Effect of Forage to Concentrate Ratio and Monensin Supplementation on cis-9, trans-11 Conjugated Linoleic Acid and trans-11 Octadecenoic Acid Concentrations of Ruminal Contents and Plasma in Sheep*

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ABSTRACT : Twenty-four cannulated Small-tailed Han×Poll Dorset wethers (BW 47.5±2.1 kg) were used to determine the effects of forage to concentrate ratio (40:60 vs. 70:30), monensin supplementation (0, 15 or 30 ppm, DM basis) and interactions of these two factors on *cis*-9, *trans*-11 conjugated linoleic acid (*cis*-9, *trans*-11 CLA) and *trans*-11 octadecenoic acid (*trans*11-$C_{18:1}$) concentrations in ruminal contents and plasma in sheep. The experiment was designed as a 2×3 factorial. The diet contained Chinese wild rye grass hay (*Aneurolepidium Chinese*), cracked corn, soybean meal, NaCl, limestone and trace mineral premix. Dietary crude fat and linoleic acid ($C_{18:2n-6}$) were adjusted with soybean oil to about 7.0% and 24.0 mg/g (DM basis), respectively. High forage diets increased ($p<0.001$) the concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in ruminal contents and plasma. Monensin supplementation increased ($p<0.001$) the concentration of *trans*11-$C_{18:1}$ in ruminal contents, but had no effect on that of *cis*-9, *trans*-11 CLA. Concentrations of *trans*11-$C_{18:1}$ ($p<0.019$) and *cis*-9, *trans*-11 CLA ($p<0.022$) in plasma increased with dietary monensin levels. Interactions of forage: concentrate ratio and monensin level tended to affect the concentrations of *trans*11-$C_{18:1}$ ($p<0.091$) and $C_{18:2n-6}$ ($p<0.083$) in ruminal contents. Increasing forage levels increased the concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in the rumen. Supplementing with monensin increased the ruminal production of *trans*11-$C_{18:1}$ and concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in plasma. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 5 : 699-704)***

Key Words : *cis*-9, *trans*-11 CLA, *trans*11-$C_{18:1}$, Forage to Concentrate Ratio, Monensin, Rumen, Plasma

INTRODUCTION

Several factors, such as oil source (Wang et al., 2002a; An et al., 2003; Choi and Wang, 2005), supplementation type of oil (Wang et al., 2002b), forage to concentrate ratio (Wang et al., 2003) and pH (Wang and Song, 2003; Qiu et al., 2004; Kim et al., 2005) affected the production of *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$ in the rumen.

The ionophore monensin inhibits lipolysis and to some extent unsaturated fatty acid biohydrogenation (Van Nevel and Demeyer, 1995), so monensin may also affect the production of *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$ in the rumen. The *cis*-9, *trans*-11 CLA has potential health benefits in humans and is also synthesized from *trans*11-$C_{18:1}$ endogenously (Griinari and Bauman, 1999; Khanal, 2004). But effects of monensin on the production of *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$ in rumen have received little attention. Limited studies (Fellner et al., 1997; Jenkins et al., 2003; Wang et al., 2005) were carried out *in vitro*, and the results were variable. There have been no reports on the effects of monensin on *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$ concentrations in plasma of sheep. Kucuk et al. (2001) reported that forage to concentrate ratio influenced duodenal flow of *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$. The objective of this study was to determine the effects of forage to concentrate ratio, monensin levels and interactions of these two factors on the concentrations of *cis*-9, *trans*-11 CLA and *trans*11-$C_{18:1}$ in ruminal contents and plasma in sheep.

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MATERIALS AND METHODS

Animals and diets

Twenty-four Small-tailed Han×Poll Dorset wethers (BW 47.5±2.1 kg) fitted with ruminal cannulas were housed in individual stalls and randomly allocated to one of six groups with four wethers per group.

The experiment was designed as a 2×3 factorial with two forage to concentrate ratios (40:60 vs. 70:30), and three monensin levels (0, 15 and 30 ppm, DM basis, as M0, M1 and M2). Dietary crude fat and linoleic acid were adjusted with soybean oil to about 7.0% and 24.0 mg/g (DM basis), respectively. Major fatty acid composition of soybean oil, soybean meal and corn were presented in Table 1, and ingredient and chemical composition were presented in Table 2. Monensin was added in the form of Rumensin™ (Elanco Animal Health, A Division of Eli Lilly & Co.). Diets were formulated to be isonitrogenous and meet the CP requirements for 100 g of gain/d (NRC, 1985). To ensure full consumption, diets were fed restrictedly and 1.2 kg

Table 1. Composition of C_{18}-fatty acid (% of total fatty acid) of soybean oil, soybean meal and corn

Items	Lipid (% DM)	Composition of C_{18}-fatty acid (%)			
		Stearic acid $C_{18:0}$	Oleic acid $C_{18:1n-9}$	Linoleic acid $C_{18:2n-6}$	Linolenic acid $C_{18:3n-3}$
Soybean oil		4.05	23.19	54.39	6.04
Soybean meal	3.23	4.66	13.89	55.55	8.05
corn	5.52	2.15	32.91	48.42	1.03

Table 2. Ingredient and chemical composition of experimental diets (% DM basis)

Items	40:60 [1]	70:30 [1]
Ingredient		
Chinese wild ryegrass hay (*Aneurolepidium Chinese*)	40	70
Cracked corn	48.9	16.7
Soybean meal	7.2	8.3
Soybean oil	2.8	4
NaCl	0.5	0.5
Groud limestone	0.4	0.3
Trace mineral premix [2]	0.2	0.2
Chemical composition		
Crude protein	11.29	11.22
Neutral detergent fiber	35.41	51.48
Acid detergent fiber	18.72	29.57
Crude fat	6.88	7.03
Ca	0.36	0.38
P	0.21	0.19
C_{18}-fatty acid composition (mg/g DM)		
Stearic acid ($C_{18:0}$)	1.67	1.95
Oleic acid ($C_{18:1n-9}$)	12.63	10.80
Linoleic acid ($C_{18:2n-6}$)	24.10	23.59
Linolenic acid ($C_{18:3n-3}$)	2.09	2.81

[1] Forage to concentrate ratio.
[2] Trace mineral premix contained (mg/g, DM basis) Fe (10), Mn (15), Zn (21), Cu (7.5), I (0.65), Co (0.125) and Se (0.05).

(DM) feed were fed at 06:00 and 16:30 in two equal allotments. Coarsely chopped (4 cm) Chinese wild rye grass hay (*Aneurolepidium Chinese*) was fed first and concentrate was fed later. The soybean oil was mixed with concentrate immediately before feeding. Water was freely available at all times.

Sample collection

The experimental period lasted 15 days, with 12 days for diet adaptation and 3 days for sample collection. On day 13, about 10 ml blood was sampled via jugular venipuncture into heparinized vacutainers from each wether before the morning feeding of the hay. The blood was immediately placed on ice and centrifuged within 1 h at 2,500×g for 15 min at 4°C and the plasma was frozen at -20°C.

On day 14, about 150 ml of whole ruminal contents was collected from each wether at 0, 3 and 6 h after the concentrate was fed in the morning. Collection time was delayed 1.5 h on day 15 to provide one sample every 1.5 h interval over a 9 h period after the concentrate was fed in the morning. Ruminal pH was measured from fresh ruminal samples. The sample was placed in a bag, and frozen at -20°C after the air was evacuated.

Sample processing and analysis

Ruminal samples were thawed and bulked on an approximately equal volume basis for each wether. The mixed sample was lyophilized and ground to pass through a 0.5 mm screen. Total lipids of ruminal content samples were extracted according to the method of Folch et al. (1957). Approximately 200 mg rumen contents were weighed into a test tube, and 8 ml chloroform-methanol (2:1, v/v) solution and 1 ml heptadecanoic acid ($C_{17:0}$, 2 mg/ml in chloroform-methanol solution, internal standard) were added. The homogenate was filtered through Whatman paper into another test tube. The first test tube and the Whatman paper were rinsed with the same chloroform-methanol solution, and the rinse fluid was filtered into the second test tube, making the final dilution volume to 20 ml. The crude extract was washed with 4 ml 0.017% $MgCl_2$ solution. The upper phase was removed and the chloroform in the lower phase was dried under the flow of N_2. Fatty acid methyl esters (FAME) were prepared according to Beaulieu et al. (2002) by incubating the lipids extracted at 50°C for 10 min in the presence of 1.2 M sodium methoxide, and after cooled the solution was incubated at 80°C for 60 min with 5% methanolic HCl (prepared by the slow addition of 10 ml acetyl chloride to 10 ml methanol). The FAME were analyzed using a gas chromatograph HP6890 (Hewlett-Packard, Avondale, PA) equipped with an automatic sampler HP15896C (Hewlett-Packard, Avondale, PA). Separations were accomplished using a 100-m CP7489 (Varian, Walnut Creek, CA) capillary column (0.25 mm i.d. and 0.2 μm film thickness). Helium was used as carrier gas. The running conditions of gas chromatograph were as follows: 250°C injector temperature; 250°C detector temperature; column oven temperature program set for 180°C for 45 min, followed by an increase of 10°C/min to 215°C, and then maintained for 17 min. The plasma was thawed, and 1 ml plasma was added to a test tube, then lipids extraction, followed by FAME preparation and analysis as described above.

Ruminal biohydrogenation of individual and total C_{18}-unsaturated fatty acids was calculated according to Wu et al. (1991).

Table 3. Ruminal biohydrogenation (%) of individual or total 18-carbon unsaturated fatty acids

Items	40:60 [3]			70:30 [3]			Effects of Monensin			Effects of ratio		SEM [4]	Pr>F [5]		
	M0	M1	M2	M0	M1	M2	M0	M1	M2	40:60	70:30		M [6]	R [7]	R×M [8]
$C_{18:1\ n-9}$ [1]	73.92	75.64	74.37	76.18	74.79	75.03	75.05	75.21	74.70	74.64	75.33	1.34	0.927	0.537	0.526
$C_{18:2\ n-6}$ [1]	87.12	85.96	83.27	94.98	94.35	92.82	91.05^{A}	90.16^{AB}	88.05^{B}	85.45^{b}	94.05^{a}	0.82	0.005	<0.001	0.579
$C_{18:3\ n-3}$ [1]	90.68	90.27	88.87	93.06	92.06	91.52	91.87	91.16	90.19	89.94^{b}	92.21^{a}	0.85	0.172	0.004	0.878
Total C_{18}-USFA [2]	83.63	83.34	81.15	89.49	88.62	87.66	86.56^{A}	85.98^{AB}	84.41^{B}	82.71^{b}	88.59^{a}	0.88	0.064	<0.001	0.783

Means in the same row with different superscripts differ significantly (p<0.05).

[1] Individual C_{18}-unsaturated fatty acid (USFA) biohydrogenation (%) = 100-100×(individual C_{18}-USFA/total C_{18}-fatty acid in ruminal contents) /(individual C_{18}-USFA/total C_{18}-fatty acid in feed).

[2] Total C_{18}-USFA biohydrogenation (%) = 100-100×(total C_{18}-USFA/total C_{18}-fatty acid in ruminal contents)/(individual C_{18}-USFA/total C_{18}-fatty acid in feed).

[3] Forage to concentrate ratio. [4] Standard error of the mean. [5] Probability levels. [6] Monensin level.

[7] Forage to concentrate ratio. [8] Interactions of forage to concentrate ratio and monensin level.

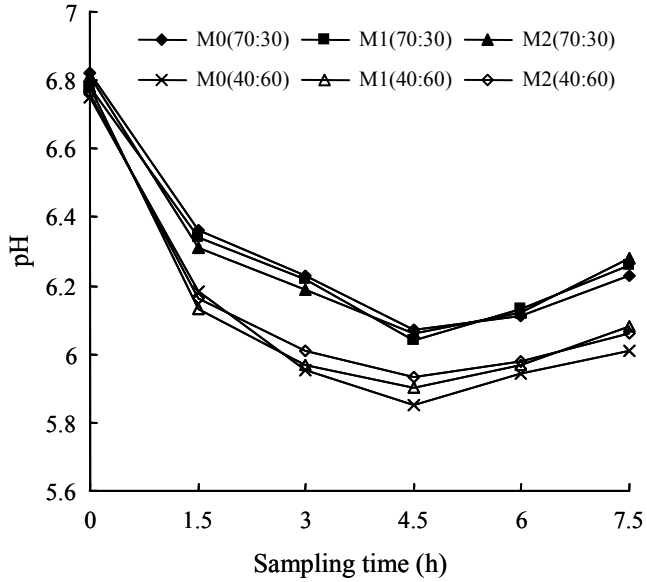

Figure 1. Ruminal pH at various sampling times.

Statistical analysis

All data were analyzed by analysis of variance for a 2×3 factorial design using a GLM procedure of SAS version 8 (SAS Inst. Inc., Cary, NC).

RESULTS

Ruminal pH was lower in low forage diets than in high forage diets, and was similar among monensin treatments.

The ruminal biohydrogenation of $C_{18:2n-6}$ (p<0.001), $C_{18:3n-3}$ (p<0.004) and total C_{18}-unsaturated fatty acids (p<0.001) was increased by high forage diets, and that of $C_{18:2n-6}$ decreased (p<0.005) with dietary monensin levels (Table 3).

*Trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA were the predominant isomers of C18:1 and CLA, respectively. Concentrations of these two intermediates in ruminal contents were increased (p<0.001) by high forage diets (Table 4). The concentration of $C_{18:2n-6}$ was decreased (p<0.001) while that of C18:0 was increased (p<0.015) by high forage diets.

The concentration of *trans*11-$C_{18:1}$ increased (p<0.001) with dietary monensin levels and was higher in the group of high forage diet with 30 ppm monensin, while that of *cis*-9, *trans*-11 CLA was unaffected by monensin supplementation (Table 4). Concentrations of total C18:1 and $C_{18:2n-6}$ were increased (p<0.001) while that of C18:0 was decreased (p<0.050) by monensin supplementation.

Concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in plasma were greater (p<0.001) in wethers fed with high forage diets than in wethers fed with low forage diets, which reflected those in ruminal contents (Table 5). Concentrations of *trans*11-$C_{18:1}$ (p<0.019) and *cis*-9, *trans*-11 CLA (p<0.022) increased with dietary monensin levels and were greater in the group of high forage diet with 30 ppm monensin than those in groups with 0 or 15 ppm monensin.

Concentrations of *trans*11-$C_{18:1}$ (p<0.091) and $C_{18:2n-6}$ (p<0.083) in ruminal contents tended to be affected by interactions between forage: concentrate ratio and monensin level (Table 4). Concentrations of other fatty acids in ruminal contents and plasma were unaffected by the interactions.

DISCUSSION

Effects of forage: concentrate ratio

Low ruminal biohydrogenation of $C_{18:2n-6}$ in groups of low forage diets might be caused by the lower rumen pH due to the high carbohydrate level in low forage diets. The main ruminal biohydrogenating bacteria are cellulolytic (Harfoot, 1981), and the biohydrogenation of unsaturated fatty acids needs free COOH radicals which form from lipolysis (Kepler et al., 1970). Low ruminal pH can depress the activity of cellulolytic bacteria (Russell and Baldwin, 1979) and Wang and Song (2001) reported the release of free $C_{18:2n-6}$ from ground oilseeds was decreased by lower pH derived from higher addition level of carbohydrate *in vitro*. Others (Kalscheur et al., 1997; Kucuk et al., 2001) also found the ruminal biohydrogenation of $C_{18:2n-6}$ was lower in low forage diets.

Table 4. Concentrations (mg/g ruminal DM) of C_{18}-fatty acid in ruminal contents

Items	40:60 [1]			70:30 [1]			Effects of Monensin			Effects of ratio		SEM [2]	Pr>F [3]		
	M0	M1	M2	M0	M1	M2	M0	M1	M2	40:60	70:30		M [4]	R [5]	R×M [6]
$C_{18:0}$	21.64	20.62	19.55	25.02	23.61	21.21	23.33^{A}	22.12^{AB}	20.38^{B}	20.60^{b}	23.28^{a}	1.22	0.050	0.015	0.764
Total $C_{18:1}$	10.23	11.70	15.15	12.26	13.56	14.42	11.25^{B}	12.63^{B}	14.79^{A}	12.36	13.41	0.87	0.003	0.156	0.235
*cis*9-$C_{18:1}$	2.44	2.35	2.70	2.50	2.63	2.53	2.47	2.49	2.61	2.50	2.55	0.17	0.667	0.685	0.449
*trans*11-$C_{18:1}$	4.09	5.99	9.41	8.13	9.40	10.22	6.11^{C}	7.69^{B}	9.82^{A}	6.50^{b}	9.25^{a}	0.73	<0.001	<0.001	0.091
$C_{18:2\ n-6}$	2.69	3.04	3.93	1.18	1.34	1.64	1.93^{B}	2.19^{B}	2.79^{A}	3.22^{a}	1.39^{b}	0.17	<0.001	<0.001	0.083
cis-9, *trans*-11 CLA	0.23	0.22	0.31	0.50	0.46	0.45	0.36	0.34	0.38	0.25^{b}	0.47^{a}	0.06	0.792	<0.001	0.564
$C_{18:3\ n-3}$	0.23	0.25	0.31	0.20	0.22	0.23	0.22	0.24	0.27	0.26^{a}	0.22^{b}	0.03	0.157	0.043	0.635

Means in the same row with different superscripts differ significantly ($p<0.05$).
[1] Forage to concentrate ratio. [2] Standard error of the mean. [3] Probability levels. [4] Monensin level.
[5] Forage to concentrate ratio. [6] Interactions of forage to concentrate ratio and monensin level.

Table 5. Concentrations (mg/100 ml) of C_{18}-fatty acid in plasma

Items	40:60 [1]			70:30 [1]			Effects of Monensin			Effects of ratio		SEM [2]	Pr>F [3]		
	M0	M1	M2	M0	M1	M2	M0	M1	M2	40:60	70:30		M [4]	R [5]	R×M [6]
$C_{18:0}$	45.53	43.85	40.26	49.61	46.67	45.46	38.79	37.82	36.20	43.21	47.25	4.02	0.516	0.235	0.957
Total $C_{18:1}$	45.63	45.21	44.48	55.63	58.22	60.15	43.07	47.50	49.20	45.11^{b}	58.00^{a}	4.69	0.936	0.003	0.834
*cis*9-$C_{18:1}$	26.99	29.52	27.29	34.57	31.79	31.22	26.43	26.99	26.74	27.93	32.53	3.39	0.883	0.114	0.730
*trans*11-$C_{18:1}$	3.95	7.63	8.95	11.27	17.56	18.76	7.24^{B}	11.88^{A}	13.02^{A}	6.84^{b}	15.86^{a}	2.10	0.019	<0.001	0.785
$C_{18:2\ n-6}$	47.12	49.31	49.29	50.48	54.53	53.10	43.64	47.06	48.46	48.57	52.70	3.79	0.696	0.199	0.968
cis-9, *trans*-11 CLA	0.63	1.05	1.16	1.95	2.30	2.39	1.23^{B}	1.58^{AB}	1.67^{A}	0.95^{b}	2.21^{a}	0.17	0.022	<0.001	0.955
$C_{18:3\ n-3}$	1.22	1.54	1.44	1.03	1.34	1.32	0.21	0.24	0.27	1.40	1.23	0.23	0.348	0.368	0.982

Means in the same row with different superscripts differ significantly ($p<0.05$).
[1] Forage to concentrate ratio. [2] Standard error of the mean. [3] Probability levels. [4] Monensin level.
[5] Forage to concentrate ratio. [6] Interactions of forage to concentrate ratio and monensin level.

Decreased concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in ruminal contents in low forage diets might also be caused by the lower ruminal pH. Wang and Song (2003) found that the production of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA increased with increasing pH when the culture was incubated with rapeseed. Martin and Jenkins (2002) reported that pH values less than 6.0 had the most influence on the production of *trans*-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in continuous culture. In the present experiment, the lower biohydrogenation of $C_{18:2n-6}$ induced by low forage diets might depress the isomerization of *cis*-9, *trans*-11 CLA from $C_{18:2n-6}$. Kucuk et al. (2001) found that the duodenal flow of *trans*11-$C_{18:1}$ increased as dietary forage increased from 45.8% to 72.9%, and the flow of *cis*-9, *trans*-11 CLA increased as dietary forage from 18.4% to 45.8%. Sackmann et al. (2003) reported that the duodenal flow of *trans*11-$C_{18:1}$ increased while that of *cis*-9, *trans*-11 CLA did not change significantly as dietary forage increased form 12% to 36%.Wang et al. (2003) found the proportion of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in ruminal fluid was not affected significantly by forage level (85% vs. 70%), which might be due to the narrow ratio of forage to concentrate.

Concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in plasma reflected those in ruminal contents as influence by dietary forage level. Wang et al. (2003) found increased tendency of *cis*-9, *trans*-11 CLA proportion in plasma collected at 9 h post feeding as dietary forage level increased. Higher concentration of total C18:1 in high forage diets was due to the higher concentration of *trans*11-$C_{18:1}$.

Effects of monensin supplementation

The main ruminal bacteria hydrogenating $C_{18:2n-6}$ were Gram-positive and monensin can inhibit the activity and growth of Gram-positive bacteria, which might be responsible for the lower biohydrogenation of $C_{18:2n-6}$ induced by monensin supplementation. Fellner et al. (1997) and Wang et al. (2005) also found monensin inhibited the hydrogenation of $C_{18:2n-6}$ *in vitro*.

Effects of monensin on the ruminal production of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA are variable. In present study, monensin supplementation increased the concentration of *trans*11-$C_{18:1}$, but did not affect that of *cis*-9, *trans*-11 CLA in ruminal contents. Jenkins et al. (2003) found *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA were both unaffected by monensin addition *in vitro*. The cultivating of Butyrivibrio fibrisolvens A38 with monensin has shown that the accumulation of *cis*-9, *trans*-11 CLA was unaffected by monensin (Kim, 2003). But Fellner et al. (1997) and Wang et al. (2005) found monensin addition increased the production of *cis*-9, *trans*-11CLA *in vitro*.

Based on the lower ruminal biohydrogenation of $C_{18:2n-6}$ and the lower concentration of C18:0 in ruminal contents, the possible reason for the accumulation of *trans*11-$C_{18:1}$ in ruminal contents might be the monensin inhibited the hydrogenation of *trans*11-$C_{18:1}$ to C18:0. Jenkins et al. (2003) also found that monensin addition increased the

proportion of total *trans*-$C_{18:1}$ and decreased that of $C_{18:0}$ *in vitro*. The exact mechanism by which monensin induced the accumulation of *trans*11-$C_{18:1}$ is required to determine further.

In spite of the similar concentration of *cis*-9, *trans*-11 CLA in ruminal contents, its concentration in plasma increased with monensin supplementation levels. The result could be due to the endogenous synthesis of *cis*-9, *trans*-11 CLA from *trans*11-$C_{18:1}$ in sheep tissues. Griinari and Bauman (1999) have proposed that a major proportion of *cis*-9, *trans*-11 CLA in tissues and milk lipids originated from endogenous synthesis from *trans*11-$C_{18:1}$ by Δ^9-desaturase in animal's tissues. Griinari et al. (2000) found *cis*-9, *trans*-11 CLA can be synthesized in bovine mammary gland. In what tissue the synthesis of *cis*-9, *trans*-11 CLA from *trans*11-$C_{18:1}$ occurs and what a role this synthesis plays in increasing the *cis*-9, *trans*-11 CLA concentration in sheep plasma and tissues are needed to examine from further research.

Interactions of forage to concentrate ratio and monensin levels

In the present study, interactions of forage to concentrate ratio and monensin levels tended to occur for concentrations of *trans*11-$C_{18:1}$ and $C_{18:2n-6}$ in ruminal contents. Jenkins et al. (2003) found that the proportions of $C_{18:0}$ and $C_{18:1}$ were affected by the interactions between soybean oil and monensin levels, and the proportions of *trans*10-$C_{18:1}$ and $C_{18:2}$ were affected by interactions of grain source, soybean oil and monensin levels. It appears that the effect of monensin may be affected by several factors, such as forage levels, grain source and other factors unknown.

CONCLUSION

Increasing dietary forage to concentrate ratio increased the ruminal biohydrogenation of $C_{18:2n-6}$ and the concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in ruminal contents and plasma of sheep. Supplementing monensin inhibit the biohydrogenation of $C_{18:2n-6}$ and increased the production of *trans*11-$C_{18:1}$ in rumen and increased the concentrations of *trans*11-$C_{18:1}$ and *cis*-9, *trans*-11 CLA in ruminal contents and plasma of sheep.

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