Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows

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The objective of the experiment was to monitor plasma levels of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2) and M1 (AFM1) in lactating dairy cows fed a single oral bolus with aflatoxin naturally contaminated corn meal (Trial 1). The possible aflatoxins (AFs) absorption through mucous membranes was also investigated using the vaginal mucosa (Trial 2). In trial 1, seven lactating Holstein dairy cows were given a single oral bolus of a naturally contaminated corn meal assuring an intake of 4.89 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2. Blood samples were collected at 0 and 5, 10, 15, 20, 25, 30 minutes after treatment. In trial 2 an aflatoxin dosage similar to that of trial 1 was provided through vaginal implant to eight lactating Holstein dairy cows. Blood samples were collected at 0 and 15, 30, 60, 180, 360 minutes after treatment. Individual milk samples of six milkings, one before and five after treatment, were also collected. Plasma and milk samples were analysed by HPLC for AFB1, AFB2, AFG1, AFG2 and AFM1 contents. In trial 1 AFB1 in plasma peaked (33.6 ng/L) as soon as 20 minutes after treatment. The plasma AFM1 was already detectable at 5 minutes (10.4 ng/L) and peaked at 25 minutes (136.3 ng/L). In trial 2 only AFB1 and AFM1 were detectable in plasma, starting from the first sampling time (15 minutes), with values of 10.7 and 0.5 ng/L, respectively. The AFB1 peaked at 30 minutes (23.9 ng/L). The AFB1 excreted in milk as AFM1 had the highest concentration (203.0 ng/L) in the first milking after treatment and decreased close to the starting values after 36 hours from treatment. The prompt appearance of studied aflatoxins, and their metabolites, in plasma suggests absorption might also take place in mouth or oesophageal mucous membranes, before the rumen compartment. Results support the hypothesis that the cytochrome P450 oxidative system, which is present in these tissues and in leukocytes, could be involved in the conversion of the AFB1 in AFM1. The absorption of AFB1 through the vaginal mucosa confirms the passive diffusion as a probable mechanism for AFB1 absorption.

Key words: Aflatoxin B1, Aflatoxin M1, Cows, Absorption, Milk.

Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows

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RIASSUNTO

ASSORBIMENTO DELLE AFLATOSSINE NEL TRATTO GASTRO INTESTINALE E NELLA MUCOSA VAGINALE DI VACCHE IN LATTAZIONE

L’obiettivo dell’esperimento è stato di monitorare il livello plasmatico dell’aflatossina B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2) e M1 (AFM1) in vacche in lattazione trattate con un singolo bolo orale a base di mais naturalmente contaminato da aflatoxine. Anche il possibile assorbimento delle aflatoxine
The liver is the main site of AFB1 bio-
transformation with the mitochondrial
Cytochrome P450 oxidative system (CYP)
converting the AFB1 into AFM1 and other
metabolites (Sudakin, 2003). The AFM1
preserves the same acute toxicity of AFB1
in rats (Pong and Wogan, 1971), with lower
carcinogenic potential (Wogan and
Paglialunga, 1974). Thus, the metabolic
pathway leading to AFM1 formation can be
considered a detoxification process
(Yannikouris and Jouany, 2002).

The IARC (2002) classified AFB1, AFB2,
AFG1, AFG2 and AFM1 as carcinogenic to
humans (Group 1). The European Union
allowed maximum limits for AFB1 concen-
tration in feed materials, complete feed-
ingstuffs and AFM1 in milk are set at 20
µg/kg, 5 µg/kg (2003/100/EC, 2003) and 0.05
µg/kg (2006/1881/EC, 2006), respectively.

The animal exposure to AFB1 occurs
mainly with the ingestion of contaminated
feeds (Sudakin, 2003), however, skin
(Rastogi et al., 2006) or inhalation (Jakab et
al., 1994) exposures might also contribute.
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When absorbed, the presence of AFs in blood is prompt (Trucksses et al., 1983; Coulombe and Sharma, 1985) and can reach organs and peripheral tissues likely through a passive mechanism (Yannikouris and Jouany, 2002). The parent toxins (AFB1, AFB2, AFG1, AFG2) or the AFM1 have been detected in plasma of cows as soon as 15 minutes from oral administration of AFs naturally contaminated corn meal (Moschini et al., 2007).

The objective of this work was to monitor the early plasma levels of AFs in lactating dairy cows following a single oral bolus of AFs contaminated corn meal. The possible AFs absorption through mucous membranes was also investigated using the vaginal mucosa.

Material and methods

Animals and samplings

Two experiments were carried out on lactating Italian Holstein Friesian dairy cows. Cows were housed at the CERZOO research and experimental centre (San Bonico, Piacenza, Italy). The research protocol and animal care were in accordance with the European Community council directive guidelines for animals used for experimental and other scientific purpose (86/609/EEC, 1986).

Cows were housed in a free stall barn and had free access to water. The diet was formulated according to the nutrient requirements of dairy cattle (NRC, 2001) for an average cow weight of 600 kg, 140 days in milk (DIM) and a 35 kg daily milk yield (3.80% fat and 3.35% protein). The bulk of the diet on a dry matter basis was: corn silage (31.2%), dehydrated alfalfa hay (16.7%), grass hay (4.1%) and energy-protein supplement (48%). The diet was fed once a day (0900h) and ad libitum (5% expected orts) as a total mixed ration (TMR).

Cows were milked twice a day (0230h and 1330h) and the individual milk yield was recorded at every milking (Afimilk system, Afimilk, Israel).

Trial 1: aflatoxin as oral drench

Seven cows averaging 185±43 DIM and 28.75±4.37 kg daily milk yield (mean±sd) were given, by oral drench and before the morning meal, a bolus of AFs naturally contaminated corn meal for a total intake of 4.89 mg, 1.01 mg, 10.63 mg and 0.89 mg, respectively, for AFB1, AFB2, AFG1 and AFG2. Blood samples were collected via jugular venipuncture at 0 and 5, 15, 20, 25, 30 minutes from all treated animals. The blood was collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer tubes (Vacutainer systems, Belliver Industrial Estate, Plymouth, UK). Then, plasma was obtained by centrifugation (3000 g for 15 minutes) and stored at -20°C until AFs analysis by High Performance Liquid Chromatography (HPLC).

Trial 2: AFs administration in vagina

Eight cows averaging 192±48 DIM and 27.78±4.13 kg daily milk yield (mean±sd) were used in the second experiment. AFs were extracted from 50 g of naturally contaminated corn meal with a methanol:water solution (80:20 v/v). Then, the methanol:water extract was dried under nitrogen, redissolved into 20 mL of water and adsorbed to a cotton wad. The cotton wad containing 4.89 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2 was implanted directly into the vagina of cows before the morning meal and removed after 24 hours.

TMR samples were collected the day before treatment for AFs content determination. Individual milk samples were collected from four randomly selected cows at each milking and for six consecutive milk-
ings starting the day before treatment. Then, samples were frozen at -4°C before HPLC analysis.

Blood samples were taken by jugular venipuncture at 0 and 15, 30, 60, 180 and 360 minutes after treatment and collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer tubes (Vacutainer systems, Belliver Industrial Estate, Plymouth, UK). Then, plasma was obtained and stored as in trial 1.

Sample analysis
Extraction of AFs from feeds was done by immunoaffinity technique according to Arranz et al. (2006). Briefly, 10 grams of dried feed were put in a methanol:water solution (80:20 v/v), shaken at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher and Schuell 595 ½ filter paper (Dassel, Germany). Then, five mL were eluted with 45 mL of bidistilled water through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

Extraction of AFs from milk was done by immunoaffinity technique according to Mortimer et al. (1987). Briefly, 50 mL of defatted milk (centrifuged at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher and Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

Chromatography
The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFs in feed and in plasma extracts were separated with a reverse-phase C18 Superspher column (4 µm particle size, 125x4mm i.d.; Merck, Darmstadt, Germany) at room temperature and isocratic conditions, with a mobile phase of water and acetonitrile: methanol solution (17:29 v/v) with a 64:36 (v/v) ratio. The flow rate
was 1 mL/minute. Then, AFs were detected by fluorescence, after postcolumn derivatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide perbromide (PBPB) at flow 0.1 mL/minute. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths. Limits of detection for AFs in feeds and plasma extracts were 0.1 ng/kg and 2 ng/L, respectively. The standard stock solution was checked for AFs concentrations according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

Results and discussion

Trial 1: aflatoxin as oral drench

This work follows a previous study on dairy cows (Moschini et al., 2007) in which the AFB1 and AFM1 were detectable in plasma as soon as 15 minutes after the ingestion of an AFs contaminated corn meal bolus.

In the current trial the TMR caused an AFB1, AFB2, AFG1, and AFG2 ingestion of 1.7±0.4, 0.3±0.1, 0.5±0.2 and 0.1±0.1 µg/cow respectively, resulting in non detectable levels of AFs in plasma (Table 1).

The AFB1 (P<0.05) was found in plasma as soon as five minutes after AFs ingestion (Table 1). The result suggests a rapid absorption of AFB1 through the gastrointestinal tract of cows and a quick oxidation of the toxin to AFM1, with a significant increase in plasma at 10 minutes compared to plasma samples collected before treatment (P<0.01). As previously reported (Moschini et al., 2007), an early AFB1 absorption before the rumen wall could be involved before the intestinal contribution for AFB1 absorption, around 120 to 180 minutes after AFB1 ingestion.

For the studied sampling schedule the AFB1 plasma level was maximum (33.6 ng/L) at 20 minutes after exposure to AFB1, whereas the AFM1 peaked at 25 minutes (136.3 ng/L). The AFB1 results agreed with our previous data in which the AFB1 plasma level peaked after 15 minutes from treatment (Moschini et al., 2007).

The AFM1 plasma levels reported by Moschini et al. (2007) were 45.3 ng/L at 15 minutes and 35.3 ng/L at 30 minutes from treatment. As in Table 1, the AFM1 plasma concentrations in the present work were 68.0 ng/L at 15 minutes and 135.3 ng/L at 30 minutes. Considering the standard deviations of data reported in these experiments, the AFM1 values at 15 minutes...
could be considered similar, while a difference could be addressed for the 30 minutes samples, with values four times higher in the present work compared to previous data (Moschini et al., 2007) obtained with the same aflatoxin dosage being given to animals (4.89 mg/cows of AFB1).

Differences in plasma AFs levels could result from a high individual variability among animals, probably related either to differences in plasma volumes or to a different passage of the toxin through gastrointestinal mucosae (Van Egmond, 1989; Veldman et al., 1992; Masoero et al., 2007).

The AFG1 was also detectable in plasma five minutes after the oral drench, with the maximum level at 30 minutes. Even in presence of higher AFG1 intake compared to AFB1 (10.63 vs. 4.89 mg/cow), the AFG1 plasma concentration was lower than AFB1. The plasma level might be related to differences on absorption dynamics of parent molecules. Very low concentrations, close to the detectable limit, were observed for the AFG2 (Table 1).

The AFM1/AFB1 plasma ratio moved from 0.99 at five minutes to 3.05 at 15 minutes and to 4.89 at 30 minutes from drenching. The pattern indicates an AFB1 conversion rate to AFM1 which overcomes the body capacity of getting rid of the AFM1, either through faeces, urine and milk (Yiannikouris and Jouany, 2002), and could lead to an accumulation of the metabolite within the blood pool.

The observed prompt absorption of considered AFs and their appearance in blood, also as metabolites, are in agreement with previous works (Polan et al., 1974; Trucksess et al., 1987; Moschini et al., 2007). Authors suggested a rumen contribution to AFs absorption before the intestinal tract.

Other ways of AFs passage to the blood compartment could be addressed too. In particular Coulombe and Sharma (1985), working with rats exposed to a single intratracheal or oral $[^3H]$AFB1 doses, measured a peak AFB1 plasma concentration after one and three hours from treatment. Authors suggested the absorption of non polar and lipid-soluble compounds, like AFs, is a rapid process either through the pulmonary tissues or in the gastro-intestinal tract.

Current results on plasma AFs and the presence of their metabolites at five minutes after treatment suggest a rapid absorption even before the rumen compartment, through the mouth or oesophageal mucous membranes.

Once absorbed, the AFB1 is converted to AFM1 and other metabolites through processes mainly microsomal CYP mediated (Coulombe, 1993; Yiannikouris and Jouany, 2002; Sudakin, 2003), and in particular in CYP3A4 and CYP2A6 forms (Gallhager et al., 1996; Pelkonen et al., 2000). These oxidative systems can be found in different tissues beyond the liver, such as the small intestine, pancreas, brain, lung, adrenal gland, kidney, bone marrow, mast cells, skin, ovary, testis and leukocytes (Krishna and Klotz, 1994; Chang and Kam, 1999; Lind et al., 2003). Thus, the oxidation of the AFB1 seems related to either hepatic or extra-hepatic CYP (Coulombe, 1993). A high oxidation activity of AFB1 within olfactory and respiratory tissues of cattle, sheep, swine and rat (Coulombe and Sharma, 1985; Tjälve et al., 1992; Larsson et al., 1994; Larsson and Tjälve, 1996) has been reported. Thus, extra-hepatic conversion of the adsorbed AFB1 might justify the prompt appearance of AFM1 in plasma (Table 1).

Trial 2: aflatoxin administration in vagina

The TMR caused an AFB1, AFB2, AFG1 and AFG2 ingestion of $2.3\pm0.5$, $0.4\pm0.1$, $0.2\pm0.2$, $0.05\pm0.01$, respectively.
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0.9±0.3 and 0.2±0.1 µg/cow, respectively, resulting in non detectable levels of AFs in plasma and in a bulk milk AFM1 contamination of 4.3±2.0 ng/L of milk from exposed dairy cows.

The AFB1 and AFM1 plasma concentrations following the aflatoxin vaginal implant are reported in Table 2. The AFB2, AFG1 and AFG2 were not detectable in collected plasma samples.

Both AFB1 and AFM1 were detected in plasma as soon as 15 minutes after treatment. For the considered sampling schedule, the maximum level for AFB1 (23.9 ng/L) was measured at 30 minutes from treatment, whereas no trend seemed detectable for AFM1, with similar concentrations in samples collected between 30 and 360 minutes from treatment (Table 2).

The low molecular weight (312.27 formula weight) and the lipophilic characteristic of AFB1 could allow a mucosal passive diffusion through tissues as previously reported in different species (Kumagai, 1989; Coulombe, 1993; Hiesh and Wong, 1994; Yiannikouris and Jouany, 2002). Indeed, there is a report in male rats of a faster pulmonary tissues AFB1 absorption compared to the gastro-intestinal tract toxin absorption (Coulombe and Sharma, 1985). This observation confirmed that the absorption and biotransformation rate of AFB1 could be affected by the administration route (Hsieh and Wong, 1994).

The prompt and continuous passage of the toxin through the vaginal mucosa observed in our trial might corroborate a passive diffusion process as a mechanism regulating the absorption of AFB1. This is in agreement with Kumagai (1989) reporting a rate of AFB1 uptake from gut to mesenteric venous blood nearly proportional to the AFB1 concentration observed after injection of [H3]AFB1 in the stomach and in other sites of the small intestine.

The highest AFM1 concentration (203.0 ng/L) was measured in milk at eight hours after the treatment (Figure 1). Then, the

Figure 1. Aflatoxin M1 (AFM1) concentration (means±SD) in milk of lactating dairy cows (n=4) at different hours from cotton wad vaginal implant with aflatoxins.
Table 1. Plasma aflatoxins concentration (means±SD; ng/L) before and after the administration of an aflatoxin contaminated oral bolus\(^1\) to lactating dairy cows (n=7).

| Minutes from the oral bolus | Aflatoxins | 0        | 5        | 10       | 15       | 20       | 25       | 30       |
|----------------------------|------------|----------|----------|----------|----------|----------|----------|----------|
| Aflatoxin B1 (AFB1)        | nd         | 10.5±9.4 | 21.1±20.2| 22.3***±16.5| 33.6***±22.8| 25.7***±11.3| 27.7***±14.7|
| Aflatoxin B2 (AFB2)        | nd         | 2.3±1.6  | 3.2±3.1  | 4.5±4.4  | 3.7±4.2  | 5.9±7.3  | 7.5±8.9  |
| Aflatoxin G1 (AFG1)        | nd         | 4.6±5.1  | 12.0±14.1| 16.3±22.7| 15.7±20.4| 24.3±36.3| 29.0±46.0|
| Aflatoxin G2 (AFG2)        | nd         | 0.5±0.7  | 0.7±0.8  | 0.3±0.6  | 0.7±0.8  | 0.9±1.0  | 0.6±0.9  |
| Aflatoxin M1 (AFM1)        | nd         | 10.4±20.5| 55.7***±49.8| 68.0***±53.9| 109.1***±54.0| 136.3***±76.6| 135.3***±81.7|

\(^1\)Aflatoxins presence in the oral bolus: 4.9 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2.
nd = not detectable (2 ng/L).
Differences with initial value (0 minute) being greater than zero (paired t-test). * P<0.05; ** P<0.01 *** P<0.001.

Table 2. Plasma aflatoxins concentration (means±SD; ng/L) before and after the aflatoxin contaminated cotton wad vagina implant\(^1\) to lactating dairy cows (n=8).

| Minutes from vaginal implant | Aflatoxins | 0        | 15       | 30       | 60       | 180      | 360      |
|------------------------------|------------|----------|----------|----------|----------|----------|----------|
| Aflatoxin B1 (AFB1)         | nd         | 10.7***±8.4 | 23.9±27.2| 11.8***±10.2| 7.8±7.0  | 4.4±5.1  |
| Aflatoxin M1 (AFM1)         | nd         | 0.5±0.6  | 4.9±6.4  | 4.4±5.2  | 2.8±3.5  | 5.2±6.2  |

\(^1\)Aflatoxins presence in the vaginal implant: 4.9 mg AFB1, 1.01 mg AFB2, 10.63 mg AFG1 and 0.89 mg AFG2.
nd = not detectable (2 ng/L).
Differences with initial value (0 minute) being greater than zero (paired t-test). * P<0.05; ** P<0.01 *** P<0.001.
pattern of the AFM1 concentration in milk was downward through consecutive milk samples (80% lower in the second sample) up to a similar pre-treatment value at 32 hours from treatment. The AFM1 excretion pattern in milk was comparable to previous reported data as consequence of a single AFB1 ingested dose (Battacone et al., 2003) with maximum concentration in the first milking (eight hours) after treatment. No AFM1 due to AFB1 administration was detected after 56 hours (fifth milking) from treatment.

A very low concentration (4.3 ng/L) of the AFB1 in milk was found only at the first milking, eight hours after treatment. The excretion of AFB1 in milk from buffalo fed an aflatoxin contaminated diet was previously reported by Pietri et al. (2003), whereas in dairy cows the parent molecule was found in milk only following a very high (300 mg/cow) AFB1 ingestion (Truckess et al., 1983). These results are in agreement with our data obtained in a preliminary experiment carried out on three dairy cows (unpublished data) showing the excretion of 7.8, 27.1 and 107.2 µg/cow of AFB1 in milk after ingestion of 5, 15 or 50 mg/cow of AFB1, respectively.

Conclusions

AFs were quickly absorbed through the gastro-intestinal tract of cows. An early absorption might also take place in mouth or oesophageal mucous membranes, before the rumen compartment. Results support the hypothesis that the CYP oxidative system, which is present in these tissues and in leukocytes, could be involved in the conversion of the AFB1 in AFM1.

The absorption of AFB1 through the vaginal mucosa confirms a passive diffusion as a probable mechanism for AFB1 absorption.

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