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エネルギー代謝関連脂質の組成に関する研究）

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Contents

Abbreviations

ANOVA: analysis of variance
BCS: body condition score
BW: body weight
CN: carbon number
COCs: cumulus–oocyte complexes
CP: crude protein
DB: double bond number
DIM: days in milk
DNA: deoxyribonucleic acid
D-PBS: Dulbecco's phosphate buffered saline
EE: ether extract
ESI: electrospray ionization
FFA: free fatty acid
h: hour
Hz: hertz
IS: internal standard
IU: international unit
kg: kilogram
kV: kilovolt
L: liter
LC/MS: liquid chromatography–mass spectrometry
MW: mature weight
Mcal: megacalories
MHz: mega hertz
min: minute
mL: milliliter
mm: millimeter
mmHg: millimeter of mercury
mmol: millimole
ms: millisecond
MS/MS: tandem mass spectrometry
NDF: neutral detergent fiber
NE_L: net energy for lactation

NFC: nonfiber carbohydrate

nmol: nanomole

NRC: nutrient requirements of dairy cattle

OPU: ovum pick up

PCA: principal component analysis

pmol: picomol

ppm: parts per million

psi: pound-force per square inch

PVA: polyvinyl alcohol

SCD: stearoyl-CoA desaturase

SD: standard deviation

SEM: standard error of the mean

TAG: triacylglycerol

TDN: total digestible nutrients

WG: weight gain

µL: microliter

µm: micrometer

µmol: micromol

Notes

The contents of Chapter I have been published in Theriogenology.

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Furukawa E, Chen Z, Kubo T, Wu Y, Ueda K, Chelenga M, Chiba H, Yanagawa Y, Katagiri S, Nagano M, Hui SP. Simultaneous free fatty acid elevations and accelerated desaturation in plasma and oocytes in early postpartum dairy cows under intensive feeding management. Theriogenology 2022;193:20-29.

Preface

Fertility in the modern dairy cow is becoming lower along with increasing in milk yields [1-3]. Pregnancy rate at the first service after parturition declined from approximately 70 to 40% over the past 50 years before 2000s [3]. Mechanisms for this impaired fertility include disrupted postpartum uterine recovery [4], embryonic loss [5], repeat breeders [6], impaired oocyte quality, delay in the first postpartum ovulation [7], and disrupted estrus expression [3]. These ovary and uterus dysfunctions are attributable to disorders in high producing cows [2] including severe negative energy balance [1], immune malfunction [8], and metabolic diseases [9]. Particularly, impaired oocyte quality and delay in the first postpartum ovulation are considered to mainly ascribed the suppression of the hypothalamic pituitary gonadal axis arising from negative energy balance [7].

Dry cows show a zero or positive energy balance until the peripartum period, and then enter a negative energy balance concurrently with delivery and the start of lactation [10, 11]. The mobilization of fat in the adipose tissue to produce energy increases the circulating levels of free fatty acid (FFA). Blood FFA levels start to increase in the peripartum period, peak typically within the first week of lactation, then decrease and return to the basal level after 6–8 weeks of lactation [12-15]. High postpartum blood FFA levels have been reported to induce lipid disorders, such as fatty liver [16], immune malfunction [17], and lipotoxicity to mammary glands [18]. Detrimental effects of oxidative stress, one of cytotoxicity induced by excessive circulating FFA, had been previously reported in the hepatocytes [19] and mammary glands [18] of lactating cows. Importantly, saturated FFA (FFA without double bond in its carbon chain, *e.g.*, palmitic and stearic acid) affects cell viability more adversely than monounsaturated FFA (FFA with one double bond in its carbon chain, *e.g.*, oleic acid) [20]. Supplementation of palmitic or stearic acid to culture medium during oocyte maturation lowered oocyte developmental competence [21], while oleic acid could reduce this detrimental effect of palmitic and stearic acid [21]. However, oocyte developmental competence was impaired when oocytes were cultured with supplementation of mixture of palmitic, oleic, and stearic acid that mimicked FFA composition and concentration in follicular fluid in postpartum cows [22].

Lipids have various biological functions in animal body including serving as components of biomembrane [23], energy source [24], and signal molecules [25]. Lipids can be classified into 8 categories based on the basic structure of lipids (fatty acyls and sterol lipids, *etc*.) and backbone structures (glycerolipids and sphingolipids, *etc*.) [26]. Among them, FFA and triacylglycerol (TAG) are the major lipid species that are involved in energy metabolism. FFA serves as a substance for synthesis of other lipids and energy production [27]. TAG, which comprises three fatty acyls linked to a glycerol backbone, is synthesized from excessive FFA

in cells, stored in lipid droplets [20], and decomposed to FFA again as necessary [28]. Storing TAG in lipid droplets is not only serve as an energy source in cells, but also protect cells from the cytotoxicity of FFA, particularly saturated FFA, by storing lipids in a non-toxic form [20]. However, when FFA levels increase beyond the ability of cells to synthesize TAG from FFA and store TAG in the cells, FFA is incorporated and accumulated, which leads to reactive oxygen species production [29], endoplasmic reticulum stress [30], ceramide accumulation [31], and, ultimately, apoptosis [32]. The protective mechanism of lipid droplets has been demonstrated in oocytes [21] and cumulus cells [33]. Cumulus cells are in direct contact with follicular fluid and protect oocytes from elevated FFA by converting them to TAG and storing lipid droplets in cumulus cells [33, 34]. However, it is not clear if and how high circulating FFA in postpartum cows affects oocyte quality through lipotoxicity.

Although some studies have reported that no differences in oocyte morphology [35] or developmental competence to blastocysts [36, 37] between different lactation stages, other studies showed the larger proportion and numbers of morphologically impaired oocytes at approximately 100 days in milk (DIM) than at 30 DIM [38]. These findings suggest that differences in the oocyte quality of cows between different lactation stages may not be readily detectable by morphology and developmental competence to blastocysts. However, developmental competence beyond blastocysts may differ between morphologically normal blastocysts [39]. For example, alterations in gene expressions including activation of pro-apoptotic pathways have been reported in post-hatching embryos matured with supplementation of high palmitic acid [39]. Therefore, to obtain detailed understanding in oocyte quality of lactating cows in relation to lipotoxicity, a study of oocytes at the molecular levels [40] that include proteins [41], gene expression [40, 42], and lipids [43] is warranted.

Oocytes can be retrieved from ovaries of living cows by using ovum pick up (OPU) method, but the number of oocytes collected per session is limited, with around 3–7 in average [35-38]. Examination on FFA and TAG compositions by high-resolution liquid chromatography–mass spectrometry (LC/MS) is a useful tool, especially for obtaining insights into lipotoxicity in oocytes of lactating cows. Recent study in our research group developed a LC/MS/MS method that enables to analyze oocyte FFA and TAG contents and compositions using a sample of 5 bovine oocytes [44].

The objective of the present study was to obtain insights into lipotoxicity in oocytes of the modern dairy cows with high milk production, particularly in their early postpartum period. Therefore, the contents and compositions of energy metabolism–related lipids (FFA and TAG) in plasma and oocytes of cows at different lactation stages were examined. Since oocyte FFA and TAG profiles of lactating cows may be affected by the feeding management systems

with different diets and milk production levels, cows under two different feeding management systems were used. Namely, cows under grazing management with the lower milk production were used in Chapter I, and those under intensive feeding management with the higher milk production were used in Chapter II.

Chapter I

High oocyte triacylglycerols concurrently with high plasma free fatty acids in postpartum cows

1. Introduction

Adverse effects of high FFA on bovine oocytes have been studied mainly *in vitro*, and cytotoxicity [43] and impaired developmental competence [21, 33, 45, 46] have been demonstrated. Furthermore, supplementation of high levels of oleic acid during maturation accelerated accumulation of lipid droplets (*i.e.*, TAG) in oocytes, although FFA levels in oocytes was not investigated in this study [21]. On the other hand, information on relationships between circulating high FFA and oocyte lipids *in vivo* is limited. A previous study indicated that the postpartum increase in blood FFA levels reflected elevated follicular fluid FFA levels [46]. Cows at 16 DIM showed a 3-fold higher blood FFA concentration and 1.5-fold higher follicular fluid FFA concentration than those at 44 DIM [46]. However, FFA concentrations and TAG contents in oocytes of postpartum cows have not been studied. A previous study examined the effects of a short-term exposure to high FFA concentrations on oocyte TAG contents [34]. This study used heifers fasted for 4 days as a high blood FFA model and demonstrated that TAG contents in oocytes did not increase despite high FFA concentrations in blood and follicular fluid [34]. However, the effects of high plasma FFA on oocytes for a longer period remain to be studied.

The present study investigated FFA and TAG compositions of plasma and oocytes of cows at different lactation stages under grazing management. Additionally, heifers, as a model animal of normal fertility, were used as a control group.

2. Materials and methods

2.1. Animals

The present study was implemented according to the animal experimental regulations of the Hokkaido University Animal Care and Use Committee (Approval No. 18-0028). Holstein cows and heifers were kept at the experimental farm of Hokkaido University (Sapporo, Japan). Eleven multiparous and 3 primiparous non-pregnant lactating cows (26–85 months of age, 1–5 parities) and 4 non-pregnant heifers with normal ovarian cyclicity (22–31 months of age) were used, and the study was conducted between June and August 2018. Cows were pastured all day and fed supplementary corn silage or housed in the barn all day and fed corn silage, hay, and concentrated feed. Heifers were kept in a free barn attached to a paddock and fed hay and wheat bran. Cows were milked twice daily (9:00 and 15:30), and the mean 305-

day milk yield of these cows was 7,710 kg.

To examine differences in the lipid compositions of plasma and oocytes between cows at different lactation stages, cows were categorized into the following three lactation groups: the early lactation group (38.7 ± 8.2, ranging between 25 and 47 DIM, n = 6); the period with a negative energy balance and high plasma FFA [47, 48], peak lactation group (62.5 ± 1.5, ranging between 61 and 65 DIM, n = 4); the period with a recovering energy balance and normal plasma FFA, and middle lactation group (175.8 ± 17.1, ranging between 160 and 202 DIM, n = 4); the period with a positive energy balance and normal plasma FFA [37, 49] (Table 1). Each lactation group included 1 primiparous cow. Mean daily milk yield for 7 days between 3 days before and after blood and oocyte collection is shown in Table 1. Body condition scores (BCS) (5-point scale) [50] were assessed by the same single assessor at the time of blood and oocyte collection (Table 1).

2.2. Sample collection

Oocyte collection was performed using the OPU method [51, 52] by a single operator. The ovary was depicted using an ultrasound imaging device (HS-2100; Honda Electronics, Toyohashi, Japan) equipped with a 9.0 MHz long-handled micro-convex probe (HCV-4710MV; Honda Electronics) designed for OPU. Cumulus-oocyte complexes (COCs) with follicular fluid were aspirated with the vacuum pressure of 100 mmHg using a single-lumen needle (17 gauge, length of 490 mm; Misawa Medical, Kasama, Japan) from follicles of 2 mm or greater in diameter. The single-lumen needle was connected to a 50-mL plastic conical tube (352070, Corning Inc., Corning, NY, USA) with a silicone tube, and the conical tube was joined to a vacuum pump with a foot-pedal switch (K-MAR-5000, Cook Medical Technology, Bloomington, IN, USA). Follicular fluid containing COCs was diluted with Dulbecco's phosphate-buffered saline (D-PBS) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO, USA) and 10 IU/mL heparin sodium (AY Pharmaceuticals Co., Ltd., Tokyo, Japan) to avoid blood coagulation. COCs were recovered under a stereomicroscope, and cumulus cells were removed by gentle pipetting with a fine glass pipette. Between 4 and 6 denuded oocytes were transferred to a 1.5-mL microcentrifuge tube (Eppendorf AG, Hamburg, Germany) with a small amount of D-PBS + 0.1% PVA (<10 μL). Oocyte samples were stored at -80°C until the lipidomic analysis.

Blood was collected by caudal venipuncture using ethylenediaminetetraacetic acid–loaded vacuum tubes (Terumo Co., Tokyo, Japan) at oocyte sampling and stored on ice. Plasma was separated by centrifugation within 4 h of collection and 100 μL of plasma was transferred

to a 1.5-mL microcentrifuge tube and stored at -80°C until the lipidomic analysis.

2.3 Lipidomic analysis by LC/MS

The solvents for lipid extraction and the LC/MS analysis were of spectral grade and purchased from Sigma-Aldrich unless otherwise specified. Authentic lipid compounds as internal standards (IS) were obtained from Sigma-Aldrich. The mixture of IS for oocyte and plasma samples was newly prepared with methanol (containing 0.006% butylated hydroxytoluene, w/v). The details of these species and concentrations are listed in Table 2.

Total lipid extraction from oocytes were performed according to Folch's method [53] as previously described [44]. Briefly, 4-6 oocytes in one Eppendorf® tube were extracted with 600 μL of ice-cold chloroform/methanol 2:1 (v/v, with IS) twice. Plasma lipids were prepared using the method of a previous study [54] with some modifications [55]. In brief, a 100-μL plasma sample was extracted with 800 μL of ice-cold chloroform/methanol 1:1 (v/v, with IS) twice. Extracted lipids were dried under a vacuum, dissolved in methanol, and filtered to remove any insoluble material prior to the LC/MS injection. To avoid lipid degradation and auto-oxidation, the extraction procedure was performed within 1 h.

LC/MS conditions were described in a previous study [44], with Prominence HPLC (Shimadzu Corp., Kyoto, Japan) coupled to an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc., San Jose, CA, USA) with an electrospray ionization (ESI) source. Sample lipids were separated on an Atlantic T3 C18 column (2.1 × 150 mm, 3 μm, Waters, Milford, MA, USA) with a flow rate of 200 μL/min. LC elution was performed using the mobile phase consisting of 5 mmol/L aqueous ammonium acetate, isopropanol, and methanol with the gradients shown in Table 3. The column and sample tray were held at 40°C and 4°C, respectively. MS data acquisition was performed under ESI positive and negative modes, with the following parameters being held constantly: MS capillary voltage, 3.0 kV; sheath gas (nitrogen) flow, 50 psi; auxiliary gas (nitrogen), 5 psi; resolving power for high-resolution MS, 60,000; scan speed, 2 Hz; scan ranges, *m/z* 150-1100 for the positive mode, *m/z* 220-1650 for the negative mode; MS/MS collision energy, 35.0; activation Q value, 0.25; activation time, 30 ms.

Spectrum processing was performed using the workstation Xcalibur 2.2 (Thermo-Fisher Scientific Inc.) with comparisons to the LIPIDMAPS database [56]. The species identified were annotated as "lipid class + total carbon number (CN) in the fatty chain(s) + total double bond number (DB) in the fatty chain(s)" (*e.g.*, FFA 14:0 and TAG 46:1) [44, 57]. TAG fatty acyl compositions were elucidated using MS/MS fragmentation as previously described [44]. The semi-quantitative amount of each lipid analyte was calibrated by its corresponding

internal standard as follows, and calculated data were exported for further analyses.

$$Analyte\ amount = IS\ amount \times \frac{Analyte\ peak\ area}{IS\ peak\ area}$$

2.4. Data analysis

In the multivariate statistical analysis, a principal component analysis (PCA) and cluster analysis were processed using R software 4.0.2 (https://www.r-project.org) and R-package mixOmics [58]. Since some lipid species are of physiological significance despite their small contents (*e.g.*, polyunsaturated fatty acids), each variance was normalized to have a unit mean value and variance before the PCA and cluster analysis by mixOmics. The Euclidean distance was used for distance measurements, and the complete linkage method was employed for the clustering method in the cluster analysis. Other statistical analyses were performed using JMP Pro 14.3.0 (SAS Institute, Cary, NC, USA). Mean values were compared using the paired *t*-test between two groups or a one-way ANOVA (using Tukey's *post hoc* test) in multiple groups. The relationship between two parameters was analyzed using Pearson's correlation coefficient, and P-values were calculated by a regression analysis. All data are shown as means ± SD, and $P < 0.05$ was considered to be significant.

3. Results

3.1. LC/MS analysis

In the present study, I detected and annotated 6 FFA and 45 TAG species from plasma and/or oocyte samples (listed in Table 4) according to their retention behavior on reversed-phase HPLC, as well as their protonated, ammoniated, or deprotonated ion signals on high-resolution MS. The lipid profile obtained by high-resolution LC/MS and MS/MS provided the identities of lipid species and their fatty acyl chains. Separated chromatographic peaks along with IS enabled the semi-quantitation of lipids for further multivariable analyses.

3.2. Lipid profiles of plasma and oocytes in different lactation groups

3.2.1. Relationship between plasma FFA, plasma TAG, and oocyte TAG

To investigate the relationship between total plasma FFA and total oocyte TAG, I performed a correlation analysis, and the results obtained revealed a positive correlation between plasma FFA and oocyte TAG ($r = 0.55$, $P < 0.05$) (Fig. 1). Plasma FFA and oocyte TAG were higher in all cows in the early lactation group than in heifers (Fig. 1). Plasma FFA and plasma TAG as well as plasma TAG and oocyte TAG showed negative correlations ($r = -0.62$ and $r = -0.72$, $P < 0.05$, respectively) when an outlier which showed high plasma TAG

(Early lactation (1) in Fig. 2AC) was excluded.

3.2.2. Clustering analysis of FFA and TAG species of cows in different lactation groups

To examine differences in lipid profiles between the lactation groups, I performed PCA with total FFA and TAG species in plasma and oocytes. Lactating cows and heifers were located in separate areas in the plasma and oocyte PCA, respectively, which indicated different lipid profiles between cows and heifers (Fig. 2AB). A hierarchical clustering analysis was performed to investigate differences in lipid profiles between the lactation groups (Fig. 2CD). Lipid species were categorized into 5 groups based on clustered lipid profiles in relation to different lactation groups: (1) saturated FFA (DB = 0); (2) unsaturated FFA (DB = 1 or 2); (3) 44–48 carbon–TAG (CN ranging between 44 and 48); (4) 50–54 carbon–TAG; and (5) 56–58 carbon–TAG (Fig. 2CD). In the cluster analysis of plasma, plasma FFA was higher in the early and peak lactation groups than in the middle lactation group and heifers (Fig. 2C). The cluster analysis of oocytes revealed that heifers showed the lowest contents of all lipids, except for saturated FFA (Fig. 2D). Among lactating cows, 50–54 carbon–TAG and 56–58 carbon–TAG were high in clusters including the early lactation group (*i.e.*, Early lactation (1)(4)(6) and (2)(3)(5), respectively, as indicated on the right side of the heat map, shown in Fig. 2D), and 44–48 carbon–TAG was high in the cluster including the peak lactation group (*i.e.*, Peak lactation (1)(3)(4)) (Fig. 2D). These results indicated that differences in lactation stages and experience of delivery were reflected in the composition of FFA and TAG species in plasma and oocytes.

3.2.3. FFA and TAG groups based on CNs and DBs in plasma and oocytes in cows in different lactation groups

FFA and TAG levels were compared between lactation groups (Fig. 3). In plasma, saturated and unsaturated FFA as well as total FFA was higher in the early lactation group than in the middle lactation group and heifers ($P < 0.05$) (Fig. 3A). In oocytes, 50–54 carbon–TAG and total TAG were higher in the early lactation group than in heifers ($P < 0.05$) (Fig. 3D). However, oocyte FFA was similar between lactation groups (Fig. 3B). In terms of the composition of these lipids, the proportion of plasma unsaturated FFA was higher in the early and the peak lactation groups (49.6% ± 6.3% and 49.9% ± 5.8%, respectively) than in the middle lactation group and heifers (32.6% ± 4.4% and 34.1% ± 5.4%, respectively) ($P < 0.05$) (Fig. 4A). In oocytes, 50–54 carbon–TAG accounted for approximately 70% of total TAG, which was similar between lactation groups (Fig. 4D).

3.2.4. FFA and TAG fatty chain compositions in plasma and oocytes

I examined differences in the fatty chain compositions of FFA and TAG between lactation groups. All FFA in plasma was significantly higher in the early lactation group than in the middle lactation group and heifers ($P < 0.05$) (Table 5). Oocyte fatty acyls 16:0, 16:1, 18:0, 18:1, 18:2, and 20:3 in TAG were significantly higher in the early lactation group than in heifers ($P < 0.05$) (Table 6). Fatty chain compositions were compared between lactation groups. Among plasma FFA, the early and peak lactation groups showed a significantly lower proportion of stearic acid (FFA 18:0) and higher proportion of oleic acid (FFA 18:1) than the middle lactation group and heifers ($P < 0.05$) (Fig. 5). However, only slight differences were observed in oocyte FFA and TAG fatty acyls between lactation groups (Fig. 5). The dominant fatty chains differed between plasma FFA (18:0, 18:1, and 16:0), oocyte FFA (18:0 and 16:0), and oocyte TAG (16:0, 18:1, and 16:1) (Fig. 5).

4. Discussion

This is the first study to describe the lipid contents and compositions of plasma and oocytes from cows at different lactation stages. Total oocyte TAG positively correlated with total plasma FFA. When FFA and TAG levels were compared between lactation groups, the early lactation group showed higher plasma FFA and oocyte TAG than heifers. A previous study that used fasted heifers as a high blood FFA model indicated that when blood FFA levels were high, FFA levels in follicular fluid and TAG contents in cumulus cells increased, whereas TAG in oocytes did not [34]. These findings suggested that the effects of elevated blood FFA were buffered at cumulus cells; therefore, oocytes were protected against the effects of high FFA [34, 43]. However, the present study suggested that high blood FFA affected and altered the quantity of oocyte TAG. Differences in the present results and previous findings [34] may be attributed to the different durations of the high blood FFA exposure period before oocyte sampling. Heifers were subjected to 4 days of fasting in the previous study [34], while cows in the early lactation stage in the present study were under high blood FFA conditions for more than 3–6 weeks between the time of peripartum and oocyte collection. Therefore, it may take between 4 days and 3 weeks for high plasma FFA levels to affect oocyte TAG contents. After the period from 4 days to 3 weeks under circulating high FFA condition, TAG contents in cumulus cells may exceed the capacity of storage in these cells, accordingly incorporated FFA in cumulus cells do not convert to TAG but overflow, and as the result, FFA may be directly or indirectly transferred from cumulus cells to oocytes via transporters such as transzonal projections [59] and/or CD36 [60]. Meanwhile, plasma and oocyte TAG showed a negative correlation, probably not because plasma TAG directly affected oocyte TAG, but because plasma TAG

correlated negatively with plasma FFA which were plausible to directly influence the increase of oocyte TAG content in the postpartum period. A more detailed investigation on blood FFA profiles after parturition and oocyte lipid compositions is needed.

Although the proportion of oleic acid to total FFA in plasma was higher in the early lactation stage than in heifers, the compositions of FFA and TAG fatty acyls in oocytes were similar between these lactation groups. Previous studies indicated that the most abundant FFA species in follicular fluid was oleic acid when the dominant blood FFA species was stearic acid [34, 46]. One possible explanation for this phenomenon is the high activity of stearoyl-CoA desaturase (SCD) at granulosa and cumulus cells [33]. SCD is an enzyme that converts saturated fatty acids (*e.g.*, 16:0 and 18:0) to monounsaturated fatty acids (*e.g.*, 16:1 and 18:1, respectively). These findings suggest that the effects of the blood FFA composition was buffered at the level of cumulus cells after blood FFA was incorporated into follicles, and support the present results showing that oocyte FFA and TAG fatty acyl compositions were stable. On the other hand, oocytes showed a higher palmitic acid proportion than plasma. The reason for this phenomenon may be the fatty acid preference of binding proteins [61] or the balance between the elongation and decomposition [62] of oocyte FFA. The amounts of fatty acyls of palmitic (16:0), stearic (18:0), and oleic (18:1) acids in oocyte TAG increased in cows in the early lactation stage compared to heifers, while the ratios of these fatty acyls in oocyte TAG were similar between lactation groups. Although it is known that oleic acids can compensate the adverse effects of palmitic and stearic acids on oocyte developmental competence *in vitro* [21], it is not clear whether the ratio or the absolute amounts of these fatty acids are more crucial to oocyte quality in oocytes in living cows. The present result suggested either possible change of oocyte quality; one possible change was that the increase of palmitic and stearic acids adversely affected oocyte quality, and the other possible change was that the increase of oleic acid by as the same ratio as palmitic and stearic acids sufficiently contributed to protecting oocytes from the adverse effects of saturated fatty acids.

Cows in the early lactation stage showed high plasma FFA and oocyte TAG, which suggested an increase in the conversion of FFA to TAG in COCs. Since FFA lipotoxicity induces harmful effects such as endoplasmic reticulum stress [30] in various non-adipocyte cells [29, 43, 63, 64], FFA is converted to TAG, which are a harmless form of lipids [33, 65]. TAG may be actively synthesized from FFA to avoid lipotoxicity in cows in the early lactation group; therefore, oocyte FFA in the early lactation group remained at similar levels to other lactation stages and heifers. Meanwhile, the low ratios of FFA 16:1/16:0 (0.051 ± 0.037, n=18) and 18:1/18:0 (0.072 ± 0.043, n=18) in oocytes regardless of lactation groups suggested that the capacity of oocytes to synthesize TAG from FFA was inherently low compared to other

tissues. FFA 16:1/16:0 and 18:1/18:0 are the product / substance ratios of the reactions SCD catalyzes, and these reactions are key steps for TAG synthesis [66]. Therefore, FFA 16:1/16:0 and 18:1/18:0 are the markers of the capacity of cells to synthesize TAG from FFA [66, 67]. As indicated in the high FFA 18:1/18:0 ratio (~4) in murine liver tissue [68], SCD activity varies between different tissues and high in such as adipocytes and hepatocytes [69]. Furthermore, a previous study indicated the lower ratios of 16:1/16:0 and 18:1/18:0 of TAG and diacylglycerols in oocytes than cumulus cells [34, 70]. The low ratios of FFA 16:1/16:0 and 18:1/18:0 in oocytes suggested the low capacity of oocytes to avoid lipotoxicity by converting FFA to TAG. In previous studies using bovine COCs, the developmental competence of oocytes decreased when they were cultured with a supplemental high FFA mixture (palmitic, stearic, and oleic acid) [45], suggesting the adverse effects of lipotoxicity on oocyte quality. Additionally, in metabolism disorder model mice fed a high lipid diet, an increase in oocyte TAG, the up-regulation of lipotoxicity biomarkers (*e.g.*, endoplasmic reticulum stress marker genes) in COCs, and a reduction in the fertilization rate have been reported [71]. A previous report found that embryos collected from lactating cows showed the darker cytoplasm, which indicated higher TAG contents in these embryos, and the lower developmental competence than those of heifers [72]. This finding suggested the relevance of lactation, TAG accumulation in oocytes, and the low quality of oocytes, and accordingly supported the potential low quality of oocytes with high TAG content in the postpartum lactating cows in the present study. The accumulated TAG in oocytes in postpartum cows is plausible to be utilized as the stored energy for oocyte and embryo development [73, 74]; however, once the incorporation of FFA into cumulus cells/oocytes exceeds their metabolizing ability, oocyte quality will be deteriorated by their lipotoxicity. The investigation of the expression of lipotoxicity was beyond the scope of this paper, accordingly it is my future problem to examine lipotoxicity in oocytes in living lactating cows by assessing endoplasmic reticulum stress marker [22] or ceramide [43].

The present study utilized 11 multiparous and 3 primiparous cows together. Although it was reported primi- and multiparous cows in the early lactation stage showed different blood FFA levels (0.3–0.4 and 0.4–0.5 mmol/L, respectively) [13-15, 75], the pattern of blood FFA levels between the different lactation stages was similar between primi- and multiparity according to previous reports. Namely, blood FFA level was higher in the early lactation stage (0.3–0.5 mmol/L) [13-15, 75] compared to the peak and the middle lactation stage (0.1–0.3 mmol/L) [14, 15, 76], and blood FFA level in the peak and the middle lactation stage was similar to or higher than that of heifers (~0.1 mmol/L) [77, 78]. Therefore, it was considered that the inclusion of primiparous cows in addition to multiparous cows did not compromise the objective of the present study. Ovum pick up was performed at random stages of ovarian cycles

in the present study. However, quality of oocytes may differ depending on the phase of a follicular wave, specifically being better in the early than the late phase [79, 80]. Therefore, quality of collected oocytes was plausible to vary to the similar degree in all lactation groups. In order to make oocyte quality as even as possible, oocytes need to be collected at the same stage of a follicular wave by using hormonal synchronization, follicular ablation, or monitoring ovarian cycles in future studies.

5. Conclusion

In conclusion, the present results indicated a positive correlation between plasma FFA and oocyte TAG, and postpartum cows with high plasma FFA had higher oocyte TAG than heifers. The accumulation of TAG in oocytes during the postpartum period may have adverse effects of lipotoxicity on oocyte quality and the negative effects may surpass the beneficial effects of the increased energy storage in oocytes. The present study provides insights to increase the fertility of high-yielding cows by improving oocyte quality through feeding and housing management for appropriate circulating FFA.

6. Tables and Figures

Table 1. Days in milk (DIM), daily milk production, and BCS of animals in different lactation groups.

Item	Early lactation, n=6	Peak lactation, n=4	Middle lactation, n=4	Heifer, n=3†
DIM	38.7 ± 8.2 (25 – 47)	62.5 ± 1.5 (61 – 65)	175.8 ± 17.1 (160 – 202)	–
Milk production (kg/day)	30.6 ± 6.3 (17.0 – 36.5)	33.1 ± 7.1 (24.5 – 41.9)	19.4 ± 1.1 (18.2 – 20.6)	–
BCS	2.6 ± 0.2 (2.5 – 3)	2.5 ± 0.4 (2 – 3)	2.8 ± 0.1 (2.75 – 3)	3.3 ± 0.0 (3.25 – 3.25)

Values are presented as the mean ± SD. Values in parentheses indicate minimum and maximum values.

Milk production (kg/day) was mean daily milk production for 7 days between 3 days before and after blood and oocyte sampling.

†The BCS of one heifer was missing.

Table 2. Contents of each lipid internal standard for oocytes and plasma.

Lipid standard	Spiked amount per tube of an oocyte sample (nmol)	Spiked amount per tube of a plasma sample (nmol)
FFA 17:0	0.281	0.627
TAG (11:0/11:0/11:0)	0.141	0.313

Table 3.1. Gradient elution program for the electrospray ionization (ESI)-positive mode.

Time /min	0	1	11	20	28	28.5	30
5 mmol/L aqueous ammonium acetate	20	20	5	0	0	20	20
Isopropanol	10	10	65	75	75	10	10
Methanol	70	70	30	25	25	70	70

Table 3.2. Gradient elution program for the ESI-negative mode.

Time /min	0	1	15	27	28	30
5 mmol/L aqueous ammonium acetate	25	5	5	0	25	25
Isopropanol	40	60	60	65	40	40
Methanol	35	35	35	35	35	35

Table 4. Identification of lipid species by LC/MS and MS/MS.

Lipid species (Molecular species)	Retention time (min)	Diagnostic ion	Calculated *m/z*	Acquired *m/z*	MS accuracy (Δppm)	Tandem MS fragments
FFA						
14:0	7.53	$[M-H]^-$	227.2017	227.2023	2.64	N/A
16:0	9.91	$[M-H]^-$	255.2330	255.2334	1.57	N/A
16:1	8.35	$[M-H]^-$	253.2173	253.2183	3.95	N/A
18:0	11.85	$[M-H]^-$	283.2643	283.2646	1.06	N/A
18:1	10.50	$[M-H]^-$	281.2486	281.2490	1.42	N/A
18:2	9.25	$[M-H]^-$	279.2330	279.2335	1.79	N/A
TAG						
44:0	13.73	$[M+NH_4]^+$	768.7076	768.7078	0.26	N/D
46:0 (14:0/16:0/16:0)	14.16	$[M+NH_4]^+$	796.7389	796.7393	0.50	523, 551
46:1 (14:0/16:1/16:0)	13.86	$[M+NH_4]^+$	794.7232	794.7230	−0.25	521, 523, 549
46:2 (14:1/16:1/16:0)	13.56	$[M+NH_4]^+$	792.7076	792.7073	−0.38	519, 521, 549
46:3	13.17	$[M+NH_4]^+$	790.6919	790.6918	-0.13	N/D
48:0 (16:0/16:0/16:0)	14.56	$[M+NH_4]^+$	824.7702	824.7711	1.09	551
48:1 (16:0/16:1/16:0)	14.25	$[M+NH_4]^+$	822.7545	822.7545	0.00	549, 551
48:2 (16:0/16:1/16:1)	13.95	$[M+NH_4]^+$	820.7389	820.7383	−0.73	547, 549
48:3 (16:1/16:1/16:1)	13.68	$[M+NH_4]^+$	818.7232	818.7227	−0.61	547
48:4	13.29	$[M+NH_4]^+$	816.7076	816.7067	-1.10	N/D
50:0 (16:0/18:0/16:0)	14.93	$[M+NH_4]^+$	852.8015	852.8022	0.82	579, 551
50:1 (16:0/18:1/16:0)	14.63	$[M+NH_4]^+$	850.7858	850.7859	0.12	577, 551
50:2 (16:0/18:1/16:1)	14.34	$[M+NH_4]^+$	848.7702	848.7699	−0.35	549, 577, 575
50:3 (16:1/18:1/16:1)	14.03	$[M+NH_4]^+$	846.7545	846.7538	−0.83	575, 547
50:4 (16:1/18:2/16:1)	13.77	$[M+NH_4]^+$	844.7389	844.7382	−0.83	547, 573
50:5	13.46	$[M+NH_4]^+$	842.7232	842.7224	−0.95	N/D
50:6	13.35	$[M+NH_4]^+$	840.7076	840.7073	−0.36	N/D
52:0 (16:0/18:0/18:0)	15.41	$[M+NH_4]^+$	880.8328	880.8336	0.91	607, 579
52:1 (16:0/18:1/18:0)	14.99	$[M+NH_4]^+$	878.8171	878.8170	−0.11	577, 579, 605
52:2 (16:1/18:1/18:0)	14.66	$[M+NH_4]^+$	876.8015	876.8010	−0.57	577
52:3 (16:1/18:1/18:1)	14.41	$[M+NH_4]^+$	874.7858	874.7861	0.34	575, 603
52:4 (16:1/18:2/18:1)	14.12	$[M+NH_4]^+$	872.7702	872.7693	−1.03	575, 573, 601
52:5 (16:1/18:3/18:1)	13.88	$[M+NH_4]^+$	870.7545	870.7544	−0.11	571, 575, 599
52:6 (16:1/18:3/18:2)	13.65	$[M+NH_4]^+$	868.7389	868.7388	−0.12	571, 573, 597
52:7	13.38	$[M+NH_4]^+$	866.7232	866.7222	−1.15	N/D
54:0 (18:0/18:0/18:0)	15.84	$[M+NH_4]^+$	908.8641	908.8632	−0.99	607

Table 4. *Continue*

Lipid species (Molecular species)	Retention time (min)	Diagnostic ion	Calculated *m/z*	Acquired *m/z*	MS accuracy (Δppm)	Tandem MS fragments
TAG						
54:1 (18:0/18:1/18:0)	15.47	$[M+NH_4]^+$	906.8484	906.8475	−0.99	605, 607
54:2 (18:0/18:1/18:1)	15.10	$[M+NH_4]^+$	904.8328	904.8320	−0.88	605, 603
54:3 (18:1/18:1/18:1)	14.77	$[M+NH_4]^+$	902.8171	902.8163	−0.89	603
54:4 (18:1/18:2/18:1)	14.52	$[M+NH_4]^+$	900.8015	900.8017	0.22	603, 601
54:5 (18:1/18:2/18:2)	14.25	$[M+NH_4]^+$	898.7858	898.7851	−0.78	599, 601
54:6 (18:1/18:3/18:2)	13.99	$[M+NH_4]^+$	896.7702	896.7692	−1.12	597, 599, 601
54:7 (18:2/18:3/18:2)	13.79	$[M+NH_4]^+$	894.7545	894.7541	−0.45	597, 599
54:8	13.46	$[M+NH_4]^+$	892.7389	892.7388	−0.11	N/D
56:4 (18:0/20:3/18:1)	14.92	$[M+NH_4]^+$	928.8328	928.8322	−0.65	605, 627, 629
56:5 (18:0/20:4/18:1)	14.66	$[M+NH_4]^+$	926.8171	926.8171	0.00	605, 625, 627
56:6 (18:1/20:4/18:1)	14.41	$[M+NH_4]^+$	924.8015	924.8017	0.22	603, 625
56:7 (18:1/20:4/18:2)	14.12	$[M+NH_4]^+$	922.7858	922.7853	−0.54	601, 623, 625
56:8 (18:2/20:4/18:2)	13.88	$[M+NH_4]^+$	920.7702	920.7699	−0.33	599, 623
56:9	13.68	$[M+NH_4]^+$	918.7545	918.7550	0.54	N/D
58:6	14.81	$[M+NH_4]^+$	952.8328	952.8325	−0.31	N/D
58:7	14.48	$[M+NH_4]^+$	950.8171	950.8171	0.00	N/D
58:8	14.34	$[M+NH_4]^+$	948.8015	948.8014	−0.11	N/D
58:9	14.04	$[M+NH_4]^+$	946.7858	946.7852	−0.63	N/D
58:10	13.71	$[M+NH_4]^+$	944.7702	944.7695	−0.74	N/D

N/A: Not available (lipid species contained only one fatty acyl, and, thus, were identical).

N/D: Not identified (MS/MS data were not obtained due to low intensity).

Table 5. Plasma FFA profiles and fatty acyl species in TAG in different lactation groups (nmol/100 μL).

Lipid	Fatty chain	Early lactation (6)	Peak lactation (4)	Middle lactation (4)	Heifer (4)
FFA	14:0	0.26 ± 0.11[a]	0.18 ± 0.02[ab]	0.10 ± 0.01[b]	0.08 ± 0.01[b]
	16:0	3.68 ± 1.04[a]	2.81 ± 0.39[ab]	1.73 ± 0.20[b]	1.38 ± 0.25[b]
	16:1	0.66 ± 0.28[a]	0.48 ± 0.26[ab]	0.13 ± 0.03[b]	0.14 ± 0.07[b]
	18:0	5.34 ± 0.79[a]	4.21 ± 0.48[ab]	3.70 ± 0.57[bc]	2.69 ± 0.24[c]
	18:1	7.57 ± 2.25[a]	5.66 ± 1.51[a]	1.99 ± 0.33[b]	1.79 ± 0.71[b]
	18:2	1.14 ± 0.24[a]	1.27 ± 0.37[a]	0.56 ± 0.07[b]	0.31 ± 0.05[b]
TAG	14:0	0.51 ± 0.42	0.28 ± 0.07	0.31 ± 0.07	0.66 ± 0.08
	14:1	0.25 ± 0.32	0.11 ± 0.01	0.12 ± 0.04	0.18 ± 0.02
	16:0	14.26 ± 6.80	9.35 ± 2.39	13.25 ± 3.40	19.06 ± 3.39
	16:1	13.47 ± 9.79	8.96 ± 1.09	10.85 ± 3.13	12.85 ± 1.43
	18:0	9.45 ± 3.90	8.61 ± 1.58	9.15 ± 2.38	9.57 ± 2.05
	18:1	21.79 ± 11.01	18.12 ± 3.06	20.44 ± 5.08	20.76 ± 3.66
	18:2	2.79 ± 0.83	3.58 ± 1.45	3.43 ± 1.10	4.13 ± 0.55
	18:3	0.46 ± 0.10[b]	0.79 ± 0.37[ab]	0.67 ± 0.26[ab]	1.05 ± 0.22[a]
	20:3	0.07 ± 0.03[b]	0.05 ± 0.01[b]	0.07 ± 0.02[b]	0.14 ± 0.03[a]
	20:4	0.34 ± 0.10[b]	0.32 ± 0.07[b]	0.47 ± 0.17[b]	1.08 ± 0.29[a]

Values are presented as the mean ± SD. The numbers in parentheses indicate the number of animals included in each lactation group.

[abc] Different letters indicate significant differences in the FFA/TAG fatty chain between lactation groups ($P < 0.05$).

Table 6. Oocyte FFA profiles and fatty acyl species in TAG in different lactation groups (pmol/oocyte).

Lipid	Fatty chain	Early lactation (6)	Peak lactation (4)	Middle lactation (4)	Heifer (4)
FFA	14:0	7.0 ± 1.4	6.2 ± 2.7	9.6 ± 4.7	5.2 ± 1.0
	16:0	131.2 ± 14.6	121.3 ± 10.6	137.6 ± 9.7	125.3 ± 8.0
	16:1	5.5 ± 1.9	9.2 ± 5.7	8.4 ± 5.7	3.1 ± 0.3
	18:0	177.2 ± 24.3	165.2 ± 14.9	182.1 ± 17.9	189.8 ± 17.5
	18:1	10.4 ± 0.9	13.7 ± 4.7	19.4 ± 11.4	7.7 ± 0.7
	18:2	1.7 ± 0.6[ab]	4.1 ± 1.9[a]	2.5 ± 1.6[ab]	1.0 ± 0.1[b]
TAG	14:0	5.0 ± 1.3	5.9 ± 2.6	6.4 ± 4.7	2.0 ± 0.3
	14:1	1.2 ± 0.4	2.3 ± 1.1	2.3 ± 2.8	0.4 ± 0.1
	16:0	91.3 ± 16.3[a]	72.1 ± 11.5[ab]	79.6 ± 37.5[ab]	43.5 ± 4.5[b]
	16:1	69.2 ± 12.7[a]	58.6 ± 10.0[ab]	62.0 ± 35.2[ab]	25.3 ± 2.5[b]
	18:0	31.7 ± 6.0[a]	20.3 ± 2.2[ab]	21.9 ± 12.7[ab]	15.1 ± 2.4[b]
	18:1	106.9 ± 15.6[a]	65.1 ± 5.8[ab]	74.6 ± 46.5[ab]	44.3 ± 7.3[b]
	18:2	14.4 ± 3.2[a]	9.9 ± 2.1[ab]	10.7 ± 5.6[ab]	6.0 ± 1.8[b]
	18:3	1.6 ± 0.6	1.4 ± 0.6	1.3 ± 0.6	0.8 ± 0.3
	20:3	0.8 ± 0.2[a]	0.4 ± 0.1[ab]	0.5 ± 0.4[ab]	0.2 ± 0.1[b]
	20:4	2.4 ± 0.7	1.6 ± 0.5	2.0 ± 1.1	1.9 ± 0.9

Values are presented as the mean ± SD. The numbers in parentheses indicate the number of animals included in each lactation group.

[ab] Different letters indicate significant differences in the FFA/TAG fatty chain between lactation groups ($P < 0.05$).

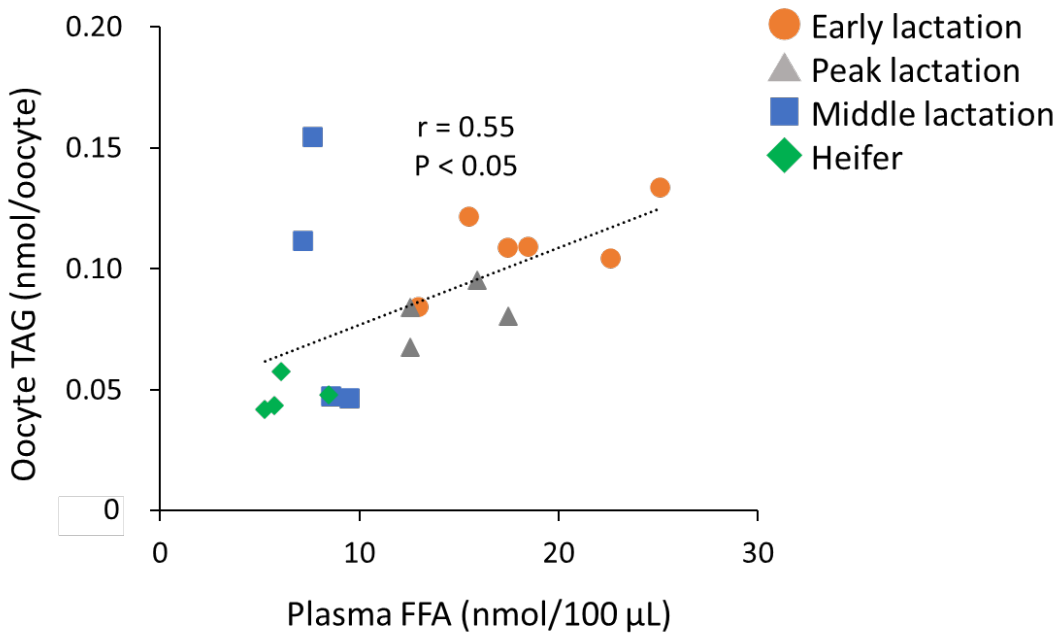

Fig. 1. Relationship between plasma FFA and oocyte TAG ($r = 0.55$, $P < 0.05$).

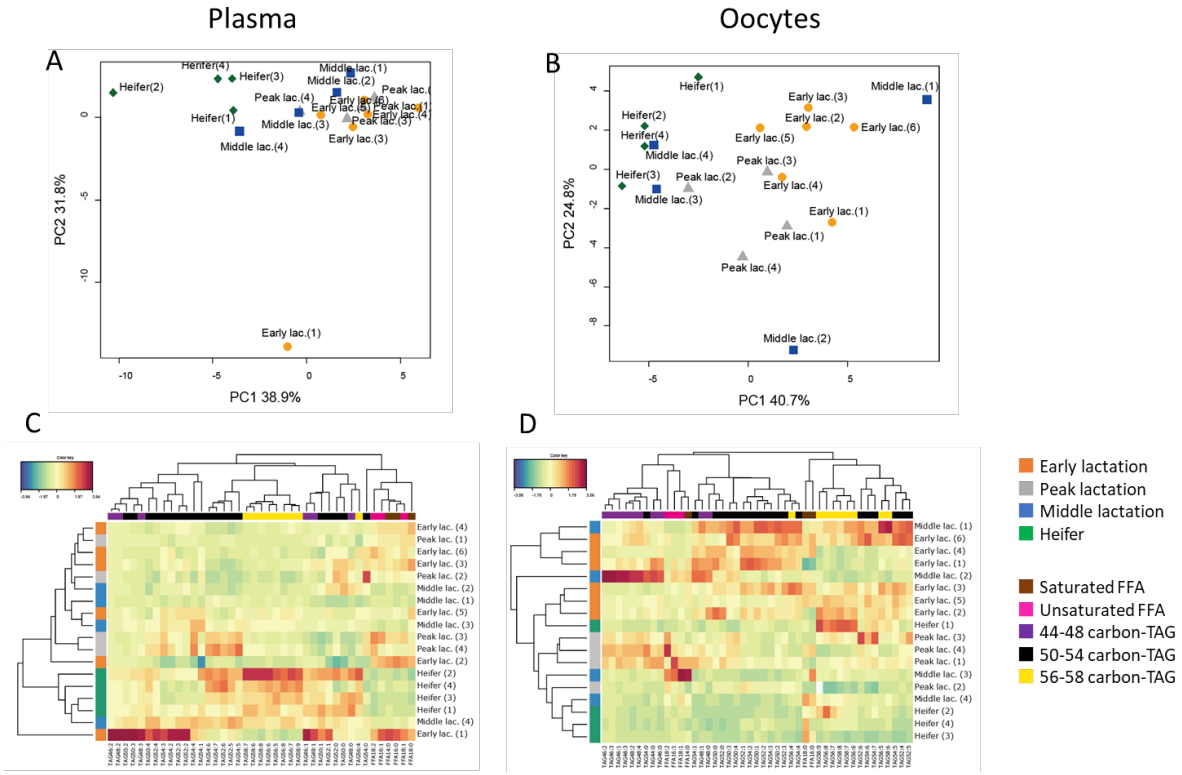

Fig. 2. Score plot in a principal component analysis (PCA) and heat map of FFA and TAG lipid species in heifers and cows at different lactation stages.

(A) Score plot of plasma, (B) score plot of oocytes, (C) heat map of plasma, and (D) heat map of oocytes.

A PCA was performed after the quantitative value was normalized.

Quantitative data were normalized before the heat map clustering procedure. The red color indicates a high value, while the blue color indicates a low value. Cows are indicated with the lactation stage [XX lac.] + the individual number [(X)]. The lactation groups to which cows belong are indicated on the left side of the column with different colors, and the lipid groups to which the lipid species belong are indicated on the top of the rows with different colors.

Saturated FFA: no DB in the fatty acyl chain, unsaturated FFA: 1-2 DB(s) in the fatty acyl chain, 44–48 carbon–TAG: a total of 44–48 carbons in the fatty acyl chains, 50–54 carbon–TAG: a total of 50–54 carbons in the fatty acyl chains, 56–58 carbon–TAG: a total of 56–58 carbons in the fatty acyl chains.

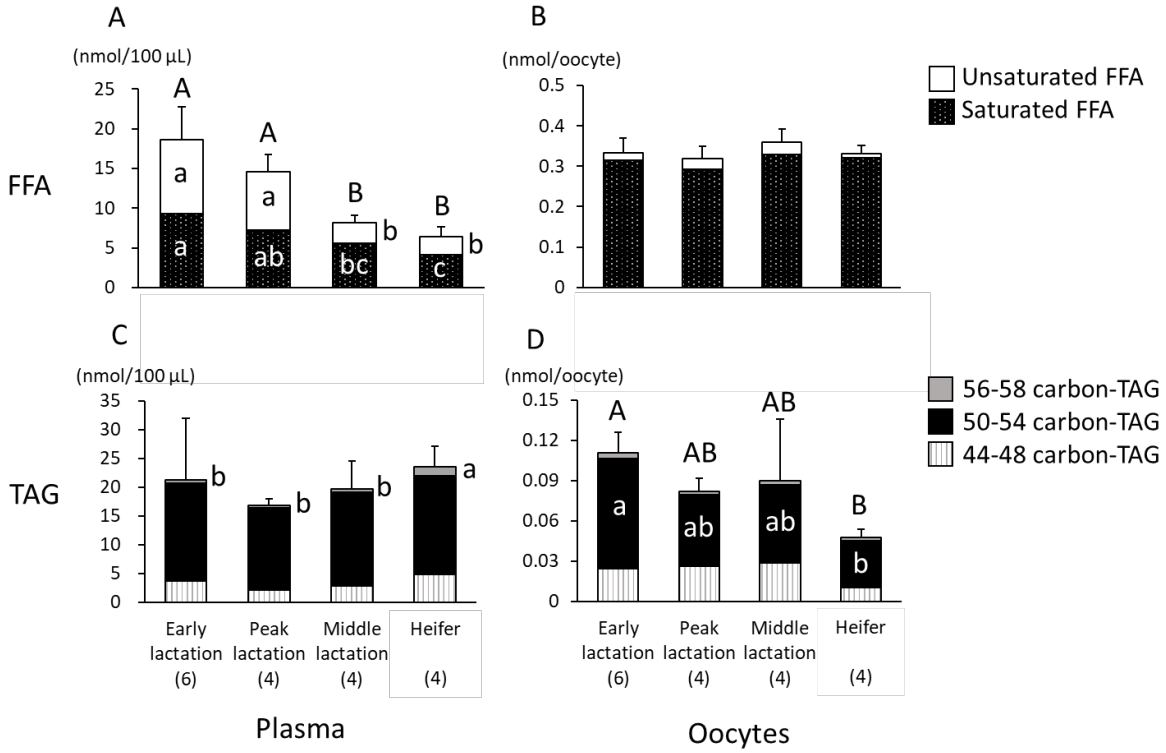

Fig. 3. Quantity of FFA/TAG lipid groups categorized based on CNs and DBs in fatty acyl chains in different lactation groups; (A) FFA in plasma, (B) FFA in oocytes, (C) TAG in plasma, and (D) TAG in oocytes.

[AB] Different letters indicate a significant difference in total FFA/TAG between lactation groups ($P < 0.05$).

[abc] Different letters indicate a significant difference in lipid groups between lactation groups ($P < 0.05$).

Error bars indicate the SD of total FFA/TAG. The numbers in parentheses indicate the number of animals included in each lactation group.

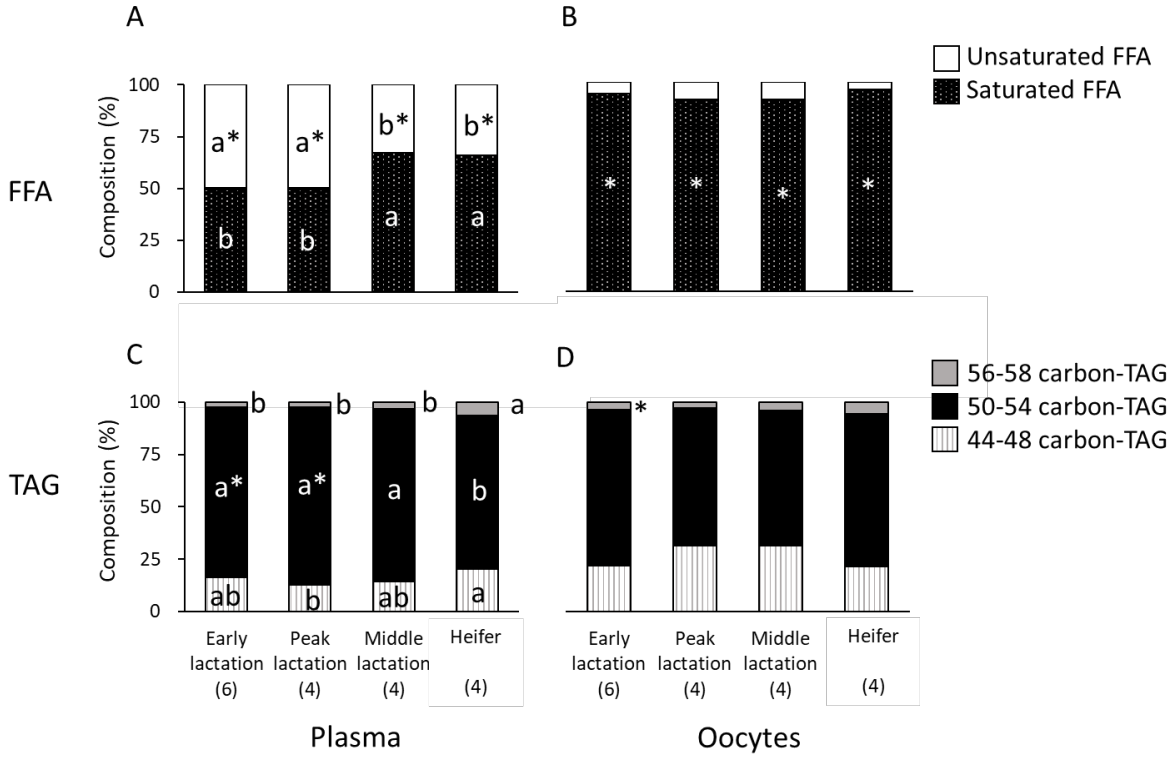

Fig. 4. Composition of FFA/TAG lipid groups categorized based on CNs and DBs in fatty acyl chains to total FFA/TAG in different lactation groups.

(A) FFA in plasma, (B) FFA in oocytes, (C) TAG in plasma, and (D) TAG in oocytes.

[ab] Different superscripts indicate a significantly different composition between lactation groups ($P < 0.05$).

* Asterisks indicate significantly different compositions between plasma and oocytes ($P < 0.05$). Mean values were compared using the paired *t*-test between plasma and oocytes or a one-way ANOVA (using Tukey's *post hoc* test) between lactation groups. The numbers in parentheses indicate the number of animals included in each lactation group.

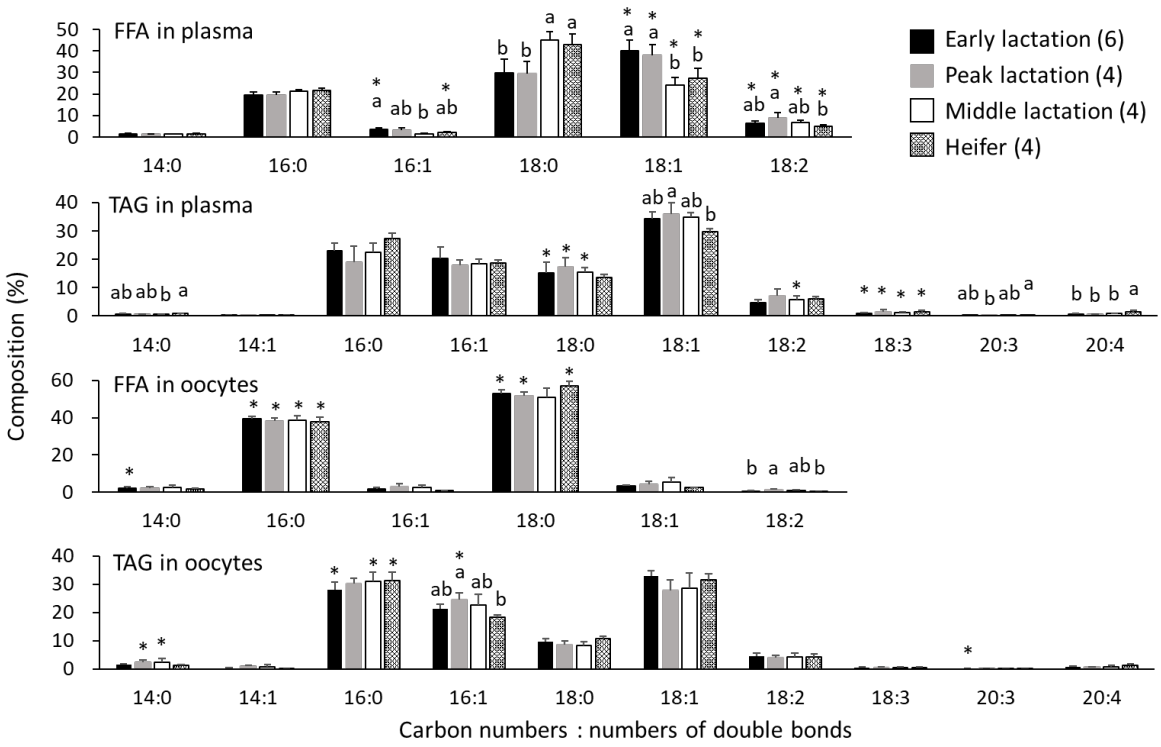

Fig. 5. Composition of fatty chains to total FFA/TAG in plasma and oocytes in different lactation groups.

[ab] Different superscripts indicate significantly different compositions between lactation groups ($P < 0.05$).

* The asterisks indicate significantly different compositions between plasma and oocytes ($P < 0.05$).

Results are shown as means ± SD (error bars). Mean values were compared using the paired *t*-test between plasma and oocytes or a one-way ANOVA (using Tukey's *post hoc* test) between lactation groups. The numbers in parentheses indicate the number of animals included in each lactation group.

7. Summary

Impaired oocyte quality is one of the main causes of low fertility in modern high-yielding dairy cows. One of the potential factors of the impaired oocyte quality is the effects of FFA cytotoxicity. In fact, high FFA supplementation to culture media exacerbated oocyte developmental competence *in vitro*. Meanwhile, artificially induced high blood FFA levels in heifers did not affect lipid compositions of oocytes *in vivo*; however, oocyte lipid compositions of postpartum cows have not yet been investigated to date. Therefore, contents and compositions of lipids involved in energy metabolism, including FFA and TAG, in plasma and oocytes were compared between cows at different lactation stages. Heifers were used as a control group that was not affected by lactation.

Plasma and oocytes were collected from 4 heifers and 14 grazed Holstein cows at different lactation stages, namely, the early lactation stage: 25–47 DIM (n = 6), peak lactation stage: 61–65 DIM (n = 4), and middle lactation stage: 160–202 DIM (n = 4). The FFA and TAG contents and compositions in plasma and oocytes were analyzed by LC/MS.

Plasma FFA positively correlated with oocyte TAG ($P < 0.05$). Plasma FFA and oocyte TAG were significantly higher in cows in the early lactation stage than in heifers ($P < 0.05$), while the peak and middle lactation stage groups had intermediate levels. However, oocyte FFA was similar regardless of lactation groups. FFA and TAG species in plasma and oocytes could be categorized into 2 and 3 groups, respectively, based on CNs and DBs, through clustering analysis. The proportion of oleic acid in plasma increased concurrently with elevations in total FFA, while the compositions of oocyte FFA and TAG fatty acyls were constant regardless of plasma FFA concentration or oocyte TAG content. The present results suggested that high postpartum plasma FFA concentrations affected the quantity of oocyte TAG, but not oocyte FFA, in cows under grazing management.

Chapter II

Simultaneous elevations and accelerated desaturation of free fatty acid in plasma and oocytes in early postpartum dairy cows under intensive feeding management

1. Introduction

In Chapter I, I studied oocyte FFA and TAG compositions of grazed lactating cows with milk production levels of ~7,700 kg/305 days, and found that blood FFA and oocyte TAG were higher in cows at 25–47 DIM than in heifers. High TAG in oocytes of these cows indicated that elevated circulating levels of FFA in the postpartum period increased the amount of FFA reaching COCs via follicular fluid [46], and also that TAG synthesis in COCs was accelerated to prevent FFA lipotoxicity. Elevated oocyte TAG suggested that the ability of oocytes to manage lipotoxic FFA may be reduced because their storage capacity for TAG was surpassed [81].

The different livestock farming systems of outdoor grazing or indoor intensive feeding have been shown to alter the contents and compositions of lipids in animal products, *i.e.*, meat [82, 83] and milk [84], which may be attributed to variations in nutrition intake [85], milk production [85], body condition dynamics [84-86], and the lipid compositions of feed [87]. Therefore, lipid compositions in oocytes in cows under indoor intensive management may differ from those in grazed cows.

Lipid compositions are important for oocyte quality. Monounsaturated FFA is synthesized from saturated FFA in cells, and this desaturation reaction is a key step in the synthesis of TAG from FFA [66]. The conversion of monounsaturated FFA to TAG is one reason for monounsaturated FFA being less cytotoxic than saturated FFA [21]. SCD catalyzes desaturation reactions, and one of the typical markers of its activity is the ratios of FFA 16:1/16:0 and FFA 18:1/18:0 as the product and substrate ratios of desaturation reactions [67]. A previous study demonstrated that the FFA 18:1/18:0 ratio increased in blood and follicular fluid with elevations in blood and follicular fluid FFA concentrations [34, 46]. These findings suggested that the increase in FFA transport from blood to follicular fluid, which is attributable to elevation of the blood FFA level, affected the FFA concentration and composition in follicular fluid. The FFA elongation reaction that long-chain fatty acids family member 6 catalyzes also affects intracellular fatty acid profiles, and the activity of the FFA elongation reaction interacts with the activities of desaturation reactions [88]. FFA 18:0/16:0 and 18:1/16:1 ratios, the product and substrate ratios of these elongation reactions, are used as elongase markers [89, 90]. Examinations of these desaturase and elongase markers are useful for obtaining information on the status of lipid metabolism in oocytes.

In the present study, the contents and compositions of FFA and TAG in oocytes at different lactation stages were examined in cows under typical modern intensive management, to obtain insights into oocyte lipotoxicity in lactating cows, particularly in the early postpartum period.

2. Materials and methods

2.1. Animals

The present study was implemented according to the animal experimental regulations of the Hokkaido University Animal Care and Use Committee (Approval No.: 18-0028 and 19-0127). Seven primiparous and 13 multiparous Holstein lactating cows (24–49 months of age, 1–3 parities) were used in this study, all of which were kept at the experimental farm of Dairy Research Center, Hokkaido Research Organization (Nakashibetsu, Hokkaido, Japan). Enrolled cows had milk production ranging between 19.8 and 43.5 kg/day (Table 1) and were without clinical issues requiring intensive treatment. The present study was conducted between January and August 2020. Cows were kept in a freestall barn and fed a total mixed ration containing a blend of grass silage, corn silage, rolled corn, soybean meal, calcium carbonate, and dicalcium phosphate (Table 2). The mean 305-day 4% fat corrected milk yield of lactation was 9,123 kg. To compare plasma and oocyte lipid profiles at different lactation stages, 5 cows per lactation stage defined based on DIM with different energy balance levels [91, 92] were used: 20–30 DIM (the severe negative energy balance group, 1 primi- and 4 multiparous), 40–50 DIM (the moderate negative energy balance group, 1 primi- and 4 multiparous), 60–80 DIM (the zero energy balance group, 3 primi- and 2 multiparous), and 130–160 DIM (the positive energy balance group, 2 primi- and 3 multiparous).

2.2. Assessment of the energy balance

Feeding management and sampling procedures of the farm used in the present study are descried elsewhere [93]. Namely, two types of total mixed rations were fed to cows depending on DIM; one is formulated for high producing cows (1–149 DIM) and the other is for low producing cows (≥150 DIM) (Table 2). Amounts of feed intake were determined daily by an automated feeder (Roughage Intake Control System, Insentec BV, The Netherlands). Dry matter contents of grass silage and corn silage were determined by drying at a 60°C forced air oven for 48 hours weekly, and the inclusion rates of ration ingredients were adjusted to account for differences in dry matter contents. Dried samples of the silages were ground in a mill to pass through a 1 mm screen, composited at 2-week intervals, and analyzed for chemical components at the Dairy Research Center, Hokkaido Research Organization. Crude protein

(CP), neutral detergent fiber (NDF), ether extract (EE), nonfiber carbohydrate (NFC), and lignin were measured by using near-infrared reflectance spectroscopy (NIRS 6500, Foss, Denmark). Milk production was recorded at every milking, and milk fat, protein, and lactose concentrations by infrared spectroscopy (MilkoScan FT2, Foss Electric, Denmark), and body weight (BW) were recorded weekly.

Calculation of energy balance per day was performed according to NRC (2001) [94] and according to Ramos-Nieves et al. [95] with slight modifications. The following equations were used, where net energy for lactation is presented as NE_L, megacalorie as Mcal, and day as d:

$$NE_L \text{ (Mcal / d) balance} = \text{energy intake (Mcal of } NE_L \text{ / d)}$$
$$- [\text{maintenance requirement (Mcal of } NE_L \text{ / d)}$$
$$+ \text{lactation requirement (Mcal of } NE_L \text{ / d)} + \text{growth requirement (Mcal of } NE_L \text{ / d)}],$$

where

$$\text{energy intake (Mcal / d)} = (0.0245 \times \text{total digestible nutrients (TDN) (\%)} - 0.12)$$
$$\times \text{ dry matter intake (kg / d)},$$
$$\text{maintenance requirement (Mcal)} = \text{metabolic BW (kg}^{0.75}) \times 0.08 \text{ (Mcal / kg}^{0.75} \cdot \text{d)},$$
$$\text{lactation requirement (Mcal)} = [0.0929 \times \text{fat (\%)} + 0.0547 \times \text{crude protein (\%)}$$
$$+ 0.0395 \times \text{lactose (\%)}] \times \text{milk production (kg)}.$$

Growth requirement was considered in the calculation regarding first and second parity;

$$\text{growth requirement (Mcal)} = 0.0635 \times \text{equivalent empty BW (EQEBW)}^{0.75}$$
$$\times \text{ equivalent empty body tissue gain (EQEBG)}^{1.097},$$

where

$$\text{EQEBW} = 0.891 \times \text{equivalent shrunk BW (EQSBW)},$$
$$\text{EQSBW} = \text{SBW} \times (478 / \text{mature SBW}),$$
$$\text{mature SBW} = 0.96 \times \text{mature weight (MW)},$$
$$\text{EQEBG} = 0.956 \times \text{weight gain (WG)}.$$

MW was defined as 700 kg, and WG was defined as 0.2 kg for first parity and 0.1 kg for second parity. The daily energy balance for 3 weeks before OPU was calculated, and the mean values of individual daily energy balances for each week (1–7, 8–14, and 15–21 days before OPU) and for 3 weeks (1–21 days before OPU) were used in analyses.

2.3. Sample collection

Oocytes were collected and prepared for the lipid analysis by the same method as Chapter I with a slight modification. OPU was performed using an ultrasound imaging device

(HS-1600V; Honda Electronics, Toyohashi, Japan) equipped with a 9.0 MHz long-handled micro-convex probe (HCV-7710MV; Honda Electronics). The mean numbers of follicles in a pair of ovaries before OPU were 19.7 ± 6.6 in total and 15.8 ± 6.4, 2.5 ± 2.2, and 1.5 ± 0.9 follicles with diameters of 2–4, 4–8, and ≥8 mm, respectively. COCs with follicular fluid were aspirated with an average of 8.2 ± 2.6 COCs per session. Cumulus cells were removed from COCs by vortexing and gentle pipetting with a fine glass pipette. Oocyte denudation was confirmed under a stereomicroscope, and 5 oocytes without the apparent atretic appearance of ooplasms (classes 1–3 according to Blondin and Sirard [96]) were selected per cow and a single sample of 5 oocytes/OPU was used in the lipidomic analysis. Five oocytes were transferred to a 1.5-mL microcentrifuge tube with a small amount of D-PBS + 0.1% PVA (<10 μL) and stored at −80°C until analyzed.

Plasma samples were also prepared in the same procedure as Chapter I. In brief, blood was collected at oocyte sampling, and separated plasma from blood was transferred to a microcentrifuge tube and stored at −80°C until the lipidomic analysis.

2.4. Lipid extraction of plasma and oocyte samples from cows

Lipid extraction of plasma and oocyte samples was performed as described in Chapter I. In brief, 5 oocytes (in one microcentrifuge tube) were extracted with 600 μL of ice-cold chloroform/methanol 2:1 (v/v) twice, and 100 μL plasma (in one microcentrifuge tube) was extracted with 800 μL of ice-cold chloroform/methanol 1:1 (v/v). After extraction, the upper layer of the sample was dried under vacuum. Total lipids were then dissolved in methanol and filtered to remove any residue prior to injection.

2.5. LC/MS-based lipid profiling of FFA and TAG

To separate and measure each lipid molecular species, the LC/MS analysis was conducted using the same Prominence HPLC and mass spectrometer systems and under the same conditions as Chapter I. Forty samples (2 types of samples [plasma and oocyte] × 5 cows × 4 lactation groups) were analyzed for lipid profiling in a single LC/MS run.

After raw data processing, lipid identification and semi-quantitation were performed as described in Chapter I. In brief, lipids were identified based on retention behavior and high-resolution MS^1 signals (in the Fourier transform mode, with tolerance ≤5.0 ppm). The semi-quantitation of each lipid species was calibrated with IS using the following equation:

$$Amount_{Analyte} = Amount_{IS} \times \frac{Peak\ area_{Analyte}}{Peak\ area_{IS}}$$

The molecular species (*i.e.*, fatty acyl composition) of each TAG were identified using the information of the acquired MS^2 fragments (in ion-trap mode), comparing with the LIPIDMAPS database and in-house library [44].

2.6. Data analysis

All statistical analyses were conducted using the statistical software JMP Pro 15.2.0. Values of BCS, BW, dry matter intake, and milk production and compositions were compared using nonparametric Kruskal-Wallis test with Steel-Dwass *post hoc* test between lactation groups. The mean values of the FFA desaturase markers (FFA 16:1/16:0 and 18:1/18:0 ratios) in plasma and oocytes were compared using the paired *t*-test. Other statistical analyses of the relationships between two parameters were performed using Pearson's correlation coefficient, and P-values were calculated by a regression analysis. A P-value < 0.05 was considered to be significant, and data are shown as means ± SD, except for data on the energy balance, which were expressed as means ± SEM.

3. Results

3.1. Energy balance transition in cows at different lactation stages

The energy balance status is a basic factor that affects lipid metabolism. Therefore, I initially examined the energy balance transition before OPU in the different lactation groups. Nutritional parameters (*e.g.*, milk production and components) were similar between the lactation groups (Table 1). The 20–30 DIM group showed a negative energy balance over 3 weeks before OPU (Fig. 1); however, it consistently increased during this period. The 40–50 DIM group showed a negative energy balance 3 weeks before OPU, followed by a consistent increase, and a nearly zero energy balance at the time of OPU. The energy balance remained near zero in the 60–80 DIM group and was maintained at a constant positive value in the 130–160 DIM group in the 3 weeks before OPU (Fig. 1). These results confirmed the different energy balance transition conditions between the lactation groups.

3.2. Transition of plasma FFA, oocyte FFA, and oocyte in relation to DIM

To clarify the relationship between lactation stages and energy metabolism-related lipids in plasma and oocytes, I examined alterations in the distribution of plasma FFA, oocyte FFA, and oocyte TAG with DIM. I confirmed the expected transition of plasma FFA with DIM, namely, an increase in the early postpartum period at 20–30 DIM (194.7 ± 53.1 μmol/L), a decrease by 50 DIM (125.5 ± 54.0 μmol/L at 40–50 DIM), and maintenance at a low level after 50 DIM (126.3 ± 28.7 μmol/L at 60–80 DIM and 85.2 ± 29.4 μmol/L at 130–160 DIM) (n = 5,

respectively) (Fig. 2A). Alterations in oocyte FFA and TAG with DIM were similar to those in plasma FFA. Oocyte FFA was 319.1 ± 26.5 pmol/oocyte at 20–30 DIM, decreased by 50 DIM (278.0 ± 20.6 pmol/oocyte at 40–50 DIM), and was maintained at a low level after 50 DIM (241.2 ± 25.0 pmol/oocyte at 60–80 DIM and 234.3 ± 25.0 pmol/oocyte at 130–160 DIM) (n = 5, respectively) (Fig. 2B). Oocyte TAG was 57.4 ± 13.5 pmol/oocyte at 20–30 DIM, decreased by 50 DIM (39.6 ± 3.5 pmol/oocyte at 40–50 DIM), and was maintained at a low level after 50 DIM (37.2 ± 6.1 pmol/oocyte at 60–80 DIM and 42.9 ± 22.0 pmol/oocyte at 130–160 DIM) (n = 5, respectively) (Fig. 2C). Despite similar transitions in plasma FFA, oocyte FFA, and oocyte TAG, 2 out of 5 cows at 130–160 DIM showed high oocyte TAG of 78.5 and 59.1 pmol/oocyte, respectively (Fig. 2C).

3.3. Oocyte FFA and TAG in relation to plasma FFA level and a long-term energy balance

I examined the relationships between plasma FFA, oocyte FFA, and oocyte TAG, which showed similar transition patterns with DIM. A correlation analysis using Pearson's correlation coefficient confirmed positive correlations between plasma FFA and oocyte FFA (Fig. 3A) and between oocyte FFA and oocyte TAG (Fig. 3C) ($r = 0.63$ and $r = 0.46$, respectively, $P < 0.05$), indicating relationships between the metabolic balance of plasma FFA, oocyte FFA, and oocyte TAG. However, no correlation was observed between plasma FFA and oocyte TAG ($r = 0.27$, $P = 0.24$) (Fig. 3B).

I then assessed the daily energy balance in different periods before OPU to clarify the relationship between the long-term nutritional status and oocyte lipid profiles. A correlation analysis using Pearson's correlation coefficient was performed, and the results obtained showed that oocyte FFA negatively correlated with the mean energy balance 1 and 21 days before OPU ($r = -0.70$, $P < 0.05$) (Fig. 4A3), 1 and 14 days before OPU ($r = -0.64$, $P < 0.05$) (Fig. 4A2), and 1 and 7 days before OPU ($r = -0.58$, $P < 0.05$) (Fig. 4A1). Therefore, the mean energy balance for a longer period before OPU showed a stronger negative correlation with oocyte FFA ($r = -0.70$, $P < 0.05$) (Fig. 4A3). In contrast, oocyte TAG did not correlate with the mean energy balance 1 and 21 days before OPU ($r = -0.34$, $P = 0.14$) (Fig. 4B3), 1 and 14 days before OPU ($r = -0.32$, $P = 0.17$) (Fig. 4B2), and 1 and 7 days before OPU ($r = -0.24$, $P = 0.30$) (Fig. 4B1).

3.4. Relationships between total FFA and FFA metabolism markers in plasma and oocytes

To investigate the relationships between the contents and compositions of FFA and TAG in plasma and oocytes, I investigated markers of desaturase (the 16:1/16:0 and 18:1/18:0 ratios) (Fig. 5) and elongase (the 18:0/16:0 and 18:1/16:1 ratios) activities, which are associated

with the metabolism of FFA and synthesis of TAG [67, 97]. As expected, the results obtained showed increases in plasma FFA desaturase markers with elevations in plasma FFA ($P < 0.05$, respectively) (Fig. 6A). Similarly, oocyte FFA positively correlated with the oocyte FFA 16:1/16:0 ratio ($r = 0.79$) and 18:1/18:0 ratio ($r = 0.56$) ($P < 0.05$, respectively) (Fig. 6B). Oocyte FFA desaturase markers also positively correlated with oocyte TAG ($P < 0.05$, respectively) (Fig. 6C). When desaturase markers were compared between plasma and oocytes, the plasma FFA 16:1/16:0 ratio (0.153 ± 0.040, n=20) and 18:1/18:0 ratio (1.415 ± 0.436, n=20) in all cows were significantly higher than the oocyte FFA 16:1/16:0 ratio (0.077 ± 0.059, n=20) and 18:1/18:0 ratio (0.094 ± 0.038, n=20), respectively ($P < 0.05$), indicating an inherently different saturated and monounsaturated FFA balance between plasma and oocytes regardless of lactation stages. Positive correlations were observed between the plasma and oocyte FFA 16:1/16:0 ratio ($r = 0.70$, $P < 0.05$) and the plasma and oocyte FFA 18:1/18:0 ratio ($r = 0.51$, $P < 0.05$) (Fig. 6D), which indicated relationships between lipid compositions in plasma and oocytes. On the other hand, regarding elongase markers, negative correlations were noted between plasma FFA and plasma FFA elongase markers (Fig. 7A), and between oocyte FFA and oocyte FFA elongase markers (Fig. 7B). Collectively, these results confirmed the relationships of lipid contents and specific metabolism markers between plasma and oocytes.

4. Discussion

4.1. Increases in oocyte FFA in early postpartum cows and potential effects on oocyte quality

The present results revealed a positive correlation between plasma and oocyte FFA and between oocyte FFA and desaturase markers. The analysis of specific lipid metabolism markers in the present study revealed a distinctly altered oocyte lipid composition with an increase in FFA. Plasma FFA showed the expected transition with DIM: an increase at 20–30 DIM, a decrease by 50 DIM, and maintenance at a low level after 50 DIM. Oocyte FFA and TAG showed similar transitions to plasma FFA. The postpartum increase in oocyte TAG was consistent with the results in Chapter I, whereas that in oocyte FFA was not; oocyte FFA was similar among heifers and cows in the early lactation (~40 DIM), peak lactation (~60 DIM), and middle lactation (~180 DIM) stages. This discrepancy was attributed to cows in the present study being subjected to more intensive management with higher milk production. I also focused on the earlier lactation stage accompanied by higher plasma FFA in the present study (20–30 DIM) than in my previous study (25–47 DIM). Furthermore, I selected oocytes based on their morphology for the LC/MS analysis, whereas oocytes without apparent morphological collapse were specifically used in my previous study. The present results suggest that high

plasma FFA in postpartum cows influenced the contents and compositions of oocyte FFA and TAG.

Oocyte FFA increased at 20–30 DIM with high plasma FFA, which indicated that oocytes in the early postpartum period were at a high risk of FFA lipotoxicity. FFA lipotoxicity may induce oocyte degeneration, which leads to impaired folliculogenesis and anovulation [98]. A severe negative energy balance in postpartum cows delays the first postpartum ovulation by suppressing the hypothalamic pituitary gonadal axis [99]. In addition to this mechanism, FFA lipotoxicity in the oocytes of postpartum cows may partly account for the negative energy balance and delay in the first postpartum ovulation [100-104]. Furthermore, the increase in oocyte FFA may explain the previously reported epigenetic changes in metabolism-associated genes in the oocytes of postpartum cows [105]. Supplemental high FFA in *in vitro* oocyte maturation was previously shown to alter the DNA methylation fingerprints of the resultant embryos [106]. This is the first study to examine the relationship between long-term energy balance and oocyte lipid profiles in lactating cows. Oocyte FFA, but not TAG, negatively correlated with the energy balance 1 and 21 days before OPU, and this correlation was stronger than those on 1 and 7 days and on 1 and 14 days before OPU. In the 20–30 DIM group, 3 weeks before OPU corresponded to the period between parturition and oocyte collection. The present results suggest that postpartum cow management to avoid a severe negative energy balance will reduce risk of elevated oocyte FFA at 20–30 DIM.

The desaturase markers, fatty acid 16:1/16:0 and 18:1/18:0 ratios, reflect the synthesis of TAG from FFA in cells [66, 67]. FFA desaturase markers are generally lower in oocytes than in tissues with a high TAG synthesis capacity, such as the liver (FFA 18:1/18:0 ratio of ~4 [68]). The low values obtained for FFA desaturase markers in oocytes in the present study were consistent with the results in Chapter I. Additionally, the protein expression of SCD1 (an abundantly expressed SCD isoform in bovine cumulus cells) was lower in bovine oocytes than in cumulus cells [33]. Therefore, the present results confirmed the inherently low ability of bovine oocytes to synthesize TAG from FFA due to low SCD activity. This low FFA-processing ability of oocytes additionally supported oocytes in the early postpartum period being vulnerable to FFA lipotoxicity. When the amount of TAG in non-adipocytes surpasses the storage capacity of cells, excess FFA are provided to cells [107]. Therefore, the increase observed in oocyte FFA in early postpartum cows in the present study may be attributed to (1) excessive FFA beyond the capacity of SCD in oocytes to convert FFA to TAG, resulting in the accumulation of FFA, and/or (2) TAG surpassing the storage capacity of oocytes, which has a negative impact on its synthesis from FFA, resulting in excess FFA in oocytes.

Cumulus cells play an important role in oocyte viability by providing nutrients and regulatory signals [108, 109]. They also exhibit high SCD activity and actively synthesize TAG in response to FFA supplementation [33]. TAG in cumulus cells were higher in heifers subjected to fasting than in control animals [34]. Accordingly, the present results showing elevated oocyte FFA in early postpartum cows prompted us to speculate that FFA and TAG profiles in cumulus cells may also be affected by high FFA in this period. Future studies are needed to investigate the role of cumulus cells in the regulation of oocyte lipids and the protection of oocytes from elevated FFA in early postpartum cows.

4.2. Altered plasma and oocyte lipid compositions with increases in plasma and oocyte lipids

The lipid analysis performed in the present study revealed a close relationship between the content and composition of lipids. A positive correlation was observed between oocyte FFA desaturase markers and oocyte TAG. Monounsaturated FFA is essential for the synthesis of TAG [67]; therefore, an increase in the proportion of monounsaturated FFA may directly contribute to active TAG synthesis. In contrast, oocyte FFA elongase markers decreased with increases in oocyte FFA in the early postpartum period. The decrease in FFA elongase markers indicates an increase in the proportion of the shorter chain FFA species, *i.e.*, less-matured FFA containing less energy [110]. Since I only examined lactating cows in the present study, further studies are needed to compare the relationship between oocyte lipid compositions and oocyte quality in lactating cows with those in heifers [72, 111] and dry cows [111], which potentially have high-quality oocytes. Plasma FFA desaturase markers positively correlated with plasma FFA and oocyte FFA desaturase markers. This result suggests that the composition of plasma FFA reflected that of oocyte FFA due to the transport of abundant FFA from blood to oocytes via follicular fluid [34, 46]. Another potential explanation for the simultaneous elevations observed in oocyte desaturase markers (FFA 16:1/16:0 and 18:1/18:0 ratios) and oocyte FFA levels was an increase in oocyte SCD activity in response to abundantly incorporated FFA, resulting in higher FFA 16:1/16:0 and 18:1/18:0 ratios and oocyte TAG contents. Although I used product and substrate ratios as markers of desaturase and elongase activities in the present study, further studies on the expression of these enzymes and their genes are needed in order to obtain a more detailed understanding of lipid metabolism in cow oocytes.

4.3. Oocyte TAG contents in the middle lactation stage

Two cows in 130–160 DIM showed higher oocyte TAG than the remaining 3 cows in the same lactation stage, while oocyte FFA was similar. These 2 cows, which were primiparous,

showed larger reductions in BCS during early lactation after parturition (*i.e.*, approximately 120 days before OPU) than the remaining 3 cows, which were multiparous (data not shown). This intensive adipose tissue mobilization during early lactation, the period corresponding to the retrospective duration of the folliculogenesis of oocytes collected at 130–160 DIM [112-114], may be associated with the elevation observed in oocyte TAG at 130–160 DIM in these two cows. The number of cows in the middle lactation stage in the present study was small; therefore, I need to investigate the long-term effects of reductions in BCS in postpartum cows on oocyte lipid profiles and their relationship with oocyte quality in a larger number of cows.

5. Conclusion

The present study revealed that cows subjected to typical modern intensive management had high oocyte FFA and TAG in the early postpartum period (Fig. 8). Consistent with the increase in oocyte FFA, the 16:1/16:0 and 18:1/18:0 ratios as desaturase markers were elevated. These lipid changes were associated with increases in plasma FFA. Based on increases in oocyte FFA in combination with the inherently low ability of oocytes to synthesize TAG from FFA, oocytes appeared to be at a high risk of FFA lipotoxicity in the early postpartum period. Milk production by the cows examined in the present study was slightly lower than the average in Japan (9,800 kg/305 days, 2020, Dairy Herd Performance Test data statistics, Livestock Improvement Association of Japan, Inc., Tokyo, Japan); therefore, oocyte lipid compositions may be affected more in cows from farms with higher milk production and a high incidence of postpartum metabolic diseases than in those used in this study. High circulating FFA in the early postpartum period may result in low fertility in modern high-yielding cows via the deterioration of oocyte quality due to lipotoxicity.

6. Tables and figures

Table 1. Average BCS and nutritional parameters of cows in different lactation stages

Item	20–30 DIM (n = 5)	40–50 DIM (n = 5)	60–80 DIM (n = 5)	130–160 DIM (n = 5)
BCS[1]	2.9 ± 0.5	2.9 ± 0.4	2.9 ± 0.4	3.0 ± 0.3
BW[23] (kg)	622 ± 53	603 ± 37	584 ± 45	649 ± 83
Dry matter intake[24] (kg/day)	17.1 ± 2.0	19.1 ± 2.2	19.2 ± 2.7	20.9 ± 2.1
Milk production[245] (kg/day)	35.5 ± 8.2 (19.8–43.5)	34.2 ± 5.7 (24.7–40.6)	32.4 ± 7.6 (23.7–42.1)	29.2 ± 3.6 (25.7–35.8)
Milk fat[23] (%)	4.57 ± 0.18	4.38 ± 0.45	4.19 ± 0.21	4.29 ± 0.27
Milk protein[23] (%)	3.17 ± 0.47	2.96 ± 0.10	2.82 ± 0.23	3.43 ± 0.26
Milk lactose[23] (%)	4.12 ± 0.27	4.30 ± 0.19	4.50 ± 0.26	4.37 ± 0.13

Values are presented as the mean ± SD.

[1]BCS was assessed by trained farm staff in the week OPU was performed.

[2]Mean values during 21 days before OPU

[3]BW and milk components were measured weekly.

[4]Dry matter intake and milk production were calculated daily.

[5]Values in parenthesis indicate the minimum and the maximum values.

Table 2. Ingredient and chemical composition of the total mixed ration fed to cows

Total mixed ration	1–149 DIM[1]	≥150 DIM[2]
Ingredient (%DM)		
Grass silage	38.7	47.1
Corn silage	22.5	20.2
Rolled corn	24.0	20.8
Soybean meal	13.1	10.5
Calcium carbonate	1.6	1.4
Dicalcium phosphate	0.0	0.1
Chemical		
DM (%)	35.0	32.8
TDN[3] (%DM)	75.3	71.8
CP (%DM)	15.7	15.1
NDF (%DM)	38.1	41.6
NFC (%DM)	35.4	31.7

[1]Mean values were calculated from 83 samples.

[2]Mean values were calculated from 10 samples.

[3]TDN were calculated following NRC 2001 with digestibility estimated by total feces collection:

$$\text{TDN} = \text{digestible CP} + 2.25 \times \text{digestible EE} + \text{digestible NFC} + \text{digestible NDF}$$

Each component was calculated using the digestibility parameters as indicated in Appendix A of Nishiura et al. [93].

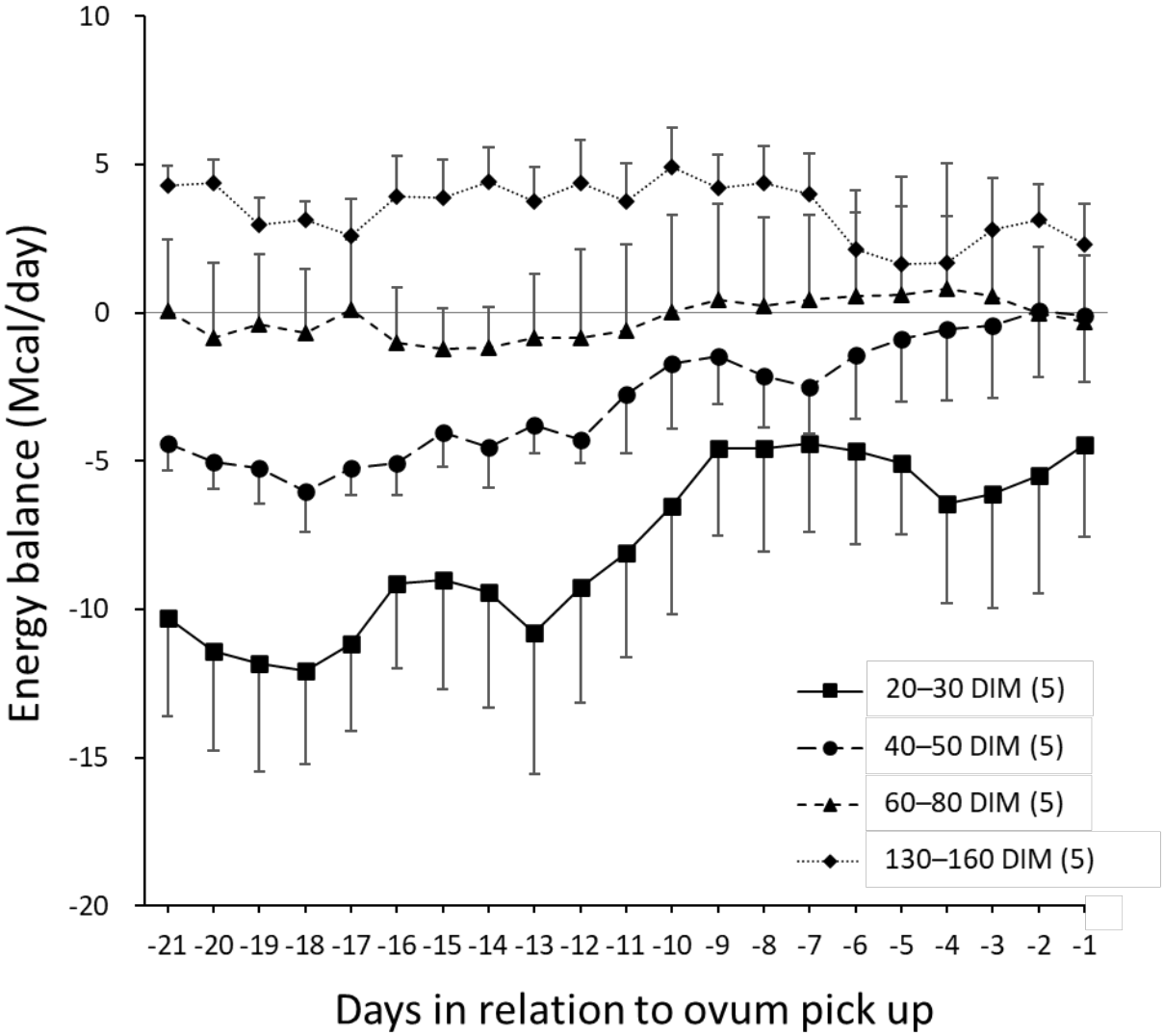

Fig. 1. Energy balance transition between 1 and 21 days before OPU.

Two types of total mixed rations were fed to cows based on DIM, and chemical components were analyzed using near-infrared reflectance spectroscopy at 2-week intervals. Daily energy balances of cows were calculated according to equations from NRC (2001). Data on the 3-day moving average of energy balances (the average values of the past 3 days) were used to remove short-term (within a few days) fluctuations in values. The values are shown in reference to the time of OPU (= Day 0). Symbols and error bars indicate mean values and SEM. The numbers in parentheses indicate the number of animals included in each lactation group.

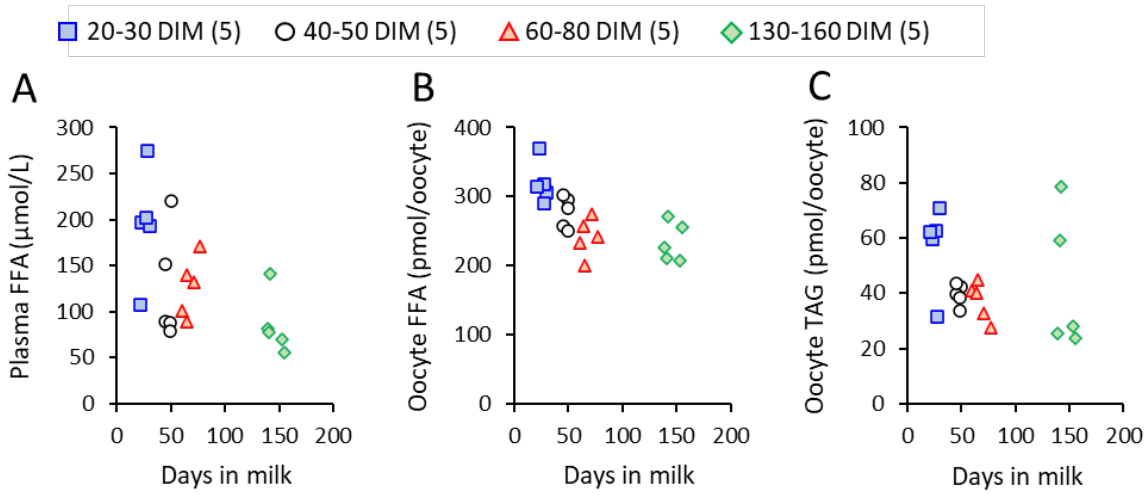

Fig. 2. Plasma FFA, oocyte FFA, and oocyte TAG profiles in relation to days in milk. Relationships between DIM and plasma FFA (A), oocyte FFA (B), and oocyte TAG (C). Blood sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation stages (20 cows in total), and FFA and TAG were analyzed using LC/MS with 100 µL of plasma and a single sample of 5 oocytes per cow. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group.

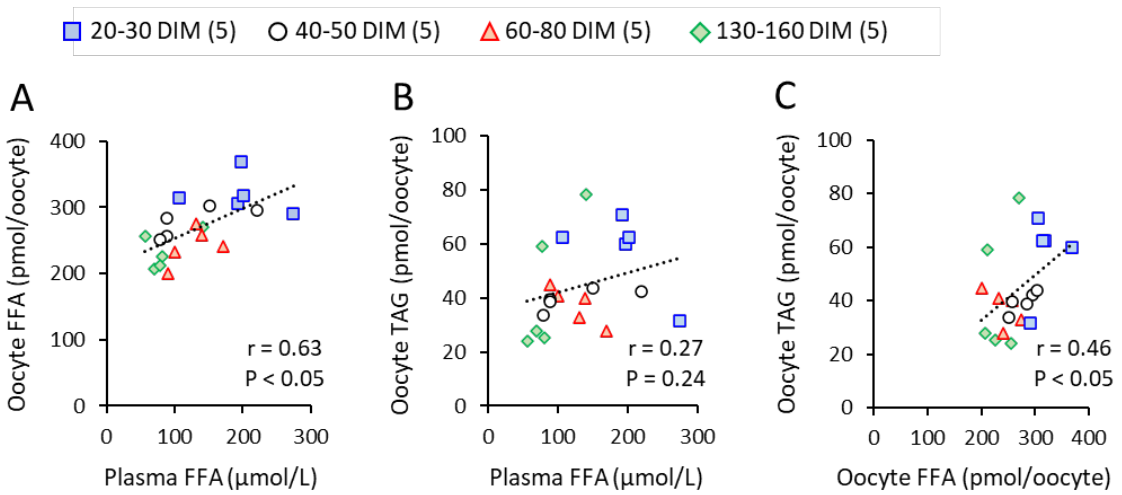

Fig. 3. Relationships between plasma FFA concentrations and oocyte FFA and TAG contents. Relationships between plasma FFA and oocyte FFA (A), plasma FFA and oocyte TAG (B), oocyte FFA and oocyte TAG (C). Sampling of blood and oocytes from cows and LC/MS analysis of FFA and TAG were performed as described in Fig. 2. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines represent the regression lines.

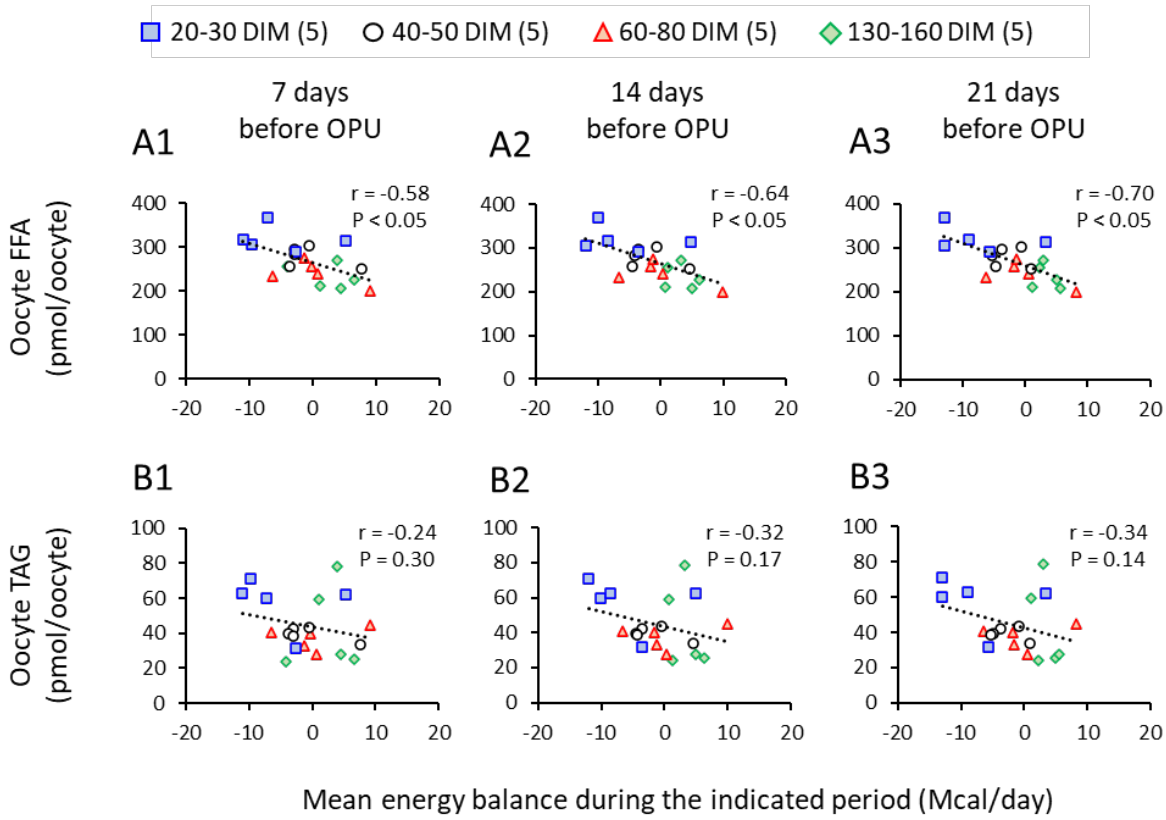

Fig. 4. Relationships between the energy balance and oocyte FFA and TAG contents. Relationships between energy balance during the indicate time before OPU and oocyte FFA (A) and oocyte TAG (B). Oocyte collection and LC/MS analysis of FFA and TAG were performed as described in Fig. 2. Energy balance was calculated daily as described in Fig. 1, and the mean energy balance during the indicated periods, 1 and 7 days before OPU (A1B1), 1 and 14 days before OPU (A2B2), and 1 and 21 days before OPU (A3B3) were calculated. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines represent the regression lines.

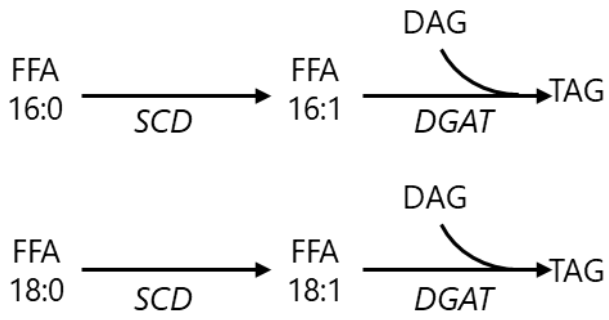

Fig. 5. Diagram of FFA desaturation reactions towards TAG synthesis in cells. FFA 16:1 and 18:1 are produced from FFA 16:0 and 18:0, respectively, by desaturation reactions which *SCD* catalyzes, and are subsequently utilized for TAG synthesis [67]. FFA, free fatty acid; DAG, diacylglycerol; TAG, triacylglycerol; *SCD*, stearoyl-CoA desaturase; *DGAT*, diacylglycerol acyltransferase

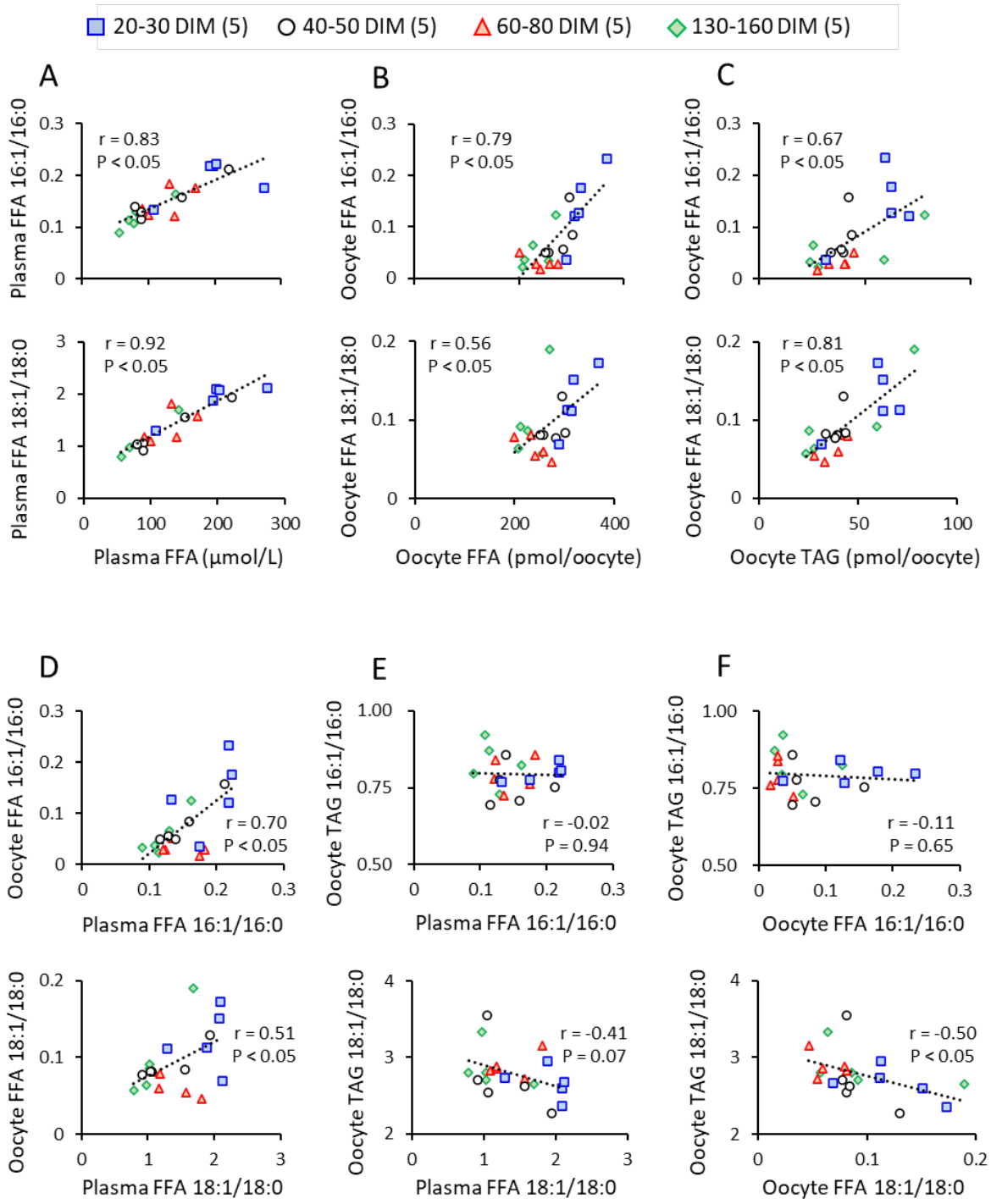

Fig. 6. Desaturase markers of fatty acid 16:1/16:0 ratio and 18:1/18:0 ratio in plasma and oocytes.

The upper panel (A–C) shows relationships between plasma FFA, oocyte FFA, and oocyte TAG and desaturase markers: plasma FFA concentrations and plasma FFA desaturase markers (A), oocyte FFA contents and oocyte FFA desaturase markers (B), and oocyte TAG contents and oocyte FFA desaturase markers (C).

The lower panel (D–F) shows relationships between desaturase markers of plasma FFA, oocyte FFA, and oocyte TAG: relationships of desaturase markers between plasma FFA and oocyte FFA (D), plasma FFA and oocyte TAG (E), and oocyte FFA and oocyte TAG (F).

Sampling of blood and oocytes from cows and LC/MS analysis of FFA and TAG were performed as described in Fig. 2. The ratios of fatty acid 16:1/16:0 and 18:1/18:0 were calculated. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines represent the regression lines.

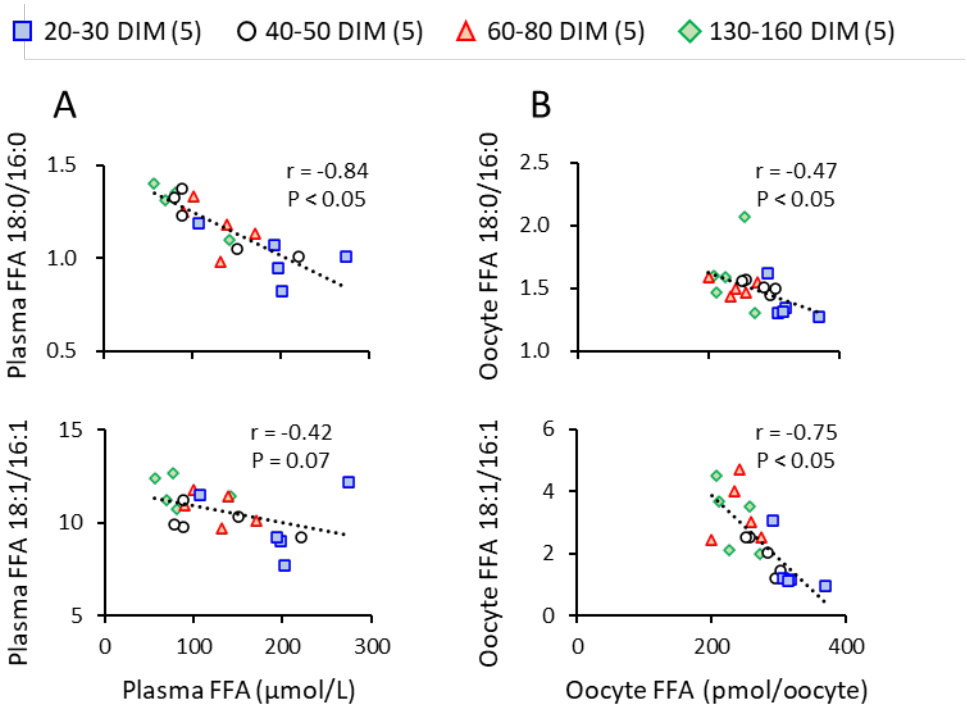

Fig. 7. Elongase markers of fatty acid 18:0/16:0 ratio and 18:1/16:1 ratio in plasma and oocytes. Plasma FFA concentrations and elongase markers (A), and oocyte FFA contents and elongase markers (B). Blood sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation stages (20 cows in total). FFA and TAG were analyzed using LC/MS with 100 µL of plasma and a single sample of 5 oocytes per cow, and the ratios of fatty acid 18:0/16:0 and 18:1/16:1 were calculated. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines represent the regression lines.

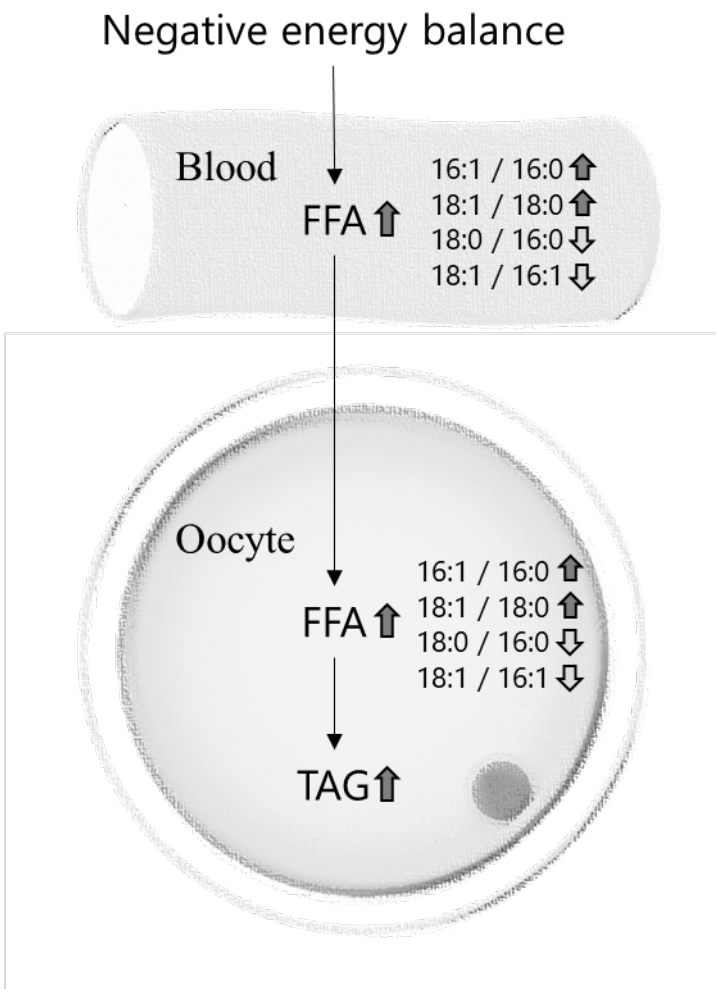

Fig. 8. Summary of the study: alterations in oocyte FFA and TAG profiles in early postpartum cows under intensive feeding management. A negative energy balance and elevated plasma FFA were observed in early postpartum cows (20–30 DIM). Plasma FFA desaturase markers (the 16:1/16:0 and 18:1/ 18:0 ratios) increased and elongase markers (18:0/16:0 and 18:1/16:1 ratios) decreased with elevations in plasma FFA concentrations. Alterations in the content and composition of oocyte FFA were similar to those in plasma FFA. The effects of abundant FFA in oocytes were reflected as an increase in TAG, the non-toxic form of lipids synthesized from FFA.

7. Summary

A severe negative energy balance and high circulating FFA in postpartum cows impair fertility. The lipotoxicity of FFA has been demonstrated to reduce the quality of bovine oocytes *in vitro*. Therefore, excess FFA in cells is converted to TAG, a non-toxic form of lipids, to protect cells from FFA lipotoxicity. The study in Chapter I found that the TAG content in oocytes was higher in postpartum lactating cows subjected to grazing management than in heifers. Since different feeding management systems are likely to affect lipid compositions in oocytes, the present study used cows under indoor intensive feeding management and investigated the compositions of the energy metabolism-related lipids, FFA and TAG, in the plasma and oocytes of cows at different lactation stages. The final aim of the study was to obtain insights into lipotoxicity in oocytes of the modern high-producing cows, particularly in early postpartum period.

Blood and oocytes were collected from 20 lactating cows categorized into the following lactation groups: 20–30 DIM (n = 5), 40–50 DIM (n = 5), 60–80 DIM (n = 5), and 130–160 DIM (n = 5). Daily energy balance data were obtained for 3 weeks prior to oocyte collection using the OPU method. The contents and compositions of FFA and TAG in plasma and oocytes were analyzed using LC/MS.

As expected, plasma FFA was high at 20–30 DIM, decreased by 50 DIM, and was maintained at a low level for the remainder of the experimental period. Similar changes were observed in oocyte FFA and TAG with DIM as plasma FFA. Oocyte FFA positively correlated with plasma FFA ($P < 0.05$), but negatively correlated with the mean energy balance 1 and 21 days before OPU ($P < 0.05$). Relationships were noted between the composition and content of FFA in plasma and oocytes, with the FFA 16:1/16:0 and 18:1/18:0 ratios positively correlating with the total amount of FFA ($P < 0.05$). The indicated oocyte FFA elevation in cows in the early postpartum period under intensive feeding management suggested that oocytes were at a high risk of FFA lipotoxicity in this period.

Summary and Conclusions

As a result of the livestock improvement prioritizing milk production over the last several decades, postpartum dairy cows experience elevation in circulating FFA that can cause multiple health disorders. Normally, excessively incorporated FFA in cells is converted to TAG, a non-toxic form of lipids. However, FFA incorporation beyond the ability of the cells to process can accumulate in cells and induce lipotoxicity. In fact, supplementation of high FFA has been demonstrated to induce lipotoxicity in oocytes *in vitro*. Investigations on FFA and TAG profiles in oocytes by LC/MS can provide a deeper understanding of lipotoxicity, however, oocyte FFA and TAG of lactating cows have not been studied to date. The objective of the present study was to investigate energy metabolism–related lipid (*i.e.*, FFA and TAG) compositions in plasma and oocytes of cows at different lactation stages, to obtain insights into lipotoxicity in the oocytes of dairy cows, particularly in the early postpartum period. Cows under grazing management with the lower milk production were explored for oocyte FFA and TAG in Chapter I, and cows under intensive feeding management with the higher milk production were examined in Chapter II.

In Chapter I, 14 cows (the mean milk yield of 7,710 kg/305 days) at three different lactation stages; the early lactation stage: 25–47 DIM (n = 6), peak lactation stage: 61–65 DIM (n = 4), and middle lactation stage: 160–202 DIM (n = 4), were enrolled. Heifers (n = 4) were used as a control group. Plasma and oocyte samples were collected by caudal venipuncture and OPU, respectively, and analyzed for FFA and TAG compositions by LC/MS. LC/MS lipid analysis detected and annotated a total of 6 FFA and 45 TAG species. Plasma FFA and oocyte TAG showed a positive correlation ($r = 0.55$, $P < 0.05$). The early lactation group showed higher oocyte TAG than heifers ($P < 0.05$), and the peak and middle lactation stage groups showed the intermediate levels of oocyte TAG. However, oocyte FFA was similar between lactation groups. Clustering analysis indicated that FFA and TAG in plasma and oocytes could be categorized into 5 groups; (1) saturated FFA (DB = 0); (2) unsaturated FFA (DB = 1 or 2); (3) 44–48 carbon–TAG (CN ranging between 44 and 48); (4) 50–54 carbon–TAG; and (5) 56–58 carbon–TAG. Regarding compositions of fatty acids, lower proportion of stearic acid (FFA 18:0) and higher proportion of oleic acid (FFA 18:1) in plasma were found in the early and peak lactation groups than in the middle lactation group and heifers ($P < 0.05$). However, only slight differences were observed in oocyte FFA and TAG fatty acyls between lactation groups. Collectively, early lactation cows under grazing management showed increase in oocyte TAG, but not in oocyte FFA, compared with heifers. This result suggested that the ability to manage lipotoxic FFA in oocytes in early lactation cows was reduced because their storage capacity for TAG was surpassed. Accordingly, oocyte lipid compositions of lactating cows under intensive feeding

management, which is the typical management style for the modern high-producing cows, should be investigated, because these cows may experience the larger impact on oocyte lipid compositions during early lactation period due to their larger energy intake and output than in grazed cows.

In Chapter II, 20 lactating cows (the mean milk yield of 9,123 kg/305 days) under intensive feeding management were used, and they were categorized into four lactation stages; 20–30 DIM (n = 5), 40–50 DIM (n = 5), 60–80 DIM (n = 5), and 130–160 DIM (n = 5). Their plasma and oocyte samples were analyzed for FFA and TAG compositions by LC/MS in the similar methods as Chapter I. Daily energy balance of the cows during 3 weeks prior to OPU was calculated. As expected, plasma FFA was high at 20–30 DIM, decreased by 50 DIM, and was maintained at a low level for the rest of the experimental period. The similar changes were observed in oocyte FFA and TAG with DIM as plasma FFA. Positive correlations were found between the pairs of plasma FFA–oocyte FFA ($r = 0.63$) and oocyte FFA–oocyte TAG ($r = 0.46$) ($P < 0.05$, respectively). Furthermore, oocyte FFA negatively correlated with the mean energy balance 1 and 21 days before OPU ($r = -0.70$), 1 and 14 days before OPU ($r = -0.64$), and 1 and 7 days before OPU ($r = -0.58$) ($P < 0.05$, respectively), thus the mean energy balance for a longer period before OPU showed a stronger negative correlation with oocyte FFA ($r = -0.70$, $P < 0.05$). Oocyte FFA positively correlated with the oocyte FFA desaturase markers, namely, FFA 16:1/16:0 ratio ($r = 0.79$) and 18:1/18:0 ratio ($r = 0.56$) ($P < 0.05$, respectively). Positive correlations were also observed between the plasma and oocyte FFA 16:1/16:0 ratio ($r = 0.70$) and the plasma and oocyte FFA 18:1/18:0 ratio ($r = 0.51$) ($P < 0.05$, respectively). Thus, relationships were noted between the composition and content of FFA in plasma and oocytes.

This was the first study that investigated oocyte lipid compositions of cows at different lactation stages and found that the increase in FFA and TAG and accelerated FFA desaturation in oocytes in the early postpartum period of cows under intensive feeding management. These findings suggested that oocytes in early postpartum period are at a risk of FFA lipotoxicity, particularly in cows under intensive feeding management for high milk production. In future studies, the existence and extent of lipotoxicity, such as reactive oxygen species production and endoplasmic reticulum stress, should be explored in oocytes in early postpartum cows, to understand its potential influence on subsequent cow fertility during the lactation.

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Summary in Japanese

過去数十年間の産乳能力を重視した乳牛の遺伝的改良により、乳期当たりの乳量は増加を続けてきた。その結果、高泌乳牛では分娩後の負のエネルギーバランスに伴い血液中の遊離脂肪酸（FFA）濃度が上昇し、この高濃度 FFA により種々の疾病および細胞の機能障害が誘発される。通常、細胞内に流入した過剰な FFA は毒性の無い脂質の貯蔵形態であるトリアシルグリセロール（TAG）に変換される。しかし、細胞の処理能力を超える過剰な FFA が流入すると、FFA は細胞内に蓄積し、細胞毒性を引き起こす。実際に、高濃度の FFA を添加して牛卵子を培養すると、卵子に脂質毒性が発現することが報告されている。液体クロマトグラフィー質量分析法（LC/MS）を用いて泌乳牛の卵子の FFA と TAG の組成を調べることは、卵子への脂質毒性の可能性について調査するために有用な方法であるが、これまでに泌乳牛の卵子の FFA と TAG の組成を調べた報告はない。そこで本研究で、特に分娩後早期の牛卵子の脂質毒性について評価するために、泌乳牛の血液と卵子のエネルギー代謝関連脂質（FFA および TAG）の組成を調べて泌乳ステージ間で比較した。第 1 章では産乳量の少ない放牧主体の牛群（平均 305 日乳量 7,710 kg）を、第 2 章では産乳量の多い濃厚飼料主体で飼養される牛群（平均 305 日乳量 9,123 kg）を用いて、卵子の FFA と TAG を調べた。

第 1 章では、3 つの泌乳ステージにあるホルスタイン種 14 頭を用いた。泌乳初期群（分娩後 25～47 日、n = 6）、泌乳ピーク群（分娩後 61～65 日、n = 4）、泌乳中期群（分娩後 160～202 日、n = 4）、および 未経産牛（n = 4、コントロール群）から血漿および卵子サンプルをそれぞれ尾静脈穿刺と経腟採卵法（OPU）により採取した。サンプル当たり 100 μL の血漿および 5 個の卵子を用いて LC/MS により FFA と TAG の組成を定性的、半定量的に解析した。その結果、血漿と卵子で合計 6 種類の FFA および 45 種類の TAG が同定された。血漿 FFA 濃度と卵子 TAG 量は正の相関を示した（$r = 0.55$、$P < 0.05$）。泌乳初期群は未経産牛よりも卵子 TAG 量が高く（$P < 0.05$）、泌乳ピーク群と泌乳中期群はその中間の卵子 TAG 量を示した。しかし、卵子 FFA 量はいずれの泌乳期においても同程度であった。クラスター解析により、血漿と卵子の FFA と TAG は 5 種類のグループ：（1）二重結合数 0 の飽和 FFA、（2）二重結合数 1 または 2 の不飽和 FFA、（3）炭素数 44～48 の TAG、（4）炭素数 50～54 の TAG および（5）炭素数 56～58 の TAG に分類された。脂肪酸の構成割合に関しては、泌乳初期群と泌乳ピーク群では泌乳中期群と未経産牛に比べて、血漿中のステアリン酸（FFA 18:0）の割合が低く、オレイン酸（FFA 18:1）の割合が高かった（$P < 0.05$）。しかし、卵子 FFA と卵子 TAG の脂肪酸組成に関しては、泌乳期のステージによる差異は殆どなかった。以上の結果より、放牧主体で飼養される泌乳初期の牛では、未経産

牛に比べて卵子 TAG 量が増加したが、卵子 FFA 量は増加しなかった。卵子内で貯蔵できる TAG 量には限界があることから、泌乳初期の牛の卵子の TAG の増加は、卵子における過剰な FFA を TAG に変換して貯蔵する能力の低下と、それに伴う卵子の脂質毒性のリスク増加を示唆した。

一方で、近年の高泌乳牛の典型的な飼養形態は濃厚飼料主体の飼養であり、放牧牛に比べて摂取エネルギー、消費エネルギーともに大きい代謝状態を示す。このようなエネルギー代謝の違いは卵子の脂質組成に影響する可能性があるため、濃厚飼料主体で飼養される牛の卵子の脂質組成を調べる必要がある。

第 2 章では、濃厚飼料主体で飼養されていた 4 つの泌乳ステージにあるホルスタイン種 20 頭を用いた。分娩後 20～30 日群（n = 5）、分娩後 40～50 日群（n = 5）、分娩後 60～80 日群（n = 5）および分娩後 130～160 日群（n = 5）から、第 1 章と同様の方法で、血漿と卵子のサンプルを採取し、LC/MS により FFA と TAG の組成を解析した。採卵前のエネルギーバランスと卵子の脂質との関係について調べるために、供試牛の OPU 前 3 週間の毎日のエネルギーバランスを計算した。血漿 FFA 濃度は分娩後 20～30 日で高く、50 日までに低下し、それ以降の供試期間中は低いレベルを維持した。卵子の FFA 量と TAG 量は、分娩後日数に伴う血漿 FFA の変化と同様の変化を示した。血漿 FFA 濃度と卵子 FFA 量（$r = 0.63$）、卵子 FFA 量と卵子 TAG 量（$r = 0.46$）の間にそれぞれ正の相関が見られた（$P < 0.05$）。さらに、卵子 FFA 量は OPU 前 21 日間、14 日間および 7 日間の平均エネルギーバランスとそれぞれ負の相関を示し（それぞれ $r = -0.70$、$r = -0.64$ および $r = -0.58$、$P < 0.05$）、その程度は OPU 前のエネルギーバランスを調べた期間が長いほど卵子 FFA 量と強い負の相関を示した（$r = -0.70$、$P < 0.05$）。卵子 FFA 量は、卵子 FFA 不飽和化マーカー（飽和 FFA を単価不飽和 FFA に変換する不飽和化反応の活性の指標）である FFA 16:1/16:0 比および FFA 18:1/18:0 比とそれぞれ正の相関を示した（それぞれ $r = 0.79$ および $r = 0.56$、$P < 0.05$）。また、血漿と卵子の FFA 16:1/16:0 比（$r = 0.70$）および血漿と卵子の FFA 18:1/18:0 比（$r = 0.51$）の間にも、それぞれ正の相関が見られた（$P < 0.05$）。すなわち、血漿と卵子で同様の FFA の量と組成の関係が示された。

本研究は異なる泌乳ステージの牛の卵子の脂質組成を調べた初の研究であり、その結果、濃厚飼料主体で飼養される牛における分娩後早期の卵子の FFA と TAG の増加および FFA 不飽和化の活性化が示された。これらの知見は、特に濃厚飼料主体で飼養される高泌乳牛において、分娩後早期の卵子に FFA の脂質毒性がある可能性を示唆するものであった。今後、分娩後早期の牛の卵子への脂質毒性がその後の繁殖性に及ぼす影響について調査するために、卵子中の活性酸素種の産生や小胞体ストレス等の脂質毒性の有無および程度について明らかにする必要がある。