder.

Toxins

Aflatoxin was produced via fermentation of rice by A. parasiticus NRRL 2999 as described by Shotwell et al (1966). The mouldy rice was autoclaved, dried and ground to a fine powder. Aflatoxins were isolated on thin-layer chromatographic (TLC) plates as described by Roberts et al (1981) and were analysed spectrophotometrically for aflatoxin content by the Nabney and Nesbitt’s (1965) method. Aflatoxin content was determined to be 0.499 mg/g of rice powder. The percentage aflatoxin of the rice powder was 83.8 AFBI, 3.7 AFB2, 12.4 AFG1 and 0.1% AFG2. It was incorporated into the basal diet to provide the desired level of 2.5 mg AF/kg of diet (2.5 ppm).

Coagulation study

Blood samples for quantification of coagulation constituents were collected from each lamb by jugular venipuncture on d 0, 7, 14, and 21 dur-
ing the intoxication period and on d 1, 2, 4, and 8 of the clearance period when the contaminated diet had been removed. Blood (2.7 ml) was immediately mixed with sodium citrate 0.11 M (0.3 ml) as an anticoagulant in a plastic tube. The citrated plasma was removed after centrifugation (1 200 g, 10 min, 4°C) and stored at −20°C before analysis. Prothrombin time (PT) and fibrinogen concentration were determined by the kit IL Test™ PT-Fibrinogen HS and the activated partial thromboplastin time (APTT) by the kit IL Test™ APTT HS (Instrumentation Laboratory, Milan, Italy). Analyses were performed using an automated coagulation instrument (ACL-2000, Instrumentation Laboratory, Milan, Italy), according to the manufacturer’s recommended procedure. All plasma samples were tested twice.

**Statistics**

The significance of data was analyzed by the Student’s t-test. A value of \( P < 0.05 \) was considered significant.

**RESULTS**

At the autopsy of the lambs, no haemorrhages or petechiae resulted from intoxication by aflatoxin. The only lesion observed was pneumonia in the most intoxicated animals (87%) with edematous and congested lungs, which affected the cranial lobules. In the control group, only 30.7% of the lambs had pneumonia.

Table I shows the PT, APTT and fibrinogen levels found during this study. The PT values increased over time in the intoxicated group. These values were elevated from d 14 of the intoxication and until the end of the experiment (\( P < 0.01 \)). The PT value was, at d 4 of the clearance period 32% higher than in the control group. The presence of aflatoxin did not significantly change the APTT value in the group of intoxicated lambs compared with the value for the control group. Although the APTT value was higher in the treated animals, it was not sufficient to give a statistically significant difference (\( P > 0.05 \)). A delayed response was noted in fibrinogen levels of the intoxicated lambs. It became statistically significant at d 21 (\( P < 0.05 \)), being higher than in the control group. This observation was related with pneumonia noted at the autopsy. Fibrinogen levels in animals with pneumonia were 730 ± 186 mg/dl, whereas animals without pneumonia had levels of 479 ± 131 mg/dl (\( P < 0.001 \)).

**Table I.** Effects of aflatoxin on the coagulation profile in lambs intoxicated with 2.5 ppm aflatoxin in their feed, during the intoxication and clearance periods.

<table>
<thead>
<tr>
<th>Day</th>
<th>PT (s) (^*)</th>
<th>APTT (s) (^*)</th>
<th>Fibrinogen (mg/dl) (^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppm</td>
<td>2.5 ppm</td>
<td>0 ppm</td>
</tr>
<tr>
<td><strong>Intoxication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>19.5 ± 1.4</td>
<td>20.5 ± 1.5</td>
<td>32.4 ± 3</td>
</tr>
<tr>
<td>7</td>
<td>19.1 ± 1.2</td>
<td>20.2 ± 2.1</td>
<td>30.1 ± 2.6</td>
</tr>
<tr>
<td>14</td>
<td>19.2 ± 1.2</td>
<td>22.2 ± 2.5**</td>
<td>32.4 ± 3.6</td>
</tr>
<tr>
<td>21</td>
<td>19.4 ± 1</td>
<td>24.6 ± 2.3**</td>
<td>34.3 ± 4.4</td>
</tr>
<tr>
<td><strong>Clearance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20.3 ± 1.1</td>
<td>23.4 ± 3.7*</td>
<td>34.9 ± 5.6</td>
</tr>
<tr>
<td>2</td>
<td>20.5 ± 1.1</td>
<td>25.1 ± 3.3**</td>
<td>33.1 ± 6.5</td>
</tr>
<tr>
<td>4</td>
<td>19.9 ± 0.7</td>
<td>26.2 ± 4.1**</td>
<td>33.8 ± 4.1</td>
</tr>
<tr>
<td>8</td>
<td>20.4 ± 1.4</td>
<td>24.6 ± 3.8**</td>
<td>32.2 ± 5.4</td>
</tr>
</tbody>
</table>

PT = prothrombin time; APTT = activated partial thromboplastin time. \(^*\) Values are means ± standard deviation. \( * P < 0.05, ** P < 0.01 \).
DISCUSSION

Spontaneous haemorrhages, petechiae and bruises have been observed in the aflatoxicosis of domestic animals such as pigs (Loosmore and Hardling, 1961) and chickens (Tung et al., 1971). In our study, we did not observe haemorrhages or petechiae in the lambs’ autopsies that would suggest disorders of the hemostatic system. Normal values of the coagulation factors and their physiological significance in sheep are not well known. Sheep have a tendency towards hypercoagulability due to decreased fibrinolytic activity and increased platelet number and adhesiveness (Gajewski and Povar, 1971). This may explain the lack of subcutaneous haemorrhages in the intoxicated lambs.

PT measurements are used to assess the extrinsic pathway of coagulation and the efficacy of factors I, II, V, VII and X (Dodds, 1989; Meyers and Wardrop, 1991). An increased PT signifies a deficiency in some of these factors (Coles, 1986; Dodds, 1989; Meyers and Wardrop, 1991). Although the PT values were higher in the intoxicated lambs, a difference of 30–50% compared with the control values is required before the level could be considered abnormal (Coles, 1986). This difference (32%) was only observed at 4th day of the clearance period. Most coagulation factors are synthesized in the liver (Dodds, 1989), which is the target organ for aflatoxins. A decrease in the synthesis of the coagulation factors would cause an increased PT as observed in this study. This is mainly due to the VII factor, which is observed early in hepatic disease (Dodds, 1989). When the toxin was removed from the diet, an increase in PT values was observed which might suggest that the liver had not been entirely cleared of toxin. Aflatoxins or its metabolites can be isolated from the liver 2–4 d post-intoxication (Fernández et al., 1994b).

APTT is commonly used in the screening tests of the intrinsic pathway and its value is increased by deficiencies in some of the factors VIII, IX, X, XI or XII (Meyers and Wardrop, 1991). In this study there were no changes in APTT levels which agrees with Harvey et al. (1991), who studied intoxicated lambs for a period of 6 weeks. Baker and Green (1987) found increased APTT in rabbits intoxicated with AFB1, but they had given them an acute dose and it is well known that rabbits are one of the most sensitive species to aflatoxins (Newberne, 1973; Pier, 1992). A 30% decrease in the synthesis of hepatic coagulation factors is necessary before the APTT values are increased (Meyer and Wardrop, 1991). The intoxicated lambs did not show an increased APTT. Perhaps there was not any significant deficiency in the factors of the intrinsic pathway synthesized in their livers.

In general, cases of hypofibrinogenemia have been found in the aflatoxicosis of animals such as broiler chickens (Doerr et al., 1976), turkeys (Witlock and Wyatt, 1981) and rabbits (Clark et al., 1986; Baker and Green, 1987), and were associated with hepatic dysfunction. However, in this study, aflatoxicosis in lambs was characterized by an increase in fibrinogen concentration. Increased fibrinogen concentration is associated with inflammatory and suppurative diseases (Coles, 1986; Dodds, 1989). The increased fibrinogen levels observed in this study in the intoxicated lambs would be due to the animals that had pneumonia, this factor being less important in the control group. Although the liver is the primary site for synthesis of fibrinogen, the reticulo-endothelial system can also synthesize fibrinogen in inflammatory diseases (Dodds, 1989). Increased fibrinogen levels are thus a more sensitive indicator of inflammation than increases in the number of leukocytes or neutrophiles in the blood (Coles, 1986).

These data suggest that aflatoxins damage hemostasis in lambs, mainly the fac-
tors affecting the extrinsic pathway. Sheep are considered to be the most resistant species to the deleterious effects of aflatoxins (Lewis et al., 1967; Miller and Wilson, 1994). Thus, prothrombin time could be used as an indicator of aflatoxicosis in lambs. It would help both in the diagnosis of the intoxication used by other authors (Doerr et al., 1976; Witlock and Wyatt, 1981; Baker and Green, 1987) and also in recuperation after intoxication (Bortell et al., 1983). More investigation is necessary to clarify which coagulation factors are affected in lambs’ aflatoxicosis.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to M Gutierrez of the Hospital Clinico de Zaragoza for his technical assistance. This project was financed by a Zaragoza University grant (No 218-75).

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