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Metabolomic analysis of alterations in lipid oxidation, carbohydrate and amino acid metabolism in dairy goats caused by exposure to Aflotoxin B1

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The purposes of this study were to investigate the systemic and characteristic metabolites in the serum of dairy goats induced by aflatoxin B1 (AFB1) exposure and to further understand the endogenous metabolic alterations induced by it. A nuclear magnetic resonance (NMR)-based metabonomic approach was used to analyse the metabolic alterations in dairy goats that were induced by low doses of AFB1 (50 μg/kg DM). We found that AFB1 exposure caused significant elevations of glucose, citrate, acetate, acetoacetate, betaine, and glycine yet caused reductions of lactate, ketone bodies (acetate, β-hydroxybutyrate), amino acids (citrulline, leucine/isoleucine, valine, creatine) and cell membrane structures (choline, lipoprotein, N-acetyl glycoproteins) in the serum. These data indicated that AFB1 caused endogenous metabolic changes in various metabolic pathways, including cell membrane-associated metabolism, the tricarboxylic acid cycle, glycolysis, lipids, and amino acid metabolism. These findings provide both a comprehensive insight into the metabolic aspects of AFB1-induced adverse effects on dairy goats and a method for monitoring dairy animals exposed to low doses of AFB1.

Keywords: Aflatoxin B1, metabolic, NMR, dairy goats.

Aflatoxin B1 (AFB1), which is a mycotoxin produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, occurs naturally in human foods and animal feeds (Zhou et al. 2006). Acute or chronic aflatoxicosis, hepatotoxicity, teratogenicity, and immunotoxicity in humans and animals as a consequence of AFB1 exposure has been reported by many researchers (Sabbioni & Sepai, 1998; Smela et al. 2001). It has been well documented that AFB1 reduces performance, alters blood profiles, decreases immune function, and influences antioxidant status as well as liver function in animals (Bennett & Klich, 2003). For this reason, AFB1 is classified as a group 1 carcinogen in humans by the International Agency for Research on Cancer (IARC), which is part of the World Health Organization (IARC, 2002). In mammals, AFB1 is metabolised to AFM1, which also has carcinogenic potency (Creppy, 2002) and can appear in milk. Therefore, AFM1 has been classified as a probable human carcinogen (IARC, 2002). The European Commission has established the maximum admissible levels of AFB1 as 20 μg/kg in dairy animal feeds, and the allowable level of AFM1 in milk and milk products should not exceed 50 ng/kg (Commission, 2006). In China, AFB1 and AFM1 are also regulated by the Standardisation Administration of China at 20 μg/kg (SAC, 2001) and 50 ng/kg (SAC, 2003), respectively.

It is widely known that AFB1 is biotransformed by the hepatic microsomal cytochrome P450 in the liver into reactive AFB1-epoxide (AFBO). AFBO binds strongly to proteins and imparts its effect, which alters the normal biochemical functions of protein macromolecules, which can lead to deleterious effects at the cellular level (Mishra & Das, 2003). AFBO binds strongly to DNA and RNA as well, causing aflatoxins to impart their effect by means of interfering with DNA replication and transcription of messenger RNA into protein at the molecular level. Mutation is

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caused by binding of the aflatoxin molecule to DNA and subsequent erroneous protein synthesis (Bedard & Massey, 2006). Therefore, AFB1 is a biosynthetic inhibitor both in vivo and in vitro, which not only affects different metabolic pathways, such as glycogenolysis/glycolysis (Kiessling & Adam, 1986) and phospholipidation (Hsieh et al. 1988), but also changes amino acid transportation (Mclean & Dutton, 1995). Smith & Moss (1985) reported that aflatoxin reduces the hepatic glycogen level. AFB1 produced various alterations in total lipid and cholesterol as well as the fatty acid composition of hepatic phosphatidylcholine and phosphatidylethanolamine in rats three weeks post-exposure (Baldwin & Parker, 1986).

Metabonomics is a powerful approach to delineating the global metabolic profiling of living organisms and capturing the subtle metabolic alterations associated with physiological and pathological stimulations. ^{1}H Nuclear magnetic resonance (NMR)-based metabonomics, with its simplicity of sample preparation and robust reproducibility, has been extensively used in metabonomic analysis of biofluids and tissues to study metabolic changes. The study of the endogenous metabolic alterations induced by heavy metals was used in NMR-based metabonomics, with results showing that the changes in lipid fraction, unsaturated lipids, and in the level of amino acids suggest perturbation of the metabolism of lipids and amino acids (Dudka et al. 2014). Sundekilde & Poulsen (2013) identified the biomarkers of somatic cell counts by NMR-based metabonomics including lactate, acetate, isoleucine, hippurate, butyrate, and fumarate. NMR-based metabonomics has also been applied to the toxicology investigation of the T-2 toxin (Wan et al. 2016), ocharatoxin A (Sieber et al. 2009), deoxynivalenol (Hopton et al. 2010), and streptozotocin (Diao et al. 2014).

For the abovementioned reasons, we conducted this study to analyse the metabolic alterations caused by AFB1 in dairy goat serum using NMR-based metabonomics approaches. The objectives of this study were to investigate the endogenous metabolic changes induced by AFB1 exposure and to further our understanding of AFB1 toxicity at the systemic level.

Materials and methods

Animals and sample collection

All animals involved in this study were cared for according to the principles of the Chinese Academy of Agricultural Sciences Animal Care and Use Committee. Twenty lactating Laoshan dairy goats (193 ± 14 d in milk, 1·36 ± 0·4 kg of milk/d, and 3·4 ± 1·0 parity) were grouped by parity (primiparous or multiparous), average daily milk yield, and day in milk and randomly assigned to one of two treatments ($n = 10$), according to a randomized block design. The treatments were 0 (control) and 50 μg/kg AFB1 (AFB1). The AFB1 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and had a purity of 98%. The experimental period consisted of one week for adaptation to the environment followed by two weeks for the feeding of mycotoxins. The goats were assigned to a free stall barn with access to fresh water, and they were fed the total mixed ration (TMR). Pure AFB1 was separately dissolved in methanol. Pelleted concentrate was used as the mycotoxin carrier, and it was top-dressed in equal portions on the TMR at the 5 : 30 a.m. feeding.

Blood samples were collected from each goat before the morning feeding *via* jugular venipuncture on 14 d. Nine millilitres of blood were collected in vacuum tubes without anticoagulant, centrifuged at $3000 \times \boldsymbol{g}$ for 15 min at 4 °C to obtain serum, and stored at −80 °C for later analysis.

Sample preparation and ^{1}H NMR spectroscopy

Serum samples were prepared by mixing 300 μl of serum with 100 μl of D_2O and 200 μl of phosphate buffer saline solution (K_2HPO_4/NaH_2PO_4, pH 7·4). After vortexing and centrifugation at 13 000 rpm for 10 min, the supernatant was pipetted into 5 mm NMR tubes for NMR analysis.

A water-presaturated Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (recycle delay-90°-$(\tau-180°-\tau)_n$-acquisition, where $\tau = 400$ μs and $n = 400$) was employed to retain NMR signals from small metabolites and to attenuate NMR signals from macromolecules with spin-spin relaxation delays ($2n\tau$) of 320 ms. CPMG spectra with a 2 ms sample time and a 3 s relaxation delay between successive scans (64 scans per spectrum, 32 K data points, spectral width 8000 Hz) were recorded using a Varian INOVA-600 spectrometer with a pulsed field gradient and three resonance probes.

Metabolites were identified by inserting the experimental spectra into the Chenomx spectral database (Edmonton, AB, Canada) for comparison with the spectra of standard compounds.

NMR spectrum processing and multivariate data analysis

After manual corrections of both the phase and baseline, the chemical shifts were referenced internally to TSP (at $\delta = 0{\cdot}0$ ppm). The chemical shift region from 0·4 to 6·0 was segmented and integrated with equal widths of 0·01 ppm. All spectral segments were normalised to the total spectral area in Excel for multivariate data analysis.

Multivariate data analysis was carried out with SIMCAP+ software (version 13.0, Umetrics, Sweden). Prior to multivariate analysis, centre scaling was applied to ^{1}H NMR data to reduce noise and artifacts in the models. Principal Component Analysis (PCA) was initially carried out on the NMR data to generate an overview. Partial least squares discriminant analysis (PLS-DA) models were applied to validate the model against overfitting through 999 random permutation tests. Discriminating variables were selected according to the loading plots, jack-knifed-based confidence intervals, variable importance in projection values (VIP > 1), and raw data plots in the orthogonal partial least squares discriminate analysis (OPLS-DA) models (Tian et al. 2015). Furthermore, independent t-tests ($P < 0{\cdot}05$)

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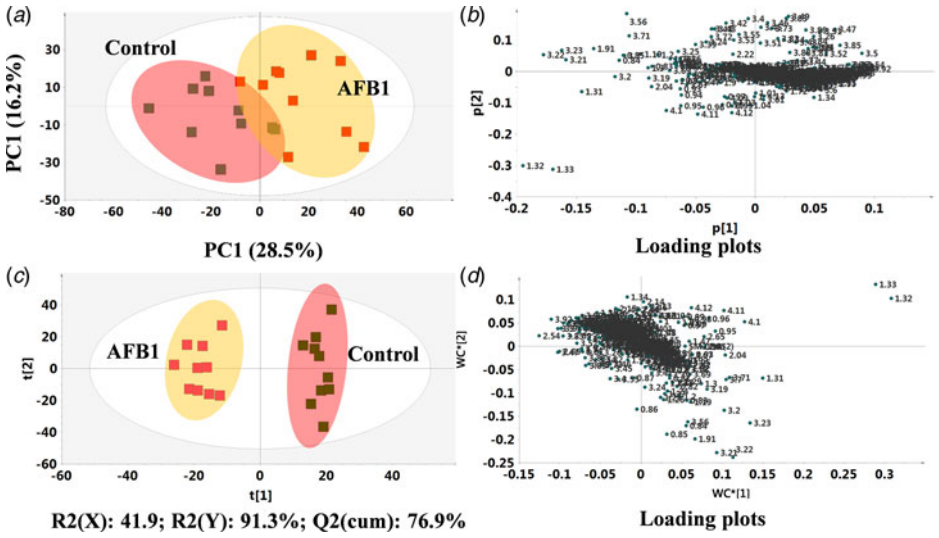

Fig. 1. (a) Score plot for the PCA model of the control and AFB1 groups. (b) Loading plots of centre-scaled ^{1}H NMR data in unsupervised PCA model. (c) Score plot for the OPLS-DA model of control and AFB1 groups. (d) Loading plots of centre-scaled ^{1}H NMR data in supervised OPLS-DA.

(SPSS version 13.0) were used to determine if the differences between the concentrations of candidate biomarkers obtained from OPLS-DA of both the control and AFB1 groups were statistically significant.

Results

Comparison of the metabolic profiles of the control and AFB1 groups

PCA models with good cluster of both the control and AFB1 groups and no strong outliers were observed (Fig. 1a). This presented apparent groupings, with only partial overlapping between the control and AFB1 data. The loading plots (Fig. 1b) reflected changes of some endogenous metabolites. To obtain improved model transparency and interpretability, OPLS-DA's for chemometric analysis were further performed to screen for differential variables. These showed a clear separation between the control and AFB1 groups without any overlap (Fig. 1c), which produced one predictive component and two orthogonal components with satisfactory modelling and predictive abilities $R^2(X) = 41{\cdot}9\%$, $R^2(Y) = 91{\cdot}3\%$, and $Q^2(\text{cum}) = 76{\cdot}9\%$. Loading plots in Fig. 1d showed changes of significantly altered endogenous metabolites. To avoid model overfitting, a default of seven rounds of cross-validation across three components was performed. Validation with 999 random permutation tests produced intercepts of $R^2(Y) = 0{\cdot}396$ and $Q^2 = -0{\cdot}244$ for the data (Supplementary Fig. S1). Overall, the results indicated that the predictive capabilities of the OPLS-DA models of the NMR data were reliable.

Identification of metabolite candidates

^{1}H NMR analyses identified a total of 17 metabolic candidates (Table 1), which were classified into carbohydrate, amino acid, and lipid metabolites, suggesting that these metabolic pathways were altered in the AFB1 group. Supplementary Fig. S2 shows the signal ^{1}H assignment of identified candidate metabolites.

Metabolic alterations

Table 1 lists the metabolic alterations between the control and AFB1 groups. AFB1-induced alterations in carbohydrate-related metabolites were observed for lactate, glucose, citrate, and acetate. The concentrations of lactate and acetate were decreased by 0·71- to 0·89-fold in the AFB1 group compared with the control group ($P < 0{\cdot}01$). The concentrations of glucose and citrate were increased by 1·21- to 2·89-fold in the AFB1 group compared with the control group ($P < 0{\cdot}01$). Citrulline, leucine/isoleucine, valine, glycine, creatine, and betaine are metabolites related to amino acids; the former three were decreased by 0·84- to 0·91-fold in the AFB1 group compared with the control group ($P < 0{\cdot}05$), and the latter three were increased by 1·32- to 1·64-fold ($P < 0{\cdot}01$). The concentrations of acetone, acetoacetate, β-hydroxybutyrate, choline, phosphatidylcholine (PC)/ glycerophosphoryl choline (GPC), lipoprotein, and N-acetyl glycoproteins are lipid-related metabolites; the former two were increased by 1·27- to 1·51-fold in the AFB1 group compared with the control group ($P < 0{\cdot}01$), and the latter two were decreased by 0·77- to 0·84-fold in the AFB1 group compared with the control group ($P < 0{\cdot}05$).

Discussion

AFB1's influence on liver damage

Choline, PC, and GPC are essential elements for structural integrity of cell membranes. The significant decreases in

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Table 1. Candidate metabolite identified by †H NMR

No.	Metabolic pathway	Metabolite	Chemical shift (ppm)	Assignment†	P‡	FC§
1	Carbohydrate	Lactate	1·33	CH_3 (d)	$4{\cdot}48 \times 10^{-4}$	0·71
2	Carbohydrate	Glucose	5·25(d), 4·66(d)	CH_2 (d)	$6{\cdot}47 \times 10^{-3}$	1·21
3	Carbohydrate	Citrate	2·54, 2·67	$2 \times CH_2$ (d)	$1{\cdot}62 \times 10^{-5}$	2·89
4	Carbohydrate	Acetate	1·91	CH_3 (s)	$7{\cdot}75 \times 10^{-3}$	0·89
5	Amino acid	Citrulline	3·71	CH (m)	$2{\cdot}62 \times 10^{-2}$	0·91
6	Amino acid	Leucine/Isoleucine	0·92–0·97	CH_3 (d)	$8{\cdot}53 \times 10^{-3}$	0·87
7	Amino acid	Valine	0·98, 1·03	$2 \times CH_3$ (d)	$3{\cdot}45 \times 10^{-2}$	0·84
8	Amino acid	Glycine	3·57	CH_2 (s)	$2{\cdot}64 \times 10^{-3}$	1·32
9	Amino acid	Creatine	3·05, 3·94	CH_2 (s)	$3{\cdot}84 \times 10^{-5}$	1·40
10	Amino acid	Betaine	3·26	$3 \times CH_3$ (s)	$3{\cdot}39 \times 10^{-3}$	1·64
11	Lipid	Acetone	2·24	$2 \times CH_3$ (s)	$2{\cdot}25 \times 10^{-3}$	1·51
12	Lipid	Acetoacetate	2·29	CH_3 (s)	$6{\cdot}58 \times 10^{-4}$	1·27
13	Lipid	β-hydroxybutyrate	1·19	CH_3 (s)	$9{\cdot}38 \times 10^{-3}$	0·80
14	Lipid	Choline	3·20	$3 \times CH_3$ (s)	$3{\cdot}03 \times 10^{-2}$	0·77
15	Lipid	PC/GPC	3·23	$3 \times CH_3$ (s)	$1{\cdot}62 \times 10^{-2}$	0·87
16	Lipid	Lipoprotein	0·82–0·88	CH_3 (t)	$2{\cdot}68 \times 10^{-2}$	0·78
17	Lipid	Nacetyl glycoproteins	2·03	CH_3 (s)	$2{\cdot}49 \times 10^{-2}$	0·83

†(d) doublet, (s) singlet, (dd) double doublet, (m) multiplet, (t) triplet
‡P, independent *t*-test for control *vs.* AFB1
§FC, fold change in the metabolite concentration (AFB1/control)

serum levels of choline, PC, and GPC in AFB1 treated goats indicated that AFB1 causes liver damage, which was in line with the decrease of lipoprotein and N-acetyl glycoproteins in the serum. As an organic osmolyte, betaine can be excreted into serum to maintain osmolarity and protect against cell damage in hypotonic conditions when hepatocytes swell and lead to the accumulation of intracytoplasmic fluids in cells (Zerbst-Boroffka et al. 2005). The observation of elevated levels of betaine in the current study suggested that both the swelling of hepatocytes and subsequent liver damage had occurred.

Previous evidence from gene expression also supports these results. Several genes that were encoding cytoskeletal organisation were up-regulated in both middle- (0·75 mg/kg body weight) and high-dose (1·5 mg/kg body weight) AFB1 groups of rats, including Rhob in both groups and Anxa1, Anxa2, Anxa4, Anxa5, Anxa7, and Arpc1b in the high-dose group (Lu et al. 2013). Additional supportive evidence of liver damage induced by AFB1 was found in rats that were dosed with 0·24 mg/(kg/day) of AFB1 for 14 d, where genes encoding the cytoskeletal constituent were up-regulated, including CRYAB α B-crystallin, RhoB, SPTA2 spectrin α chain (fodrin α chain), and TMSB 10 thymosin β–10 (Ellinger-Ziegelbauer et al. 2004).

AFB1's impact on carbohydrate metabolism

An increase in citrate excretion together with decreased lactate excretion was observed with ^{1}H NMR detection, which suggested impairments in both Cori and Krebs cycle metabolism in periods of altered energy production, which may be linked to damaged mitochondrial function. The major function of electron transport is ATP synthesis in mitochondrion. The depletion of ATP that was seen resulted from impaired energy metabolism. A previous investigation also reported that ATP synthesis pathways are more affected in chicks fed 2 mg of AFB1/kg, leading to decreased energy production and the genes being down-regulated, such as in the adrenodoxin mitochondrial precursor, cytochrome P450 2C5, cytochrome P450 2P3, and nicotinamide adenine dinucleotide phosphate-cytochrome b5 reductase (Yarru et al. 2009). Consistent with the findings in broilers (Tessari et al. 2010) and ducks (He et al. 2013), our goats that were fed diet supplements of 50 μg/kg of AFB1 had a 1·21-fold changes of the glucose level in serum, indicating that liver function had been damaged and that a consequence of AFB1 exposure is an accelerated rate of glycogenolysis. Such depletion of hepatic glucose has also been observed in rats dosed with 0·32 mg/(kg body weight/day) of AFB1 (Zhang et al. 2011).

AFB1's impacts on lipid metabolisms

A previous investigation found increases in the relative liver weights of chicks that were fed aflatoxin (Ortatatli & Oğuz, 2001). In this study, we observed decreased levels of β-hydroxybutyrate and acetate in the serum after AFB1 treatments, indicating that AFB1 exposure alters the lipid metabolism of goats. These results were consistent with the findings of Yarru et al. (2009), who found that genes involved in fatty acid metabolism were downregulated in chicks that were fed 2 mg/kg of AFB1. Acetoacetate and acetone are the products of the β-oxidation of fatty acid in mitochondria, hence, their elevations in this study suggested the promotion of β-oxidation of fatty acid with AFB1 exposure. In agreement with our findings, a previous investigation found that AFB1 exposure caused up-regulation of

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peroxisome proliferator-activated receptor R, a key regulator of lipid metabolism (Yarru et al. 2009).

AFB1's impact on amino acid metabolism

In this study, AFB1 induced an increase in the levels of citrulline, leucine/isoleucine, and valine and a decrease in the level of glycine, which implies that AFB1 altered hepatic amino acid metabolism. Moreover, a noticeable increase in creatine levels was observed in the serum of AFB1-treated goats. Phosphocreatine, an energy reservoir, can be rapidly converted to creatine by creatine kinase and the release of adenosine triphosphate (ATP) and creatine in response to high energy demands (Diao et al. 2014). Suppression of the tricarboxylic acid (TCA) cycle caused by AFB1 exposure will inevitably result in insufficient energy production. Hence, the accumulated creatine in the serum of AFB1 exposed goats may be the result of the accelerated conversion of phosphocreatine to creatine to meet this energy demand. The decreased serum levels of citrulline implied that the urea cycle was interrupted by AFB1 because it is the intermediate metabolite in the urea cycle. This is inconsistent with the AFB1-induced down-regulation of gene expression of carbamoyl-phosphate synthetase I in mice (Zhuang et al. 2014).

Conclusion

In conclusion, dairy goats' exposure to low doses of AFB1 caused significant alterations in several metabolic pathways, including lipid oxidation, TCA cycle, and carbohydrate and amino acid metabolism. Our results also show that ^{1}H NMR-based metabolomic analysis of the serum provided an effective method for understanding AFBI's adverse effects on goats' health at low levels as well as a method for preventing AFM1 contamination of milk.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000590.

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