



## NOTE

Internal Medicine

# Relevance of serum concentrations of non-esterified fatty acids and very low-density lipoproteins in nulli/primiparous and multiparous cows in the close-up period

Shin OIKAWA<sup>1)\*</sup>, Haruka SAITOH-OKUMURA<sup>1)</sup>, Masaki TANJI<sup>1)</sup> and Ken NAKADA<sup>1)</sup><sup>1)</sup>Department of Veterinary Herd Health, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

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**ABSTRACT.** Serum concentrations of non-esterified fatty acids (NEFA) and very low-density lipoprotein (VLDL) in close-up dairy cattle were compared in relation to parity. Data were obtained from 37 nulli/primiparous (NP) and 24 multiparous (MU, parity: 2–7) cows between 14 days and 1 day prepartum. A positive correlation ( $r=0.684$ ,  $P<0.01$ ) was found between serum NEFA and VLDL concentrations in NP cows. Among the VLDL constituents, the NEFA concentration was particularly correlated with the triglyceride (TG) concentration ( $r=0.658$ ,  $P<0.01$ ). However, no significant correlation was found between the concentrations of NEFA and VLDL or VLDL-TG in MU cows ( $r=-0.028$  and  $0.307$ ). These results suggest the presence of higher hepatic secretion of NEFA-derived VLDL in NP cows.

**KEY WORDS:** cattle, non-esterified fatty acids (NEFA), parity, very low-density lipoprotein (VLDL)

Various periparturient diseases of dairy cattle are closely interrelated [5], and fatty liver development is considered to be a particularly important underlying factor in the onset of the diseases [13, 14, 16]. Hepatic lipidosis in dairy cattle is mainly triggered by a negative energy balance caused by stress, lactogenesis, and inadequate periparturient feed intake [4, 19]. When a dairy cow is in negative energy balance, triglyceride (TG) stored in the adipose tissue is mobilized as an energy source to compensate for the energy shortage [6, 9]. Briefly, triglyceride is hydrolyzed into non-esterified fatty acid (NEFA) and glycerol by hormone-sensitive lipase. Subsequently, NEFA is mobilized to the liver to be used as energy source via two pathways. In one, mobilized NEFA undergoes beta-oxidation in hepatocyte mitochondria and becomes acetyl-CoA, which enters the tricarboxylic acid cycle to produce energy. In the other pathway, NEFA entering the liver is re-esterified as triglyceride and then secreted into the periphery as very low-density lipoprotein (VLDL) together with apolipoprotein B-100 (ApoB-100), phospholipids, and cholesterol, where it is used as energy. However, when the liver is flooded with NEFA, the excess triglyceride that cannot be processed into VLDL accumulates in the liver. The bovine liver is especially physiologically inept at secreting VLDL [18], and develops hepatic lipidosis when such a situation persists. Moreover, TG accumulated in hepatocytes physically impedes the organelles, decreasing apolipoprotein production, VLDL production and secretion, and consequently the blood apolipoprotein concentration as well [12–14, 16, 21].

Vandehaar *et al.* reported that there was a positive correlation in cows and heifers between the plasma NEFA concentration from 1 to 2 days prepartum and liver triglyceride content within 1 day of parturition [22]. They found lower values of postpartum liver triglyceride content in heifers than in cows, even though no difference was found in the prepartum blood NEFA concentration. This suggests that even though their livers are exposed to similar levels of NEFA prepartum, heifers, unlike cows, are able to metabolize TG without having to store it. That is, their study indicates that heifers have relatively high hepatic lipid metabolism capacity, and that the ability to process NEFA is affected by parity. From the above, we derived the following hypotheses: the correlation between serum NEFA and VLDL concentrations will be low in dairy cattle with higher parity because the NEFA entering their livers will be easily accumulated as TG. In contrast, NEFA and VLDL values will correlate highly in dairy cattle with lower parity because their livers will easily process incoming NEFA into VLDL and secrete it into the blood. The purpose of the study reported here was to investigate the validity of these hypotheses by analyzing the NEFA and VLDL concentrations in nulli/primiparous (NP) and multiparous (MU) cows.

\*Correspondence to: Oikawa, S.: oishin@rakuno.ac.jp

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**Table 1.** Body condition, rumen fill scores, serum nonesterified fatty acids, very low-density lipoprotein the concentration of each of its constituents in nulli/primiparous and multiparous cows

Variables	BCS		RFS <sup>a)</sup>		NEFA (mEq/l)		VLDL (mg/dl)		VLDL-TG (mg/dl)		VLDL-PL (mg/dl)		VLDL-TC <sup>a)</sup> (mg/dl)		VLDL-Protein (mg/dl)	
	NP	MU	NP	MU	NP	MU	NP	MU	NP	MU	NP	MU	NP	MU	NP	MU
Mean	3.41	3.45	2.7	2.3	0.505	0.404	10.962	10.535	5.595	5.503	1.505	1.323	1.754	1.270	2.108	2.437
SE	0.04	0.08	0.1	0.1	0.068	0.051	0.751	0.912	0.565	0.543	0.121	0.115	0.169	0.174	0.182	0.727
Q1	3.25	3.00	2.0	2.0	0.245	0.253	7.820	7.582	3.070	3.618	1.040	0.970	0.800	0.680	1.320	1.075
Q2	3.50	3.50	3.0	2.0	0.370	0.330	9.920	9.445	4.920	5.360	1.250	1.130	1.780	0.990	1.950	1.585
Q3	3.63	3.75	3.0	3.0	0.595	0.498	12.010	12.420	6.540	6.750	1.945	1.583	2.370	1.863	2.660	1.986

a)  $P < 0.05$ , when nulli/primiparous (NP) and multiparous (MU) cows were compared. SE=standard error, Q1=25th percentile, Q2=50th percentile, Q3=75th percentile. BCS=body condition score, RFS=rumen fill score, NEFA=nonesterified fatty acids, VLDL=very low-density lipoprotein, TG=triglycerides, PL=phospholipids, TC=total cholesterol. The VLDL concentration is expressed as the total amount of each lipid and protein.

This study was conducted on a single dairy farm with approximately 200 parous cows milked two times daily in Ebetsu City, Hokkaido, Japan, from January 2010 through June 2011. We carried out regular health examinations of the cows and analyzed their blood profiles every other Monday or Tuesday. The dairy herd consisted of six groups: postfresh, high-yield, first-calf heifer, low-yield, far-off and close-up cattle. The close-up cattle and others were housed in a loose barn and 2-row free-stall pens, respectively. All animals were fed based on the National Research Council recommendations and treated appropriately, following the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which essentially conform to the guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the U.S.A. (NIH publication No. 86-23, revised 1996). The average 305-day milk yields of the primiparous and multiparous cows were 9,115 and 11,318 kg, respectively. A total of 61 Holstein dairy cattle in the close-up period were involved. These animals were at 14 days to 1 day prepartum of their late dry period, and did not have clinical signs after moving to far-off pens. They were split into 37 NP (parity of  $0.4 \pm 0.5$ ; parity 0=24, parity 1=13) and 24 MU cows (parity of  $2.7 \pm 1.2$ ; parity 2=15, parity 3 to 7=9). No animal had a clinical history of displaced abomasum, and they did not experience clinical ketosis, milk fever or retained placenta.

Caudal vein blood was collected from clinically healthy cattle at around 9:30 AM before feeding them total mixed rations, and stored immediately at 4°C. For blood collection, plain tubes were used for VLDL isolation and determination of the NEFA concentration [20]. Sera were separated and stored at 4°C and -20°C respectively, until analysis.

Assessments of the body condition score (BCS) and rumen fill score (RFS) were performed together with blood sampling. For the BCS, the method of Ferguson *et al.* was used [3]. According to the method of Zaaijer *et al.*, the RFS was used as an index to assess the adequacy of the dry matter intake (DMI) and passage rate of the feed consumed [10]. Briefly, the left paralumbar fossa each cow was observed from behind to assess the tension by DMI, which was scored on a scale from 1 to 5 (1=very poor, 2=poor, 3=good, 4=very good, 5=excellent).

Serum VLDL separation was performed based on the method described previously [17], using a fixed-angle rotor (TLA-110; Beckman Coulter, Fullerton, CA, U.S.A.) in an ultracentrifuge (OPTIMA TLX; Beckman Coulter) at 16°C.

The concentrations of TG, phospholipids, total cholesterol (TC) and protein in the VLDL fraction were measured using commercial kits (Wako Pure Chemicals, Osaka, Japan) [17]. The VLDL concentration was calculated as the total sum of TG, phospholipids, TC, and the protein concentration. The concentration of NEFA was measured using an automatic analyzer (Bio Majesty JCA-BM2250, JEOL Ltd., Tokyo, Japan) [15].

The occurrence of periparturient diseases (displaced abomasum, clinical ketosis, milk fever and retained placenta) was investigated using the farm's clinical data obtained within 30 days postpartum.

Measurements were statistically analyzed using computer software (SPSS version 17.0; SPSS, Chicago, IL, U.S.A.). The normality of data was verified by the Shapiro-Wilk test. Concentrations of NEFA and various VLDL constituents (TG, phospholipids, TC, and protein) were log-transformed for data processing [15]. The Pearson product-moment correlation coefficient was used for the correlation between the log-transformed NEFA concentration and various measurements. Student's or Welch's *t*-test was used to test for differences in mean values. Fisher's exact test was used for the rate of periparturient disease occurrence, and the odds ratio was calculated.

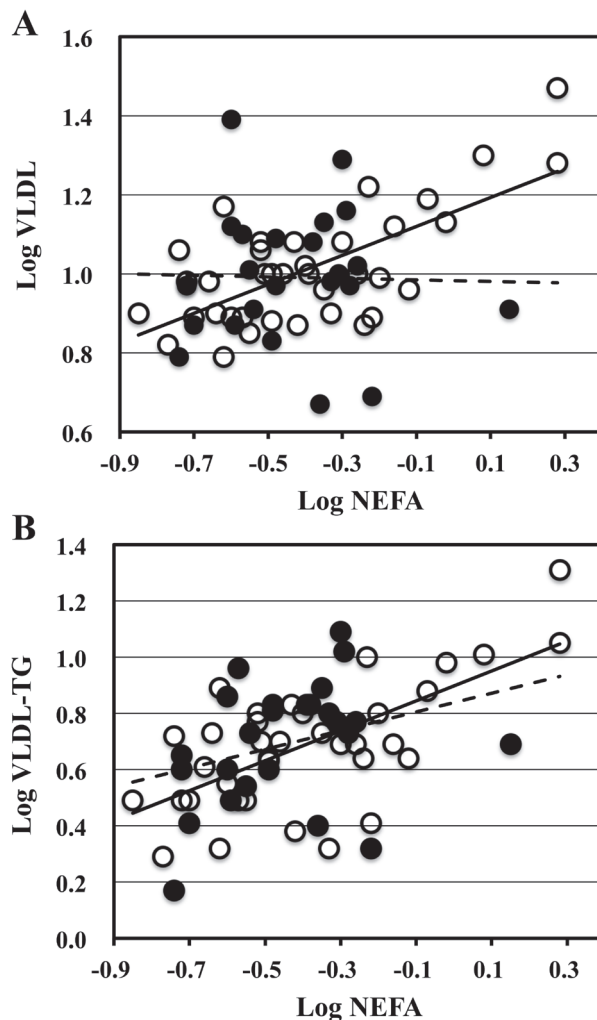
The BCS and RFS, as well as the concentrations of NEFA and VLDL, are shown in Table 1. The RFS of the MU cows was lower than that of the NP cows ( $P < 0.05$ ). Among the VLDL constituents, the TC concentration of NP cows was higher than that of MU cows ( $P < 0.05$ ). No other differences were observed between the two groups.

A significantly positive correlation was found between the NEFA and VLDL concentrations in NP cows ( $r = 0.684$ ,  $P < 0.01$ ; Fig. 1A). Among the VLDL constituents, the TG concentration was positively correlated with NEFA ( $r = 0.658$ ,  $P < 0.01$ ; Fig. 1B). In contrast, no correlation was found between NEFA and the VLDL/VLDL-TG concentrations in MU cows ( $r = -0.028$  and  $0.307$ , respectively). As shown in Table 2, a negative correlation was found between the NEFA concentration and RFS in both groups. A mild positive correlation was found between the NEFA concentration and BCS in MU cows.

The occurrence rates of periparturient diseases within 30 days postpartum were 21.6% (8 of 37 cows) for NP cows and 50.0% (12 of 24 cows) for MU cows. The odds of disease occurrence were high in MU cows (odds ratio: 3.6; 95% confidence interval: 1.2–11.1).

In NP cows, a significantly positive correlation was found between the NEFA and VLDL concentrations (Fig. 1A). Among the VLDL constituents, the TG concentration was positively correlated with NEFA (Fig. 1B). However, such correlations were not observed in MU cows. This suggested that hepatic synthesis and secretion of NEFA-derived VLDL occurred easily in NP cows as opposed to MU cows [22], which might be related to the observed difference in the postpartum occurrence rates of periparturient diseases between the two groups. Although the bovine liver is known to generally have inferior VLDL secretion ability compared to the rat liver [18], our study clarified the extent to which this ability differed according to parity. No significant differences were observed between the concentrations of each blood item in nulli- and primiparous cattle or between cows with parity 2 and those with parity  $\geq 3$  (data not shown).

Various organelles, enzymes, and proteins are necessary for hepatic VLDL synthesis [6]. Blood NEFA provides fatty acid to the liver, where it is esterified into TG in the cytoplasm. The other lipid components of VLDL, namely cholesterol and phospholipids, are also synthesized in the liver. Meanwhile, ApoB-100, which binds these lipids, is synthesized in the rough endoplasmic reticulum. The lipids and proteins are assembled by microsomal transfer protein (MTP) in the smooth endoplasmic reticulum, and then finally modified into VLDL particles in the Golgi apparatus to be secreted out of the liver. ApoB-100 is especially crucial to the particle formation, and its synthesis, along with VLDL secretion, is decreased in cattle with hepatic lipidosis [17, 21]. In a study comparing livers of calves and rats, VLDL-apolipoprotein production was similar whereas the secretion rate was lower in calves than rats, suggesting that VLDL secretion depends on factors other than ApoB-100 [7]. The lack of a difference between the VLDL protein concentrations in NP and MU cows observed in our study (Table 1) also supports a previous report that ApoB-100 production is not directly relevant to VLDL secretion. Bernabucci *et al.* investigated the mRNA expression of ApoB and MTP in pre- and postpartum cows, finding that the ApoB mRNA expression at 3 days postpartum was lower than that at 35 days prepartum, while the MTP mRNA expression at 3 days postpartum was higher than that at 35 days prepartum [1]. Such changes may be responsible for the lower hepatic VLDL secretion in the early postpartum period. In the present study we found a lower VLDL-TG concentration in MU cows than in NP cows (Table 1), which showed that there was a difference in the ratio of VLDL particle constituents. On the other hand, it has been reported in experiments using bovine hepatocytes cultured *in vitro* that sterol regulatory element-binding protein (SREBP) 1c overexpression increases lipid synthesis and decreases lipid oxidation and VLDL export, resulting in the induction of TG accumulation [11], and that low SREBP-1c expression can decrease lipid synthesis, increase lipid oxidation, and decrease the TG and VLDL contents [2]. When further pursuing the mechanism of change in VLDL secretion due to



**Fig. 1.** Relationship between serum NEFA and VLDL (A) or VLDL-TG (B) concentrations. ○=nulli/primiparous (NP), ●=multiparous (MU). Solid line=predicted for NP, dotted lines=predicted for MU. A  $\log_{10}$  transformation was applied to serum NEFA (mEq/l), VLDL (mg/dl) and VLDL-TG (mg/dl) concentrations because of their lognormal distribution.

**Table 2.** Correlation coefficients ( $r$ ) between serum NEFA concentration and body condition/rumen fill scores, or serum nonesterified fatty acids, very low-density lipoprotein and the concentration of each of its constituents in nulli/primiparous and multiparous cows

Cows	BCS	RFS	VLDL	VLDL-TG	VLDL-PL	VLDL-TC	VLDL-Protein
NP	0.295	-0.487 <sup>b</sup>	0.684 <sup>b</sup>	0.658 <sup>b</sup>	0.339 <sup>a</sup>	0.219	0.039
MU	0.595 <sup>b</sup>	-0.425 <sup>a</sup>	-0.028	0.307	0.039	-0.187	-0.247

a)  $P < 0.05$ , b)  $P < 0.01$ . NP=nulli/primiparous, MU=multiparous, BCS=body condition score, RFS=rumen fill score, NEFA=nonesterified fatty acids, VLDL=very low-density lipoprotein, TG=triglycerides, PL=phospholipids, TC=total cholesterol. The VLDL concentration is expressed as the total amount of each lipid and protein. Concentrations of NEFA, VLDL and its constituents (TG, PL, TC and protein) were log-transformed for data processing.

parity, mRNA expression of SREBPs and the aforementioned proteins involved in the assembly of VLDL will need to be taken into account.

A positive correlation was observed between the blood NEFA concentration and BCS in MU cows (Table 2). Decreased DMI leads to a low-energy state, which causes adipose tissue TG to be broken down by hormone-sensitive lipase and increases NEFA in the blood [6, 9]. Cattle with a high BCS in their dry period show an earlier DMI decrease than cattle with an adequate BCS [8]. Our data provide support for a series of connections between the BCS, DMI, and NEFA (Table 2). However, as there was no clear relation between the NEFA concentration and BCS in NP cows, we cannot simply assume that the NEFA concentration is increased just because the BCS is high during the dry period. On the other hand, the RFS showed a negative correlation with the NEFA concentration in both groups (Table 2). It seems that the BCS and RFS are relatively well linked in MU cows, but that the RFS is more effective than the BCS for assessing the low energy state in NP cows. In conclusion, we have illustrated the differences in hepatic VLDL secretion due to parity, confirming our initial hypotheses.

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