

High-Fat Diet-Induced Obesity Primes Impaired Fatty Acid β -Oxidation and Consequent Ovarian Dysfunction During Early Pregnancy

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Abstract

Background: Obesity is associated with many adverse effects on female fertility. Obese women are more likely to have ovulatory dysfunction due to dysregulation of the hypothalamic-pituitary-ovarian axis. However, ovarian function in obese women during early pregnancy still needs further assessment.

Methods: Obesity was induced in C57BL6/J mice using high-fat diets (HFD) for 12 weeks; *In vitro* high-fat model was established with KGN cells treated with Oleate acid and Palmitic acid. Ovarian morphology of obese mice in early pregnancy was assessed by Hematoxylin and Eosin staining and its function was assessed using ELISA, Western blotting and Immunohistochemistry. The Oil Red O staining and Transmission electron microscopy were used to detect fatty acid accumulation and specific markers relating to ovarian functional mechanism were assessed by Real time PCR, Western blotting, Lactate detection, ATP detection, Biochemical analyses and ELISA.

Results: The results of this study showed that during early pregnancy, the number of corpus luteum, serum estradiol and progesterone levels, and the expression of genes CYP19A1, CYP11A1 and StAR, which are related to steroid biosynthesis, were significantly increased in HFD female mice. HFD-fed mice also showed a significant increase in ovarian lipid accumulation on day 7 of pregnancy. Genes involved in fatty acid synthesis (*Acs14* and *Elovl5*) and fatty acid uptake and transport (*Slc27a4*), together with the β -oxidation rate-limiting enzyme (*Cpt1a*) were significantly upregulated in HFD-fed mice. Specifically, there was abnormal elevation of ATP level and aberrant expression of tricarboxylic acid cycle (TCA) and electron transport chain related genes in the ovary of HFD pregnant mice. Treatment of KGN cells with etomoxir targeting β -oxidation of fatty acid, showed decrease tricarboxylic acid cycle and electron transport chain. The elevated ATP level and the increased estradiol and progesterone levels were reversed.

Conclusions: This study indicated that during early pregnancy, high-fat diet and induced-obesity increased fatty acid β -oxidation, which in turn increase the tricarboxylic acid cycle and the electron transport chain, and consequently increases ATP production and ovarian dysfunction.

Introduction

The prevalence of obesity has increased dramatically around the world. It has been reported that more than 650 million adults worldwide are affected by obesity [1]. Obesity is an increase in body weight due to excessive accumulation of fat tissue, which contributes to an increased prevalence of obesity-related metabolic dysfunction, cardiovascular diseases, diabetes, musculoskeletal disorders and some cancers [2]. In addition, obesity exerts a negative influence on female fertility; and it is associated with many adverse maternal and fetal effects prenatally [3]. The rates of many pregnancy complications – such as hypertensive disorders, gestational diabetes, preterm births and the other health challenges that warrant cesarean delivery – are higher in obese women [3]. However, the underlying mechanisms of obesity's adverse effect on female fertility have not been well elucidated.

The ovary is the female gonad and an important endocrine organ. The main functions of the ovary include production, maturation and release of the oocyte, and synthesis of female sex steroid and peptide hormones that regulate reproductive and non-reproductive functions [4]. Environmental exposures can exhaust the oocyte pool and cause depletion of follicular cells, premature ovarian failure, early menopause, and infertility. Obese women are more likely to suffer ovulatory dysfunction due to dysregulation of the hypothalamic-pituitary-ovarian axis [5, 6]. However, the impact of obesity on ovarian functions, during early pregnancy, is less clear.

In women, about 15% of miscarriages occur during early pregnancy [7]. During early pregnancy, female sex steroid hormones, especially estrogen and/or progesterone, directly regulate the expression of several genes in the uterus to help maintain pregnancy. Appropriate levels of estrogen and progesterone reduce the risk of miscarriages [7-9]. The regulatory effect of estrogen and progesterone on the ovaries and the uterus, during early pregnancy, renders the endometrium more receptive to blastocysts by inducing endometrial secretory changes that are essential for successful implantation and decidualization in mice. Hence, investigations of the impact of obesity on ovarian functions can help to clarify the mechanistic bases of the association between obesity and female infertility.

Glucose and free fatty acids (FFAs) are the major energy sources for the body [10]. Mitochondrial β -oxidation of FFAs is also a major source of ATP production [11]. Whenever the metabolism of glucose and FFAs are disturbed – as a result of inappropriate dieting, unhealthy lifestyle or other factors – the functions of tissues and organs are dysregulated. It is well established that lots of tissues and organs, such as the skeletal muscle, exhibits dysregulation of glucose uptake, lipid metabolism, and mitochondrial function in obese individuals [12, 13]. However, whether ovarian glucose and fatty acid homeostasis is dysregulated in diet-induced obesity, during early pregnancy, has not been extensively investigated.

Thus, in this study, we investigated ovarian functions and assessed the integrity of ovarian glucose and fatty acid homeostasis in high-fat diet-induced obesity in maternal mice during early pregnancy. Our findings would provide theoretical bases for exploring the effects and mechanisms of ovarian dysfunction in obese maternal mice during early pregnancy, and offer a scientific basis for a comprehensive evaluation of obesity as a risk factor for female infertility.

Materials And Methods

Animals

C57BL6/J mice (4-week-old) were purchased from Charles River Laboratories. The animals were housed in cages with a 12:12 h light:dark cycle set at ambient temperature (22°C-25°C). After acclimation of ordinary food for one-week, female mice were fed either chow diet (Research Diets, D12450J) or high-fat diet (HFD) (Research Diets, D12492) for 12 weeks respectively. Caloric composition of chow diet consisted of 20% protein, 10% fat and 70% carbohydrates, while high-fat diet had 20% protein, 60% fat and 20% carbohydrates. The mice were weighed weekly. After 12 weeks, the female mice were mated with

fertile males of the same strain, which were fed with normal diet. The appearance of a vaginal plug was marked as day 1 of pregnancy (D1). The serum and tissue samples on D7 were collected and stored for further studies. All animal procedures were approved by the Ethics Committee of Chongqing Medical University, China.

Cell culture

The human ovarian granulosa cell line KGN was purchased from EK-Bioscience (Shanghai, China) and authenticated through STR analysis by Cellcook Biotech (Guangzhou, China). The cells were cultured at 37°C in DMEM/F-12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Beyotime, Shanghai, China). In order to establish the high-fat cell model, we treated the cells with 400µM Oleate acid (OA, Sigma, USA) and 200µM Palmitic acid (PA, Sigma, USA) for 24h. After that, luteinization was induced in the cells with 1IU human Chorionic Gonadotropin (hCG, Merck, Germany) for 12h.

Real time PCR

Total RNA was extracted from the ovaries and KGN cells with the Trizol reagent (Invitrogen, USA). cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Real-time RT-PCR reactions were performed using GoTaq® qPCR Master Mix Technical Manual (Promega, USA) and ABI ViiATM7 real-time fluorescence quantitative PCR instrument. Data was analyzed using the $\Delta\Delta C_t$ method; and were normalized to β -actin. Primer sequences are listed in Supplemental Table 1 and 2.

Western blot

Tissues and cells were homogenized in RIPA buffer (Beyotime, Shanghai, China) with protease and phosphatase inhibitor cocktail (Thermo Fisher, USA). The BCA kit (Beyotime, Shanghai, China) was used to measure the protein concentration. Equal amounts of proteins were separated using 10% SDS-PAGE and then transferred onto a PVDF membrane. The nonspecific binding sites on the membranes were blocked with 5% non-fat milk at room temperature for 1 h. Then, the membranes were incubated with primary antibodies (Supplemental Table 3) at 4 °C overnight. After washing with PBST, the membranes were incubated with anti-rabbit or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase. Finally, the bands on the membranes were visualized using an ECL detection kit (Millipore, Germany). The light density and grey level were analyzed and calculated by using the ImageJ software. β -actin was used as a loading control.

Biochemical analyses

Blood was centrifugated at 3000 rpm for 10 min under 4 °C to obtain serum. Serum levels of triglyceride (TG) and total cholesterol (TC) were determined using specific assay kits (Nanjing Jiancheng, Nanjing, China) through glycerol-3-phosphate oxidase p-aminophenol (GPO-PAP) and cholesterol oxidase-peroxidase coupling (COD-PAP) methods. Ovarian tissue and KGN cells were homogenated with absolute ethyl alcohol over ice, then TG was tested according to Triglyceride test kit (Nanjing Jiancheng, Nanjing,

China) protocols, by GPO-PAP method. TG level in tissues and cells were expressed as mol/g protein. Protein concentration was detected with the BCA protein assay kit.

ELISA

Serum and cell medium estradiol and progesterone levels were determined using commercial enzyme-linked immunosorbent assay kits (ELISA, Yanhui Biotech, Shanghai, China) according to the manufacturer's protocols. Absorbance was read at 450 nm.

Hematoxylin and eosin (H&E) staining

Ovarian tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Embedded tissues were sectioned at 5 μ m and stained with H&E prior to the assessment of the morphological changes in the condition of high-fat diet.

Immunohistochemistry

Paraffin-embedded sections (5 μ m thickness) were deparaffinized and treated with citrate repair solution in microwave oven for 5 min at high fire and 15 min at low fire. After repairing, the rabbit SP kit for immunohistochemistry (ZSGB-BIO, Beijing, China) was used for the subsequent procedures. Firstly, endogenous peroxidases were inhibited via incubation with 3% hydrogen peroxide for 10 min at room temperature. Secondly, the nonspecific binding sites were blocked with 10% goat serum for 30 min at 37°C and then incubated with primary antibodies (Supplemental Table 3) overnight at 4°C. Then, the sections were incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at 37°C. Finally, peroxidase activity was detected using the 3,3-diaminobenzidine substrate (DAB, ZSGB-BIO, Beijing, China). The sections were counterstained with hematoxylin. Positive area quantification was calculated by using ImageJ.

Transmission electron microscopy

Specimens were fixed in 2.5% glutaraldehyde, dehydrated in graded alcohol and stained with OsO₄ and uranyl acetate. The sections were polymerized and cut to prepare for use on a Hitachi H-7650 transmission electron microscope.

Oil Red O staining

Ovarian tissues were fixed in 4% paraformaldehyde for 24 h and 30% sucrose for 1 h, then embedded in OCT and serially sectioned at 4 μ m. KGN cells were cultured on 13mm round glass coverslips. After incubating in 60% isopropyl alcohol for 5 min, both sections and cells were stained with freshly prepared 0.15% Oil Red O (Solarbio, Beijing, China) for 10 min. Then, sections and cells were washed in 60% isopropyl alcohol to remove unspecific attachments. Finally, the sections and cells were counterstained with hematoxylin and mounted with glycerol jelly.

Lactate detection

Ovarian tissue was homogenized on ice, centrifugation at 12000g at 4°C for 5 min and the supernatant taken for lactate detection. For KGN cells, culture medium was directly used for lactate detection. Lactate content was determined by a lactate test kit (Nanjing Jiancheng, Nanjing, China) according to the instructions of the manufacturer. Briefly, 1 ml enzyme reagent, and 0.2 ml Color-substrate solution were added to the samples, mix well and incubated in 37 ° C water bath for 10 min. 2 ml stop buffer was then added and the absorbance measured at 530nm. The lactate content was then normalized to total protein content.

ATP detection

ATP levels were measured using ATP Assay Kit (Beyotime, Shanghai, China). Tissue and cells were homogenized on ice, centrifugation at 12000g at 4°C for 5 min and the supernatant taken. ATP detection reagent add to the supernatant and measured with luminometer at 10 s RLU and concentration calculated based on an ATP standard curve and expressed as mol/mg. Protein concentration was determined using the BCA protein assay kit.

CCK-8 assay

KGN cells were seeded on a 96-well microtiter plate (5.0×10^3 cells/well) and the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) was used to determine the number of viable cells. The CCK-8 reagent was added to each well and incubated for 2 h. Then, the absorbance of each well was measured at 450 nm.

Statistical analysis

Differences between two groups were determined using unpaired Student's *t*-test (2-tailed). Differences among three groups were calculated using one-way analysis of variance (ANOVA), while differences within groups were assessed using the Mann-Whitney test. All data are shown as mean \pm standard error of the mean (SEM). GraphPad Prism version 5.4 was used for all statistical analyses, and differences were considered significant when $P < 0.05$. All experiments were repeated, at least, 3 times.

Results

Ovarian function during early pregnancy is impaired by high-fat diet-induced obesity.

After a 12-week feeding period, female C57BL/6J mice fed with HFD diets showed higher body weights (Fig. 1a and b). Compared with controls, HFD female mice had significantly elevated fasting levels of circulating serum triglycerides and total cholesterol (Fig. 1c and d). Since embryo implantation and decidualization, are related to estrogen and progesterone levels, which are produced by the corpus luteum of the ovary [14]. We determined the effects of HFD-induced obesity on the ovary during early pregnancy. Assessment of the luteal structure on D7 by H&E staining showed that the number of corpus luteum was significantly increased in the HFD group (Fig. 1e and f). Serum E2 and P4 levels on D7 were further measured to explore the effect of HFD-induced obesity on luteal function. As shown in Fig. 1g and h,

serum estradiol and progesterone levels were considerably increased by HFD-induced obesity. It is well known that CYP19A1, CYP11A1 and steroidogenic acute regulator (StAR) are linked to the regulation of steroid biosynthesis. The CYP19A1 is a cytochrome P450 enzyme responsible for the aromatization of testosterone into estradiol [15]. StAR and the cholesterol side chain cleavage enzyme (P450_{scc}), which is encoded by CYP11A1, catalyzes the first and rate-limiting steps of steroid biosynthesis [16, 17]. Our results showed a significant increase in CYP19A1, CYP11A1 and StAR on D7 in the HFD-fed pregnant mice when compared with those of the control group (Fig. 1i-j). Taken together, our results indicate that ovarian function was impaired by the HFD-induced obesity.

Ovarian glycolysis is not altered by high-fat diet-induced obesity during early pregnancy.

Hyperglycemia and dyslipidemia are two metabolic alterations of obesity, a condition which is associated with dysregulated glucose and fatty acid metabolism in tissues and organs [18]. To understand the possible mechanism by which high-fat diet-induced obesity impairs ovarian function during early pregnancy, we first explored whether glycolysis, the central pathway of glucose metabolism [19], is affected by obesity. Previous reports showed that highly expressed pyruvate dehydrogenase kinase 1 (PDK1) reduces the activity of pyruvate dehydrogenase (PDH) by inducing the phosphorylation of its E1 α subunit (PDHA1) and subsequently, shifts the energy metabolism from oxidative phosphorylation to aerobic glycolysis [20]. In addition, hypoxia-inducible factor-1 (HIF1) can transform cells from aerobic oxidation to anaerobic glycolysis by activating the switch protein: PDK1 [21]. Our results showed that, there was no significant difference in ovarian HIF1 α , PDK1, PDP1 and PDHA1 expression between the HFD group and the control group on D7 (Fig. 2b-e). This suggests that the impaired ovary, during early pregnancy, exhibited no obvious energy metabolic shifts between aerobic and anaerobic glycolysis. In addition, the *Slc2a1* (code glucose transporter 1 protein) and *Slc2a4* (code glucose transporter 4 protein), and the glycolysis key enzymes Hexokinase 2 (*Hk2*), Pyruvate kinase M2 (*Pkm2*) and phosphoglycerate kinase 1 (*Pgk1*) were similarly expressed in the pregnant mice ovary on D7 in both the HFD group and the control group (Fig. 2a). Moreover, the absence of significant changes in ovarian lactate production in the HFD group (Fig. 2f) confirms that ovarian glycolysis is not significantly altered by high-fat diet-induced obesity during early pregnancy.

High-fat diet-induced obesity enhances fatty acid β -oxidation in the ovary during early pregnancy

Based on ATP availability, ATP can trigger different cell responses, including cell proliferation, differentiation and apoptosis [22-24]. In this study, the ovarian tissue concentration of ATP was significantly increased by high-fat diet-induced obesity on D7 (Fig. 3a). Due to the stability of ovarian glycolysis in high-fat diet-induced obesity during early pregnancy, we speculated that the abnormally elevated ATP level in the dysfunctional ovary mainly comes from disordered fatty acid metabolism. Thus, we first examined the effect of high-fat diet-induced obesity on ovarian lipid accumulation. According to our results, high-fat diet caused a significant increase in ovarian lipid accumulation, as evidenced by the quantitative results of lipid content and Oil Red O staining and transmission electron microscopy of the ovary tissue on D7 (Fig. 3b-d). Moreover, genes involved in fatty acid synthesis (*Acs14* and *Elovl5*) and

fatty acid uptake and transport (*Slc27a4*), together with the β -oxidation rate-limiting enzyme (*Cpt1a*), were significantly upregulated by high-fat diet-induced obesity on D7 (Fig. 3e-g). This suggests that high-fat diet-induced obesity enhances fatty acid β -oxidation in the ovary during early pregnancy. Mitochondrial fatty acid β -oxidation, the electron transport chain, and the tricarboxylic acid cycle are interrelated pathways involved in the cellular bioenergetics of eukaryotes [25]. Specifically, the expressions of some genes involved in the tricarboxylic acid cycle and electron transport chain were also abnormal on D7 in the HFD pregnant mice ovary (Fig. 3h). Taken together, these results suggest that during early pregnancy, high-fat diet-induced obesity primes fatty acid β -oxidation, which in turn increases the tricarboxylic acid cycle and the electron transport chain, and consequently increases ATP production and ovarian dysfunction.

Fatty acid β -oxidation contributes to the impairment of ovarian function by high-fat diet-induced obesity.

Palmitic acid (16:0, PA) is the most abundant non-esterified fatty acid in the western diet, and in the blood [26-28], while oleic acid (18:1, OA) is the most abundant monounsaturated non-esterified fatty acid in human serum [27, 28]. Cell loading with free fatty acids have been commonly used to develop *in vitro* models of obesity. In the present study, a mixture with 400 μ M OA and 200 μ M PA was used at a non-cytotoxic concentration to probe further into the role of high-fat diet induced obesity in the compromised ovarian function *in vitro* (Fig. 4a). We found that exocellular estradiol and progesterone levels significantly increased in OA- and PA-treated KGN cells (Fig. 4b and c). Exposure to OA and PA, significantly increased the protein expressions of steroid biosynthesis regulatory genes, including CYP19A1, CYP11A1 and StAR, in the luteinized KGN cells (Fig. 4d and e).

We further detected the concentration of ATP in the KGN cells, and the results showed that ATP levels were significantly increased following OA and PA treatments (Fig. 6d). Consistent with the *in vivo* results, both the lactate production and the expression of the factors regulating glycolysis in OA and PA-treated KGN cells were not significantly changed (Fig. 5). This suggests that the glycolysis of luteinized KGN cells is not obviously altered by OA and PA treatment *in vitro*. Given our earlier observation of the elevated ATP levels and the stable glycolysis, we hypothesized that the abnormally elevated ATP levels in the dysfunctional KGN cells also mainly came from disordered fatty acid β -oxidation. According to the results, the accumulation of lipid droplets and the intracellular TG content significantly increased in OA and PA-treated KGN cells (Fig. 6a-c). Moreover, the β -oxidation rate-limiting enzyme CPT1A in KGN cells was also dramatically upregulated by OA and PA treatments when compared with that of the control group (Fig. 6e and f). Etomoxir is a widely used small molecule that is able to inhibit fatty acid oxidation. It achieves this by exerting an irreversible inhibitory effect on CPT1A [29]. Following interference of β -oxidation of fatty acid with 100 μ M etomoxir, the compromised tricarboxylic acid cycle and electron transport chain, and the elevated ATP level by OA and PA were also reversed by etomoxir (Fig. 6g and h). Treatment with etomoxir further reversed the high-fat-induced dysfunctional state of the KGN cells, as indicated by a significant decrease in the levels of exocellular estradiol and progesterone than those in the cells treated with OA and PA alone (Fig. 6i and j). Steroid biosynthesis regulatory genes, including CYP19A1, CYP11A1 and StAR, were also downregulated (Fig. 6k and l).

Discussion

It has been reported that obese women have an increased risk of pregnancy complications, such as miscarriage [3]. However, the underlying mechanisms have not been well elucidated. In early pregnancy, the competent blastocyst adheres to the decidualized endometrium to invade the uterine tissue and vessels and to establish pregnancy. During this time, the corpus luteum of the ovary secretes estrogen and progesterone [30, 31], so as to ensure the success of decidualization. Obese women are more likely to have ovulatory dysfunction due to dysregulation of the hypothalamic-pituitary-ovarian axis [3]. However, ovarian functionality of obese women during early pregnancy is not very clear. In this study, we used high-fat diet to induce obesity in maternal mice and then assessed ovarian functions in them. According to our study, female mice fed HFD diets showed increased numbers of corpus lutea and elevated serum estradiol and progesterone levels during early pregnancy. Significantly increased exocellular estradiol and progesterone levels were also found in luteinized KGN cells after OA and PA treatments. All these *in vivo* and *in vitro* results indicate that ovarian function was impaired by high-fat diet induced-obesity during early pregnancy.

Obesity has been found to be associated with dysregulated glucose and fatty acid metabolism in tissues and organs, such as adipose tissues, liver, muscle, pancreas and brain [32, 33]. To understand the mechanisms by which high-fat diet induced-obesity impairs ovarian function during early pregnancy, we first tested whether impaired glycolysis is involved in it. According to the *in vivo* and *in vitro* results, ovarian glycolysis is not altered by high-fat diet-induced obesity. Also, the impaired ovary exhibited no obvious energy metabolic shifts between aerobic and anaerobic glycolysis. About 90-95% ATP production comes from oxidative phosphorylation of mitochondria and the rest from glycolysis. Mitochondrial fatty acid oxidation is the main source of ATP in many tissues and organs [34]. Fatty acid oxidation is a complex biological process involving mitochondrial β -oxidation, tricarboxylic acid cycle activity, and the electron transport chain [35]. Alterations in fatty acid metabolism can contribute to a variety of pathologies and cell dysfunction [36]. Mitochondrial fatty acid oxidation is the main source of ATP in many tissues and organs [34, 37]. Maintaining lipid homeostasis is a highly complex process which involves lipid storage, synthesis and utilization. During obesity, when the levels of free fatty acids exceed the lipid storage capacity of the adipose tissue, they begin to accumulate in the organs. This condition – known as “ectopic lipid deposition” – has several pathologic consequences [38]. Given our earlier observation of the stability of glycolysis, we hypothesized that dysfunctional ovary mainly results from disordered fatty acid metabolism. When high-fat diet was used to induce obesity in the mice, we found that lipid droplets were accumulated in the ovary during early pregnancy. The levels of triglycerides in the ovary were also elevated. These data indicate that ectopic lipid deposition in the ovary is involved in high-fat diet-induced obesity. To identify the lipid metabolism-associated genes whose expressions are altered in the ovary following the obesity induction, we tested several genes involved in fatty acid synthesis, transportation and β -oxidation. The results showed that ovarian *Acs14* and *Elovl5* (which are involved fatty acid synthesis) and *Slc27a4* (which is involved in fatty acid transportation) were dramatically up-regulated in the obese mice during early pregnancy. CPT1A is the rate-limiting enzyme that transfers the longchain fatty acyl CoA to the mitochondria for β -oxidation [39]. This enzyme was also observed to be upregulated

in the obese mice. The upregulated expression of CPT1A *in vivo* and *in vitro* suggests that high-fat diet-induced obesity primes the aberrantly increased fatty acid β -oxidation in the ovary during early pregnancy. Etomoxir is widely used as a potent irreversible inhibitor of CPT1A [40]. In this study, when etomoxir was used to interfere with β -oxidation of fatty acid, the accumulation of lipid droplets and the levels of exocellular estradiol and progesterone levels in the OA- and PA-treated KGN cells were improved. All these suggest that high-fat diet-induced obesity enhances fatty acid β -oxidation in the ovary of mice during early pregnancy.

ATP is the direct energy for all kinds of life activities. Depending on its decrease or increase, ATP can trigger many different cell responses, including cell proliferation, differentiation and apoptosis [18, 22-24]. Here, the ovarian tissue concentration of ATP, during early pregnancy, was significantly increased by high-fat diet-induced obesity. Consistent with the *in vivo* results, ATP levels were significantly increased in the OA- and PA-treated KGN cells. The mitochondria meet the energy demands of cells through the uptake and oxidation of various substrates. This leads to the generation of acetyl CoA in order to produce ATP via the tricarboxylic acid cycle oxidation [41]. Transfer of electrons via mitochondrial co-factors NAD(H) and FAD(H₂) to the electron transport chain creates a proton gradient across the inner mitochondrial membrane generating the mitochondrial membrane potential [41]. This drives oxidative phosphorylation, which generates ATP by the reduction of oxygen to water and the phosphorylation of ADP [41]. In this study, we found that some genes involved in the tricarboxylic acid cycle and electron transport chain were also abnormally expressed in the ovaries of the obese pregnant mice. The compromised tricarboxylic acid cycle and electron transport chain, and the elevated ATP level in the OA- and PA-treated KGN cells were reversed following etomoxir treatment. All these suggest that during early pregnancy, high-fat diet induced obesity can prime impaired fatty acid β -oxidation, which will in turn compromise the tricarboxylic acid cycle and the electron transport chain, and consequently increase ATP production and ovarian dysfunction.

In conclusion, our findings indicate that ovarian functions are impaired by high-fat diet-induced obesity during early pregnancy in mice. Abnormal fatty acid β -oxidation, but not abnormal glycolysis, contributes to impairing ovarian functions during obesity induced by high-fat diet. Our findings help in understanding the mechanistic effect of obesity on the ovary and female fertility; and reinforces the need to regulate the intake of high-fat diets so as to avoid obesity and its associated effects. Nevertheless, the confirmation of our findings in humans is highly recommended.

Declarations

Ethics approval and consent to participate

The present study was approved by the Chongqing Medical University ethics committee.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used in the present study are available from the corresponding author upon reasonable request.

Competing interests

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

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Authors' contributions

Conceptualization, R.G. and Q.L.; data curation, Q.L. and S.G.; writing—original draft preparation, Q.L.; writing—review and editing, R.G.; supervision, C.Y., X.L., X.C. and J.H.; project administration, Y.D., Y.G., X.M., T.L. and F.L.; funding acquisition, Y.W. and R.G. All authors have read and agreed to the published version of the manuscript.

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Figures

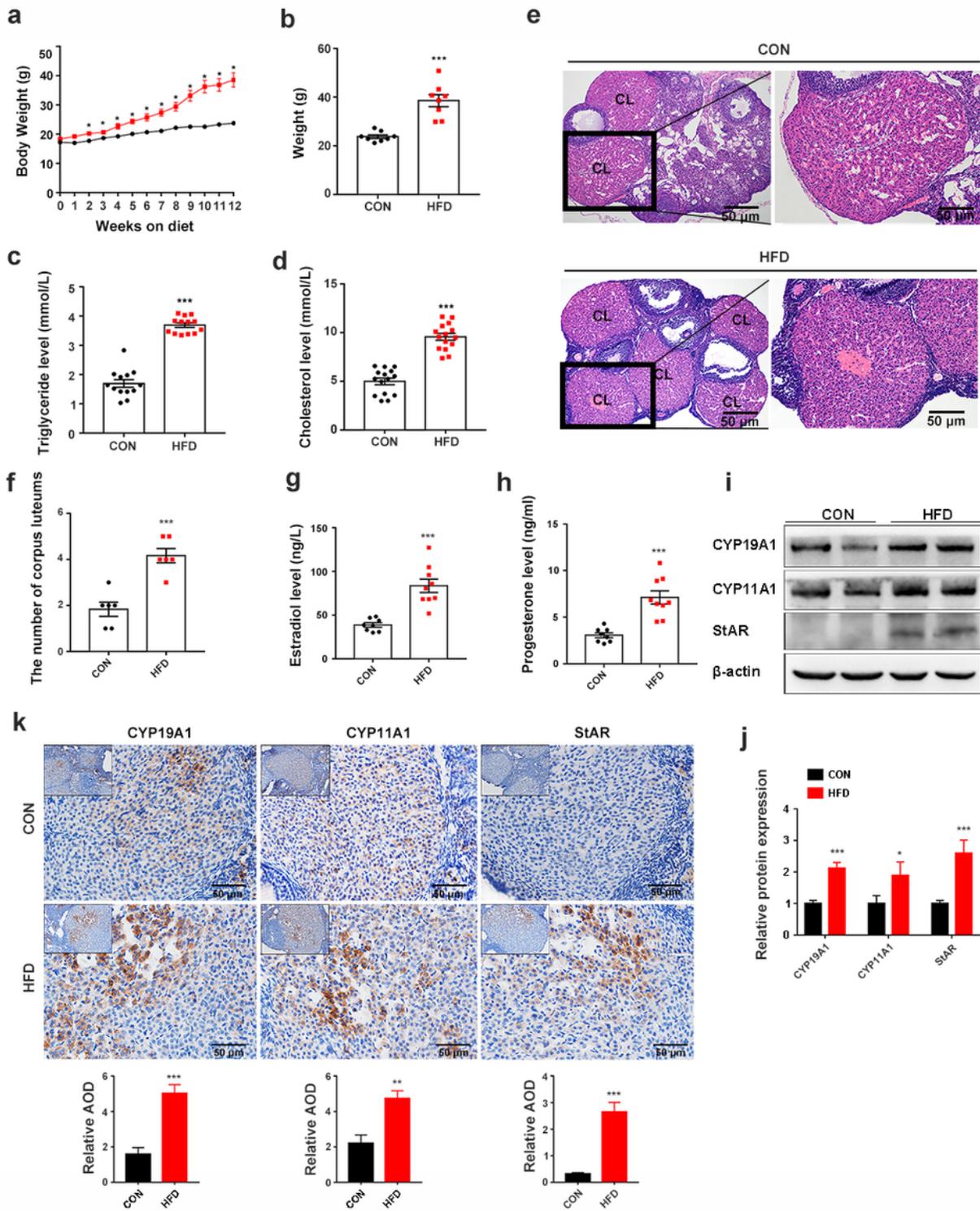


Figure 1

The high-fat diet-induced obese mice exhibited aberrant ovarian function in early pregnancy. (a) Body weights of female mice fed either chow diet or high fat diet for 12 weeks respectively. (b) Body weights of female mice on day 7 of pregnancy. Serum (c) triglyceride and (d) total cholesterol levels in mice fed with chow diet or high-fat diet on day 7 of pregnancy. (e). Ovarian morphology was assessed by H&E staining on day 7 of pregnancy, the time essential for decidualization. CL: corpus luteum. (f) The number of

corpus lutea in control and HFD treated groups. Serum (g) estradiol and (h) progesterone levels on day 7 of pregnancy was detected by ELISA. (i) Western blot showed the protein expression of CYP19A1, CYP11A1 and StAR in ovary on day 7 of pregnancy. (j) The histogram showed the statistical analysis of western blot on E. (k) Immunolocalization showed the CYP19A1, CYP11A1 and StAR expression in corpus lutea. Average optical density (AOD; IOD [Integrated Optical Density]/area) was used for the quantitative analysis of immunohistochemical staining, and the data are shown in bar graphs. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. CON: chow diet group; HFD: high fat diet group.

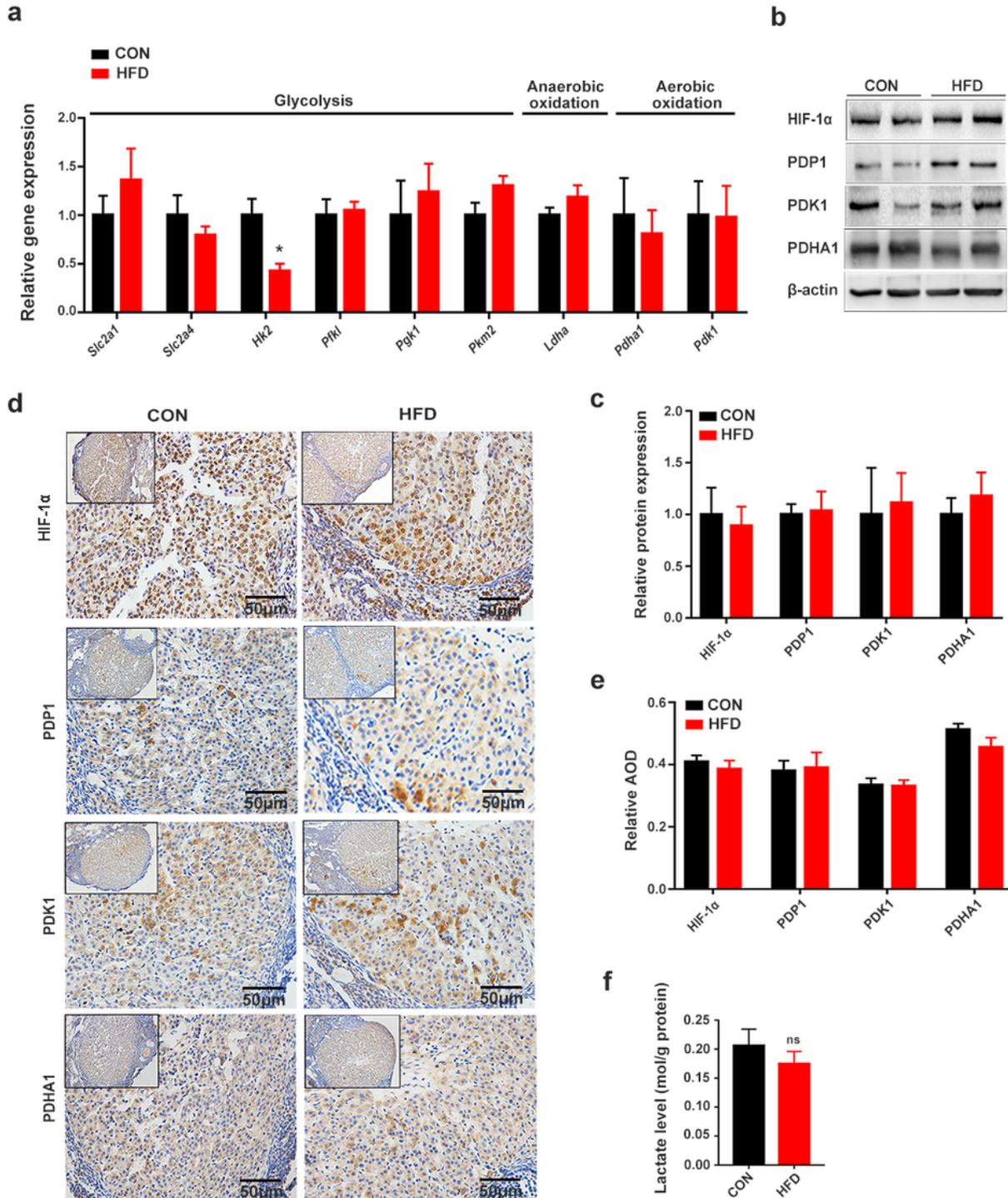


Figure 2

Ovarian glycolysis is not altered by high-fat diet-induced obesity during early pregnancy. (a) Real time PCR showed that the key glycolysis-regulatory genes including *slc2a1*, *slc2a4*, *hk2*, *pkm2*, *pgk1*, *pfkl*, *ldha*, *pdha1* and *pdk1* were stably expressed in the pregnant mice ovary on D7 in the HFD group. For each group, n = 15. (b) Western blot showed that, the ovarian expression of proteins in HFD group, which shifts the energy metabolism between aerobic and anaerobic glycolysis, was comparable with that in control on D7. (c) The histogram showed the statistical analysis of western blot on B. Each group n = 6. (d) According to immunohistochemistry, after treatment with high-fat diet the expression and the location of proteins HIF1 α , PDK1, PDH and PDHA1 were not obviously altered on D7. (e) The histogram showed the statistical analysis of immunohistochemistry on D. (f) There was not significantly change of ovarian lactate production in HFD group when compared with that in control on D7. Each group n = 8-10. *P < 0.05. CON: chow diet control group; HFD: high fat diet group.

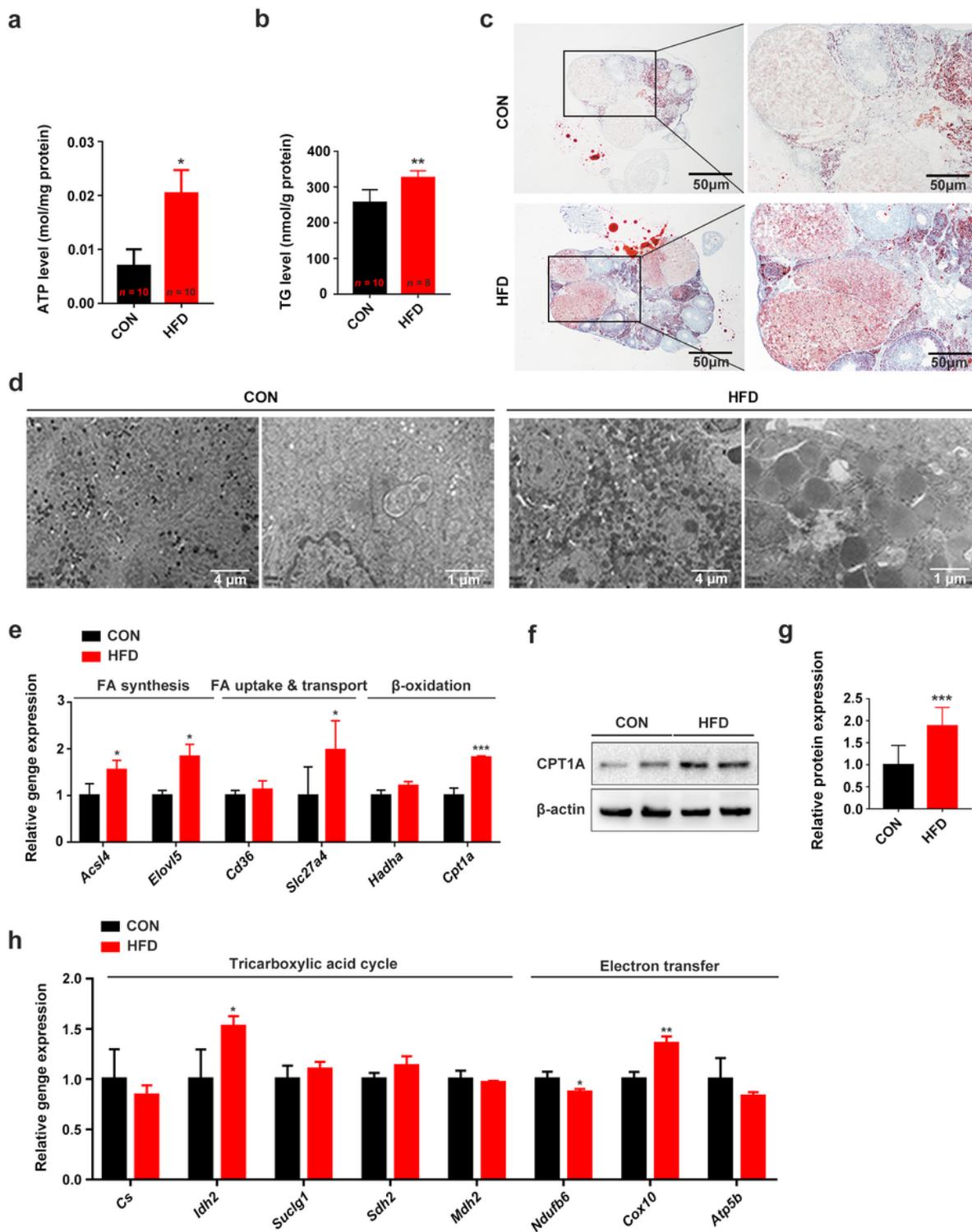


Figure 3

High-fat diet-induced obesity enhances fatty acid β -oxidation in the ovary during early pregnancy. Ovary tissues on day 7 of pregnancy were examined to show the role of high-fat diet-induced obesity in fatty acid metabolism during early pregnancy. (a) ATP production in the ovarian tissues in the pregnant mice on D7. According to the (b) quantitative results of lipid content, (c) Oil Red O staining, and (d) transmission electron microscopy image analysis, high-fat diet caused a significant increase in ovarian

lipid accumulation. (e) Genes involved in fatty acid metabolism were detected by Real time PCR. Each group n =15. (f) Fatty acid β -oxidation rate-limiting enzyme CPT1A was examined by western blot. (g) The histogram showed the statistical analysis of western blot on F. Each group n = 6. (h) The expression of genes involved in the tricarboxylic acid cycle and electron transport chain were detected by Real time PCR. *P<0.05, **P<0.01 and ***P<0.001.

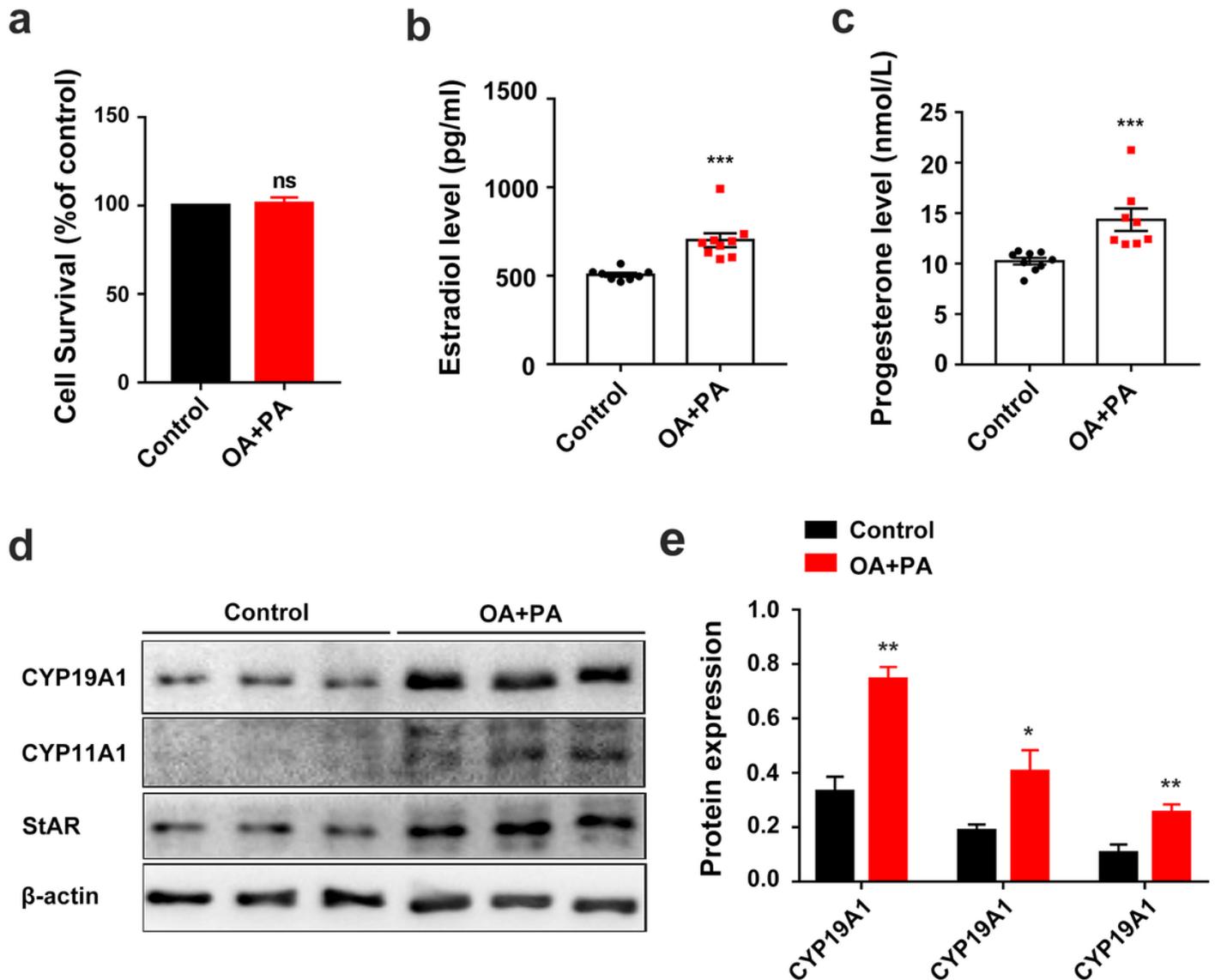


Figure 4

Palmitic acid and oleic acid induced ovarian dysfunction in luteinized KGN cells. Luteinized KGN cells were treated with a mixture of 400 μ M OA and 200 μ M PA (oleic acid/palmitic acid, 2:1) for 24 h. (a) Cell viability. Each group n = 5. Exocellular estradiol (b) and progesterone (c) levels significantly increased in OA- and PA-treated KGN cells. Each group n = 8-9. (d) The expression of steroid biosynthesis genes, including of CYP19A1, CYP11A1 and StAR, was detected by western blot. Each group n = 6. (e) The histogram showed the statistical analysis of western blot on D. *P<0.05, **P<0.01, ***P<0.001. OA: oleic acid. PA: palmitic acid.

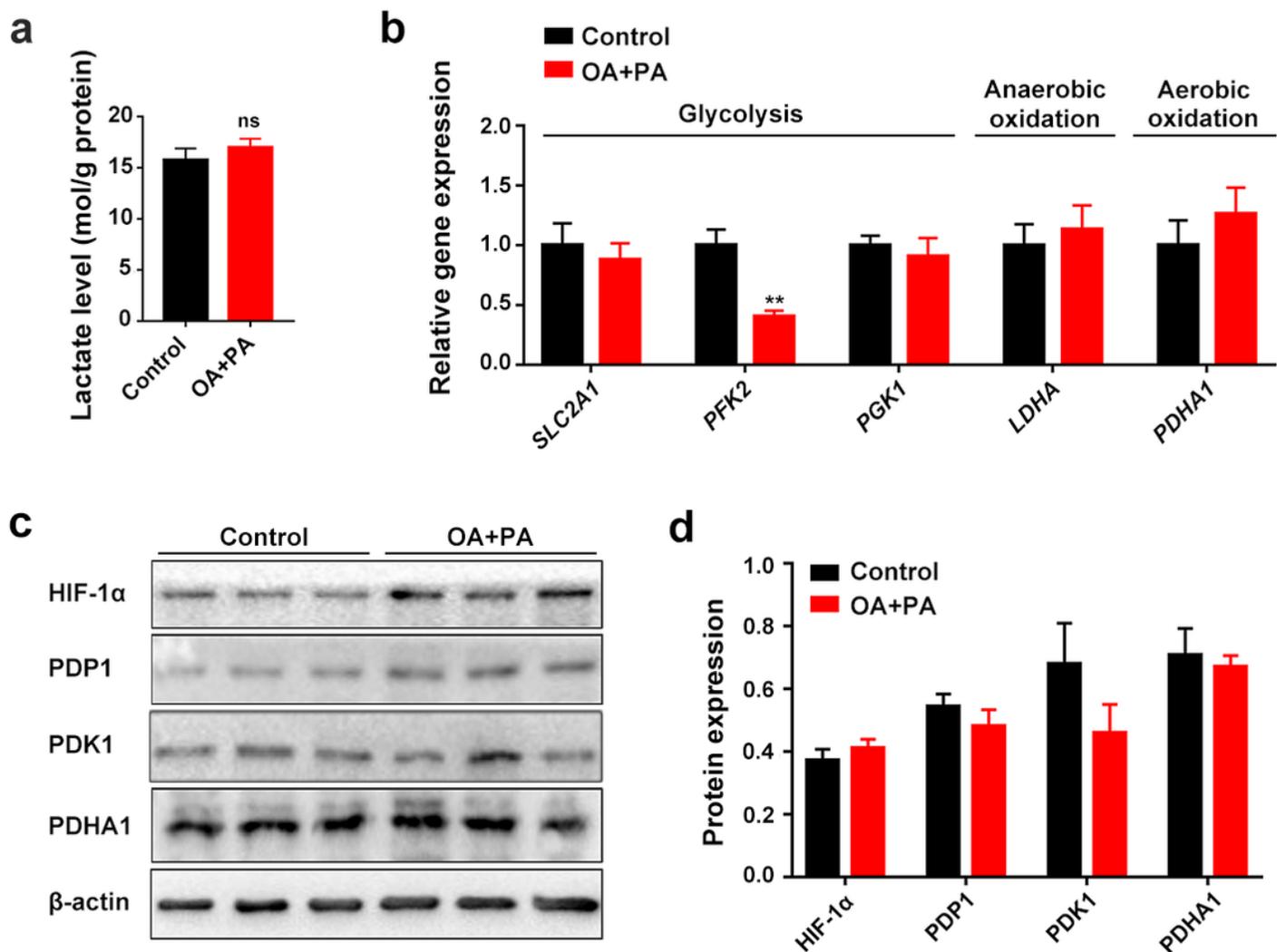


Figure 5

Glycolysis in luteinized KGN cells was not apparently altered by palmitic acid and oleic acid. (a) Lactate production. (b) Real time PCR showed the key genes regulated glycolysis including SLC2A1, PGK1, PFK2, LDHA and PDHA1 were stably expressed in luteinized KGN cells treated with palmitic acid and oleic acid. (c) Western blot showed the expression of HIF1α, PDK1, PDP1, PDHA1 was not apparently altered by palmitic acid and oleic acid. (d) The histogram showed the statistical analysis of western blot on C. **P<0.01. OA: oleic acid. PA: palmitic acid.

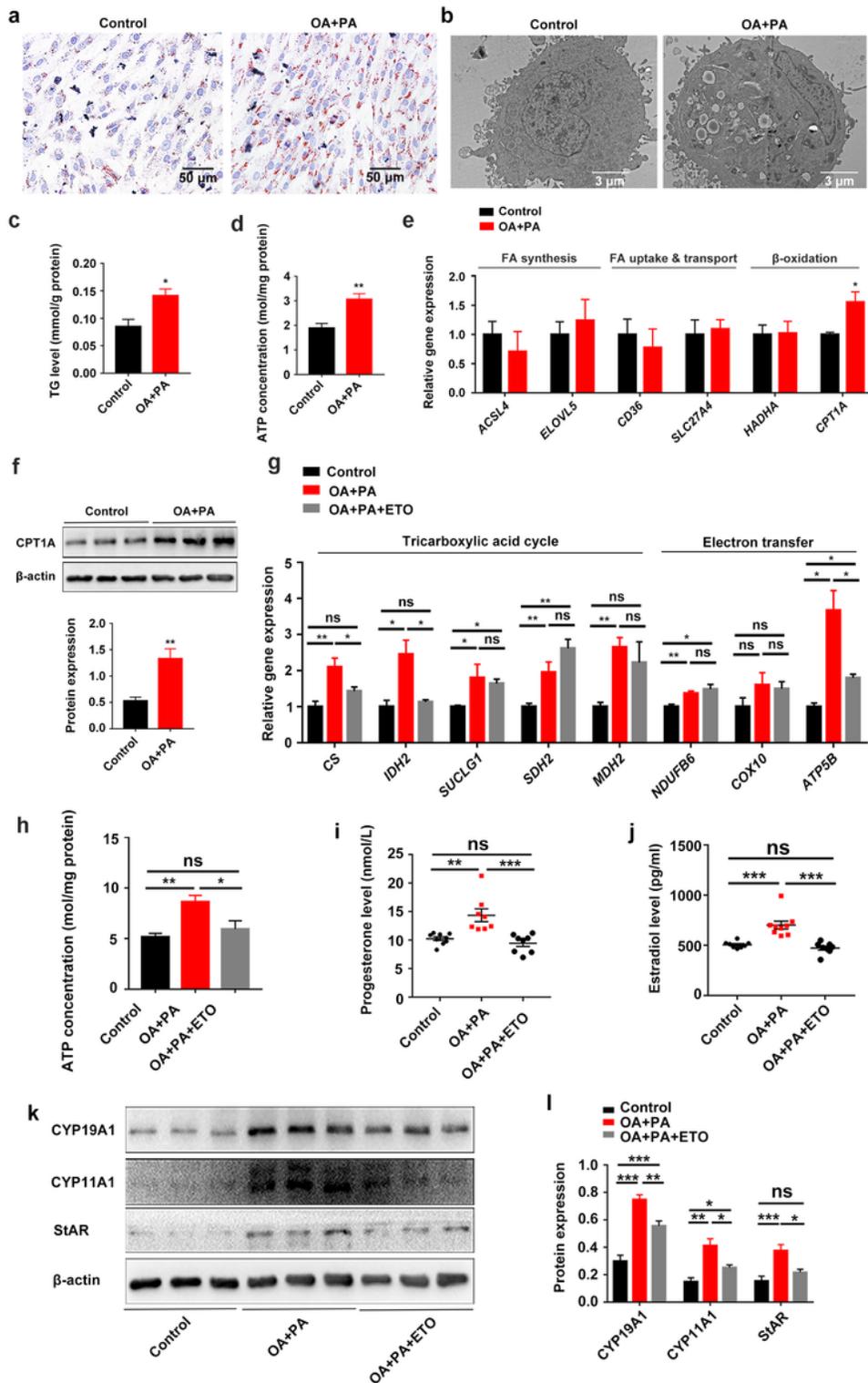


Figure 6

Fatty acid β -oxidation contributes to impaired ovarian function in luteinized KGN cells treated with palmitic acid and oleic acid. The accumulation of lipid droplets in KGN cells was stained by (a) Oil Red O and (b) transmission electron microscopy. Each group $n = 5$. (c) Intracellular TG content was significantly increased in OA- and PA-treated KGN cells. Each group $n = 8$. (d) The concentration of ATP in KGN cells treated with palmitic acid and oleic acid. Each group $n = 8$. (e) Real time PCR and (f) western blot showed

the expression of CPT1A in KGN cells were dramatically upregulated by PA and OA. Each group n = 10. (g) The compromised tricarboxylic acid cycle and electron transport chain, and (h) The elevated ATP level by PA and OA was also reversed in the condition of etomoxir. Each group n = 8. Treated with etomoxir, exocellular (i) estradiol and (j) progesterone levels were significantly decreased than those in the cells treated with PA and OA alone. Each group n = 7-8. (k, l) The expression of steroid biosynthesis genes, including of CYP19A1, CYP11A1 and StAR, was dramatically downregulated in the role of etomoxir. Each group n = 6. *P<0.05, **P<0.01, ***P<0.001. OA: oleic acid. PA: palmitic acid. ETO: etomoxir.

Supplementary Files

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