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The effect of condensed-tannins in fresh Sulla *(Hedysarum coronarium)* on the net flux of fatty acids across the mammary gland and their secretion in the milk of lactating ewes

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ABSTRACT

Twelve sheep were surgically prepared with catheters in the mesenteric artery and mammary vein and a transonic flow probe around the pudendal artery to quantify the effect of condensed-tannins (CTs) on net mammary flux of fatty acids (FAs) and their secretion in milk. Two weeks later (Day 0), sheep were fed fresh Sulla (1500 g DM/d), which contains medium to high concentration of CTs (43.7 g CTs/kg DM). Six ewes were orally drenched (four times/d) with polyethylene glycol (160 g/d in water) to remove the effects of CTs; the remaining ewes received water. On Day 28, the mammary plasma flow and net flux of most FAs were similar between treatments. Arterial supply of C23:0, C18:2, C18:3, C20:1 and C22:5 to the mammary gland was increased ($P<0.10$) with CTs. The increase for C18:2 and C18:3 was concomittant with increased ($P<0.10$) concentrations in milk. No effect of CTs on total fat concentration and yield in milk was observed. Conclusions from this study indicate that CTs in Sulla increased the milk concentration of C18:2 and C18:3 by increasing arterial supply of these FAs to the mammary gland.

Keywords: milk; plasma net flux; fatty acids; condensed tannins; sheep.

INTRODUCTION

Feeding ewes with *Lotus corniculatus* reduces milk fat concentration (Wang *et al*., 1996) but this has not been observed in dairy cows (Harris *et al*., 1998; Woodward *et al*., 1999). The mechanism behind the reduction in milk fat concentration in lactating ewes is unclear and to our knowledge, there are no indications that this effect is specific for particular fatty acids (FAs).

Plant complex lipids are rapidly hydrolysed in the rumen, with the resultant unsaturated FAs suffering extensive biohydrogenation to FAs and their isomers (Bikerstaffe *et al.,* 1972). These free FAs pass from the rumen adsorbed to partly digested plant material. How CTs interact with biohydrogenation of FAs in the rumen is largely unknown. *In vitro* studies have shown that adding CTs extracted from *Lotus corniculatus* to rumen bacterial cultures reduces the specific growth of some, but not all, proteolytic rumen bacteria (Min, 1999). We hypothesized that CTs in Sulla affect rumen microbes involved in biohydrogenation and that this may change the profile of FAs leaving the rumen. Our objective was to investigate the effects of CTs on the net flux of FAs across the mammary gland of ewes and on their secretion in milk.

MATERIALS AND METHODS

Experimental animals

Experimental procedures were reviewed and approved by the Crown Research Institute, Animal Ethics Committee in Palmerston North (New Zealand). Four days after lambing, 12 Romney ewes were transferred indoors to metabolism crates and fed Lucerne pellets and chaffed Lucerne hay (1200:800 g/d). The ewes were milked twice daily with milk let down assisted by an intravenous injection of oxytocin (1 IU).

Surgical preparations

Eight days after lambing, a catheter was placed in the mesenteric artery (Huntington *et al*., 1989) and a transonic flow probe was fitted around the pudendal artery. Two days prior to sampling, a catheter was inserted into the mammary vein for blood sampling.

Treatments

After recovering from surgery (Day 0; third week of lactation), all ewes were offered fresh Sulla (1500 g DM/d; 43.7 g CTs/kg DM) for 28 days. Half the ewes were orally drenched (four times/d) with polyethylene glycol (PEG; 160 g/d in water) to remove the effects of the CTs (CT inactive; PEG group) whilst the remaining ewes received a drench of water (CT active; CT group). The treatments were applied according to a completely randomised block design.

Concentration of FAs in feed and in milk

The FAs were extracted from freeze-dried ground Sulla samples and methylated (Slover & Lanza, 1979; AOCS, 1992) after addition of 1 mL of an internal standard (C13; 4 mg/mL). They were quantified using a Hewlett Packard gas-liquid chromatograph equipped with a flame-ionisation detector (GLC; model 6890; SGE BPX70 column, 120 m length, 0.25 mm internal diameter, 0.25 μm thick; split ratio, 50:1).

Whole milk (50 mL) from each milking was collected on two consecutive days, kept at 4°C and then pooled for each ewe for analysis of FA concentration. The pooled samples were preserved with 10% Bronopol (2-Bromo-2-nitro-1, 3-propanediol; 10 μL/mL milk) before being frozen at –20°C until analysis. Milk fat was obtained by centrifuging the milk samples at 750 g for 4 min. The cream was removed and centrifuged at 25,000 g for 3 min, then melted at 60°C and re-centrifuged at 25,000 g for 3 min. The FAs in the fat were processed and analysed with the procedures described for feed except 0.1 μL of sample was injected onto a BPX 70 column (10 m length, 0.1 mm internal diameter, 0.1 μm thick).

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Whole milk (4 mL) from four consecutive milkings was collected each week and mixed with 25 µL of sodium azide (2%) and frozen until analysis for protein, fat and lactose by near-infrared spectroscopy in transmission mode (Back *et al*., 2001).

Net FA fluxes

On Day 28, 1,500 units of ovine heparin/h were infused intravenously to allow continuous removal of blood for three consecutive 2-hour periods from the mesenteric artery and the mammary vein. Blood samples were centrifuged at 4000 g for 15 min (4°C) and 2 mL of plasma from each sample was pooled for each vessel and ewe and kept at -20°C until analysis for FA concentration. On the day of analysis, a FA internal standard (C21:0) was added to pooled plasma samples and the lipids were extracted using a two-step chloroform/methanol (1:1 v/v) procedure. The FAs in the choloroform layer were methylated by adding 2 mL of a methanolic boron trifluoride solution (14%). The concentrations of methylated FAs were measured using a GLC according to the procedure as described previously except 1 µL of sample was injected onto a BPX 70 column (30 m length, 3.2 mm internal diameter, 0.25 µm thick).

Mammary blood flow was continuously measured during blood sampling using a transonic flow probe. Arterial supply of FAs to the mammary gland was calculated by multiplying the plasma flow (mL/min) by the plasma arterial concentration of FAs (mg/mL). Net plasma flux of FAs across the mammary gland was calculated as plasma flow (mL/min) x (venous concentration - arterial concentration; mg/mL). A negative flux indicated removal, whereas a positive flux indicated release of FAs by the mammary gland.

Statistical analysis

The profile of total FAs extracted from feed, milk and plasma according to the extraction procedure described in this study represents the FAs in all lipid fractions (triacylglycerol, phospholipids, cholesterol and free FAs). Data were analysed using the GLM procedure of SAS (SAS Institute, Inc. 1999-2001) according to a completely randomised block design. Paired t-tests were used to compare arterial and venous FA concentrations. Least-squares means (± standard error of the means) are presented in the tables. Significant statistical differences between treatments were indicated by a $P<0.05$ whilst a $P<0.1$ was considered to represent a trend. Two CT ewes were discarded for some calculations: one ewe had a slow recovery from surgery and for the other ewe, the mammary vein catheter was not working on the day of sampling.

RESULTS

Feed and milk parameters

The FA composition (mg/g DM) of Sulla was: 3.60 for C16:0; 0.55 for C18:0; 0.65 for C18:1; 2.55 for C18:2; 15.40 for C18:3; 0.11 for C20:0; 0.08 for C20:4; 0.21 for C22:0; 0.20 for C24:0.

Dry matter and FA intakes were not affected by the CTs in Sulla (data not shown). Milk fat concentration (CT: 7.84 *vs*. PEG: 8.31 (SEM 0.78) %), milk yield (CT: 1200 *vs*. PEG: 1106 (SEM 100.33) g/d) and total milk fat yield (CT: 85.7 *vs*. PEG: 84.4 (SEM 13.17) g/d) were similar for the CT and PEG ewes before the experimental period. The CTs in Sulla did not affect milk fat concentration, milk yield or milk fat yield after 27 days of feeding (Table 1). On Day 27, a trend to lower ($P<0.10$) C4:0 and C17:0 but to increase ($P<0.10$) C14:0, C18:2 and C18:3 concentrations in milk was observed in CT ewes compared to PEG ewes (Table 1).

TABLE 1. Effect of condensed tannins in Sulla on milk yield and milk fatty acid concentration (least-square means) in mid to late lactation in ewes.

	Treatments[1]		SEM	P
	PEG ewes (n = 6)	CT ewes (n= 5)		
Whole Milk (g/d)	773.6	789.6	76.12	0.88
Fat (%)	6.43	6.76	0.218	0.30
Fat (g/d)	43.6	55.1	5.13	0.18
Saturated Fatty Acid (wt %)				
C4:0	2.33	2.14	0.049	0.02
C6:0	2.79	2.66	0.078	0.25
C8:0	2.87	2.88	0.125	0.95
C10:0	9.08	9.52	0.359	0.39
C12:0	4.94	5.42	0.240	0.18
C14:0	11.14	12.01	0.280	0.05
C15:0	1.36	1.24	0.054	0.14
C16:0	26.41	27.25	0.957	0.53
C17:0	0.82	0.75	0.016	0.01
C18:0	12.65	11.44	0.512	0.12
Total	74.39	75.30	1.278	0.61
Unsaturated Fatty Acid (wt %)				
C16:1	0.75	0.66	0.035	0.11
trans Vaccenic	1.27	1.40	0.178	0.61
cis Vaccenic	0.65	0.57	0.057	0.32
C18:1	19.19	17.65	0.793	0.19
trans C18:1	0.35	0.32	0.019	0.33
C18:2	1.25	1.47	0.082	0.08
C18:3	1.75	2.32	0.099	0.004
CLA[2]	0.56	0.57	0.104	0.96
Total	25.76	24.96	1.203	0.64

[1]PEG and CT ewes mean that ewes fed Sulla were either drenched with polyethylene glycol (PEG) to remove the effect of condensed tannins (CT) or drenched with water (CT active).
[2]CLA: *cis*-9 *trans*-11 conjugated linoleic acids.

Blood parameters

Plasma concentration of most saturated and unsaturated FAs in the mesenteric artery (Table 2) and mammary vein (data not shown) were similar for CT and PEG ewes. The CTs tended to increase ($P<0.10$) the arterial plasma concentration of C23:0 and C18:3 and increased ($P<0.05$) the plasma concentration of C18:2, C20:1 and C22:5. The concentration of C14:0 and *trans* C18:1 in the mammary vein tended to decrease whereas that of C18:2, C18:3, C20:4 and C24:0 tended to increase ($P<0.10$) in CT ewes compared to PEG ewes (data not shown).

Mammary plasma flow was not affected by the treatments (PEG: 259.7 *vs*. CT: 355.6 (SEM 41.29) mL/min). The mammary arterial supply of C18:2, C18:3, C20:1, C20:4, C22:5 and C22:6 tended to be higher ($P<0.10$) for CT ewes than for PEG ewes (Table 3).

Results from the paired t-tests for the venous-arterial

TABLE 2: Effect of condensed tannins in Sulla on arterial plasma fatty acid concentration (least-square means) in mid to late lactation in ewes.

Concentration (μg/mL)	Treatments[1]		SEM	P
	PEG ewes (n = 6)	CT ewes (n= 5)		
Saturated Fatty Acid				
C14:0	6.20	6.80	1.042	0.67
C15:0	9.36	9.84	1.197	0.77
C16:0	182.63	202.86	18.945	0.45
C17:0	14.93	15.87	1.736	0.70
C18:0	222.95	269.66	26.090	0.22
C23:0	2.17	2.96	0.313	0.10
C24:0	1.78	2.23	0.245	0.20
Total	440.02	508.68	83.134	0.31
Unsaturated Fatty Acid				
C16:1	13.67	9.52	2.212	0.20
trans Vaccenic	11.46	14.16	2.219	0.39
cis Vaccenic	9.56	9.39	0.924	0.90
C18:1	257.85	230.00	30.098	0.51
trans C18:1	4.93	4.38	0.800	0.62
trans C18:2	4.33	4.23	0.552	0.90
C18:2	144.48	208.06	20.532	0.05
g C18:3	4.40	3.92	0.886	0.70
C18:3	127.97	188.42	21.726	0.08
CLA[2]	6.17	6.49	1.288	0.85
C20:1	1.64	3.34	0.498	0.04
C20:3	3.21	3.21	0.308	1.00
C20:4	24.92	29.86	2.071	0.12
C20:5	40.09	45.66	3.202	0.24
C22:5	36.71	46.51	3.060	0.05
C22:6	27.57	38.30	4.842	0.14
Total	718.92	843.65	83.134	0.30
Total Fatty Acid	1158.94	1352.33	129.344	0.31

[1]PEG and CT ewes mean that ewes fed Sulla were either drenched with polyethylene glycol (PEG) to remove the effect of condensed tannins (CT) or drenched with water (CT active).
[2]CLA: *cis*-9 *trans*-11 conjugated linoleic acids.

TABLE 3: Effect of condensed tannins in Sulla on arterial plasma supply (least-square means) of fatty acids to the mammary gland in mid to late lactation in ewes.

Arterial Supply (mg/min)	Treatments[1]		SEM	P
	PEG ewes (n = 6)	CT ewes (n= 5)		
Saturated Fatty Acid				
C14:0	1.58	2.19	0.439	0.31
C15:0	2.40	3.43	0.611	0.24
C16:0	47.20	72.59	12.705	0.17
C17:0	3.85	5.69	0.981	0.19
C18:0	57.90	97.55	17.089	0.12
C23:0	0.56	1.14	0.244	0.11
C24:0	0.46	0.75	0.164	0.21
Total	113.96	182.73	31.718	0.14
Unsaturated Fatty Acid				
C16:1	3.48	3.09	0.789	0.72
trans Vaccenic	3.08	5.50	1.404	0.23
cis Vaccenic	2.46	3.35	0.563	0.26
C18:1	67.04	82.79	18.680	0.53
trans C18:1	1.28	1.46	0.312	0.67
trans C18:2	1.12	1.42	0.297	0.45
C18:2	37.40	77.68	15.436	0.09
g C18:3	1.13	1.33	0.308	0.62
C18:3	33.20	69.60	13.776	0.08
CLA[2]	1.65	2.56	0.822	0.41
C20:1	0.43	1.32	0.304	0.06
C20:3	0.82	1.10	0.202	0.32
C20:4	6.38	11.25	1.665	0.06
C20:5	10.37	16.29	2.417	0.10
C22:5	9.40	17.01	2.393	0.05
C22:6	7.02	15.33	2.911	0.07
Total	186.26	310.33	59.674	0.16
Total Fatty Acid	296.24	487.90	90.418	0.15

[1]PEG and CT ewes mean that ewes fed Sulla were either drenched with polyethylene glycol (PEG) to remove the effect of condensed tannins (CT) or drenched with water (CT active).
[2]CLA: *cis*-9 *trans*-11 conjugated linoleic acids.

concentration differences of FAs indicate they were not different from zero (data not shown) therefore, the effect of CTs on the net flux of FAs across the mammary gland should be interpreted with care. The data suggest that total saturated FAs were removed by the mammary gland in CT ewes whereas they were released in PEG ewes (Table 4). Only the venous-arterial concentration difference and the net flux of C24:0 were affected ($P<0.05$) by the CT treatment with a release in CT ewes and a marginal uptake in PEG ewes (Table 4). The net flux of total unsaturated FAs indicates a release in CT ewes compared to a removal of these FAs by the mammary gland in PEG ewes. Only the venous-arterial concentration difference and the net flux of g C18:3 tended to be different ($P<0.10$) between CT and PEG ewes. Overall, the FAs (saturated and unsaturated) were released by the mammary gland in CT ewes whereas a removal was observed with PEG ewes. These changes were not significant (Table 4).

DISCUSSION

This study reports for the first time that CTs in Sulla increased the concentration of C18:2 by 18% and C18:3 by 33% in milk. These FAs are essential for the animal as they cannot be synthesized *de novo* (Maynard *et al.*, 1969), so their increase in milk must be the consequence of increased arterial supply of triacylglycerides and/or free FAs to the mammary gland. Indeed, the effects of CT on concentrations of C18:2 and C18:3 in milk are reflective of changes in their arterial plasma concentration and mammary arterial supply.

From these results it would be expected that the mammary gland would take up more C18:2 and C18:3 from plasma and incorporate it into the milk fat. The venous-arterial concentration difference for C18:2 and C18:3 were not, however, different from zero in both CT and PEG ewes because the increase in C18:2 and C18:3 concentration in arterial plasma was matched by an increase in C18:2 and C18:3 concentrations in venous plasma. The mammary venous-arterial concentration difference of FAs is the balance between the following processes: arterial supply; synthesis; the secretion into milk fat, lymph or other compounds; use as precursors for synthesis of other FAs; oxidation and release from the mammary cells into the venous circulation. Two of these processes are nonexistent or negligible in the

TABLE 4: Effect of condensed tannins in Sulla on net fatty acid flux (least-square means) in mid to late lactation in ewes.

Net Flux (mg/min)[1]	Treatments[2] PEG ewes (n = 6)	CT ewes) (n= 4	SEM	P
Saturated Fatty Acid				
C14:0	0.62	0.20	0.556	0.57
C15:0	-0.10	0.03	0.143	0.50
C16:0	0.86	0.82	2.282	0.99
C17:0	0.16	-0.31	0.403	0.40
C18:0	-1.20	-4.97	2.454	0.27
C23:0	-0.051	0.09	0.158	0.52
C24:0	-0.002	0.41	0.092	0.02
Total	0.28	-4.05	4.874	0.51
Unsaturated Fatty Acid				
C16:1	-0.19	0.19	0.340	0.41
trans Vaccenic	-0.13	-0.75	0.328	0.19
cis Vaccenic	0.03	-0.11	0.152	0.50
C18:1	-2.1	1.70	4.419	0.53
trans C18:1	0.09	-0.18	0.238	0.42
trans C18:2	-0.03	-0.001	0.198	0.92
C18:2	-0.81	3.69	2.599	0.22
g C18:3	-0.08	0.20	0.105	0.08
C18:3	-0.49	2.42	1.918	0.28
CLA[2]	-0.04	-0.32	0.130	0.14
C20:1	0.19	0.48	0.556	0.69
C20:3	-0.10	-0.006	0.059	0.23
C20:4	0.052	0.17	0.221	0.68
C20:5	-0.13	0.27	0.215	0.20
C22:5	-0.05	-0.25	0.212	0.50
C22:6	0.06	0.56	0.412	0.37
Total	-3.75	8.10	9.402	0.36
Total Fatty Acid	-3.46	4.05	13.942	0.69

[1]A negative flux indicates a removal whilst a positive value indicates a release of fatty acids by the mammary gland.
[2]PEG and CT ewes mean that ewes fed Sulla were either drenched with polyethylene glycol (PEG) to remove the effect of condensed tannins (CT) or drenched with water (CT active).
[3]CLA: *cis*-9 *trans*-11 conjugated linoleic acids.

mammary gland: C18:2 and C18:3 cannot be synthesized by mammalian cells (Maynard *et al*., 1969) and mammary oxidative degradation of FAs is negligible (Annison *et al*., 1967). It is possible that C18:2 and C18:3 liberated from the triacylglycerides of chylomicrons and lipoproteins by the action of lipoprotein lipase present at the surface of the blood capillaries were not all taken up by mammary cells and some appeared in the venous plasma. This would result in a net release of these FAs in the mammary vein (West *et al*., 1972; Miller *et al*., 1990). Our net flux data suggest a similar mechanism of uptake and bypass for C18:2 and C18:3, both of which were released by the mammary gland in CT ewes concomitant with an increase in C18:2 and C18:3 concentrations in milk.

The arterial flow of other unsaturated FAs such as C20:1, C20:4, C22:5 and C22:6 also tended to be increased by the CTs. The C20:4 and C22:5 can be synthesized *de novo* from C18:2 and C18:3, respectively (Maynard *et al*., 1969). These FAs were not present in detectable amounts in milk.

Although specific FAs were affected by CTs, no effect of CTs in Sulla was observed on milk fat concentration. Our results agree with those obtained from studies using dairy cows which have shown that CTs in *Lotus corniculatus* does not affect milk fat concentration (Harris *et al*., 1998; Woodward *et al*., 1999). The composition of milk FAs reported in this study is comparable in most instances to data reported for dairy cows (Chouinard *et al*., 1999; Enjalbert *et al*., 2000).

Overall, our results suggest that CTs alter the metabolism of FAs somewhere between the rumen and the mammary gland and this alteration results in a greater supply of C18:2 and C18:3 to the mammary gland. Earlier studies have reported that 80 to 95% of the dietary C18:2 and C18:3 were hydrogenated to C18:0 in the rumen (Bickerstaffe *et al*., 1972). Min (1999) has shown *in vitro* that CTs inhibit the growth of some rumen microbes whilst other strains are relatively resistant to the effect of CTs. Most likely, the increased C18:2 and C18:3 concentrations in milk resulted from the action of CTs on the metabolic activities of some microbes in the rumen such that more C18:2 and C18:3 appeared in the abomasum. Alternatively, it is possible that lipid synthesis and oxidation in the liver has been altered by CTs allowing increased C18:2 and C18:3 release into the blood. The flow of FAs appearing in the abomasum and the net flux of the FAs across the small intestine, the portal drained viscera and the liver were also measured in this study and will provide more information on the movements of FAs from the rumen to the mammary gland.

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