Effects of facial eczema on skatole detoxification efficiency in dairy cows

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ABSTRACT

Facial eczema (FE) is a disease caused by the fungal toxin sporidesmin, which upon ingestion by dairy cows can cause liver damage in susceptible animals. Skatole is formed in the rumen by the breakdown of dietary protein, absorbed into blood and is cleared by metabolism in the liver to 3-methyloxindole (MOI) and 3-hydroxy-3-methyloxindole (HMOI) and products of further oxidation and conjugation. In this study we have determined the concentrations of skatole, MOI and HMOI in the blood of dairy cows selected for their "high" gamma-glutamyltransferase (GGT) and "low" GGT in autumn and spring. Significant differences in skatole and MOI median concentrations were found in the autumn blood between the high and low GGT groups (skatole: 11.9 and 1.1 ng/mL respectively, $P < 0.001$; MOI: 23.9 and 11.9 ng/mL respectively, $P < 0.05$) although there was no difference in blood HMOI levels. Similar trends were observed for animals with high GGT in both autumn and spring when compared to low autumn and spring GGT. Animals with high autumn GGT and low spring GGT also contained increased median concentrations of skatole and MOI in the spring blood compared to the low autumn and spring GGT group, suggesting a lingering effect of FE damage on liver detoxification efficiency not detected by measuring spring GGT levels.

Keywords: detoxification; skatole; facial eczema; 3-methyloxindole; 3-hydroxy-3-methyloxindole; cattle.

INTRODUCTION

Facial eczema (FE) is a disease of ruminants which affects the liver and is caused by ingestion of toxin-containing spores of the saprophytic fungus *Pithomyces chartarum*. The severity of liver damage induced by the *P. chartarum* toxin, sporidesmin, is directly related in susceptible animals to the serum concentrations of the enzyme gamma-glutamyltransferase (GGT). This parameter is commonly used to monitor the incidence and severity of sub-clinical FE (Towers, 1978; Towers & Stratton, 1978). We have reported (Fraser *et al*., 2006) that concentrations of the “pastoral” flavour compound skatole may be highly elevated in blood and milk from dairy cows suffering from clinical or sub-clinical FE as judged by GGT concentrations.

Skatole is formed from the breakdown of tryptophan in dietary protein in the rumen (Tavendale *et al*., 2005), absorbed from the rumen and transported to the liver. It is largely cleared by liver metabolism but can transfer rapidly between the blood and mammary tissue (Roy *et al*., 2004). Levels of skatole in milk have been linked to dietary protein intake by the animal (Lane *et al*., 2002).

Our hypothesis is that impaired liver metabolism is the causal link between elevated concentrations of skatole and FE. In dairy cattle, a reduction in milk yield associated with FE derived liver damage and clinical disease is well known (Smith & Towers, 2002). After the initial toxic exposure, the liver begins regenerating within a few weeks and a proportion of liver function returns within 1 to 2 months. However, regeneration may not be complete, especially after repeated exposure to sporidesmin over several years (Smith & Towers, 2002). As the liver is the major detoxification route for skatole (Roy *et al*., 2004), a poorly functioning liver may not efficiently remove it from the circulatory system resulting in higher levels of skatole in blood, and in milk causing adverse flavour effects. Incomplete liver regeneration may account for the carry-over effects of FE in autumn on skatole concentrations in milk in the following spring which we observed previously (Fraser *et al*., 2006).

Two oxidation products of skatole, 3-methyloxindole (MOI) and 3-hydroxy-3-methyloxindole (HMOI) have been reported as the major products of liver metabolism of skatole found in the plasma of ruminants (Smith *et al*., 1993). In healthy dairy cows on diets differing in protein content we have observed that high levels of skatole in milk and plasma are associated with elevated levels of the skatole oxidation products MOI and HMOI in both plasma and skim milk (G.A. Lane, Unpublished data). By measuring concentrations of MOI and HMOI as well as skatole we reasoned it should be possible to distinguish high skatole levels due to diet from high skatole levels due to impaired liver function, and indeed obtain a measure of the efficiency of skatole metabolism.

The objectives of this study were to firstly investigate further whether the FE history and “status” of a dairy cow was reflected in the levels of skatole in blood plasma in spring, or whether

elevated levels could be simply attributed to the higher protein content of the diet. Our second objective was to determine whether FE damage to the liver of dairy cows can affect the conversion of skatole to MOI and HMOI. To elucidate these questions, samples from two studies investigating the incidence of FE in dairy herds from two different years were analysed for GGT and skatole, and measurements of MOI and HMOI in plasma.

MATERIALS AND METHODS

Animals and sampling

Trial 1: As part of a study investigating genetic variation in resistance to FE in dairy cattle (Cullen *et al*., 2006), two herds of dairy cows in the Waikato and two herds in the Bay of Plenty were selected in autumn 2005. Each herd contained at least 3% clinical cases of FE and at least 45% of cows with elevated GGT activity. GGT was measured in a total of 1,223 blood samples taken from every cow in each herd, regardless of the clinical FE status of the individual cow. Samples were drawn from the tail into heparinised evacuated tubes, which were then transported at ambient temperature to a commercial laboratory in Hamilton for GGT analysis. In one herd from the Bay of Plenty (Herd 8, bled on 30 March 2005) 93% (165 out of 177) of the samples had an elevated GGT level with 18% of the cows (32 out of 177) exhibiting clinical symptoms. To take advantage of the wide range in GGT within this herd samples of residual plasma from 10 cows with a high GGT (>400 iu/L) and 10 cows with a low GGT (<100 iu/L) cows were set aside for analysis of skatole and metabolites.

Trial 2: In autumn 2007, dairy herds in the Waikato province with at least 2 to 3% cases of clinical FE were included in our study. In three such herds (#38, 39 and 42), all cows present were blood-sampled in April/May (n = 941 cows) as in Trial 1, and a further round of blood-sampling in each herd was carried out for the determination of GGT and glutamate dehydrogenase (GDH) activity in October 2007. Cows which were outliers for GGT activity were categorised into three groups; low-low (LL), high-low (HL) and high-high (HH), on the basis of autumn and spring blood GGT levels where the threshold between high and low GGT concentration was 500 iu/L in autumn and 200 iu/L in spring. Samples of plasma were stored at -20ºC for subsequent analysis of skatole.

Analytical methods

Blood samples were analysed for GGT by the method of Towers and Stratton (1978), and GDH, an indicator of hepatocyte damage (Cullen *et al.*, 2006), by Gribbles Veterinary Pathology Laboratory, Hamilton.

Plasma concentrations of skatole (ng/mL) were measured, after sample cleanup using two partitioning steps, by high performance liquid chromatography with fluorescence detection (Claus *et al*., 1993).

Concentrations of 3-methyloxindole and 3-hydroxy-3-methyloxindole (ng/mL) in plasma were determined by gas chromatography-mass spectrometry (GC-MS) with a stable isotope internal standard. Briefly, to 1 mL of plasma was added 10 µL of internal standard (0.508 µg, 3-hydroxy-3-d_3-methyloxindole) and 2,000 units of β-glucuronidase (Type H-5) in 1 mL 0.2 M sodium acetate/acetic acid buffer (pH 4.8) and the mixture incubated at 38ºC for 16 hours. The sample was diluted with 1 mL of water and loaded onto a preconditioned C18 cartridge. The cartridge was washed with 2 x 4 mL of water, briefly vacuum dried (10 to 15 seconds) and eluted with 4 mL of acetonitrile. The acetonitrile was dried by passing through anhydrous sodium sulphate and then concentrated to 0.5 mL using a vacuum centrifuge. Aliquots (2 µL) were analysed by GC-MS (QP-5050A, Shimadzu, Kyoto, Japan) on a 50% polydimethylsiloxane-50% phenyl column (ZB-50, Phenomenex, CA) in selected ion mode.

Statistical analysis

All data were statistically analysed using Minitab version 15.1.0.0 (Minitab Inc., PA) using non-parametric methods as the data were not normally distributed. The Kruskal-Wallis test was used in Trial 1 and the Mann Whitney test was used in Trial 2 to test if the concentrations of the analytes differed between the status groups.

RESULTS

Trial 1

From within the herd, two groups of ten animals were selected as either low or high outliers on the basis of plasma GGT concentration in autumn. The high outlier group included two animals with clinical FE. The median and range of GGT concentrations for the selected groups (low outlier or high outlier FE status) are shown in Table 1, together with the concentrations in the blood of skatole, MOI and HMOI. Blood GDH levels were not analysed on these animals, but the correlation of $\log_e$ GDH and $\log_e$ GGT is very high (r = 0.88, Cullen *et al.*, 2006), so that the GGT data here are indicative of the hepatocyte damage. The median blood concentrations of skatole and MOI were significantly higher in the high outlier group than the low outlier group ($P < 0.001$ and $P < 0.05$ respectively). There was no significant difference in HMOI concentrations between the two groups.

TABLE 1: Median value (range) of gamma-glutamyltransferase (GGT), skatole, 3-methyloxindole (MOI) and 3-hydroxy-3-methyloxindole (HMOI) in blood samples of 10 dairy cows with low GGT and 10 dairy cows with high GGT sampled during autumn 2005.

Analyte	Facial eczema status		P value
	Low outliers	High outliers	
GGT (iu/L)	22 (7 – 23)	3,308 (3,100 – 5,090)	
Skatole (ng/mL)	1.1 (0.3 – 2.5)	11.9 (5.2 – 69.0)	0.001
MOI (ng/mL)	12 (7 – 32)	24 (12 – 57)	0.010
HMOI (ng/mL)	54 (27 – 102)	52 (18 – 89)	0.705

Trial 2

From across the three herds monitored, the median and range of autumn GGT for each of the selected groups is shown in Table 2, together with the corresponding data for spring GGT and blood skatole concentrations. Spring blood GDH levels were also monitored and showed a positive correlation with spring GGT levels (r = 0.85, P <0.001). The median blood skatole concentrations were significantly (P <0.001) higher for both high GGT autumn groups compared to the low autumn GGT group. The median skatole concentration for the HH group was more than three-fold higher than that of the HL group (P <0.001).

A subset of blood samples with predominantly higher blood skatole concentrations were selected from the LL and HL groups, along with all samples from the HH group for measurement of MOI and HMOI concentrations. The median and range of autumn GGT for the subsampled groups within the herds are shown in Table 3, together with the corresponding data for spring GGT, skatole, MOI, and HMOI concentrations measured in the blood of these cows. The median blood concentrations of MOI were approximately 50% greater for both the HH and HL groups compared to the LL group. The difference between the HL and LL groups was statistically significant (P = 0.029), and that between the HH and LL group medians approached significance (P = 0.058). The median HMOI concentrations for the HH group and the LL group did not differ significantly, but the median HMOI concentration for the HL group was significantly higher than for the HH and LL groups (P = 0.028 and 0.002 respectively).

DISCUSSION

Trial 2 provided further evidence that liver damage resulting from FE can result in elevated skatole concentrations in cows in the following spring, as well as immediately after exposure to sporidesmin. It also provided new direct evidence of impaired metabolism of skatole in the liver in FE-affected cows.

The clear evidence from these trials linking high levels of both skatole and GGT in the blood suggests a strong relationship between sporidesmin-induced liver damage and reduced efficiency of liver catabolism of skatole. However the occurrence of high blood GGT was not always associated with elevated blood skatole, suggesting some level of

TABLE 2: Median value (range) of gamma-glutamyltransferase (GGT) in autumn and spring and skatole in spring in blood samples of all dairy cows sampled during autumn and spring 2007. Spring skatole concentrations for all groups were highly significantly different (P <0.001) from each other. The threshold between high and low groups was 500 iu/L in autumn and 200 iu/L in spring.

Analyte	Season	Facial eczema group		
		Low-low	High-low	High-high
Number of cows		43	112	25
GGT (iu/L)	Autumn	21 (0 – 467)	1,976 (564 – 3,953)	1,880 (869 – 2,825)
	Spring	17 (5 – 124)	43 (3 – 196)	453 (204 – 2,028)
Skatole (ng/mL)	Spring	6.6 (0.2 – 44.6)	15.0 (0.5 – 111.0)	50.8 (7.4 – 308.8)

TABLE 3: Median value (range) of gamma-glutamyltransferase (GGT) in autumn and spring, and skatole, 3-methyloxindole (MOI) and 3-hydroxy-3-methyloxindole (HMOI) in spring in blood samples of dairy cows selected for higher spring skatole levels that were sampled in 2007.

Analyte	Season	Facial eczema group		
		Low-low	High-low	High-high
Number of cows		19	28	25
GGT (iu/L)	Autumn	21 (0 – 467)	1,746 (609 – 3,916)	1,880 (869 – 2,825)
	Spring	16 (5 – 124)	54 (10 – 177)	453 (204 – 2,028)
Skatole (ng/mL)	Spring	13.1 (0.5 – 44.6)	35.7 (0.5 – 111.0)	50.8 (7.4 – 308.8)
MOI (ng/mL)	Spring	43^{a} (27 – 506)	63^{b} (34 – 121)	70^{ab} (28 – 161)
HMOI (ng/mL)	Spring	264^{a} (129 – 506)	409^{b} (205 – 1,027)	351^{a} (92 – 517)

Median values within rows with a different superscript are significantly different (P <0.05).

liver damage can occur without impacting on skatole metabolism and diet can also be a factor affecting skatole concentrations (Lane *et al*., 2002).

The measurements of the major blood metabolites, MOI and HMOI, provided clear evidence that the elevated concentrations of skatole in FE-affected cows were indeed due to impaired metabolism of skatole rather than other factors. While a 5 to 10 fold difference in skatole levels in the blood between groups of different FE status was observed in these trials, the differential for MOI levels was only 1.5 to 2 fold and there was no, or only a slight, difference in HMOI levels. The implications of this are made clearer by considering the mole ratios of total measured skatole metabolites to skatole in the blood for the different groups. Thus in Trial 1, the median ratio was 27:1 for the low outlier group, and 3:1 for the high outlier group, and in Trial 2 the median ratios were 26:1, 12:1 and 8:1 for the LL, HL and HH groups. Thus the proportion of skatole remaining un-metabolised was much higher in the blood of FE-affected cows.

The significant margin between the median blood concentrations of skatole of the group of cows that had high levels of GGT in the autumn but low levels in the spring (HL) and the control group (LL) suggests a lingering effect of FE damage on liver detoxification efficiency not necessarily observable in the spring GGT data. This is supported by the metabolite ratio analysis above. These data suggest that liver function of some of the high FE status animals from the previous autumn is still impaired, with potential adverse effects on milk quality, and also on milk-solids yield (C.A. Morris, Unpublished data), despite an apparently healthy liver function as assessed by GGT measurement.

Thus measurements of plasma skatole can provide indicative evidence of residual impaired liver function in spring in animals suffering FE in the preceding autumn that complements evidence from GGT analyses. With a low GGT in spring not being unambiguous evidence of healthy liver function, additional measurements of blood concentrations of products of skatole oxidation in the liver, namely MOI and HMOI, can help to clarify the significance of elevated skatole concentrations.

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