Short communication: A decrease in diameter of milk fat globules accompanies milk fat depression induced by conjugated linoleic acid supplementation in lactating dairy cows

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ABSTRACT

Milk fat is secreted from the mammary gland in the form of milk fat globules (MFG). Although milk fat depression has been studied since the beginning of the last century, the extent to which this phenomenon alters MFG synthesis is not fully understood. The aim of this study was to evaluate the effect of conjugated linoleic acid (CLA) on the size and distribution of MFG during milk fat depression in dairy cows. Twelve Holstein cows in mid lactation (145 ± 31 d in milk, 583 ± 34.6 kg of body weight, and 27.2 ± 2.4 kg of milk/d) were randomly assigned to a control diet or control plus Ca-protected CLA at 15 g/kg of dry matter for a 6-d period. The average diameter and particle size distribution of MFG were measured using a Mastersizer 3000 laser particle size analyzer (Malvern Instruments Ltd., Malvern, UK). Feeding CLA did not affect dry matter intake (16.2 ± 0.4 kg/d), milk production (28.4 ± 0.4 kg/d), milk protein, or lactose, but it decreased milk fat content (3.46 vs. 2.52%). In addition, surface area-related mean diameter of fat globules in cows fed CLA was lower compared with controls (3.02 vs. 3.45 μ m). The percentage of large fat globules decreased and that of small fat globules increased in response to CLA. Overall, the data suggest that the milk fat depression induced by CLA is accompanied by a decrease in average diameter of MFG.

Key words: lactation, lipid droplet, fatty acids, nutrition

Short Communication

Milk fat depression (MFD) is a syndrome characterized by a reduction of milk fat content (Bauman and Griinari, 2000) often caused by feeding high-grain diets alone or in combination with vegetable or fish oil (Bau-

man and Griinari, 2003; Bichi et al., 2013). Although many theories have been proposed to explain MFD, the bulk of available in vivo data to date provides strong support for the *trans* fatty acid theory as major cause of MFD (Bionaz and Loor, 2008). This theory states that *trans*-10,*cis*-12 CLA (an intermediate of ruminal biohydrogenation; Loor et al., 2002) directly inhibits mammary synthesis of milk fat through direct effects on lipogenic enzymes (Bauman et al., 2008). Since 2000, numerous studies have confirmed the negative effect of CLA on the synthesis of milk fat (Baumgard et al., 2000; Loor and Herbein, 2003; Viswanadha et al., 2003; Harvatine et al., 2018).

Milk fat is secreted from mammary cells as milk fat globules (MFG), unique structures composed mainly of a triacylglycerol core surrounded by a lipid bilayer membrane similar to the apical membrane of mammary epithelial cells. The diameter of MFG in secreted milk varies depending on breed (Michalski et al., 2005), genetics (Argov-Argaman et al., 2013), lactation stage (Mesilati-Stahy and Argov-Argaman, 2014), and diet (Lopez et al., 2008). For instance, diets with different concentrate-to-forage ratios reduced MFG diameter of milk accompanied by decreased milk fat (Argov-Argaman et al., 2014). Therefore, mechanisms controlling MFG size are also plausibly a component of MFD.

We hypothesized that reduction of milk fat in response to feeding CLA is associated with altered MFG size. We used a rumen-protected product rich in *trans*-10,*cis*-12 CLA (Han et al., 2012) to induce MFD and measured MFG size using a laser particle size analyzer.

All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care Committee of Henan Agricultural University. Twelve multiparous Holstein cows (145 ± 31 DIM, 583 ± 34.6 kg of BW, 27.2 ± 2.4 kg of milk/d) were randomly divided to receive either a control basal diet or the control plus Ca-protected CLA at 15 g/kg of DM (Qingdao Auhai Biotech Co. Ltd., Qingdao, China). All cows were adapted to the respective diets for 3 d followed by 6 d of sampling. Rations were formulated

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to meet the cows' predicted nutrient requirements according to the National Research Council (NRC, 2001). Ingredients and chemical composition of the diets are reported in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17845>). The commercial rumen-protected CLA contained similar amounts of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17845>). Cows were housed in a naturally ventilated barn, fed individually, and milked 3 times daily at 0600, 1400, and 2000 h. They had ad libitum access to their respective diets as a TMR after each milking. Feed offered and refused was measured daily for each cow and recorded through the experimental period to calculate DM intake. Milk yield was recorded daily. To shorten the interval between sampling and analysis of MFG, milk samples were taken at the 1400 h milking. Milk samples were divided into 2 samples; one sample was sent to Henan DHI Testing Center to analyze milk fat, milk protein, and milk lactose concentrations using infrared spectrophotometry (Foss 120 Milko-Scan, Foss Electric, Hillerød, Denmark). The second sample was processed immediately to measure diameter and size distribution of MFG using a Mastersizer 3000 laser particle size analyzer (Malvern Instruments Ltd., Malvern, UK).

Based on the Mastersizer 3000 operating instructions, the parameter of refractive index was set at 1.560 with stirrer speed at 2,000 rpm. In preliminary experiments, we found that adding EDTA to dissociate casein micelles (~150 nm) was unnecessary. A total of 400 mL of water was added to a beaker. After measuring background, 1 mL of a pure milk sample was added to the beaker. The system automatically measured MFG parameters 3 times and calculated an average value. The values obtained for each sample were analyzed using Mastersizer software, which yielded volume-re-

lated equivalent diameter ($D_{[4,3]}$), surface area-related equivalent diameter ($D_{[3,2]}$), a particle size distribution diagram, and a table showing the percentage of size distribution.

Milk yield, milk composition and yield, and MFG diameter and distribution were all analyzed using the general linear model (GLM) and SPSS 25 software (SPSS/IBM Corp., Chicago, IL) for a full factorial model with repeated measures. The model contained time (1, 2, 3, 4, 5, and 6 d), treatment (control and CLA), and time $\times$ treatment interactions. Significant difference was declared as $P < 0.05$, and $0.05 \leq P \leq 0.10$ was defined as statistical trends.

No time effect ($P > 0.05$) or differences between treatments ($P > 0.05$) were observed for cow DMI, milk yield, milk protein and yield, or milk lactose and yield (Table 1). Supplementation with CLA led to a significant reduction of milk fat and yield ($P < 0.01$). Most research investigating CLA effects on milk composition has consistently demonstrated that the inhibitory effects are specific to milk fat; other components and milk yield are generally unaffected (Baumgard et al., 2001; Perfield et al., 2002; Viswanadha et al., 2003), which is consistent with results from the present study.

The size distribution graph in Figure 1 clearly shows that the peak of particle distribution gradually shifted from large to small diameter with time of CLA feeding. The size of MFG across mammalian species can vary from 200 nm to over 15 μm (Argov et al., 2008). We detected MFG sizes ranging mainly from 1.0 to 10.0 μm , while whereas MFG $< 1.0 \mu\text{m}$ and $> 10.0 \mu\text{m}$ were rare (Figure 1). Previous research reported an effect of diet on small ($D_{[4,3]}$ of 3.44 μm) and large MFG ($D_{[4,3]}$ of 4.53 μm ; Couvreur et al., 2007). For instance, extruded linseed supplementation (i.e., providing additional linolenic acid) to dairy cows decreased $D_{[3,2]}$ (3.66 μm) and

Table 1. Effects of a 6-d Ca-CLA supplementation to mid-lactation Holstein cows on lactation performance and milk composition

Item	Treatment ¹		SEM	P-value		
	Control	CLA		Treatment	Time	Treatment $\times$ time
DMI, kg/d	16.53	15.98	0.39	0.37	0.60	0.71
Milk yield, kg/d	28.76	28.03	0.37	0.19	0.22	0.12
Milk protein, %	3.35	3.17	0.09	0.21	0.56	0.51
Milk protein yield, g/d	966.45	887.15	31.39	0.10	0.72	0.23
Milk fat, %	3.46	2.52	0.14	< 0.01	0.02	< 0.01
Milk fat yield, g/d	998.76	702.51	42.84	< 0.01	0.11	< 0.01
Milk lactose, %	4.94	4.82	0.05	0.15	0.18	0.53
Milk lactose yield, g/d	1,421.72	1,353.21	30.19	0.14	0.11	0.22
TS yield, g/d	3,606.85	3,122.36	68.04	< 0.01	0.02	0.22
$D_{[4,3]}$, ² μm	4.12	3.73	0.13	0.07	0.08	0.50
$D_{[3,2]}$, ³ μm	3.45	3.02	0.11	0.02	0.54	0.02

¹n = 6 cows each in the control and CLA groups.

²Volume-related equivalent diameter.

³Surface area-related equivalent diameter.

increased $D_{[4,3]}$ ($4.73 \mu\text{m}$) MFG (Lopez et al., 2008). In the present study, compared with the control diet, feeding CLA led to lower $D_{[3,2]}$ (3.45 vs. $3.02 \mu\text{m}$, $P = 0.02$, Table 1) and tended to lower $D_{[4,3]}$ (4.12 vs. $3.73 \mu\text{m}$, P

$= 0.07$) MFG, suggesting a negative association among exogenous CLA, milk fat content, and MFG size.

Using different dietary treatments and doses of CLA, researchers have reported MFD specifically with the

Figure 1. Representative examples of milk fat globule size distribution between the control (control basal diet) and CLA (control plus Ca-protected CLA at 15 g/kg of DM) groups from d 1 to 6 of the experiment. The particle size distribution graph was generated by Mastersizer 3000 software (Malvern Instruments Ltd., Malvern, UK).

Table 2. Effects of a 6-d Ca-CLA supplementation to mid-lactation Holstein cows on the size distribution of fat globule particles (% of total fat globules)

Size, μm	Treatment ¹			P-value		
	Control	CLA	SEM	Treatment	Time	Treatment × time
1.19	0.10	0.58	0.10	0.01	0.31	0.21
1.36	1.00	2.03	0.21	<0.01	0.12	0.09
1.54	2.84	4.17	0.29	0.01	0.07	0.07
1.75	5.08	6.41	0.38	0.03	0.05	0.07
1.99	7.07	8.41	0.48	0.07	0.02	0.04
2.27	8.41	9.17	0.49	0.29	0.05	0.10
2.58	9.17	9.60	0.46	0.52	0.06	0.14
2.93	9.80	9.88	0.41	0.89	0.05	0.25
3.33	10.68	10.32	0.39	0.53	0.01	0.58
3.78	11.73	10.81	0.39	0.12	0.01	0.47
4.29	12.37	10.88	0.38	0.02	0.07	0.13
4.88	11.96	10.11	0.41	0.01	0.07	0.11
5.55	10.39	8.51	0.49	0.02	0.12	0.12
6.30	8.11	6.46	0.59	0.07	0.11	0.14
7.16	5.61	4.36	0.64	0.19	0.06	0.14
8.14	3.22	2.49	0.60	0.41	0.01	0.11
9.24	1.50	1.25	0.45	0.70	0.01	0.23

¹n = 6 cows each in the control and CLA groups.

trans-10,*cis*-12 isomer. For example, Baumgard et al. (2001) infused 0.0, 3.5, 7.0, and 14.0 g/d of *trans*-10,*cis*-12 CLA during a 5-d period (Baumgard et al., 2001), and Loor and Herbein (2003) infused 15 g/d for 2 d in cows fed high-oleic or high-linoleic oil. In the present study, CLA was the only source of PUFA and the amount fed was beyond the expected physiological levels that could reach the small intestine. Thus, future studies using other PUFA such as linoleic acid or physiological doses of CLA could provide more in-depth understanding of the mechanisms regulating MFG size and associations with milk fat content.

Feeding CLA led to greater percentage of small MFG (size from 1.19 to 1.99 μm) and lower percentage of large MFG (size from 4.29 to 6.30 μm; $P < 0.05$ or $0.05 \leq P \leq 0.10$, Table 2). Other MFG were not affected by feeding CLA ($P > 0.01$). The decrease in the average diameter of MFG following CLA supplementation was associated with an increase in small MFG and a decrease in large MFG (Table 2). Therefore, the decrease in the size of fat globule particles due to CLA was accompanied by changes in the size distribution of MFG.

Bovine mammary epithelial cells synthesize lipid droplets (LD) and secrete them as MFG into the milk, supporting the view that they serve as an indicator of mammary synthesis capacity for milk fat (Altenhofer et al., 2015). To our knowledge, this report is the first to document changes in MFG size during CLA-induced MFD. The data suggest that an increase in small fat globules along with a decrease in large fat globules was due to reduced milk fat synthesis capacity of the mammary gland during CLA-induced MFD. Molecular mechanisms linking CLA and MFD in ruminants have

mainly been focused on the downregulation of mRNA abundance of key de novo fatty acid synthesis enzymes (Gervais et al., 2009; Han et al., 2012; Harvatine et al., 2018). Although the precise mechanisms controlling the size of MFG are unclear, MFG size has been suggested to be determined by the size of LD produced before and during secretion (Argov-Argaman, 2019). Thus, a co-regulatory mechanism involving intracellular triacylglycerol synthesized, membrane material availability, and LD fusion has been proposed to exist in the control of LD size (Argov-Argaman, 2019). Clearly, more studies are needed to more precisely understand the mechanisms involved in controlling LD and MFG size along with their secretion by the mammary gland.

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