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Assessment of genotoxicity and cytotoxicity of AFB_1 by peripheral blood mononuclear cells in goats.

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ABSTRACT

Aflatoxins are known as natural substances highly toxic and carcinogenic for humans and animals. Nevertheless, limited knowledge is available on effects of this fungi's metabolites on immunology of small ruminants. For this reason, a study was carried out to ascertain the effects of Aflatoxin B_1 (AFB_1) on peripheral blood mononuclear cells (PBMC) functions in goats. Eight non-lactating and non-pregnant Saanen goats were exposed to different levels of AFB_1 (0, 25, 50 and 100 $\mu g AFB_1\ kg^{-1}$ body weight) given orally in a single dose. Individual blood samples were collected at 0, 24, 72, 120 and 192 h after AFB_1 administration. PBMC were isolated and submitted to a multi-level assay scheme to evaluate the effects of AFB_1 on cell proliferation (DNA synthesis quantification), DNA damage (Comet Assay genotoxicity test) and cell viability (MTT test). DNA synthesis and cells viability were severely depressed in a dose and time dependent manner ($P<0.01$). For all AFB_1 levels tested, tail moment showed higher levels ($P<0.01$) after 24 h from AFB_1 administration. Only for the highest dose of AFB_1, tail moment was higher ($P<0.01$) also 72 h after the administration. These findings confirm the high cytoxicity and genotoxicity of AFB_1, and suggest that exposure to AFB_1 may compromise the immune response of goats.

INTRODUCTION

Aflatoxins (AFs) are natural substances produced by fungi of the genus *Aspergillus* growing on a variety of feed and foodstuffs. Like many secondary metabolites of filamentous fungi, AFs are capable to cause disease and death in humans and other animals (Bennet & Klich, 2001). From early 1960s to date, a great interest is playing around this family of mycotoxins especially due to the ascertained carcinogenicity for human of Aflatoxin B_1 (AFB_1) (Palmgren & Hayes, 1987; IARC, 1993) and for the heavy impact on feed and food crops availability and economic losses imputable to the AFs contaminations (Bhat, 1988; Coulibaly 1989). Moreover, lactating animals tend to excrete a fraction of the ingested AFB_1 through the milk as Aflatoxin M_1 (AFM_1) (Allcroft *et al.*, 1966; Rodricks & Stoloff, 1977), which is considered a potential carcinogenic compound (IARC, 1993). To date, several studies have been conducted on AFs toxicity, mostly concerning laboratory animals (Newberne *et al.*, 1969; Eaton & Groopman, 1994; Cullen & Newberne, 1994) or livestock species (Miller & Wilson, 1994). Among a plethora of effects and signs of alflatoxicoses, in many cases was emphasized the decreasing of humoral and cellular immunity in turkey, cattle, guinea pigs, rabbit, trout, swine and some laboratory animals (Pier & Heddleston, 1970, Pier, 1973a; Pier, 1973b; Pier

et al., 1977, Miller *et al.*, 1978, Cysewski *et al.*, 1978, Pugh *et al.*, 1984; Bondy & Pestka, 2000). Several reports associated the increased incidence of infectious diseases with AFs contamination of diets (Pier, 1973; Richard, 1978). On the basis of published data, ruminants appear to be more resistant than other livestock species in respect to the AFs exposure effects (Osuma *et al.*, 1977; Miller *et al.*, 1978; Bryden *et al.*, 1980; McKenzie *et al.*, 1981, Hatch *et al.*, 1982; Colvin *et al.*, 1984; Clark *et al.*, 1984; Hoerr *et al.*, 1986; Harvey *et al.*, 1988, Brucato *et al.*, 1989; Abdelsalam *et al.*, 1989). However, some reproductive traits, growing patterns, productive levels and products quality (especially milk) may be affected by the ingestion of AFs contaminated feedstuffs (Colvin *et al.*, 1984, Hussein & Brasel, 2001). Up to now, limited knowledge is available on effects of AFs on immunology of small ruminants in general and goat in particular. This study was performed to assess whether PBMC isolated from goats differ in response to acute exposure with different AFB_1 doses at 24, 72, 120 and 196 h after treatment. Peripheral blood mononuclear cells (PBMC) have been widely used to assess the immunotoxicity of several chemical compounds and stress conditions (Atkinson & Miller, 1984; Dijkmans *et al.*, 1988; Viora *et al.*, 1996; Smits *et al.*, 1996; Lacetera *et al.*, 2006) and therefore in the present study PBMC have been selected as cellular target. DNA synthesis as indicator of lymphocytes proliferation (Tizard, 1992, Profit & Unteregger, 2001), DNA damage (Sasaky *et al.*, 2000) and mitochondrial dehydrogenase activity as a cyto-functional end-point (Mosmann, 1983; Lindl & Bauer, 1994) have been considered to evaluate PBMC function.

MATERIAL AND METHODS

Experimental design

Eight 18 months old non-lactating and non-pregnant Saanen goats, were used and housed in a free barn. The animals (56.1±0.9 kg BW) were fed on mixed hay *ad libitum* supplemented with 200 g/day of commercial concentrate before and during the entire trial. Concentrate and hay were checked for AFB_1 natural contamination. Experimental treatments consisted in the administration of different amount of AFB_1. Twenty milligrams of AFB_1 (Sigma, UK) were dissolved in DMSO (dimethyl sulfoxide) (Sigma, Germany) to obtain a contamination solution at 10 mgAFB_1/ml. Fifty grams aliquots of AFB_1-free concentrate, were contaminated with the AFB_1-DMSO solution to the final dosage showed in Table 1. Such doses were selected on the basis of our previous trials with goats (Ronchi *et al.*, 2005) and level tested by others (Hatch *et al.*, 1982). Experimental groups were formed selecting two animals per group, on the basis of the BW. Contaminated feeds were administrated with ration at the start of the trial.

Table 1 – Experimental design and multi-test time frame.

		Time (h)				
Treatment	Dose (µgAFB_1/kg BW)	0	24	72	120	192
Control	0	DS, MTT, CA	DS, MTT, CA	DS, MTT, CA	CA	DS, MTT
High	100	DS, MTT, CA	DS, MTT, CA	DS, MTT, CA	CA	DS, MTT
Medium	50	DS, MTT, CA	DS, MTT, CA	DS, MTT, CA	CA	DS, MTT
Low	25	DS, MTT, CA	DS, MTT, CA	DS, MTT, CA	CA	DS, MTT

DS= DNA synthesis, MTT= mitochondrial dehydrogenase activity test , CA= Comet Assay (single cell electrophoresys)

Blood samples were collected via jugular venipuncture using evacuated glass tubes coated with sodium heparin. Samples collection was performed prior to starting the experiment (time t_0) and then at 24, 72, 120 and 192 hours after treatment (time t_1, t_2, t_3 and t_4, respectively). DNA synthesis (DNA synthesis test), DNA damage (Comet Assay) and cell viability assay (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] test) were tested in peripheral blood mononuclear cells (PBMC) stimulated with PHA (phytohemagglutinin).

Samples pre-treatment

The PBMC were isolated by density gradient centrifugation following the method reported by Lacetera *et al.* (2001). Blood diluted in RPMI-1640 medium containing 25 mM HEPES (Sigma, Milano, Italy) was layered over Ficoll-Paque PLUS (APB, Milano, Italy) and centrifuged (600 × *g* for 45 min at 20°C). The mononuclear cell band was recovered and washed twice in PBS using centrifugation (400 × *g* for 10 min at 4°C). Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. The PBMC recovery and viability were determined by hemocytometer count using the trypan blue exclusion method. The PBMC were re-suspended at 1×10^6 cells/ml in RPMI 1640 medium containing 25 mM HEPES supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B/ml (Sigma, Italy).

DNA synthesis assay

The DNA synthesis was evaluated as previously described by Lacetera *et al.* (2001). After isolation the PBMC (100 µl) were added to 96-well tissue culture plates at final concentration of 2×10^5 cells/well. The PBMC were stimulated with phytohemagglutinin (PHA 1.5 µg/ml). Control wells contained 100 µl of PBMC suspension (2×10^5 cells/ml) without PHA (unstimulated). According to the guidelines provided by the manufacturer (APB, Milan), additional control wells contained 100 µl of complete RPMI 1640, or 100 µl of PBMC suspension without 5-bromo-2′-deoxyuridine (BrdU, see below). The tissue culture plates were incubated for 48 h at 39 °C in an atmosphere of 95% air and 5% CO_2. Thereafter, 100 µM of the pyrimidine analogue BrdU (in 10 µl of RPMI-1640) were added to each well to give a final concentration of 10 µM BrdU. Following a further 18-h incubation, the culture medium was removed by centrifuging the tissue culture plates at 300 × *g* for 10 min. Thereafter, plates were dried at 60 °C for 1 h. Cell proliferation was measured by ELISA using a commercial kit for the measurement of BrdU incorporation during DNA synthesis in proliferating cells. Tests were performed according to the guidelines provided by the manufacturer (APB, Milan). The incubation time with peroxidase-labelled monoclonal anti-BrdU antibody was 90 min. Values for DNA synthesis were expressed as the optical density recorded at 450 nm wavelength, both in unstimulated and stimulated wells, minus the optical density recorded in control wells without BrdU. Data mean values were expressed as percent control response. Data were expressed as percent of control.

Cell viability assay (MTT Test)

After isolation the PBMC (100 µl) were added to 96-well tissue culture plates at final concentration of 2 x 10^5 cells/well. The PBMC were stimulated with phytohemagglutinin (PHA 1.5 µg/ml). Control wells contained 100 µl of PBMC suspension (2×10^5 cells/ml) without PHA (unstimulated). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium

bromide – Sigma Chemicals, St. Louis, USA] was dissolved in PBS (phosphate buffer saline) at 5 mg/ml and filter-sterilized to remove any insoluble residue present. According to Mosmann (1983), MTT stock solution was added to each well at final concentration of 0.5 mg/ml and culture plates were incubated for 8 h at 39°C in an atmosphere of 95% air and 5% CO_2. The supernatant was removed and 100 µl of 0.04 N HCl in isopropanol was added to the wells and mixed thoroughly to completely dissolve the crystalline material. The plates were read on a microtiter plate (ELISA) reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Data were expressed as percent of control.

Single Cell Electrophoresis (Comet Assay) method

Microscope slides were pretreated, 150 µl of 1% normal agarose in PBS was spread out on microscope slides, covered with a coverslip and kept at 4 °C until their use. Five µl of whole blood was mixed with 75 µl of low melting point agarose (0.75% in PBS). Immediately after mixing all solution was pipetted on to the pre-treated slides, covered with coverslip and kept at 4° C until solidification. The coverslip was removed and the slides were soaked in ice-cold lysis solution (2.5M NaCl, 100mM EDTA, 10 mM Tris-HCl, 1% v/v Triton X-100, 10% v/v DMSO, pH 10) overnight at 4°C. The slides were then placed in the electrophoresis chamber, covered with cooled electrophoresis buffer (NaOH 300mM, EDTA 1 mM, pH 13.0) and incubated for 10 minutes at room temperature (unwinding). The slides were subsequently electrophoresed in the same buffer for 20 min at 300 mA and 25 V (Singh *et al*., 1988). After electrophoresis the alkaline samples were neutralized in 0.4 M Tris-HCl pH 7.4 buffer. The slides were rinsed in distilled water, air dried, fixed in methanol and stored until analysis. DNA was stained with ethidium bromide (20 µg/ml) and the comets were examined at 400× magnification with an automatic image analyzer (Comet Assay III; Perceptive Instruments, U.K.) connected to a fluorescence microscope (Eclipse E400; Nikon). To evaluate the amount of DNA damage, computer-generated tail moment values was used. A total of 100 cells were scored from two different slides of the same sample. Data were expressed in tail moment arbitrary units.

Statistical analysis

Data for all variables measured were analyzed as repeated measures using the MIXED procedure of SAS (SAS, 1999). The model included fixed effects (AFB1 concentration: 1,..4), time of sampling (1, ..4), random effects (goat) and the error term. For each analyzed variable, goat was subjected to 3 covariance structures: compound symmetric, autoregressive order one, and unstructured covariance. The covariance structure that had the largest Akaike's information criterion and Schwarz's Bayesian criterion was considered the most desirable analysis. Least squares means were separated with the PDIFF procedure of SAS (SAS, 1999). Data are reported as least squares means with standard errors.

RESULTS

DNA synthesis has shown to be treatment dependent being depressed at all AFB_1 doses tested (Figure 1a). Compared to the control, higher dosages (100 and 50 µg/kg BW) gave a similar response indicating a decrement ($P<0.01$) of the cell proliferation greater than 60%. Also the exposure to 25 µg/kg BW dosage inhibited ($P<0.01$) the DNA synthesis of 30%. At the same time, data showed a clear time/effect relationship (Figure 1b) with a higher depression ($P<0.01$) of DNA synthesis 24 h after the AFB_1 administration. DNA synthesis increased till

to the end of trial. Nevertheless, after 192 h, no complete recovery in DNA synthesis capacity of lymphocytes was observed (P<0.01). Only the lowest dose tested at 192 h after the start of the trial showed a DNA synthetic activity comparable to that of the control (Table 2). Similar DNA synthesis depression has been observed both at 24 h after the treatment with 25 $\mu gAFB_1$/kg BW and 192 h after the treatment with high dosages (50 and 100 $\mu gAFB_1$/kg BW) (Table 2).

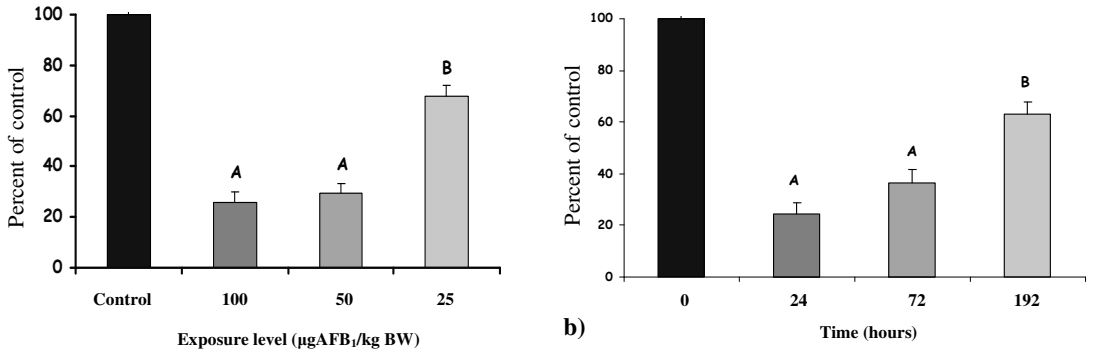

Figure 1 - Effects of AFB_1 exposure level (a) and time after treatment (b) on lymphocytes DNA synthesis (LSmeans ± SE expressed as % of control). Means with different letters mean significant differences; A, B = P<0.01.

Table 2 - DNA synthesis. Time x treatment interaction (LSmeans ± SE expressed as % of control). Means with different letters mean significant differences: A, B, C, D= P<0.01

	Time		
Treatment	24 hours	72 hours	192 hours
Control	100 ± 6.2^{D}	100 ± 6.8^{D}	100 ± 6.2^{D}
100 $\mu gAFB_1$/kg BW	11 ± 6.5^{A}	19 ± 6.8^{A}	47 ± 6.5^{B}
50 $\mu gAFB_1$/kg BW	19 ± 6.2^{A}	24 ± 7.6^{A}	47 ± 6.5^{B}
25 $\mu gAFB_1$/kg BW	43 ± 6.8^{B}	66 ± 7.1^{C}	95 ± 6.5^{D}

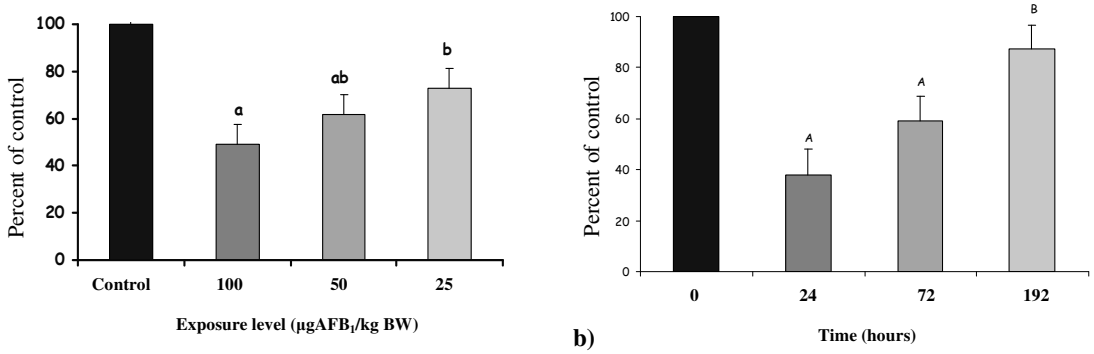

Figure 2 - Effects of (a) AFB_1 exposure level and (b) time after treatment on mitochondrial dehydrogenase activity (LS means ± SE expressed as % of control). Means with different letters mean significant differences: a, b = P<0.05; A, B = P<0.01.

Mitochondrial dehydrogenase activity was depressed (P<0.05) from around 30% by lower dosage to near 50% by 100 $\mu gAFB_1$/kg BW exposure (Figure 2a). Lymphocytes viability was

almost entirely recovered at the end of the trial (192 h), while was heavily depressed (P<0.01) 24 h and 72 h after treatment (Figure 2b) showing a clear time/effect relationship. Time *x* treatment interaction was not significant (P>0.05). Compared with control, after 24 h from AFB_1 administration lymphocytes DNA was highly (P<0.01) damaged at all levels of AFB_1 tested, although no clear dose/response relationship was observed for the whole range of exposure (Figure 3a).

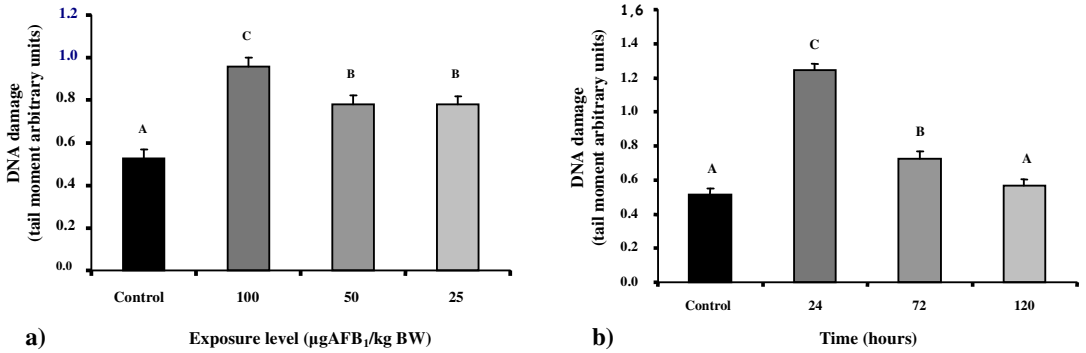

Figure 3 - Effects of AFB_1 exposure level (a) and time after treatment (b) on DNA damage (LSmeans ± SE expressed as % of control). Means with different letters mean significant differences: a, b = P<0.05; A, B = P<0.01.

The comet assay showed a DNA damaged until 72 hours after dosage, while at 120 after the treatment no DNA damage was observed (Figure 3b). This was probably due to the actions of DNA repair enzymes that remove covalent adducts produced by AFB_1 and their metabolites. A significance time *x* treatment interaction has been found (Figure 4). For low and medium AFB_1 levels exposure the tail moment was higher 24 hours after administration, but in goats expose to the highest AFB_1 dose DNA was still damaged at 72 hours (P<0.05). The damage of DNA and the time to repair itself are proportional with the severity of exposure to AFB_1.

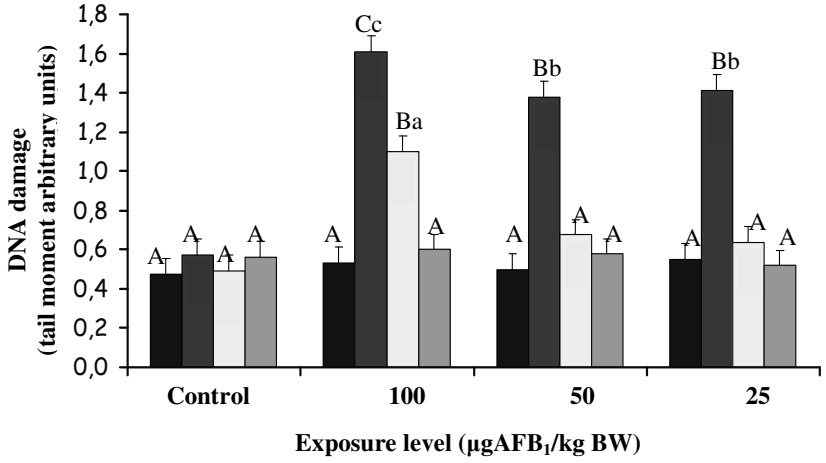

Figure 4 – DNA damage. Effects of AFB_1 exposure level and time after treatment (LSmeans ± SE expressed as % of control). Means with different letters means significant differences: a, b, c = P<0.05; A, B, C = P<0.01. Bars color: black = 0 h, dark gray = 24 h, light grey = 72 h and medium grey = 120 h after AFB_1 administration.

DISCUSSION

The immunotoxic effect of AFB_1 was studied in several animal species. AFB_1 has been found to reduce antibody production, to inhibit the phagocytic ability of macrophages, to reduce complement and decrease T-cell number and function (Richard *et al.*, 1978, Pier, 1986; Reddy *et al.*, 1987). Raisuddin *et al.* (1993) on weaning rat exposed to 60, 300 and 600 µg AFB_1/kg of BW for four weeks, showed higher susceptibility to infections and cancer genesis. Watzl *et al.* (1999) in rat fed for one week with contaminated diet (1 mg AFB_1/kg BW) found a decrease in uptake of thymidine analogue 5-bromo-2′-deoxyuridine. Moreover, Qureshi *et al.* (1998) in broilers fed with a diets containing 0, 0.2, 1, 5 and 10 mg AFB_1/kg BW, reported a suppression of humoral and cell-mediated immunological response, which lead to increased susceptibility to candidiasis, coccodiosis, salmonellosis and general immunologic deficiency in those animals. One of the first measurable effect of AFB_1 on cells and tissues is the inhibition of DNA synthesis. It would appear that AFB_1 blocked the initiation step in DNA replication rather then next step processes. In agreement with Kumimoto (1974) and Hsieh (1987) assumptions, it is possible to hypothesize that the reduction of DNA synthesis observed in our work is probably due to a direct action of covalent binding of AFB_1 against the DNA and to the enzymes responsible of cells replication and/or a direct action against membranes proteins involved in the uptake of thymidine analogue 5-bromo-2′-deoxyuridine. DNA damage detected for single strand breaks and expressed by tail moment disappeared at all dosages tested after 72 h. This might be explained by the fact that single strand breaks are quickly repaired. Therefore, we suppose that the DNA damage is not the main responsible for the alteration of DNA synthesis. At the same time we can not exclude that high values of DNA migration found in the first 24 h after treatment, could have an indirect action in delaying and/or decreasing the synthesis of enzymes involved in the cell replication. Finally, restoration of functionality as indicated by mitochondrial dehydrogenase activity recovery a long time after (192 h) the exposure event, was likely imputable both to the actions of cellular repair mechanisms and to the decrease of haematic concentration of AFB_1 following metabolism and excretion mechanisms.

CONCLUSIONS

Results of the present study confirm the immonotoxicity effect of AFB_1 as a consequence of feeding animals with contaminated diets. In particular, goats exposed to a single dose of AFB_1 showed an impairment of lymphocyte functions measured by DNA synthesis, mitochondrial dehydrogenase and DNA single strand breaks, that lead to decrease in the immune response. Therefore, the exposure to AFB_1 of goats may increase susceptibility to infections and cause general immunologic deficiency resulting in decreased productivity and possibly increased mortality.

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