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Impact of short-term high-fat feeding on lipid droplet content in mouse oocytes

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Abstract. Mature mammalian oocytes contain lipid droplets (LDs), which are neutral lipid storage organelles critically important for energy metabolism. In mice, maternal obesity, induced by long-term (> 3 months) high-fat feeding, contributes to the accumulation of LDs in mature oocytes. However, few studies have investigated the influence of short-term high-fat feeding on LD content. In this study, we demonstrated that 3 weeks of high-fat feeding is sufficient to increase LD content and intracellular triacylglycerol levels. Using a two-step centrifugation technique to release LDs into the perivitelline space, we found that short-term high-fat feeding increased the level of LDs in MII oocytes and that 3 days of high-fat feeding were sufficient to increase efficiency of LD release. Collectively, our study suggests that short-term high fat feeding can have a higher impact on lipid metabolism during oocyte maturation.
Key words: High-fat diet (HFD), Lipid droplet, Mouse, Oocyte, Short-term overfeeding

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Maternal obesity has a major impact on oocyte competence, and is associated with meiotic spindle abnormalities and mitochondrial dysfunction [1–4]. In addition, maternal obesity causes epigenetic alterations in developing oocytes [5], resulting not only in the impairment of early embryonic development but also in defects in fetal and postnatal development [6], thus implying dependence of oocyte competence on maternal nutritional and metabolic conditions.

A widely used model for studying the effect of maternal obesity on oocyte competence involves feeding female mice a high-fat diet (HFD) for a long period (> 3 months) [3, 6, 7]. However, short-term (3–7 days) high-fat overfeeding impairs insulin sensitivity and glycemic control before a substantial increase in body weight and fat mass is observed [8, 9]; hence, we speculated that short-term high-fat feeding could influence oocyte competence in terms of lipid metabolism.

Lipid droplets (LDs) are ubiquitous cellular organelles involved in lipid storage. They store neutral lipids mainly consisting of triacylglycerols (TGs) and sterol esters (SEs), utilized as a reservoir of metabolic energy or as precursors for membrane synthesis [10, 11]. Mammalian oocytes and embryos contain LDs, although their content varies substantially among species: for example, mature porcine oocytes contain ~10-fold more lipids than mouse oocytes [12].

In our previous study, we used a method termed forced lipophagy for the autophagic degradation of LDs, and demonstrated that proper amounts of LDs are essential for preimplantation embryonic development [13]. More recently, using a novel technique for isolating almost all LDs from mouse MII-oocytes, we revealed adequate maintenance of LD mass during early embryonic development [14]. These findings imply that LDs play a pivotal role even in mouse oocytes, which contain low levels of LDs. It is plausible that studies focusing on maternal environmental factors affecting LD content in mature oocytes may reveal the unknown mechanisms involved in oocyte competence. In this study, we sought to determine whether short-term high-fat feeding changes LD content in MII mouse oocytes.

In preliminary studies, to investigate if short-term high-fat feeding affects oocyte maturation in response to hormonal stimulus, C57BL/6 females were fed either HFD or a control low-fat diet (LFD) for 3, 7, 14, or 21 days and then superovulated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to collect mature MII oocytes. The average number of MII oocytes collected over the entire experimental period was similar between the HFD- and LFD-fed females (Fig. 1A), whereas body weight increased linearly in the HFD group except in the 3-day HFD group, with significant differences between the HFD and LFD groups (Fig. 1B). These results suggest no effect of short-term HFD on hormonally induced oocyte maturation.

To explore whether short-term high-fat feeding increases TG content in MII oocytes, we collected MII oocytes from superovulated C57BL/6 females fed either HFD or LFD for 21 days. The levels of TG species in MII oocytes were analyzed using liquid chromatography/ Fourier transform mass spectrometry (LC/FT-MS), and higher levels of all TG species in MII oocytes were observed in HFD-fed females
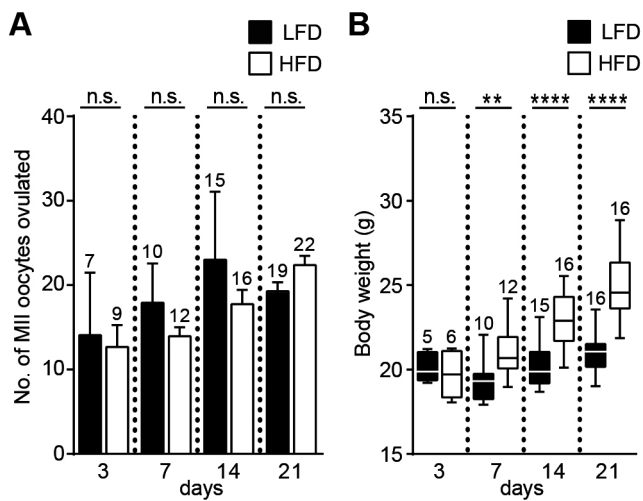
A

B

Fig. 1.   Changes in the number of ovulated MII oocytes and body weight of female mice fed short-term high-fat diet (HFD). (A) Average number of MII oocytes collected from female mice fed HFD or low-fat diet (LFD) for 3, 7, 14, or 21 days. (B) Body weight of female mice fed LFD or HFD for the indicated time period. Numbers above bars indicate numbers of female mice analyzed. Data represent means ± SEM of at least three independent experiments. n.s. non-significant, ** $P < 0.01$, **** $P < 0.0001$, Student's *t*-test.
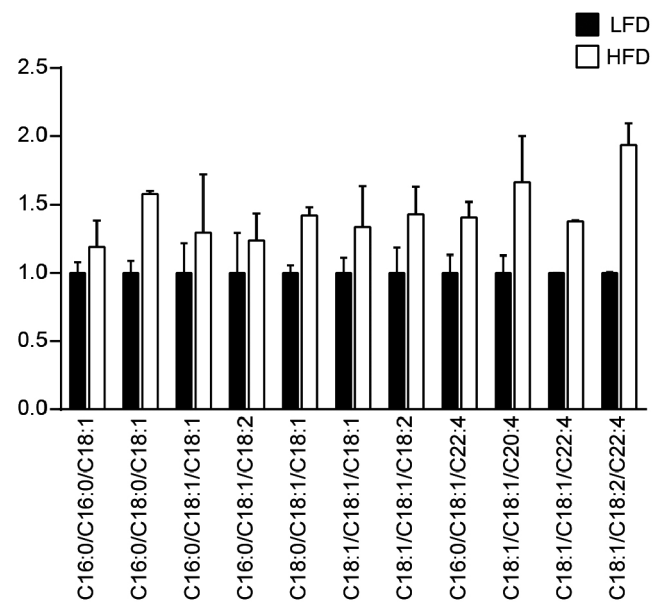
Fig. 2.   Three weeks of high-fat feeding increases triacylglycerol (TG) levels in MII oocytes. Quantification of the relative levels of TG species measured using liquid chromatography/Fourier transform mass spectrometry (LC/FT-MS) in MII oocytes derived from female mice fed low-fat diet (LFD) or high-fat diet (HFD) for 21 days. The x-axis represents triacylglycerol molecular species composed of three fatty acids esterified to a glycerol backbone. Data represent means ± SEM of two independent experiments with 100 MII oocytes in each condition.

than in LFD-fed females (Fig. 2), indicating 21 days of high-fat feeding as sufficient duration to increase TG content in MII oocytes. As TGs are the major storage lipids in LDs, these results also imply elevated LD mass in MII oocytes.

Labeling LDs with fluorescent dyes like BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-Indacene) is a convenient way to observe LD distribution in MII oocytes/embryos. However, these dyes may not be suitable for quantification of LD levels, especially when the LDs tend to aggregate in MII oocytes, as it may result in fluorescence saturation or strong autofluorescence (Supplementary Fig. 1: online only). In a previous study, we developed a method to remove almost all LDs from MII oocytes without cell disruption [14]. This method is based on two-step centrifugation combined with hyperosmotic treatment: the cytoplasmic LDs are aggregated at the cell peripherally after the initial centrifugation (4,200 *g* for 10 min), and the resulting LDs are released into the perivitelline (PV) space, which is expanded by hyperosmotic treatment, after the second centrifugation (9,500 *g* for 10 min). We hypothesized that increased LD content may cause an increase in LD release efficiency after two-step centrifugation, and the efficiency of LD release is related to the LD content in MII oocytes.

To test this hypothesis, we collected MII oocytes from C57BL/6 females fed either HFD or LFD for 3, 7, 14, or 21 days and performed a two-step centrifugation (Fig. 3A). Notably, over the entire experimental period, the proportion of LD release after a two-step centrifugation was markedly higher in MII oocytes from HFD-fed females than from LFD-fed females (Fig. 3B). Consistent with this, we found a significantly larger area occupied by LDs within the PV space in MII oocytes from HFD-fed females than those from LFD-fed females

(Fig. 3C), whereas the cytoplasmic area remained unchanged, except in the 7-day HFD group (Fig. 3D). These results indicate that 3 days of high-fat feeding is sufficient to increase the TG content in MII oocytes, increasing the LD release rate and thereby expanding the area of PV space occupied by LDs. Furthermore, these results suggest that the increased LD release rate may be a true reflection of the level of LDs in MII oocytes.

Our previous finding that LDs are newly synthesized soon after LD removal (delipidation) [14] led us to explore the influence of short-term high-fat feeding on the production of new LDs. For this, MII oocytes collected from female mice fed HFD or LFD for 3, 7, 14, or 21 days were delipidated by a two-step centrifugation method, followed by culture for 1.5 h or 25 h without a fatty acid (FA) supply. The MII oocytes were then stained with BODIPY 493/503 to visualize new LDs. BODIPY staining revealed newly synthesized LDs at 1.5 h after delipidation, as previously reported by us [14], and the LDs became markedly more abundant during culture (Supplementary Fig. 2: online only). Importantly, these changes in LDs were observed in all experimental periods without detectable differences between HFD- and LFD-fed groups, indicating that short-term high-fat feeding influences LD synthesis during *in vivo* oocyte maturation.

We previously demonstrated that the proportion of successful delipidation was dependent on mouse strain [14], probably due to differences in food intake and nutrient regulation system, with C57BL/6 mice more susceptible to diet-induced obesity than other
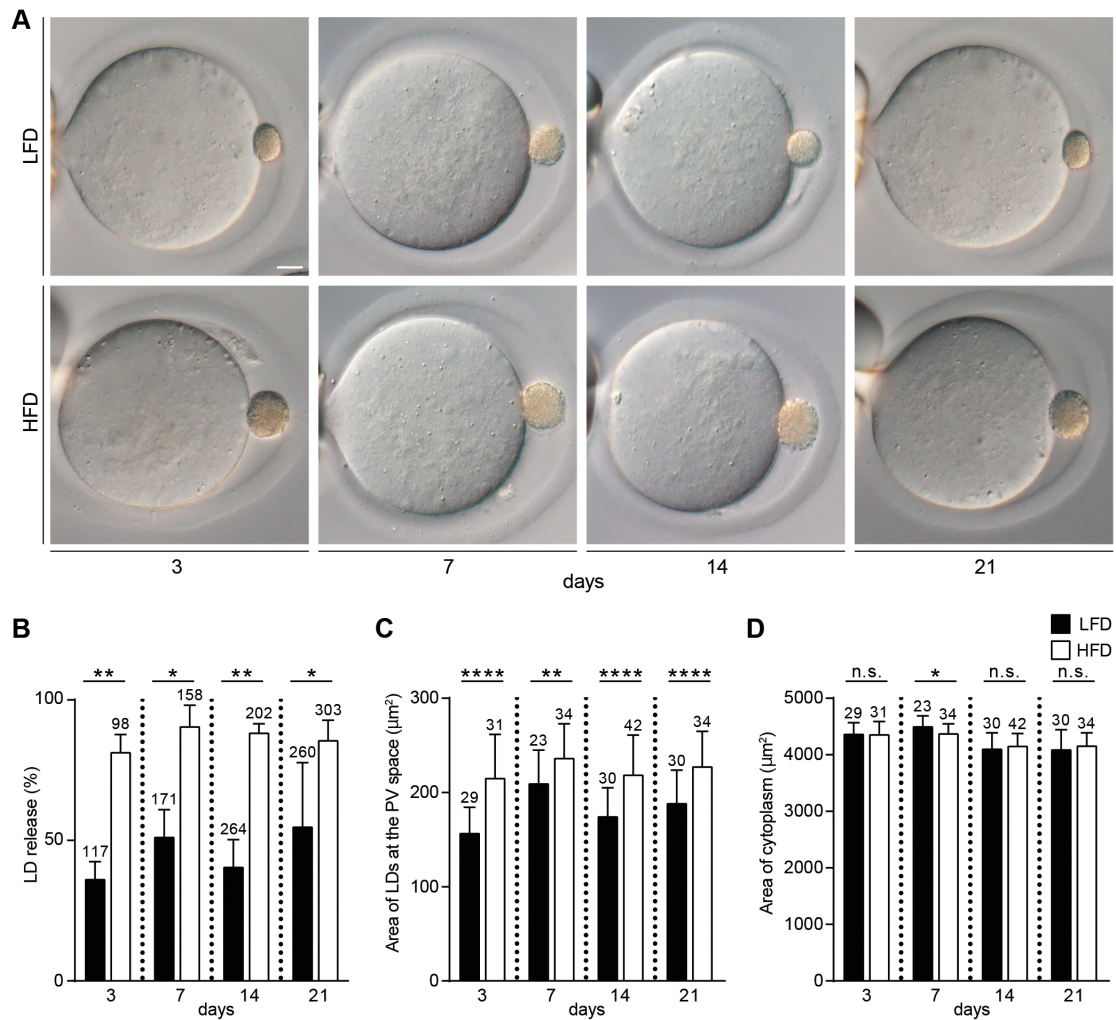
Fig. 3. Short-term high-fat feeding increases lipid droplet (LD) size and their release rate after a two-step centrifugation. (A) Representative images of MII oocytes after a two-step centrifugation. MII oocytes were collected from female mice fed high-fat diet (HFD) or low-fat diet (LFD) for 3, 7, 14, or 21 days and centrifuged to release the cytoplasmic LDs into the perivitelline (PV) space. Scale bars, 10 μm. (B) Percent of efficiency of LD release into the PV space after a two-step centrifugation. (C) Quantification of total LD area (at the PV space) in MII oocytes after a two-step centrifugation. (D) Quantification of total cytoplasmic area in MII oocytes after a two-step centrifugation. Numbers above bars indicate total numbers of MII oocytes analyzed. Data represent means ± SEM of at least three independent experiments. n.s. non-significant, * P < 0.05, ** P < 0.01, **** P < 0.0001, Student's *t*-test.

mice [15, 16], contributing to the higher rate of successful delipidation in C57BL/6 mice. Hence, we examined whether the physiological response to short-term overfeeding is conserved in other mice with a lower rate of successful delipidation as well, such as the outbred stock ICR. Therefore, we repeated the experiments using MII oocytes collected from ICR mice. The results demonstrated increase in the area of PV space occupied by LDs after two-step centrifugation due to 7-day high-fat feeding (Supplementary Fig. 3: online only), implying conservation of physiological response to short-term overfeeding, although the delipidation rate differed substantially between mouse strains.

Extensive studies investigating the effect of long-term (> 3 months) high-fat maternal overfeeding on oocyte competence have been conducted in mice [3, 6, 7]. However, only few studies have described the effect of short-term (4 weeks) high-fat feeding on oocyte maturation, with the studies reporting increased lipid content in mature oocytes and/or their surrounding cumulus cells [17, 18]. In contrast, our study is the first to demonstrate that high-fat feeding for less than 3 weeks is sufficient to increase LD content in MII oocytes. Such an increase in LDs contributes to a higher proportion of LD release after a two-step centrifugation, indicating a correlation between increased LD mass and LD release efficiency. Further, we revealed that short-term high-fat feeding had little effect on new LD synthesis after delipidation, implying an important role of the intrafollicular environment in lipid metabolism in developing oocytes.

To determine the influence of increase in LD mass due to short-term

HFD on oocyte competence, further studies such as IVF and embryo transfer experiments are required. However, given our previous findings that excess LDs are rapidly hydrolyzed to normalize LD content, probably through lipase activity [14], it is reasonable to speculate that overabundance of LDs, induced by short-term HFD, does not greatly affect their competence.

Fully-grown oocytes are found in antral follicles that are characterized by the presence of an antrum filled with follicular fluid. Lipids constitute a major component of the follicular fluid, and maternal obesity causes an increase in lipid content, especially in TG levels [19], which may result in lower oocyte quality. Therefore, short-term high-fat feeding may cause a rapid increase in the lipid content of the follicular fluid, facilitating FA uptake by somatic follicular cells, particularly the cumulus cells. As a result, these FAs may be stored as TGs in cumulus cells or may be provided directly to the oocytes through gap junctions, where they are utilized for LD production. Follicular fluid also contains many metabolites, including steroid hormones, enzymes, and small molecules other than lipids [20, 21]. These components may be altered by metabolic stress like short-term overfeeding or obesity, and these changes can influence lipid metabolism, leading to LD accumulation in oocytes inside the follicle.

The fundamental mechanisms by which short-term high-fat feeding increases TG content remain unknown. To resolve these issues, it will be important to understand when and how LDs are generated and stored during oogenesis.

Methods

Mice

Eight-week old C57BL/6J female mice (Japan SLC, Shizuoka, Japan) were group-housed (4–5 mice per cage) under a 12 h light:12 h dark cycle and specific pathogen-free conditions. The mice were allowed to adapt to the housing conditions for 1 week. During this period, all mice were fed a standard diet (MBR-1, Funabashi form, Chiba, Japan). The mice were fasted for 1 day, followed by feeding of either a high-fat diet (HFD; Research Diets D12492 containing 5.2 kcal/g, 20% kcal protein, 60% kcal fat, and 20% kcal carbohydrate) or a control low-fat diet (LFD; Research Diets D12450J containing 3.8 kcal/g, 20% kcal protein, 10% kcal fat, and 70% kcal carbohydrate, containing similar FA composition and sucrose levels to HFD [22]) with *ad libitum* access to food and water for 3, 7, 14, or 21 days before MII oocyte collection. ICR female mice (Japan SLC) fed HFD or LFD for 7 days were used for the experiment described in Supplementary Fig. 3. Body weights were measured prior to MII oocyte collection. All animal experiments were performed in accordance with the relevant guidelines and were approved by the Animal Care and Use Committee of the National Institute of Quantum and Radiological Science and Technology.

MII oocyte collection and LD removal by a two-step centrifugation

MII oocytes were collected from superovulated females as described previously [23] and maintained in FHM medium (270 mOsm) containing 10 µg/ml cytochalasin B (C2743, Sigma-Aldrich, St. Louis, MO, USA) and FA-free BSA (4 mg/ml, A8806, Sigma-Aldrich) for more than 30 min. A two-step centrifugation for releasing

cytoplasmic LDs into the PV space was performed as described in our previous report [14]. After the two-step centrifugation, the area of the cytoplasm and LDs (occupied in the PV space) were measured using the cellSens software (Olympus, Waltham, MA, USA). Images were acquired with a 40x UPLSAPO 0.95 NA or 20x UPLSAPO 0.75 NA objectives (Olympus) and recorded with a CCD camera (Prime 95B; Photometrics, Tucson, AZ, USA). LDs released into the PV space were removed using a micropipette when necessary, as described previously [14].

Fluorescent labelling of LDs in MII oocytes

To visualize LDs, MII oocytes were incubated for 30–60 min with BODIPY 493/503 (1–2 µg/ml, D3922, Thermo Fisher Scientific, Waltham, MA, USA) in PB1 FA-free BSA, washed once, and transferred to PB1 FA-free BSA in a glass-bottom dish (D11140H; Matsunami Glass, Osaka, Japan) covered with mineral oil. Fluorescence images were acquired using a spinning disk confocal (X-Light V2; Molecular Devices, San Jose, CA, USA) as previously described [14].

TG analysis

TG species analysis was performed as previously described [14]. Briefly, 100 MII oocytes collected from female mice fed either LFD or HFD for 21 days were washed several times with PBS supplemented with PVP, transferred into a sterile microtube containing 20 µl ultrapure water (43001-1B; Kanto Chemical, Tokyo, Japan), and stored at –80°C until analysis. Sample preparation and LC/FT-MS analysis were performed at the Environmental Technology Department at the Chemicals Evaluation and Research Institute, Japan (CERI, Tokyo).

Statistical analysis

A two-tailed Student's *t*-test was used to calculate P values. Statistical analysis was performed using Prism 6 (GraphPad). Data represented mean ± SEM. Significance levels: n.s., non-significant, $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$, $**** P < 0.0001$.

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