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# THE EFFECTS OF CONDENSED-TANNINS ON THE NET FLUX OF SKATOLE AND INDOLE ACROSS THE MAMMARY GLAND AND THEIR SECRETION IN MILK OF LACTATING EWES FED FRESH SULLA (*HEDYSARUM CORONARIUM*)

*N.C. ROY [A], K. FRASER [B], G.A. LANE [B], G.W. REYNOLDS [C], M.H. DEIGHTON [A], J.S. PETERS [A], B.R. SINCLAIR [A], A.F. DEATH, [A]. and W.C. McNABB [A]*

[A] Nutrition and Behaviour Group, AgResearch Limited, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand
[B] Plant Breeding and Genomics, AgResearch Limited, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand
[C] Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.

## SUMMARY

The effects of condensed tannins (CT) in fresh Sulla (*Hedysarum coronarium*) on the net flux of skatole and indole from the gastrointestinal tract to the mammary gland were investigated in ewes during mid to late lactation. Twelve ewes were prepared with catheters in the mesenteric artery and the mesenteric, portal, hepatic and mammary veins. A transonic flow probe was fitted around the pudic artery for measuring mammary blood flow. All ewes were fed Sulla (2000 g DM $d^{-1}$; 80 g CT $d^{-1}$) for 28 days. Half the ewes were orally drenched (4 times per d) with polyethylene glycol (PEG; 160 g $d^{-1}$ in water) to remove the effects of CT whilst the remaining ewes received water. Milk yield was measured twice daily for three weeks. Milk samples were taken at the morning and evening milkings and pooled for measuring skatole and indole concentration. Overall, the CT in Sulla did not affect ($P>0.05$) milk yield and milk fat yield. Milk indole and skatole concentration and yield tended to be lower ($P<0.1$) with the CT treatment at day 14 but overall, only skatole parameters were reduced by the CT. On day 28, blood flow was measured and blood samples were taken to determine concentration of indole and skatole in plasma so their net plasma flux could be calculated. The plasma flow across the mammary gland was unaffected ($P>0.05$) by CT. The CT in Sulla decreased the concentration of indole ($P<0.05$) and skatole ($P<0.1$) in the mesenteric artery and mammary vein, but did not affect ($P>0.05$) the net flux of indole and skatole across the mammary gland. Results from this study indicate that CT in a Sulla diet reduces the concentration of skatole in plasma and milk, and that circulating skatole exchange into milk, but that it does not appear to be an active process.

*Keywords*: skatole, indole, condensed tannins, plasma net flux, milk

## INTRODUCTION

The indole compounds, 3-methyl-indole (skatole) and indole are amines that give a faecal taint to milk and dairy products and are perceived in Asian and European markets as negative flavours (Keen 1998). Studies in Australia indicated that these compounds are not present in any significant quantity in forages (Conochie 1953). The amines found in milk are most likely the result of rumen metabolism of the amino acid tryptophan and waste detoxification and excretion (Keen 1998). There is a scarcity of information in the literature on how these amines produced in the rumen, are metabolized by the gastrointestinal tract and the liver of lactating animals before being secreted in milk.

Preliminary research has shown that feeding legumes that contain condensed tannins (CT) reduces the concentration of indole and skatole in milk (Lane *et al.*, unpublished results). The mechanism behind this action is not completely understood but previous studies have shown that CT decreases the degradation of protein in the rumen (McNabb *et al.*, 1996). We hypothesized that the reduced concentration of skatole and indole in milk is a consequence of the reduction in rumen protein degradation that occurs when CT-containing forages are fed to ruminants. Our objective was to investigate the effect of CT on the flux of skatole and indole across the small intestine, portal-drained viscera, the liver and the mammary gland of lactating ewes and on their secretion in milk. The data reported in this paper focus on the mammary gland and milk only.

## MATERIALS AND METHODS

*Experimental animals*

The Animal Ethics Committee of AgResearch Limited approved the experimental protocol. Four days after lambing, 12 Romney ewes were transferred to metabolism crates, held indoors and fed twice daily with lucerne pellets (600 g $d^{-1}$) and chaff (400 g $d^{-1}$) for the week before surgery. Water was available *ad libitum*. The ewes were milked twice daily using a portable milking machine with milk letdown assisted with an intravenous injection of oxytocin (1 iu).

*Surgical preparations*

Surgery occurred about 8 d after lambing. Twelve ewes were prepared with a permanent cannula in the abomasum under isofluorane anesthesia. During surgery, permanent indwelling catheters were placed in the mesenteric (2 sites), portal and hepatic veins, and in the mesenteric artery (Huntington *et al.* 1989). A transonic flow probe was fitted around the pudic artery. Three days prior to the start of the sampling period, a temporary catheter was inserted into the mammary vein for blood sampling.

*Treatments*

After recovering from surgery, all ewes were offered fresh Sulla (2000 g DM $d^{-1}$; 80 g CT $d^{-1}$) for 28 days. Half the ewes were orally drenched (4 times per d) with polyethylene glycol (PEG; 160 g $d^{-1}$ in water) to remove the effects of the CT (CT inactive; PEG group) whilst the remaining ewes received a drench of water (CT active; Control group). The treatments were applied according to a completely randomized block design. Each block included two ewes from each treatment.

*Skatole and indole concentrations in milk*

Milk yield was measured twice daily. Whole milk (50 ml) from each milking was collected on two consecutive days, kept at 4ºC and pooled for each ewe, for the analysis of skatole and indole concentration on days 7, 14 and 21. The pooled samples were frozen at –20ºC until analysis. Indole and skatole concentration was measured in milk using an adaptation of the method of Denhard *et al.* (1993). Milk fat was separated from whole milk (100 ml) by centrifugation at 10,800 *g* in a refrigerated centrifuge for 30 minutes. A sub-sample of the milk fat was melted in a water bath (55°C) and a 100 µl aliquot was dissolved in 1 ml of hexane. Internal standard was added (2-methylindole), and the solution was partitioned with acetonitrile/water (75:25 v/v). An aliquot (50 µl) of the aqueous fraction was analyzed by high performance liquid chromatography (HPLC) with fluorescence detection.

*Net indole and skatole fluxes*

On day 28, blood was continuously sampled for three consecutive 2-hour periods from the mesenteric artery and the mammary vein. These blood samples were centrifuged at 4000 *g* for 15 minutes (4ºC) and the plasma was removed and stored at –20ºC until analyzed for skatole and indole. Mammary blood flow was continuously measured during the blood sampling using the transonic flow probe. The plasma skatole and indole concentrations were measured in the sample taken during the last 2-h sample by the method of Claus *et al.* (1993), utilizing ether extraction, and HPLC with fluorescence detection. Net plasma flux of indole and skatole across the mammary gland was calculated as plasma flow (ml/min) x (Arterial concentration-Venous concentration; ng/ml). A positive flux indicated a removal, whilst a negative flux indicated a release by the mammary gland.

*Statistical analysis*

Data were subjected to the GLM procedure of SAS (SAS Institute, Inc. 1996) according to a completely randomized block design. Paired t-tests were used to verify whether the arterio-venous concentration differences for skatole and indole were different from zero. Least squares means ± overall standard deviations are presented in the tables. Significant statistical differences between treatments were declared at a probability less than 0.05.

## RESULTS

The CT contained in Sulla did not affect ($P>0.05$) milk yield (Table 1) and milk fat yield (data not shown) over the three weeks of feeding. Milk concentration and yield of indole and skatole were similar ($P>0.05$) between treatment groups on day 7 although a trend ($P<0.1$) for CT to decrease skatole concentration and yield in milk was observed (Table 1). At day 14, the indole and skatole parameters were lower ($P<0.05$) in CT ewes than in the ewes receiving PEG (Table 1). This decrease

did not however persist to day 21. No effect (P>0.05) of CT on milk indole concentration was observed for the overall period of Sulla feeding (Table 1). But in contrast, milk skatole level and yield were lower in CT ewes (P<0.1) for the overall period (Table 1).

**Table 1. Effect of condensed tannins on milk yield and milk skatole and indole concentration and yield in mid- to late-lactating ewes fed fresh Sulla.**

| Milk Parameters | Treatments | | Standard Deviation | Probability |
|---|---|---|---|---|
| | PEG (n = 6) | Control (n = 5) | | |
| Milk Yield (g/d) | | | | |
| D0 | 1106 | 1200 | 219.8 | 0.50 |
| D7 | 1152 | 1230 | 189.5 | 0.53 |
| D14 | 1005 | 1010 | 222.5 | 0.97 |
| D21 | 889 | 972 | 189.6 | 0.50 |
| Overall | 1016 | 1071 | 179.8 | 0.63 |
| Indole (ng/g) | | | | |
| D7 | 38.5 | 46.5 | 11.19 | 0.28 |
| D14 | 36.1 | 21.2 | 4.06 | 0.0006 |
| D21 | 27.3 | 30.1 | 7.64 | 0.58 |
| Overall | 34.0 | 32.6 | 4.39 | 0.62 |
| Skatole (ng/g) | | | | |
| D7 | 111.9 | 45.7 | 45.69 | 0.06 |
| D14 | 134.4 | 75.3 | 29.87 | 0.01 |
| D21 | 149.5 | 133.3 | 44.07 | 0.57 |
| Overall | 131.9 | 84.7 | 38.04 | 0.08 |
| Indole Yield (µg/d) | | | | |
| D7 | 47.2 | 64.8 | 18.80 | 0.17 |
| D14 | 37.1 | 21.0 | 9.91 | 0.03 |
| D21 | 24.4 | 29.8 | 9.84 | 0.40 |
| Overall | 36.2 | 38.6 | 9.46 | 0.70 |
| Skatole Yield (µg/d) | | | | |
| D7 | 140.0 | 53.9 | 66.18 | 0.07 |
| D14 | 130.9 | 71.9 | 30.15 | 0.02 |
| D21 | 132.5 | 126.3 | 38.91 | 0.80 |
| Overall | 134.4 | 84.1 | 42.08 | 0.09 |

Blood and plasma flows were not affected (P>0.05) by the treatments (Table 2). Arterial and venous concentrations of indole and skatole were lower (P<0.05) in ewes fed Sulla compared to the ewes drenched with PEG (Table 2). The arterial inflow of indole (PEG: 726.6 vs. CT: 279.7 SD (253.08) µg $min^{-1}$; P=0.05) and skatole (PEG: 1732.8 vs. CT: 747.6 SD (774.01); P=0.12) was lower in the control ewes. Results from the paired t-tests for the arterio-venous concentration differences indicate that they were not different (P>0.05) from zero (data not shown). Therefore, the effect of CT in Sulla on the net flux of indole and skatole should be interpreted with care. The data show a net removal of indole by the mammary gland and a net release of skatole, but these trends remain to be confirmed as the difference in arterio-venous concentrations were not significant.

**Table 2. Effect of condensed tannins on plasma flow, arterio-venous concentration difference and net flux of skatole and indole across the mammary gland in mid to late lactating ewes fed fresh Sulla.**

| Mammary Gland Measurement | Treatments | | Standard Deviation | Probability |
|---|---|---|---|---|
| | PEG (n = 4) | Control (n= 4) | | |
| Blood Flow (mL/min) | 342.9 | 400.1 | 131.41 | 0.53 |
| Plasma Flow (mL/min) | 265.5 | 316.2 | 105.11 | 0.49 |
| Plasma Indole (ng/mL) | | | | |
| Mesenteric Artery | 2.8 | 0.8 | 0.69 | 0.02 |
| Mammary Vein | 2.7 | 0.7 | 0.49 | 0.005 |
| Plasma Skatole (ng/mL) | | | | |
| Mesenteric Artery | 6.5 | 2.2 | 2.86 | 0.08 |
| Mammary Vein | 6.5 | 2.2 | 2.57 | 0.06 |
| Net Plasma Flux ( g/min) | | | | |
| Indole | 22.5 | 29.1 | 59.84 | 0.89 |
| Skatole | -14.4 | -8.3 | 149.48 | 0.95 |

## DISCUSSION

This study reports for the first time that CT in Sulla reduced the secretion of indole and skatole in milk. This reduction could have been achieved by CT reducing the supply of these two amines to the mammary gland and/or by an indirect effect of CT on fat secretion, as these amines are far more soluble in fat than in blood (Conochie 1953). The latter explanation can be discarded because there was no effect of CT on milk fat secretion, and the effects of CT on concentrations in milk reflected effects on concentrations in circulating plasma. More likely, the effect of CT was due to a reduction in the degradation of dietary protein in the rumen and therefore, a reduction in the supply of indole and skatole to the blood circulation.

This study also provides new evidence of the net fluxes of indole and skatole across the mammary gland. The arterio-venous concentration differences of indole and skatole across the mammary gland were not significantly different from zero indicating that the balance of the processes (production, catabolism, storage, secretion) dealing with indole and skatole in the mammary gland results in a outflow of these compounds in the vein draining the mammary gland similar to their inflow to this tissue. This suggests that the transfer of indole and skatole into milk from the plasma is essentially passive. Blood constituents such as albumin and other smaller molecules often appear in milk without the need for active transport mechanisms (Larson 1985). This phenomenon is called transport through "leaky tight junctions" and the significance of this process to milk composition increases as lactation progresses (Larson 1985). It is most likely that indole and skatole "leak" into milk directly from the blood. However, these net flux results do not exclude a role of the mammary gland in the production or metabolism of these compounds, nor allow us to distinguish between the processes that might occur in the mammary gland.

The effect of CT on reducing indole and skatole secretion in milk did not persist beyond 14 days of feeding Sulla. This suggests that either the rumen adapted to the presence of the CT in the diet or the liver became less effective at removing the indole and skatole as lactation progressed. The present results disagree with those of Conochie (1953) who showed that high levels of indole in blood are correlated with high levels in milk. Indeed, the decrease in milk indole and skatole concentration and yield did not persist to day 21, but the plasma concentrations of indole and skatole were reduced in the CT ewes at day 28. Assuming a similar concentration and yield of indole and skatole in milk between treatments on day 21 and day 28 (not measured), the lower arterial inflow observed with the CT treatment at day 28 might not have been as marked as that occurring at day 14 (not measured) and could therefore, have prevented an effect on milk concentration and yield at days 21 and 28.

Alternatively, it is possible that other tissues such as the small intestine or liver were involved in the metabolism of indole and skatole. The flux of skatole and indole across the small intestine and portal-drained viscera and liver also measured in this experiment will provide more information on the movements of these amines from the rumen to the mammary gland. The integration of the data from the small intestine, portal-drained viscera and liver with the mammary and milk data reported here will allow us to determine which tissue(s) are likely to be the major site(s) of metabolism of indole and skatole in the lactating ruminant.

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Email:nicole.roy@agresearch.co.nz and warren.mcnabb@agresearch.co.nz