The role of the adiponectin system in acute fasting-impaired mouse ovaries

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Abstract

Adiponectin (ADIPOQ, encoded by *Adipoq*) is an important white adipose-derived adipokine linked to energy homeostasis and reproductive function. This study aims to reveal the expression and role of the adiponectin system in the ovaries under acute malnutrition. In this study, 48-h food deprivation significantly inhibited ovarian growth by suppressing cell proliferation and inducing cell apoptosis in the ovaries of gonadotrophin-primed immature mice. It was also accompanied by significantly decelerated basic metabolism (glucose, triacylglycerol and cholesterol), varied steroid hormones (follicle-stimulating hormone, luteinizing hormone and estradiol) and vanishment of the peri-ovarian fat. It is noteworthy that after acute fasting, the adiponectin levels in ovaries rather than in blood were significantly elevated. Immunohistochemical study demonstrated that adiponectin and its receptors (ADIPOR1 and ADIPOR2) primarily appeared in ovarian somatic and/or germ cells, and their protein expressions were upregulated in the ovaries from fasted mice. Further *in vitro* study verified that ADIPOR1/2 agonist obviously inhibited follicle-stimulating hormone-induced oocyte meiotic resumption, while the antagonist significantly enhanced the percentage of oocyte maturation in the absence of follicle-stimulating hormone. Furthermore, the build up of peri-ovarian fat under physiological status in mice showed a positive correlation with both the hypertrophy of adipocytes and growth of ovaries. Taken together, these findings indicate that the upregulation of the adiponectin system disturbs the normal female reproductive function under the malnutrition status, and it may be associated with the loss of peri-ovarian fat depots.

Reproduction (2019) 158 429–440

Introduction

Female reproduction is considered as an energy consuming process, consisting of follicle development, oocyte maturation, ovulation, fertilization, parturition and lactation (Della Torre *et al.* 2014). Numerous studies have proved that either overnutrition or undernutrition affects normal reproductive function, leading to serious reproductive disorders (Yan *et al.* 2008, Garcia-Garcia *et al.* 2011, Wu *et al.* 2015). Though obesity or overnutrition has aroused huge attentions worldwide, nutrient deficiency still plagues some low- and middle-income countries and is one of the greatest threats to the survival of wildlife. As reported, malnutrition disturbs ovarian development primarily by regulating hypothalamic–pituitary–gonadal (HPG) system and the involved biological activities (Li *et al.* 2014, Wang *et al.* 2016). Previous studies proved that acute food deprivation in mice would result in oocyte maturation inhibition and fertility reduction by regulating glucose transport and utilization (Han *et al.* 2012). However, whether other energy metabolism-related factors

participate in this undernutrition-related ovarian impairment remains unclear.

White adipose tissue is a critical energy reservoir, consisting of various fat depots. A growing number of studies have indicated that fat present around abdominal viscera was different from that present in subcutaneous areas, depicting critical functions in inflammation, insulin resistance, type 2 diabetes as well as metabolic syndrome (Fontana *et al.* 2007, Hayashi *et al.* 2008). Rodents are most frequently used pre-clinical models of human metabolic diseases, whereas fat deposition in rodents is not identical to those in humans. Gonadal fat depot tissue refers to a major composition of visceral white fat in rodents, covering peri-ovarian adipose tissues (POAT) in females and epididymal fat depots in males. However, humans do not exhibit gonadal fat depots in their bodies. In most literature, the gonadal adipose tissues of rodents are considered as the corresponding visceral fat depots in humans. Recently, POAT in female mice was reported as the critical factor for ovarian development (Wang *et al.* 2017,

Yang *et al.* 2018a). However, the function of POAT during female reproduction under malnutrition condition has been insufficiently known.

Besides energy storage, white adipose tissue is another vital endocrine organ that secretes a number of peptide hormones named adipokines (e.g., leptin, adiponectin, resistin and visfatin) (Scherer 2006), mostly linked with reproductive function. Among them, the 30 kDa protein adiponectin is the most abundant adipokine being critical to glucose and lipid metabolism (Berg *et al.* 2001, Combs *et al.* 2004, Lihn *et al.* 2005). Unlike those of most adipokines, circulating adiponectin levels are inversely correlated with body mass index (Arita *et al.* 1999, Yamamoto *et al.* 2002). Adiponectin is primarily bound to its seven transmembrane protein receptors, ADIPOR1 and ADIPOR2, and all of those, forming the adiponectin system. Recent studies have found that adiponectin system expressed in various cerebral and reproductive tissues, including hypothalamus, pituitary, testis, ovary and placenta (Caminos *et al.* 2008, Tsatsanis *et al.* 2015, Sartori *et al.* 2016, Rak *et al.* 2017). Accumulating evidences indicated that hypoadiponectinemia in female was often associated with the metabolic dysregulations and reproductive dysfunctions (e.g., polycystic ovary syndrome) (Artimani *et al.* 2016). In ovary, adiponectin and its receptors are abundantly expressed, whereas the subcellular localizations are inconsistent in different species (Chabrolle *et al.* 2007a,b, 2009). It has been extensively proved that adiponectin inhibited proliferation or stimulated apoptosis in various mammalian cancer tissues (Diedonne *et al.* 2006, Nakayama *et al.* 2008). Adiponectin is found working in the processes of granulosa cells steroidogenesis and proliferation as well as oocyte maturation (Pierre *et al.* 2009, Maillard *et al.* 2010, Oliveira *et al.* 2017), whereas the effects of adiponectin on ovarian cell proliferation and apoptosis in female reproduction remain controversial.

In the current study, POAT was found closely associated with the ovarian development, and it disappeared in fasted immature female mice. Based on these findings, a hypothesis was proposed that the abnormality of ovarian function under malnutrition might be attributed to the loss of POAT and locally secreted adipokines. To test this hypothesis, the ovarian cell proliferation and apoptosis, blood metabolic and hormonal status, and ovarian adiponectin system were analyzed in the control and fasted mice. Furthermore, experiments to test the effects of ADIPOR1/2 agonist and antagonist on oocyte maturation *in vitro* were designed. Results obtained here will help gain more insights into the role of gonadal fat and adiponectin system in female reproduction, especially under the condition of energy metabolism imbalance.

Materials and methods

Materials

All chemicals used were purchased from Sigma-Aldrich Corp. unless otherwise indicated. Acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulfate, glycine, SDS-PAGE pre-stained molecular weight standards, protein assay kits and horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulins (IgG) were obtained from Beijing ComWin Biotech Inc. (Beijing, China). The Click-iT Plus TUNEL Assay kit, SlowFade Gold Antifade Mountant with 4',6'-diamidino-2-phenylindole (DAPI), Medium 199 (M199) and TRIzol Reagent were purchased from Thermo Fisher Scientific Inc.. PVDF membranes and Immobilon Western Chemiluminescent HRP Substrate kit were from Millipore. Anti-rabbit PCNA, caspase-3 (CASP3), cleaved caspase-3 and PARP were purchased from Cell Signaling Technology Inc.. Diaminobenzidine, 2-(4-Benzoylphenoxy)-*N*-[1-(phenylmethyl)-4-piperidinyl] acetamide (ADIPOR agonist), anti-rabbit cyclin D1, D2, D3 (CCND1, CCND2, CCND3) and p15 polyclonal (CDKN2B) antibodies, anti-mouse p21 (CDKN1A), p27 (CDKN1B), β -actin (ACTB) and vinculin (VCL) monoclonal antibodies and anti-goat ADIPOR1 and ADIPOR2 antibodies were purchased from Santa Cruz Biotechnology Inc.. Anti-mouse adiponectin antibody was purchased from Abcam Inc.. AdipoRon (ADIPOR antagonist) was purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). The mouse adiponectin ELISA kit was obtained from Cusabio Biotech Co. Ltd. (Wuhan, China).

Animals

Kunming female mice were purchased from China Institute of Laboratory Animal Science and were housed at a constant temperature (24–26°C) and under a controlled light cycle (14-h light and 10-h darkness). All animal work was approved by the Policy on the Care and Use of Animals of the Ethics Committee of Beijing Forestry University.

To study the physiological growth of POAT, new born, 1, 2, 3 and 6-week-old mice were utilized; all these animals had *ad libitum* access to food and water. In the acute fasting experiment, immature female mice (3 weeks) were injected intraperitoneally with equine chorionic gonadotrophin (eCG, 5 IU/mouse) for one time and killed by cervical dislocation 48 h later. In each experimental replicate, 30 mice were taken and divided into two groups (15 mice per group). One group was called the fasted group (F group), and the other group was called the control group (C group). The C group had *ad libitum* access to food and water, while the F group mice were only given water *ad libitum*.

Biochemical and hormonal assays

Blood was collected and centrifuged at 825 *g* for 15 min. Plasma was separated and transferred to a sterile tube and stored at –80°C until assayed. Plasma and ovarian protein sample were used for detection of adiponectin levels by the mouse adiponectin ELISA kit according to the manufacturer's

instructions. Absorbance measurements at 450nm were performed using microplate spectrophotometer. Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estrogen (E2) were assayed by direct solid-phase radioimmunoassay (RIA) using commercially available kits (Northern Biotechnology, Beijing, China). Concentration of glucose (GLU), triglyceride (TG), and cholesterol (CHO) were determined by Technicon RA-1000 using commercially available kits (Northern Biotechnology, Beijing, China). The intra- and inter-assay coefficients of variation were 10%.

Histology, immunohistochemistry and immunofluorescence

Ovaries and POAT were fixed in cold 4% (w/v) paraformaldehyde overnight. After fixation, tissues were dehydrated through an alcohol series and embedded in paraffin, and subsequently sectioned at 5 μ m using a microtome. After dewaxing and rehydration, sections were processed for hematoxylin-eosin (HE) staining, immunohistochemistry, immunofluorescence or TUNEL assay.

Tissues for HE staining were stained with hematoxylin for 5 min and eosin for 3 min. Ovaries for immunohistochemistry were immersed in 10 mM sodium citrate buffer (pH 6.0) and heated in a microwave oven for antigen retrieval. Thereafter, sections were incubation in 3% H_2O_2 to inhibit endogenous peroxidase. Then, all tissues samples were rinsed in PBS and incubated with primary antibodies at 4°C at least for 16 h. Nonimmune serum was used as a negative control. All primary antibodies were used at a dilution of 1:100. After washes in PBS, the sections were incubated with the biotinylated secondary antibody for 30 min at 37°C, and then were rinsed in PBS. The staining was revealed by incubation with 3,3'-diaminobenzidine tetrahydrochloride at room temperature. Finally, sections were counterstained with hematoxylin and observed under an Olympus BX51 microscope.

Sections for immunofluorescence were performed generally the same as immunohistochemistry described earlier, and normal donkey serum (5% v/v) was used as blocking solution and fluorescein-conjugated donkey anti-goat or anti-mouse secondary antibodies were used at a dilution of 1:1000. Sudan black (1% w/v) was used to block the unspecific stain before counterstaining with DAPI and sections were observed under an Olympus BX51 fluorescence microscope.

Real-time quantitative polymerase chain reaction analysis

Total RNAs of ovaries were extracted using TRIzol Reagent according to the manufacturer's protocol, and then proceeded

for cDNA generation. The mRNA abundance profiles of target genes were analyzed using real-time quantitative polymerase chain reaction (qPCR) and normalized against *Actb* mRNA. The primer pairs used in this study were listed in Table 1. The final data were analyzed by the $2^{-\Delta\Delta CT}$ method as previously mentioned (Livak & Schmittgen 2001).

Western blot

Total proteins were extracted from the whole ovaries on ice in lysis buffer containing 10 μ mol/L protease inhibitors. Homogenates were centrifuged at 12000 rpm for 6 min at 4°C, and the protein concentrations in the supernatants were determined using BCA protein assay kit. Aliquots of proteins were boiled in 100°C water for 10 min and then stored at -20°C until analyzed. Protein extracts (10–40 μ g) were separated by SDS-PAGE with 5% (w/v) stacking gel and 10% (w/v) separating gel at 100V for approximately 1.5 h and electrically transferred to 0.45 μ m PVDF membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) to block nonspecific binding for 2 h at room temperature, and then incubated with primary antibody in TBST with 0.5% non-fat milk at 4°C at least 16 h. After washes in TBST, the membranes were then incubated with HRP-conjugated secondary antibody for 2 h at room temperature. After washing again in TBST, the blots were visualized by Immobilon Western Chemiluminescent HRP Substrate kit, and protein content was determined by densitometrically scanning the exposed x-ray film. Primary antibodies were diluted as follows: ADIPOR1, 1:1000; ADIPOR2, 1:1000; adiponectin, 1:5000; CCND1, 1:800; CCND2, 1:800; CCND3, 1:800; p15, 1:1000; p21, 1:1000; p27, 1:1000; PCNA, 1:2000; caspase-3, 1:2000; cleaved caspase-3, 1:1000; PARP, 1:1000; ACTB, 1:5000; and VCL, 1:5000.

TUNEL assay

Analysis of apoptotic cells in the ovaries was identified by the TUNEL technique according to the manufacturer's instructions. Paraffin-embedded ovaries sections (5 μ m) were deparaffinized and treated with paraformaldehyde (4% w/v) after washing with PBS, the slides were incubated with proteinase K at room temperature for 15 min. After washing with PBS, the positive control was treated with Dnase I (1 unit Nasel diluted in 20 mmol/L Tris-HCl, pH 8.4, 2 mmol/L $MgCl_2$ and 50 mmol/L KCl for 30 min at room temperature). Then, all tissue samples were rinsed in PBS and incubated with TdT for 60 min at 37°C and Click-iT Plus reaction at 37°C

Table 1 Primer sequences used for real-time quantitative polymerase chain reaction in the control and fasted mice.

Target gene	Primer sequences		Product size (bp)
	Forward	Reverse	
<i>Cyp19a1</i>	5'-TCGTCGCAGAGTATCCAGAGGT-3'	5'-CGCATGACCAAGTCCACAACAG-3'	163
<i>Star</i>	5'-GTGACGGTGGCACACGACTT-3'	5'-TCTCCTTCCATCTCTGTGGCTTCT-3'	238
<i>Esr1</i>	5'-TGAAAGGCGGCATACGGAAGAC-3'	5'-GCATCCAACAAGGCACTGACCAT-3'	214
<i>Esr2</i>	5'-CCACTGCCAATCATCGCTTCTCT-3'	5'-TTCACAGGACCAGACACCGTAATG-3'	229
<i>Actb</i>	5'-GAGATTACTGCTCTGGCTCTCTA-3'	5'-GGACTCATCGTACTCTGCTTG-3'	150

for 30 min after wash with PBS. Subsequently, sections were counterstained with DAPI and observed under the Olympus BX51 epifluorescence microscope.

Cumulus cell-oocyte complexes (COCs) culture and maturation assay

Ovaries were obtained from 46 h eCG-primed mice without fasting treatment, and then the ovaries were placed in hypoxanthine (HX)-M199 medium. COCs were collected by puncturing large antral follicles with hypodermic needle (27-gauge). COCs were washed three times and equal size with several layers of cumulus cells were collected. In each experiment, one group of COCs were cultured in an 80 μ L drop covered with mineral oil in 35 mm culture dish. Cultures were incubated in a humidified modular incubator at 37°C with 5% CO₂ for 22–24 h. At the end of the culture period, oocytes were denuded mechanically by repeated pipetting to remove cumulus cells and the number of oocytes with a germinal vesicle (GV), germinal vesicle breakdown (GVBD) or polar body 1 (PB1) were determined under the inverted microscope.

Statistical analysis

All experiments were repeated a minimum of three times and results were presented as the mean $\pm$ S.E.M. Data were analyzed by *t*-test or one-way ANOVA followed by Student–Newman–Keuls method (SNK, SigmaPlot, version 12.0 software). Significant differences were defined at *P* values of <0.05.

Results

Acute fasting suppressed basic metabolism, ovarian growth and POAT expansion

As shown in Fig. 1A, the plasma GLU, CHO and TG concentrations in the acute fasting group (F group) were markedly decreased compared with those in the control group (C group). Concurrently, mouse ovarian mass in the F group decreased significantly (38% decrease, *P* < 0.05, Fig. 1B). Consistent with the mentioned result, smaller ovarian size, looser and thinner cumulus cell layers in the F group mice were observed (Fig. 1C), and all the ovarian sections employed for HE staining were taken from the middle of tissues. The interesting finding is that the POAT completely vanished in the F group compared with that in the C group (Fig. 1D). At the same time, the enlarged uterus in the F group was not observed in the C group after 48 h eCG treatment (Fig. 1D).

Characteristics of POAT accumulation in vivo

The physiological characteristic of the accumulation of per-ovarian adipose tissues is illustrated in Fig. 2A. The figure showed that POAT did not show up during the early postnatal life, for example, in the newborn,

Figure 1 Effects of acute fasting on the basic metabolism, ovarian growth and POAT expansion. (A) Plasma glucose (GLU), cholesterol (CHO) and triglyceride (TG) concentrations in the mice from F and C groups. (B) Mouse ovarian mass in the C groups and F groups after 48-h food deprivation. (C) HE staining of paraffin-embedded sections of mouse ovaries from C and F groups. (D) Morphological characteristics of POAT and uterus between C and F groups. F group, the fasting group; C group, the control group. Scale bars represent 1 mm. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates (number of mice *n* = 30 per experiment). The different letters denote a statistical difference at *P* < 0.05 using two-sample *t*-test.

Figure 2 Characteristics of peri-ovarian adipose tissue (POAT) accumulation *in vivo*. (A) Photographs of the internal reproductive organs in female mice of the newborn, 1, 2, 3 and 6 weeks old. (B) Hematoxylin-eosin (HE) staining of paraffin-embedded sections of POAT from 3 and 6 weeks mice. (C) Mouse body and ovarian weights in female mice of 2, 3 and 6 weeks old. Scale bars represent 100 μ m. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates (number of mice $n=15$ per experiment). The different letters denote a statistical difference at $P<0.05$ using one-way ANOVA.

1 or 2-week mice; it started to build up in the mice around prepubescent period (3 weeks) and increased to maximum in the sexually mature stage (6 weeks). During the expansion of the adipose tissues, the hypertrophy of the white adipocytes occurred as shown in Fig. 2B. In the meantime, both the body and ovarian weights of mice were noticeably upregulated ($P<0.05$, Fig. 2C).

Acute fasting stimulated cell apoptosis and suppressed cell proliferation in ovaries

Cell apoptosis and proliferation of the ovaries of control and acute fasting groups were compared. As shown in Fig. 3A, the positive staining of TUNEL in granulosa and theca cells obviously increased in the ovaries of the acute-fasted mice. Though the protein expressions of caspase-3 and PCNA in ovaries were not affected by acute fasting (Fig. 3B), immunohistochemical results suggested that PCNA in the antral follicles from the F group were obviously

Figure 3 Effects of acute fasting on the ovarian cell growth. (A) Acute fasting increased the TUNEL-positive staining in the ovarian follicles of mice from F groups. (B) Proteins expression of caspase-3 (CASP3) and PCNA in mouse ovaries from F and C groups, and all protein contents were normalized against vinculin (VCL). (C) Immunolocalizations of caspase-3 and PCNA in the mouse ovarian follicles from F and C groups. (D) Proteins expression of cleaved caspase-3 and PARP in mouse ovaries from F and C groups, and all protein contents were normalized against β -actin (ACTB). F group, the fasting group; C group, the control group. Scale bars represent 100 μ m. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates, the number of mice was $n=30$ per experiment in (B). The different letters denote a statistical difference at $P<0.05$ using two-sample *t*-test.

downregulated (Fig. 3C). Moreover, in ovaries from fasted mice, the protein expression of both cleaved caspase 3 and PARP were significantly upregulated (Fig. 3D).

The immunolocalizations for CCND1, CCND2 and CCND3 were primarily observed in granulosa cells of the antral follicles (Fig. 4A), and the ovarian protein expression of CCND3 (Fig. 4D) rather than that of CCND1 (Fig. 4B) or CCND2 (Fig. 4C) was inhibited in F group. Likewise, the cell cycle-inhibited protein CDKN2B, CDKN1A and CDKN1B were all detected in the granulosa cells of antral follicles (Fig. 5A), whereas the ovarian protein expression of CDKN2B (Fig. 5B) rather than that of CDKN1A (Fig. 5C) or CDKN1B was significantly upregulated after acute fasting treatment (Fig. 5D).

Acute fasting disturbed hormonal secretion and relative mRNA expression

According to the RIA results, acute fasting significantly downregulated blood FSH and LH concentrations but elevated E2 level (Fig. 6A). Consistent with the mentioned result, the mRNA expression levels of *Cyp19a1* and *Star* in ovaries were significantly upregulated in F group (Fig. 6B). Besides, the C and F groups depicted no significant differences in ovarian *Esr1* and *Esr2* mRNA expression (Fig. 6C).

Acute fasting enhanced the ovarian protein expression of adiponectin system

Adiponectin concentrations in plasma and ovaries were ascertained using both ELISA and Western blot. As shown in Fig. 7A, 48-h food deprivation did not affect

the plasma adiponectin content. Note that, the ovarian adiponectin level was significantly improved after the acute fasting ($P < 0.05$, Fig. 7A). Moreover, the F group showed significantly up-regulated protein expressions of adiponectin (Fig. 7B), ADIPOR1 (Fig. 7C) and ADIPOR2 (Fig. 7D) in mouse ovaries, as compared with those in the C group. Figure 7E showed that adiponectin was expressed in both the germ cells (oocytes) and the somatic cells (granulosa cells and theca cells); ADIPOR1 was primarily located in theca cells, while ADIPOR2 was identified in granulosa cells.

Effects of AdipoRs agonist and antagonist on oocyte maturation in vitro

As shown in Fig. 8A, the high concentration (≥ 10 $\mu\text{mol/L}$) of 2-(4-Benzoylphenoxy)-N-[1-(phenylmethyl)-4-piperidinyl] acetamide (ADIPOR agonist) significantly suppressed FSH-induced oocyte meiotic resumption (35% decrease, $P < 0.05$). Though AdipoRon (ADIPOR antagonist) had no statistical differences in FSH-induced oocyte maturation, it facilitated oocyte meiotic resumption without FSH addition (12% increase, $P < 0.05$, Fig. 8B). However, ADIPOR agonist and antagonist did not affect cumulus expansion (Fig. 8C and D).

Discussion

Undernutrition is associated with reproductive dysfunction. Previous studies proved that acute fasting

Figure 4 Effects of acute fasting on the expression of CCND1, CCND2 and CCND3 in ovaries. (A) Immunolocalizations of CCND1, CCND2 and CCND3 in the mouse ovarian follicles from F and C groups. (B, C and D) Proteins expression of CCND1, CCND2 and CCND3 in mouse ovaries from F and C groups, and all protein contents were normalized against vinculin (VCL). F group, the fasting group; C group, the control group. Scale bars represent 100 μm . Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates (number of mice $n = 30$ per experiment). The different letters denote a statistical difference at $P < 0.05$ using two-sample t -test.

Figure 5 Effects of acute fasting on the expression of cyclin-dependent kinase inhibitors in mice ovaries. (A) Immunolocalizations of CDKN2B, CDKN1A and CDKN1B in the mouse ovarian follicles from F and C groups. (B, C and D) Proteins expression of CDKN2B, CDKN1A and CDKN1B in mouse ovaries from F and C groups, and all protein contents were normalized against vinculin (VCL). F group, the fasting group; C group, the control group. Scale bars represent 100 μ m. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates (number of mice $n=30$ per experiment). The different letters denote a statistical difference at $P<0.05$ using two-sample t -test.

Figure 6 Effects of acute fasting on the hormonal secretion and relative mRNA expression. (A) Plasma FSH, LH and E2 concentrations in the mice from H and C groups. (B) mRNA expression of *Cyp19a1* and *Star* in mouse ovaries from F and C groups, and the relative mRNA contents were normalized against *Actb*. (C) mRNA expression of *Esr1* and *Esr2* in mouse ovaries from F and C groups, and the relative mRNA contents were normalized against *Actb*. F group, the fasting group; C group, the control group. Scale bars represent 100 μ m. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates, the number of mice was $n=30$ per experiment in (A). The different letters denote a statistical difference at $P<0.05$ using two-sample t -test.

suppresses oocyte maturation and reduces mouse fertility by inhibiting glucose transport and utilization (Yan *et al.* 2008, Han *et al.* 2012). In this study, the findings implied that the loss of POAT and upregulation of the adiponectin system might be link to the food deprivation-induced impairments of mouse ovaries.

In female mammals, successful reproduction is dependent on a highly complex and intricately regulated process of folliculogenesis. In this process, granulosa cell proliferation and differentiation are vital to follicular initiation, recruitment, selection, dominance, ovulation, and luteinization, while apoptosis of theca and granulosa cells induces follicular atresia. In this study, acute fasting significantly suppressed the protein expression of cyclin D3 in ovary, instead of those of cyclin D1 or cyclin D2. Cyclin D3 has been found to be upregulated in response to high fat diet in mice (Wu *et al.* 2015). We speculate that cyclin D3 may be sensitive to the nutritional condition and specially regulate mouse ovarian cell proliferation. In female, CDKN1A and CDKN1B are extensively studied cyclin-dependent kinase inhibitors (CDKIs), considered critical negative regulators for follicle development. However, neither protein CDKN1A nor CDKN1B was changed in ovaries after acute-fasted treatment. Besides, another CDKI, CDKN2B was significantly stimulated in the ovaries of fasted mice. To the best of our knowledge, the physiological role of CDKN2B in ovaries has been rarely reported (Cho *et al.* 2016), the role and the intricate mechanism of CDKN2B in female reproduction require further studies. Adiponectin has been reported inhibiting proliferation or stimulated apoptosis in different cancer cells (Diedonne *et al.* 2006,

Figure 7 Effects of acute fasting on adiponectin system in mice. (A) The relative concentration of adiponectin in the plasma and ovaries of mice from F and C groups. (B, C, D and E) Protein expressions of adiponectin (ADIPOQ), ADIPOR1 and ADIPOR2 in mouse ovaries from F and C groups, and all protein contents were normalized against vinculin (VCL). F group, the fasting group; C group, the control group; ADPN, adiponectin. Scale bars represent 100 μm. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates, the number of mice was $n=30$ per experiment in (A). The different letters denote a statistical difference at $P<0.05$ using two-sample *t*-test.

Nakayama *et al.* 2008, Katira & Tan 2016). In this study, it was also demonstrated that acute fasting-induced ovarian cell apoptosis by detecting more positive TUNEL staining in ovaries of mice. Caspase-3 is generally known as a major executioner protease participating in granulosa cell apoptosis during follicular atresia (Matsuda *et al.* 2012). Though the control and fasted groups displayed no significant difference in the protein expression of caspase-3, the upregulated active caspase-3 had been observed. To further verify the activation of caspase-3, the protein expression pattern of PARP, a classical substrate for active caspase-3, was also

Figure 8 Effects of adiponectin receptors agonist and antagonist on cumulus cell-oocyte complexes (COCs) meiotic resumption and cumulus cell expansion. Mouse COCs isolated from eCG-primed mice were cultured in HX-M199 medium with or without FSH (50IU/L), adiponectin receptors agonist, and antagonist. (A and B) The percentage of oocyte maturation (the ratio of GVBD and PB1) at the end of 22 h of culture. (C and D) Photographs of the cumulus cell expansion at the end of 22 h of culture. Error bars represent the mean $\pm$ S.E.M. for three groups of experimental replicates, the number of COCs was $n=50$ per experiment. The different letters denote a statistical difference at $P<0.05$ using one-way ANOVA.

detected (Henning *et al.* 2018). Likewise, the protein expression of cleaved PARP was significantly elevated in ovaries from F group. Based on these findings, it was concluded that acute fasting-induced ovarian cell apoptosis in a caspase-3-dependent manner. Thus, our results strongly demonstrated that acute fasting inhibited the mouse ovarian development by disturbing the process of ovarian cell proliferation and apoptosis.

In the ovary, the hypothalamic–pituitary–gonadal (HPG) axis and its secreted sex hormones act as the major regulator of cell growth and death. FSH and LH, two pituitary-secreted gonadotropins, are essential for promoting follicle growth, ovulation and the production of estrogen (E2) and progesterone (P4) (Richards 2018). In this study, noticeably decreased blood FSH and LH levels were ascertained in the acute fasting-induced POAT deficient mice, while the circulating E2 level was improved. The low levels of FSH and LH in acute-fasted mice might not be able to support big antral follicles and fully matured oocytes and could ultimately result in impaired ovarian development under malnutrition condition. Recently, Wang *et al.* have demonstrated that removal of POAT result in reduced FSH and E2 levels but enhanced LH content (Wang *et al.* 2017). Similarly, FSH displayed a corresponding decrease manner in the status of fasting-induced loss of POAT. However, the variations of E2 and LH were completely opposite to those reported by Wang *et al.*, presumably due to the special treatment of acute fasting. Consistent with our findings, existing studies in rats and hamsters proved that food deprivation could improve plasma estrogen levels and suppress the pulsatile release of LH (Jones *et al.* 2002, Sarookhani *et al.* 2014). *In vivo*, the inhibiting effects of adiponectin and its paralog C1q/TNF-related protein 9 on FSH and LH were observed in various animals, including rat, Nile tilapia and porcine (Cheng *et al.* 2011, Kiezun *et al.* 2014, Yang *et al.* 2018a). It is suggested that the acute fasting-induced decrease in circulating FSH and LH may be attributed to the improvement of adiponectin levels in ovaries. Furthermore, studies in bovine ovaries reported that adiponectin suppressed the gene expression of *Cyp11a* and *Cyp17a1* in theca cells (Dobrzyn *et al.* 2018), revealing that adiponectin might affect steroidogenesis. In this study, upregulated ovarian adiponectin was observed to be accompanied by decreased levels of circulation estrogen under acute fasting. The gene expression of *Cyp19a1* and *Star* mRNA levels in ovaries was markedly upregulated in the fasted mice, suggesting the increased plasma E2 in fasted mice. It has been reported that E2 can act as a negative feedback regulator of LH secretion (Mostari *et al.* 2013, Munoz *et al.* 2017), probably leading to a decrease in the circulating LH level here. Besides, it was found that acute fasting did not affect the mRNA expression of *Esr1* and *Esr2* in ovaries, which further confirmed that the function of E2 would be enhanced under the acute fasting. Nevertheless, the role of increased estrogen

levels remains unknown. It has been reported that the circulating estrogen participates in pain transmission and modulation of nociceptive responses (Coulombe *et al.* 2011), so it is speculated that the increase in estrogen might be a physiological protective mechanism against the damage derived from food deprivation.

Undernutrition strikingly interrupts mammalian basic metabolic status (Zha & Qian 2017). In this study, acute fasting significantly downregulated the blood GLU, CHO and TG concentrations in female mice. White adipose tissue is the body's largest energy reservoir, consisting of various fat depots. It has been confirmed that fat distribution and function varied dramatically throughout life (Tchonia *et al.* 2010). Increasing researches (e.g., studies in perinodal, perivascular, pericardial, perirenal and POAT) have focused on the area of the site-specific properties and functions of white adipose tissues (Lohn *et al.* 2002, Pond 2003, Fox *et al.* 2009, Wang *et al.* 2017, Yang *et al.* 2018b). POAT is a major composition of visceral white adipose tissue; it plays special roles in inflammation and metabolic disorders of female rodents (Nteeba *et al.* 2013). In this study, it was revealed that mouse POAT did not accumulate in the early postnatal life, and it started to accumulate during prepubescence stage and expanded greatly in sexual maturity, revealing a positive relationship between the hypertrophy of adipocytes and growth of ovaries. According to the findings of Wang *et al.* and Yang *et al.*, the removal of the POAT of mice exhibited abnormal estrous cyclicity, inhibited steroidogenesis, decreased fertility and less ovulated mature eggs (Wang *et al.* 2017, Yang *et al.* 2018b). Collectively, these findings implied that appropriate storage of POAT is essential for successful female reproduction. However, our knowledge remains insufficient about the detailed function and mechanism of POAT on female reproduction.

Adiponectin is a white adipose-secreted adipokine linking metabolism and reproduction; it is negatively correlated with adiposity and body mass index. In the present study, 48-h food deprivation significantly decreased the body weight of mice. However, there was no difference in the plasma adiponectin levels between the control and fasted groups. In fact, other rodent studies have also demonstrated that adiponectin levels were not affected by acute food deprivation or short-term food restriction (Johansson *et al.* 2008, Turyn *et al.* 2008). Similarly, studies in humans have proposed that serum adiponectin concentrations remain remarkably stable during acute fasting (Gavrilu *et al.* 2003, Imbeault *et al.* 2004). In this study, ELISA and Western blot results suggested that acute fasting significantly enhanced the ovarian rather than blood adiponectin content. Likewise, study in cattle showed that the adiponectin concentrations in serum and follicular fluid are not correlated (Heinz *et al.* 2015). Recently, different structural and functional properties of various white adipose tissue depots have been uncovered, including

adipokine secretions (Hocking *et al.* 2010, Wronska & Kmiec 2012). In humans and rodents, subcutaneous white adipose tissues are reported to be a major source of adiponectin (Wronska & Kmiec 2012). Considering that the POAT completely vanished following food deprivation in this study, we suggest that the increase in endogenous adiponectin synthesis and/or its secretion in ovaries might be influenced by the peripheral adipose tissues through a paracrine and/or autocrine regulation. In brief, these findings encourage us to propose that there are adipose tissue depot-specific differences in the secretion of adiponectin in some specific organs and tissues.

Thus far, the role of adiponectin in female reproduction remains unclear, but its expression in reproductive tissues has been observed in humans and various animals. It is demonstrated that the localization of adiponectin and its receptors are abundant in oocyte, granulosa cells and theca cells of various animals, such as bovine, ewes and humans (Pierre *et al.* 2009, Maillard *et al.* 2010, Wang *et al.* 2016). Some researches indicated lower expressions of the adiponectin system in granulosa cells compared with that in theca cells (Tabandeh *et al.* 2010). In this study, we show that adiponectin was abundant in the whole ovary while ADIPOR1 was mainly expressed in theca cells and ADIPOR2 was mainly present in granulosa cells of mouse ovaries. The presence of the adiponectin in oocyte suggests a potential involvement in oocyte maturation. However, different findings have been reported on the function of adiponectin on oocyte maturation. For example, studies in goats and pigs demonstrate that adiponectin addition stimulates oocyte meiotic maturation (Chappaz *et al.* 2008, Oliveira *et al.* 2017), whereas bovine adiponectin has no influence on oocyte maturation (Maillard *et al.* 2010). In this study, we show that ADIPOR- agonist suppressed FSH-induced oocyte meiotic resumption *in vitro*, while the antagonist significantly enhanced oocyte meiotic resumption without FSH addition. These functional differences of adiponectin could possibly be due to the species-specific response of experimental animal. Furthermore, this study showed that food deprivation elevated ovarian expressions of both ADIPOR1 and ADIPOR2, suggesting that adiponectin may directly modulate mouse ovarian development. In granulosa cells, ADIPOR1 is reported to be the primary adiponectin receptor regulated by gonadotropin (Chabrolle *et al.* 2007b), and its knockdown in KGN cell lines leads to an arrest of cell proliferation and apoptosis phenotype. It seems that low concentration of circulating adiponectin is disadvantageous for ovarian cell growth. In contrast, it was demonstrated that adiponectin system was upregulated under low gonadotrophin (FSH and LH) after undergoing food deprivation, implying that high concentration of adiponectin can also be disadvantageous. Collectively, all these suggest that the adiponectin system may participate in regulating

ovarian development through the HPO axis under acute malnutrition.

In conclusion, this study suggests a specific upregulation of ovarian adiponectin system accompanied by a deficiency of POAT and impaired HPG axis-regulated ovarian development following 48-h food deprivation. These findings implicate that the POAT and adiponectin system may involve regulation of reproductive function through the paracrine/autocrine pathway. However, to reveal the role and mechanism of POAT and/or adiponectin system in ovarian physiology, further research is required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities (NO. 2018ZY31); the National Natural Science Foundation of China (NO. 31500943); the Beijing Natural Science Foundation (NO. 8182039) and Collage Student Innovation and Entrepreneurship Training Program (NO. G201910022039).

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Received 25 February 2019

First decision 19 March 2019

Revised manuscript received 8 September 2019

Accepted 13 September 2019