REPRODUCTION

High-fat diets exaggerate endocrine and metabolic phenotypes in a rat model of DHEA-induced PCOS

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Abstract

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder with unclear etiology and unsatisfactory management. Effects of diets on the phenotype of PCOS were not fully understood. In the present study, we applied 45 and 60% high-fat diets (HFDs) on a rat model of PCOS induced by postnatal DHEA injection. We found that both DHEA and DHEA+HFDs rats exhibited reproductive abnormalities, including hyperandrogenism, irregular cycles and polycystic ovaries. The addition of HFDs, especially 60% HFDs, exaggerated morphological changes of ovaries and a number of metabolic changes, including increased body weight and body fat content, impaired glucose tolerance and increased serum insulin levels. Results from qPCR showed that DHEA-induced increased expression of hypothalamic androgen receptor and LH receptor were reversed by the addition of 60% HFDs. In contrast, the ovarian expression of LH receptor and insulin receptor mRNA was upregulated only with the addition of 60% HFDs. These findings indicated that DHEA and DHEA+HFDs might influence PCOS phenotypes through distinct mechanisms: DHEA affects the normal function of hypothalamus–pituitary–ovarian axis through LH, whereas the addition of HFDs exaggerated endocrine and metabolic dysfunction through ovarian responses to insulin-related mechanisms. We concluded that the addition of HFDs yielded distinct phenotypes of DHEA-induced PCOS and could be used for studies on both reproductive and metabolic features of the syndrome.

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathy associated with reproductive and metabolic disorders in women of reproductive age, characterized by a clustering of hyperandrogenism, hyperinsulinemia, menstrual dysfunction, hirsutism, acne and infertility complications (Franks 1995, Qiao & Feng 2011). Hyperandrogenism occurs in 60–80% of the patient population (Franks 2006) and is critically involved in the occurrence and development of PCOS (Goodarzi et al. 2011). Dysfunctional hypothalamus–pituitary–ovarian axis is responsible for the anovulatory infertility and polycystic ovaries in PCOS (Burt Solorzano et al. 2012), and results in abnormal negative feedbacks of estrogen and progesterone, increased serum luteinizing hormone (LH) but decreased follicle-stimulating hormone (FSH) levels (Daniels & Berga 1997, Ascoli et al. 2002, Tsutsumi & Webster 2009). Several rodent PCOS models have been established by mimicking the hyperandrogenism with pre or postnatal treatment with androgen, estrogen or aromatase inhibitors (McCarthy &

Brawer 1990, Kafali et al. 2004, Manneras et al. 2007, Shi & Vine 2012, Walters et al. 2012, van Houten & Visser 2014). Long-term postnatal injection of DHEA, a metabolic intermediate in the biosynthesis of androgen abundant in the circulation, has been widely utilized to generate PCOS models in rodents. DHEA-treated rats exhibit some human PCOS characteristics, including hyperandrogenism, acyclicity, anovulation and polycystic ovaries (Roy et al. 1962, Ward et al. 1978, Lee et al. 1991, Anderson et al. 1992, Sander et al. 2006). Metabolic disturbances, however, are not commonly reported in this model (Walters et al. 2012).

Indeed, a large proportion of PCOS women exhibit metabolic abnormalities such as insulin resistance (IR), dyslipidemia and obesity (Diamanti-Kandarakis & Dunaif 2012). Diets and fat metabolism at least partially account for PCOS phenotypes (Al-Azemi et al. 2004, Azziz et al. 2004, Franks 2006). Elevated androgen levels are related to increased amount of adipose tissue, particularly in visceral and abdominal regions, and induce obesity in 30–70% of PCOS women (Lim et al. 2013,

Borruel *et al*. 2013). Obesity in turn increases the risk of dyslipidemia, IR, type II diabetes, hypertension, metabolic syndrome and other complications (Yilmaz *et al*. 2005, de Groot *et al*. 2011). Loss of body weight significantly improves the ovulatory rate of PCOS women and ameliorates hyperandrogenism-related symptoms (Morris *et al*. 2006, Tsagareli *et al*. 2006, Panidis *et al*. 2008). Though high-fat diets (HFDs) have been shown to cause obesity and infertility in female rodents (Omagari *et al*. 2008, Powell *et al*. 2014), how fat affects PCOS phenotypes is not fully elucidated. To gain a better understanding of interactions between hyperandrogenism and fat in PCOS, we applied HFDs with different fat contents (45% and 60%) in a rat model of PCOS induced by DHEA injection and observed the resultant changes of body weight, body fat content, menstrual cycles, ovarian morphology, serum hormone levels, glucose tolerance and several hormones and their receptor levels in the ovary and hypothalamus. We showed that the addition of HFDs exaggerated a number of PCOS phenotypes than DHEA alone and that DHEA and DHEA+HFDs might influence PCOS phenotypes through distinct mechanisms.

Materials and Methods

Animals and grouping

Twenty two-day-old female Sprague-Dawley rats were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. Rats were housed four to six per cage with standard laboratory conditions (12 h light:12 h darkness cycle) and free access to rodent feed and water. All animal experimental procedures were approved by the Animal Care and Use Committee of Peking University Third Hospital according to the national legislation for animal care.

All rats were randomly divided into four groups (n=22–23 each group): control, DHEA, DHEA+45% HFD and DHEA+60% HFD groups. The DHEA group received daily s.c. DHEA injection (6 mg/100 g body weight dissolved in 0.1 ml of sesame oil) from day 27 to day 46 to establish PCOS (Anderson *et al*. 1992), and was fed with normal rodent diet containing 10% fat. The DHEA +45 and +60% HFD groups received the same DHEA injection and were fed with 45 or 60% fat-containing diets (45 and 60% of calories from fat; Research

Diets, New Brunswick, NJ, USA) respectively. The control group received normal rodent diet and daily s.c. sesame oil injection for an equivalent length of time.

After 20 days of treatment, half of the animals randomly chosen from each group (n=12–13 each group) were assessed for body composition analysis, vaginal smears, oral glucose tolerance tests (OGTTs), serum, ovarian and hypothalamic substance levels and ovarian morphology. The left ovaries were weighed and processed for staining with hematoxylin and eosin (H&E) (details below). The right ovaries and the hypothalamus were quickly frozen and stored at $-80\,°C$ for RNA extraction and qPCR analysis as described below. The rest animals received serum ovulation induction for oocyte collection.

Body weight and body composition analysis

The body weight was measured every 6 days from day 27 until day 45.

By the end of the injection schedule, MRI was performed with rats placed in a clear plastic holder without sedation and inserted into an Echo MRITM device (Echo Medical Systems, Houston, TX, USA) for measurement of the body fat mass composition.

Vaginal smears and estrus cycle determination

The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from days 37–46 following Shorr staining (Shorr 1941): pro-estrus (round, nucleated epithelial cells), estrus (cornified squamous epithelial cells), metestrus (cornified squamous epithelial cells and predominance of leukocytes) and diestrus (nucleated epithelial cells and predominance of leukocytes) (Marcondes *et al*. 2002).

Rats from DHEA and DHEA+HFD groups were completely acyclic and remained in constant metestrus/diestrus cycles while control rats had normal cycles. To eliminate the effects of estrus cycles on other measurements, only control rats in the metestrus/diestrus cycles were used for further examination.

Oral glucose tolerance test

At the end of schedule, the rats were fasted 8 h before oral administration of glucose (2 mg/kg body weight), as previously described (Lee *et al*. 1991). Tail blood samples were obtained

Table 1 Primer sequences for real-time PCR used in the study.

| Target genes	Primer sequence	Tm (°C)
Androgen receptor	Sense: 5′-AACTCAGTGGCTGGGATTATG-3′	60
	Antisense: 5′-CCAAATCAGGACCCTAAGGAAG-3′	
Insulin receptor	Sense: 5′-CAGTGTCGTGATCGGAAGTATT-3′	60
	Antisense: 5′-CTGAGGTACTCTGGGTTTGAAG-3′	
LH receptor	Sense: 5′-AACTCAGTGGCTGGGATTATG-3′	60
	Antisense: 5′-CCAAATCAGGACCCTAAGGAAG-3′	
GnRH receptor	Sense: 5′-CTGAGCAAGTTTGGCTGTTATG-3′	60
	Antisense: 5′-GAGGGACAAGCACGTAACTATT-3′	
GAPDH	Sense: 5′-GGAGAAACCTGCCAAGTATGA-3′	60
	Antisense: 5′-TTGAAGTCACAGGAGACAACC-3′	

Tm, melting temperature.
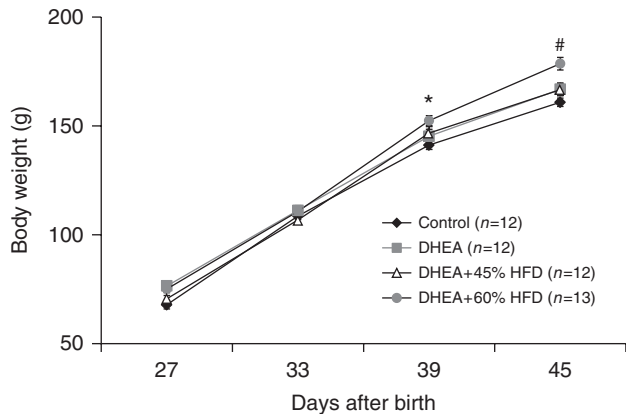
Figure 1 Body weight curves of rats during the 3-week treatment period. DHEA+60% HFD significantly increased the body weight. *$P < 0.05$ vs the control group; #$P < 0.05$ vs the other three groups, two-way ANOVA followed by Tukey's *post hoc* tests.

on multiple time points: before and 30, 60, 90 and 120 min after administration for measurement of blood glucose levels with a glucometer (Roche Diagnostics).

Serum and hypothalamic levels of hormones and other substances

After treatment for 20 days, blood samples were obtained after an 8-h fasting. The levels of testosterone, 17β-estradiol (E_2), LH, FSH, HDL, LDL, insulin and homocysteine (HCY) were determined with ^{125}I-labelled RIA kits (Beijing North Institute of Biological Technology, Beijing, China). Total cholesterol (TCh) and triglyceride (TG) levels were determined by biochemical kits (China Diagnostics Medical Corporation, Beijing, China). Protein concentrations of testosterone and LH in the hypothalamus were measured with the Bradford method as described (Shao *et al*. 2007).

Ovarian morphology

By the end of the experiment, rats ($n = 12$–13 each group) were sacrificed with left ovaries removed and weighed, then fixed overnight in 4% formaldehyde, placed in 70% ethanol, dehydrated and embedded with paraffin. The sections were prepared and stained with H&E (Beisuo Biotech, Company, Beijing, China). The ovaries were longitudinally and serially sectioned at 5 μm (LEICA CM1850, Heidelberg, Germany), every fifth section ($n = 6$ per ovary) was mounted on a glass slide and observed under the light microscopy for histomorphologic

examinations. The resulting slides were further confirmed by a pathologist. Meanwhile, the area of the ovary, the thickness of follicular wall and granulosa cell layer were measured (by NIS-Elements 3.2, Nikon Eclipss 80 i; Nikon, Tokyo, Japan). The analyses of ovarian follicles at different stages of development, corpora lutea (CL) and regression follicles were performed by two persons independently to avoid duplicate counting. The results were confirmed by a pathologist.

Oocyte collection and immunofluorescence

For oocyte collection, rats were superovulated by i.p. injection of 25 IU of pregnant mare's serum gonadotropin (PMSG) (Hua Fu Biotechnology Company, Tianjin, China) after DHEA treatment (day 47), followed by 20 IU of human chorionic gonadotropin (hCG) (Hua Fu Biotechnology Company) 48 h later. Cumulus–oocyte complexes (COCs) were collected 15–16 h after hCG injection (Masumi *et al*. 2001). Surrounding cumulus cells were removed by a brief exposure to 0.2% hyaluronidase and repeated pipetting. Mature oocytes at metaphase II were selected by the extrusion of the first polar body (Zhou *et al*. 2003, Ubaldi & Rienzi 2008). The oocyte maturation rate was analyzed.

Spindle assembly of MII oocytes was observed by an immunofluorescence method, as previously described (Qi *et al*. 2013). In brief, for spindle and chromosome analysis, denuded MII oocytes were fixed in 4% paraformaldehyde for 30 min at room temperature after twice PBS rinsing. Then oocytes were permeabilized with 1% Triton X-100 for 30 min, incubated with 1% BSA in PBS for 1 h at room temperature and transferred to a droplet containing monoclonal anti-α-tubulin-FITC antibody (1:100 dilution in BSA) for 1 h. After three washes, the nuclei of oocytes were stained with 10 μg/ml of Hoechst 33342 for 10 min. Finally, the oocytes were mounted on glass slides and examined with a laser scanning confocal microscope (LSM710 Carl Zeiss, Oberkochen, Germany).

Quantitative real-time PCR

Right ovaries and the hypothalamus were dissected from 47-day-old rats, quickly frozen and stored at −80 °C for RNA extraction. cDNA was synthesized with the first-strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada), and real-time qPCR analysis was performed using SYBR Green PCR master mix (Invitrogen); expression levels were assessed by the ABI 7500 real-time PCR system (Applied Biosystems). Details of the primer sequences were listed in Table 1.

All qPCRs were carried out in a final volume of 20 μl following the manufacturer's instructions (Invitrogen). Each sample's reaction was triplicate, and the mean value was

Table 2 Body composition analysis. Values are presented as means ± s.e.m.

	Control ($n = 12$)	DHEA ($n = 12$)	DHEA+45% HFD ($n = 12$)	DHEA+60% HFD ($n = 12$)
Body weight (BW, g)	160.9 ± 1.9	166.8 ± 3.0	166.7 ± 2.6	178.6 ± 2.9[†]
Body fat (% of BW)	4.3 ± 0.4	4.6 ± 0.1	8.6 ± 0.2*	11.9 ± 0.2[†]
Body lean (% of BW)	88.7 ± 0.8	88.5 ± 1.1	86.4 ± 1.6	85.3 ± 1.4
Free water (% of BW)	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0*	0.2 ± 0.0*

*$P < 0.01$ vs control and DHEA groups; [†]$P < 0.01$ vs control, DHEA and DHEA+45% HFD groups, one-way ANOVA followed by Tukey's *post hoc* tests.

Table 3 Serum and hypothalamic substance levels. Values are presented as means±s.e.m.

	Control ($n=12$)	DHEA ($n=12$)	DHEA+45% HFD ($n=12$)	DHEA+60% HFD ($n=13$)
Serum				
E$_2$ (pg/ml)	12.7±2.3	52.7±7.8*	48.5±11.4*	48.0±7.6*
Testosterone (ng/ml)	0.3±0.1	35.5±5.0*	31.6±6.0*	31.4±7.5*
LH (mIU/ml)	22.1±2.3	35.9±2.3*	34.1±2.7*	32.1±3.1*
FSH (mIU/ml)	10.6±0.7	11.6±1.2	11.7±1.1	12.5±1.2
Insulin (IU/ml)	26.1±2.8	24.4±2.6	32.3±2.0	42.4±5.8[‡]
TCh (mmol/l)	1.4±0.2	1.3±0.2	1.2±0.2	1.4±0.1
HDL (mmol/l)	0.5±0.1	0.5±0.2	0.6±0.1	0.6±0.3
TG (mmol/l)	0.8±0.1	1.0±0.1	1.2±0.2[†]	1.6±0.3[†]
LDL (mmol/l)	0.4±0.1	0.5±0.2	0.6±0.1*	0.7±0.1[†]
HCY (μmol/l)	4.8±0.5	3.3±0.4	8.7±0.8*	11.7±2.5[‡]
Hypothalamus				
Testosterone (ng/ml)	3.5±0.6	8.9±1.0*	7.8±0.2*	7.3±0.3*
LH (mIU/ml)	35.9±0.9	47.3±0.6*	37.9±1.2*	36.3±0.7

*$P<0.01$ vs control group; [†]$P<0.01$ vs control and DHEA groups; [‡]$P<0.01$ vs control, DHEA and DHEA+45% HFD groups, one-way ANOVA with Tukey's *post hoc* tests.

normalized by housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The changes in the expression level were assessed by the method of $2^{-\Delta\Delta CT}$.

Statistical analysis

All results were presented as means±s.e.m. and analyzed by one-way ANOVA with Tukey's *post hoc* tests by SPSS 19.0. In all statistical comparisons, P values <0.05 were considered to be statistically significant.

Results

DHEA+HFD treatment increased body weight and body fat content

With 3-week DHEA+60% HFD treatment, rats had gained significantly more weight than controls (group: $F_{3,45}=4.53$, $P<0.01$; day: $F_{3,45}=3053.49$, $P<0.01$; group by day: $F_{9,45}=4.67$, $P<0.01$, two-way ANOVA with Tukey's *post hoc* tests, Fig. 1, Table 2). No group differences were detected between DHEA and DHEA+45% HFD groups.

As for the body fat mass composition measurements (Table 2), the addition of HFDs significantly increased the body fat ($F_{3,26}=224.43$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests), but decreased free water within the body weight ($F_{3,26}=130.11$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Table 2). In total, 60% HFDs had a stronger effect than 45% HFDs on body fat.

These results indicated abnormal fat accumulation with the addition of HFDs in DHEA-treated rats.

HFDs exaggerated endocrine and metabolic dysfunction induced by DHEA treatment

DHEA injection, with or without HFDs, induced a clear increase of serum E$_2$ ($F_{3,49}=6.08$, $P<0.01$), testosterone ($F_{3,49}=9.04$, $P<0.01$) and LH ($F_{3,49}=3.50$, $P<0.05$) but not FSH ($F_{3,49}=0.78$, $P>0.05$, one-way ANOVA with Tukey's *post hoc* tests, Table 3) levels. DHEA+60% HFDs increased insulin levels compared with all other three groups ($F_{3,49}=5.56$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Table 3). While TCh ($F_{3,49}=0.82$, $P>0.05$) and HDL ($F_{3,49}=2.1$, $P>0.05$, one-way ANOVA) levels remained unchanged, DHEA with HFDs increased TG ($F_{3,49}=4.26$, $P<0.05$), LDL ($F_{3,49}=4.19$, $P<0.05$) and HCY ($F_{3,49}=19.19$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Table 3) levels.

In the hypothalamus, the testosterone level was also elevated with DHEA treatment, with or without HFDs ($F_{3,36}=358.97$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Table 3). In contrast, the elevation of hypothalamic LH level by DHEA was reversed by the addition of 60% HFDs ($F_{3,36}=358.97$, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Table 3).

These data indicated not only the successful establishment of the rat PCOS model with DHEA injection but also more severe metabolic dysfunction induced by HFDs.
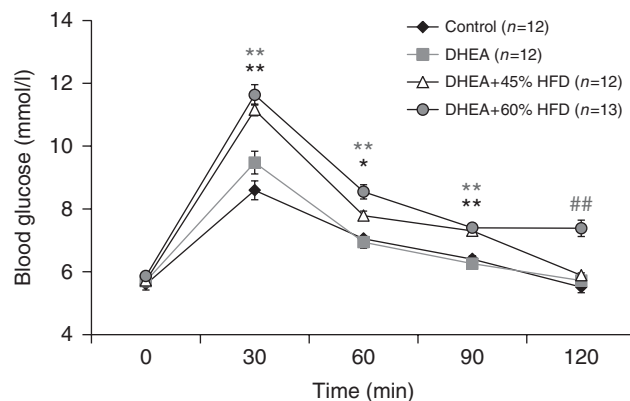

Figure 2 OGTTs revealed impaired glucose tolerance in DHEA+HFD-treated rats. *$P<0.05$, **$P<0.01$ vs the control and DHEA groups; ##$P<0.01$ vs the other three groups, two-way ANOVA followed by Tukey's *post hoc* tests.
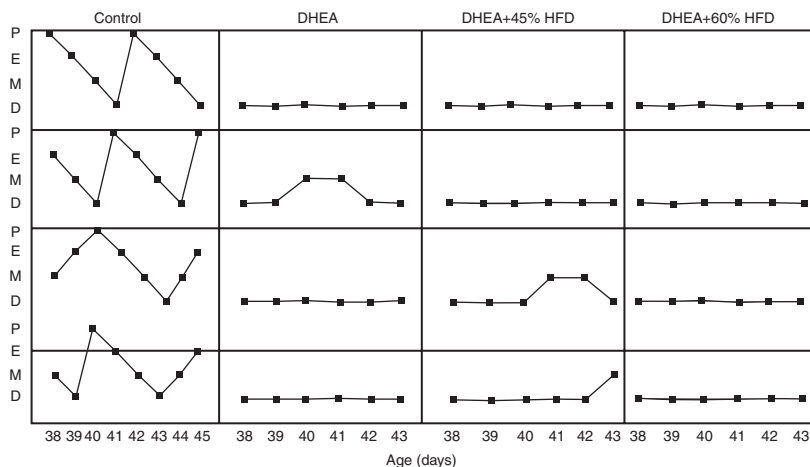
Figure 3 DHEA injection, with or without HFDs, disrupted the normal estrus cycle. P, pro-estrus; E, estrus; M, metestrus; D, diestrus.

DHEA + HFD treatment impaired glucose tolerance

We further examined the impact of DHEA and HFDs on glucose tolerance with the OGTT. While fasting glucose levels were similar across groups, treatment of DHEA + HFDs, but not DHEA alone, resulted in markedly increased glucose levels after administration of glucose for 30, 60 and 90 and 120 min in the DHEA + 60% HFD group (groups: $F_{3,45} = 40.74$, $P < 0.01$; time: $F_{4,180} = 401.5$, $P < 0.01$; groups by time: $F_{12,180} = 9.34$, $P < 0.01$, two-way ANOVA with Tukey's *post hoc* tests, Fig. 2), suggesting impaired glucose tolerance in DHEA + HFD-treated rats.

DHEA treatment disrupted normal menstrual cycles

Daily examination of vaginal smears from days 37 to 46 showed that the normal 4-day estrus cycle in control rats was disrupted by DHEA injection (Fig. 3). Both DHEA and DHEA + HFDs rats remained irregular throughout the examination period. Vaginal smears showed leukocytes, the dominant cell type of the diestrus phase. Occasionally, manifestations of the metestrus phase could be observed in DHEA and DHEA + 45% HFD rats, indicating complete acyclic rats after DHEA treatment.

DHEA induced morphological changes of the ovary

Control rats showed normal morphology: follicles at different stages were observed in these rats, with normal theca and granulosa cells (Fig. 4A). In contrast, DHEA-treated rats showed remarkable cyst-like appearance of follicles along with follicle collapse possibly induced by atresia (Fig. 4B, C and D). With a higher magnification, DHEA and DHEA + HFD groups exhibited more cystic follicles characterized by thickened follicular walls and diminished granulosa cell layers (Table 4 and Fig. 5) (Manneras *et al*. 2007). These ovaries contained fewer antral follicles but more fluid-filled cystic

follicles, indicating the absence of ovulation. Histological examination of ovaries from DHEA-treated rats revealed scant granulosa cells layers with some apoptotic cells (Fig. 5F). The number of apoptotic cells increased with higher fat content (Fig. 5G and H). Occasionally, apparently normal follicles were observed, similar to women with PCOS (Azziz 2006). It should be noted that DHEA + HFD-treated rats had thinner granulosa cell layers but thicker theca cell layers than controls, which were not obvious in DHEA rats (Table 4). These data indicated ovarian abnormalities in DHEA-treated rats, which were more serious with the addition of HFDs.
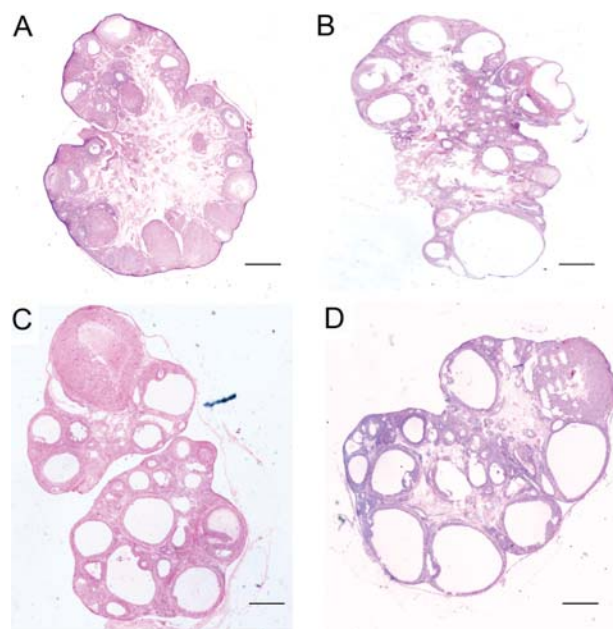

Figure 4 Survey views showing ovaries from control (A), DHEA (B), DHEA + 45% HFD (C) and DHEA + 60% HFD (D) rats (scale bar: 200 μm).

Table 4 Effects of DHEA, DHEA+45% HFD and DHEA+60% HFD on ovarian weight, ovarian area, and follicle development.

	Control (n=12)	DHEA (n=12)	DHEA+45% HFD (n=12)	DHEA+60% HFD (n=12)
Ovary weight (g)	0.19±0.02	0.14±0.01	0.23±0.02[†]	0.26±0.01[†]
Ovary area (mm²)	13.9±0.2	14.2±0.1	28.6±0.2[†]	31.9±1.2[‡]
Cystic follicles per section (n)	0.78±0.58	3.55±0.16*	5.58±0.56*	6.53±0.84*
Corpora lutea per section (n)	2.41±0.1	1.54±0.7*	1.42±0.5[†]	0.82±0.1[‡]
Thickness of granulosa layer (μm)	58.2±0.3	43.5±0.89*	35.4±0.5[†]	34.3±5.4[†]
Thickness of follicular wall (μm)	81.3±5.1	92.5±0.75	101.42±3.57[†]	119.82±6.75[†]

Values were means±S.E.M. *$P<0.01$ vs control group; [†]$P<0.05$ vs control and DHEA groups; [‡]$P<0.01$ vs control, DHEA and DHEA+45% HFD groups, one-way ANOVA followed by Tukey's *post hoc* tests. Follicular wall=theca interna and granulosa cell layer surrounding the antrum.

HFDs exaggerated MII oocyte abnormalities induced by DHEA treatment

To determine whether the spindle organization and chromosome alignment in metaphase II oocytes were affected by DHEA and HFD, we collected oocytes from each group after ovulation. Spindle morphology (green) and chromosome alignments (blue) were shown in Figure 6A, B, C and D. While the number of oocytes was not changed (Fig. 6E), DHEA treatment, with or without HFDs, significantly decreased the percentage of MII oocytes (Fig. 6F) but increased the rate of abnormal MII oocytes (Fig. 6G). Control rats displayed normal chromosome assembly and spindle configuration, including exclusive localization of chromosomes on the equatorial plate. In contrast, enlarged perivitelline space, fragmented cytoplasm or giant polar bodies were considered to be morphological abnormalities.

Cumulus expansion in the COC is necessary for oocyte maturation and following embryo development (Uyar *et al.* 2013). With DHEA and DHEA+HFD treatment, the cumulus cells were closely associated with oocytes and difficult to be removed, suggesting cumulus expansion dysfunction caused by DHEA+HFD treatment.

HFDs modified DHEA treatment-induced changes of ovarian and hypothalamic hormone receptor mRNA expression

DHEA injection induced a significant increase in androgen receptor (AR) ($F_{3,22}$=6.57, $P<0.01$) and LH receptor ($F_{3,25}$=14.01, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Fig. 7A and C) mRNA in the hypothalamus. Interestingly, both these increases were reversed by the addition of 60% HFDs. In contrast, the ovarian expression of LH receptor mRNA was not upregulated without the addition of HFDs ($F_{3,27}$=4.61, $P<0.05$, one-way ANOVA with Tukey's *post hoc* tests, Fig. 7F). DHEA and HFDs also upregulated the IR mRNA expression in the ovary but not in the hypothalamus ($F_{3,26}$=6.99, $P<0.01$, one-way ANOVA with Tukey's *post hoc* tests, Fig. 7B and E).
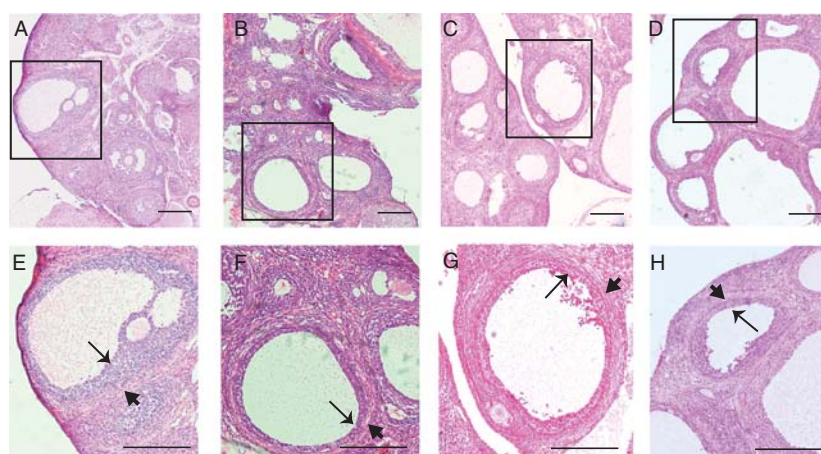

Figure 5 Histological sections of representative ovaries. Survey views showing rat ovaries of control (A), DHEA (B), DHEA+45% HFD (C) and DHEA+60% HFD (D) groups (scale bar: 200 μm). (E, F, G and H) were magnified photos of (A, B, C and D) (scale bar: 200 μm). (E) Ovaries from normal cycling 47-day-old rat showed CL and follicles at different stages. The theca (short arrow) and membrane granulosa layers (long arrow) appeared normal. (F) Ovaries of DHEA-treated rats showed many cyst follicles with few CL. (G) Ovaries of DHEA+45% HFD-exposed rats contained multiple cystic follicles, showing thinner granulosa layers (long arrow) with apoptotic bodies. Short arrows indicated a thicker follicular wall. (H) Ovaries of DHEA+60% HFD-exposed rats showed the largest number of cysts, with thinner granulosa layer (long arrows) with partially luteined and many apoptotic cells. Follicular walls in this group were the thickest among the four groups.
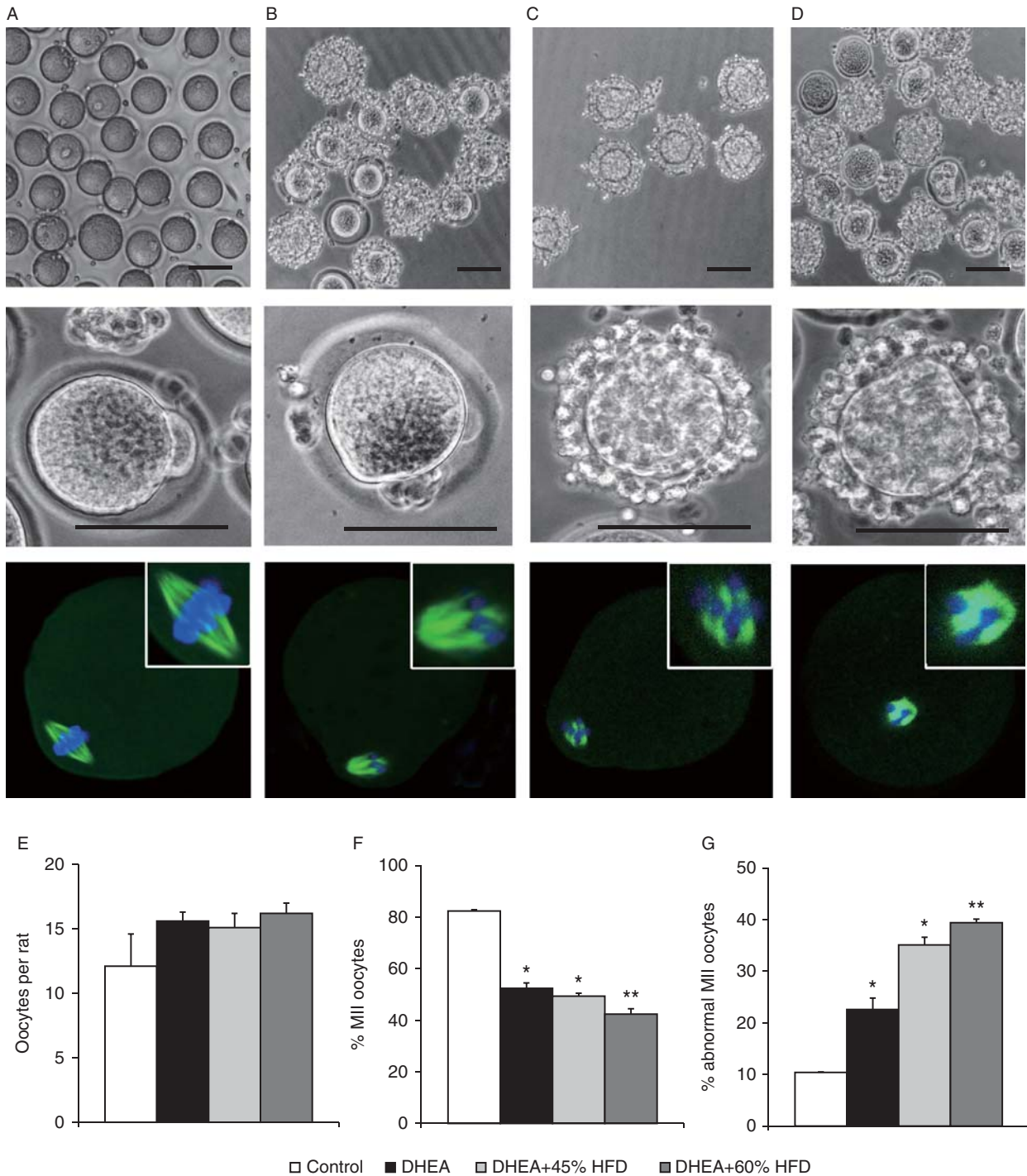
Figure 6 Ovulatory functions of control, DHEA, DHEA +45 and +60% HFD groups (*n*=10 each). First and second lines: microscopic images of oocytes removed from fallopian tube with magnifications of ×4 and ×10 (scale bar: 100 μm) respectively. Third line: under stereomicroscopy, confocal microscopy with double staining of α-tubulin (green) and Hoechst-tubulin (blue) to show spindle morphology and chromosome alignment. (A) In the control group, most of the oocytes were in the MII phase, with normal polar bodies, spindles and nuclei found in a larger magnification. (B) DHEA treatment significantly decreased the recovered oocyte number per rat, with two different stages of oocytes observed with increased rate of oocyte stayed at MI phase and decreased rate of normal MII oocytes. In DHEA+45% HFD (C) and DHEA+60% HFD (D) groups, more granulosa cells were closely associated with oocytes. (E) The number of oocytes was not affected by DHEA or HFDs, but DHEA treatment, with or without HFDs, significantly decreased the percentage of MII oocytes (F) and increased the rate of abnormal MII oocytes (G). *$P<0.01$ vs control group, **$P<0.01$ vs control and DHEA groups, one-way ANOVA followed by Tukey's *post hoc* tests.
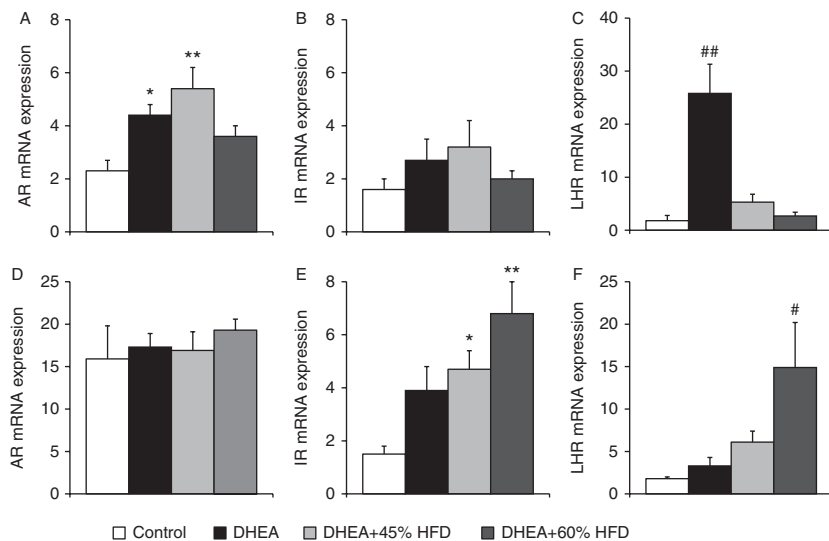
Figure 7 Hypothalamic and ovarian mRNA levels of hormone receptors. Hypothalamic mRNA expressions of AR (A), IR (B) and LH receptors (C) were modulated by DHEA and HFDs. Ovarian mRNA expression of AR (D), IR (E) and LH receptors (F) were modulated in a distinct pattern. $*P < 0.05$; $**P < 0.01$ vs control group; $^{\#}P < 0.05$ vs control, DHEA and DHEA + 45% HFD groups; $^{\#\#}P < 0.01$ vs control, DHEA + 45% HFD and DHEA + 60% HFD groups, one-way ANOVA followed by Tukey's *post hoc* tests.

These findings indicated that the addition of HFDs modified the mRNA expression patterns of DHEA treatment-induced changes of ovarian and hypothalamic hormone receptors, which could underlie the phenotypic differences revealed above.

Discussion

Obesity and metabolic syndrome are common phenotypes in PCOS women, and adiposity positively correlates with symptom severity (Al-Azemi *et al.* 2004, Azziz *et al.* 2004, Franks 2006). Here we aimed to explore the influence of HFDs on PCOS phenotypes in a rat model. Almost no rodent models display all clinical manifestations of PCOS (van Houten & Visser 2014). The DHEA model used in the present study had high stability, with disturbed cyclicity, hyperandrogenism and poly-cystic ovaries repeatedly demonstrated in different reports, including the present study (Roy *et al.* 1962, Ward *et al.* 1978, Lee *et al.* 1991, Anderson *et al.* 1992). Limited data, however, were present pertaining meta-bolic abnormalities to this model (Walters *et al.* 2012). One inevitable disadvantage of the DHEA model is the reversal of endocrine and reproductive abnormalities when DHEA injection is suspended (Walters *et al.* 2012). But in the present study, all experiments were performed during or immediately after DHEA injection, ensuring the reliability of our findings. In general, we showed that the addition of HFDs, especially 60% HFDs, exagger-ated a number of endocrine and metabolic dysfunction in addition to that caused by DHEA alone.

Previous studies have shown that normal rats fed with HFDs exhibit a bimodal pattern in body weight gain similar to that observed in humans (Omagari *et al.* 2008, Powell *et al.* 2014). But these studies apply long-term HFDs, usually for several months. In our pilot experi-ment, 20-day HFDs induced few metabolic changes in normal rats, similar to one previous study (Lai *et al.* 2014). However, the addition of HFDs in conjunction with DHEA induced significantly increased body weight, body fat content and glucose tolerance. Though IR has been reported in several PCOS models, e.g., the DHT model (Movérare-Skrtic *et al.* 2006, Manneras *et al.* 2007), the presence of body weight or IR changes in DHEA-treated rats is controversial (Sander *et al.* 2006, Walters *et al.* 2012). These phenotypical differences across models could result from strain variations (mice vs rats) or treatment duration (3 months vs 20 days). For example, DHEA injection induces glucose tolerance in BALB/c mice (Huang *et al.* 2015), but not in C57 mice (Lai *et al.* 2014). In the present study, a mild and insignificant increase of glucose level could be observed in the DHEA group at 30 min revealed by OGTTs, which was exaggerated by the addition of HFDs. Several other metabolic measures, including serum insulin, TG, LDL and HCY, also changed only in the presence of HFDs, indicating their additive effects on metabolism over DHEA.

Sex hormones and fat metabolism possess complex interactions (Diamanti-Kandarakis *et al.* 1995, Silfen *et al.* 2003). In DHEA-treated rats, ovarian cysts had a flattened epithelioid cell layer facing the antrum, which might reflect the transformation of outer granulosa cells during atresia. The polycystic appearance of ovaries was also exaggerated by HFDs. Exposure to DHEA + HFDs induced PCOS-like conditions with morphological differences from those of DHEA-treated rats, including much larger cysts, thinner granulosa and thicker theca cell layers, stimulated interstitial tissue and increased ovarian weight and size. Previous studies indicated that the thickened theca wall consisted of a vascularized layer of luteinzed and atretic granulosa cells (Manneras *et al.* 2007). These changes might reflect increased ovarian androgen synthesis (Lee *et al.* 1991). Hyper-insulinemia could be responsible for this change, as

insulin acted as a co-gonadotropin with LH (Poretsky *et al.* 1999). *In vitro* studies have shown that ovarian androgen production is stimulated by insulin through the IR (Nestler *et al.* 1998). Treatments targeting hyperinsulinemia decreased androgen levels and restored normal ovulation in women and adolescents with PCOS (Dunaif *et al.* 1996, Ehrmann 1999, Baillargeon *et al.* 2003). Thus, hyperinsulinemia might predispose to excess androgen production and exaggerate PCO phenotypes. Insulin also promoted the proliferation of theca cells, the secretion of LH and the number as well as affinity of LH receptors on granulosa cells. The high LH level would terminate the proliferation of granulosa cells and cause anovulatory infertility (Kuscu *et al.* 2006). These mechanisms were consistent with findings in the present study: serum insulin and LH levels, as well as ovarian LH and IR levels, were significantly higher in the DHEA + 60% HFD group.

Increased gonadotrophin-releasing hormone (GnRH) pulse frequency, increased LH pulsatility and relative FSH deficiency have been consistently reported in PCOS (Berger *et al.* 1975, Balen *et al.* 1995, Laven *et al.* 2002, Hsu *et al.* 2009, Burt Solorzano *et al.* 2012). Normal slowing of GnRH and LH pulse frequency resulted from feedback inhibition by increased progesterone levels during the luteal phase, which could be impaired by hyperandrogenism (Daniels & Berga 1997, Pielecka *et al.* 2006). This would cause LH over-production over FSH, which in turn promoted theca cell production of androgens, interfered with granulosa cell aromatization to estrogen and impaired follicle maturation and ovulation (Burt Solorzano *et al.* 2012). This vicious cycle of androgen over-production pushed forward the pathogenesis of PCOS. Androgens have diverse roles in reproduction and affect brain development (Gao *et al.* 2005, Foecking *et al.* 2008). Previous studies have shown that long-term androgen treatment upregulated AR mRNA expression in the hypothalamus and further affected the activities of the hypothalamus–pituitary–ovarian axis (Feng *et al.* 2010). This result was replicated in the present study. LH receptors expressed in the hypothalamus were upregulated by DHEA stimulation, but were differentially modulated by HFDs. The increased ovarian expression could result from hyperinsulinemia (Akamine *et al.* 2010) and correlate with morphological changes around this area in these rats, whereas the decreased hypothalamic expression might represent impaired negative feedback processes. The hyperinsulinism and LH stimulated the proliferation of ovarian stromal cells and resulted in the significant cumulus expansion disorder. DHEA and DHEA + HFDs affected the chromosome configuration of superovulated oocytes and the normal-to-abnormal spindle ratio, which could be one reason of ovulation failure. Assembly, rotation and elongation of meiotic spindles are crucial for the correct separation of chromosomes,

which guarantees the stability of genomes during reproduction (Sun & Schatten 2006).

Thus, DHEA and DHEA + HFD treatment might influence PCOS phenotypes through distinct mechanism. The former primarily affects the normal function of the hypothalamus–pituitary–ovarian axis, resulting in increased serum androgen and LH levels. The addition of HFDs induced significant IR as well as morphological and LH and IR expression changes in the ovary, which were consistent with the PCOS subtypes in humans (Dale *et al.* 1992, Grulet *et al.* 1993, Silfen *et al.* 2003). Hyperinsulinemia may aggravate PCOS phenotypes through abnormal GnRH function, excessive LH secretion and abnormal ovarian androgen production (Nestler *et al.* 1998, Poretsky *et al.* 1999, Gamba & Pralong 2006, Baptiste *et al.* 2010, Pralong 2010). More recent studies have revealed that HFDs affect normal hypothalamic functions through insulin signaling pathways (Oh *et al.* 2013). The complex interactions might also raise the possibility that PCOS could be differentiated into sub-categories characterized by endocrine or metabolic phenotypes.

Conclusions

In summary, treatment of female rats with DHEA induced reproductive disorders similar to human PCOS. The addition of HFDs further brought about metabolic dysfunction in these rats. The DHEA + HFD treatment protocol thus represented a rodent model more similar to clinical situations and could be used to investigate the pathogenesis and mechanisms of PCOS, as well as the interactions between lipid metabolism and hyperandrogenism.

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.

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Author contribution statement

H Zhang, M Yi, Y Zhang, H Jin, W Zhang and J Yang performed the experiment; H Zhang, M Yi, L Yan, R Li, Y Zhang and J Qiao designed the experiment; and M Yi and H Zhang wrote the manuscript. All authors modified and approved the manuscript.

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