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Vitamin D has therapeutic effects on obesity and hyperandrogenemia in PCOS mouse model induced by low dose DHEA and high-fat diet

Huiling Xu[1†], Shumin Qiu[1†], Peiyang Lin[1], Xiuhua Liao[1], Yunhong Lin[1], Yan Sun[1,2*] and Beihong Zheng[1,3*]

Abstract

Polycystic ovary syndrome (PCOS) is the most complex and common reproductive endocrine disease among reproductive age women. This study aimed to investigate the effects of vitamin D (Vit.D) in a PCOS mouse model induced by low dose DHEA and high-fat diet. Prepubertal female mice were divided into 4 groups randomly: control, PCOS, PCOS with low dose Vit.D(LDVD), and PCOS with high dose Vit.D(HDVD) groups ($n=10$ per group). PCOS mice were administrated with high-fat diet and subcutaneous injection with 6 mg/kg/day dehydroepiandrosterone throughout the study. After the first 30 days, 1,25(OH)2D3 was intend to be administered by intraperitoneal injection for 40 consecutive days, 1.3 μg/kg/week in LDVD group, and 13 μg/kg /week in HDVD group. However, the mice in HDVD group appeared to be fatigue and anorexic after the Vit.D injections, then all died within two weeks. The body weights and testosterone levels in PCOS group were significantly higher than those in the control and LDVD groups ($P<0.001$). The total cholesterol levels in the control group were lower than those in PCOS and LDVD groups ($P<0.001$). Further, the ratio of liver to body weight was different among groups ($P<0.001$). Our data illustrates that Vit.D has therapeutic effects on obesity and hyperandrogenemia in PCOS mouse model induced by low dose DHEA and high-fat diet. However, over dose of Vit.D is toxic. Further researches are needed to elucidate the mechanisms.

Keywords Vitamin D, Polycystic ovary syndrome, Obesity, Hyperandrogenemia

Introduction

Polycystic ovary syndrome (PCOS) is the most complex and common reproductive endocrine disease among reproductive age women [1, 2]. The major endocrine features of PCOS are hyperandrogenemia and insulin resistance, they establish a vicious cycle that stimulates each other, and obesity is a common complication in women with PCOS [3]. Multiple factors of heritability and environment conjointly participate in the pathomechanism of PCOS; however, the underlying mechanisms remain to be understood [4–7]. A well-founded hypothesis is that excess androgen induces insulin resistance and

†Huiling Xu and Shumin Qiu contributed equally to this work.

*Correspondence:
Yan Sun
sunyanteam@163.com
Beihong Zheng
zhengbeihong2010@163.com
[1]Center of Reproductive Medicine, Fujian Maternity and Child Health Hospital College of Clinical Medicine for Obstetrics and Gynecology and Pediatrics, Fujian Medical University, No.18 Daoshan Road, Fuzhou, China
[2]Fujian Maternal-Fetal Clinical Medicine Research Center, Fuzhou, China
[3]Fujian Key Laboratory of Prenatal Diagnosis and Birth Defect, Fuzhou, China

compensatory hyperinsulinemia, which promote visceral adiposity and abdominal adipose tissue deposition, and hyperinsulinemia further facilitates androgen secretion from the adrenal glands and ovaries, resulting in PCOS [1]. Therefore, obesity and hyperandrogenemia are prevalent in women with PCOS. Of note, obesity is also an independent risk factor for infertility [8–10].

Vitamin D (Vit.D) concentrations are significantly lower in PCOS patients than controls, and this is associated with abnormal androgenic and calcium status [11]. Vit.D deficiency has been speculated to induce disrupted ovarian maturation [12]. Calcium signals play essential roles in oocyte activation and maturation [13, 14]. Meanwhile, Vit.D and calcium therapy normalize the menstrual cycles in PCOS [15]. Further, studies have shown the therapeutic effects of Vit.D (ranging from 1000 IU/d to 60,000 IU/weekly) on hormone imbalances and metabolic disorder in women with PCOS, such as hyperandrogenism, hyperlipemia and insulin resistance, and high dose of Vit.D (4000 IU/d) was recommended [16–18]. These findings support the hypothesis that Vit.D deficiency may contribute to the development of PCOS, and Vit.D may be a feasible treatment for PCOS.

However, multiple studies have demonstrated that long-term administration of a high dose Vit.D did not have any significant benefits for women with PCOS [19–22]. There is still controversy in the effect of Vit.D administration in PCOS. The objective of this study was to investigate the effects of Vit.D administration in PCOS mouse model induced by low dose dehydroepiandrosterone (DHEA) and high-fat diet.

Materials and methods

Ethics statements

This study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (approval number: 2018−232). All experiments were performed in accordance with relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines.

Animals and experimental protocols

Female mice at 21 days of age were obtained from Laboratory Animal Center of Fujian Medical University, and maintained in a 12-hour light cycle, temperature and humidity controlled, environment with access to water and food ad libitum. The mice were divided into 4 groups randomly: control, PCOS, PCOS with low dose Vit.D(LDVD), and PCOS with high dose Vit.D(HDVD) groups ($n=10$ per group). PCOS mice were administrated with high-fat diet and subcutaneous injection with 6 mg/kg/day DHEA throughout the study. The high-fat diet, which comprised 60% fat, 14.1% protein, and 25.9% carbohydrate, with an energy density of 5 kcal/g, was obtained from TROPHIC Animal Feed High-Tech Co. Ltd, China(TP23400). Controls fed a normal diet, and were injected with placebo(oil used as the solvent for DHEA and vitamin D). After the first 30 days, 1,25(OH)2D3 was intend to be administered by intraperitoneal injection for 40 consecutive days, 1.3 μg/kg/week in LDVD group, and 13 μg/kg /week in HDVD group. The mice in the control and PCOS groups were given intraperitoneal injections of placebo. The mice were weighed every couple of days.

Assessment of estrous cycle

The estrous cycle was detected by observation of vaginal epithelial cell smears under light microscope during the last 14 consecutive days of the study. The smears were fixed and stained with Wright-Giemsa Stain (Baso, Zhuhai, China). The stages of the estrous cycle were identified according to the presence or absence of leukocytes, nucleated epithelial, and cornified epithelial cells. Proestrus stage is characterized by predominant nucleated epithelial cells, estrus stage is indicated by the presence of mostly cornified squamous epithelial cells, metaestrus stage is indicated by both cornified epithelial cells and leukocytes, and diestrus stage is indicated by primarily leukocytes.

Sacrifice and specimen collection

At the end of the study, all of the mice were sacrificed after anesthesia through intraperitoneal injection with 3% pentobarbital sodium (30 mg/kg). Blood samples were reserved by cardiac exsanguination under anesthesia, and the ovaries and livers were taken and weighed at the end of the study. Serum was obtained for analysis of 25(OH)D, testosterone, cholesterol, triglyceride, and glucose. Ovaries were fixed with 4% paraformaldehyde, embedded in paraffin wax and sectioned at 5 μm. Then, the sections were stained with hematoxylin and eosin (H&E) and examined with microscope (Olympus, Tokyo, Japan).

Statistical analysis

Analysis of variance (ANOVA) was used for comparison among groups, with post hoc test using Fisher's LSD Multiple-Comparison Test. SPSS 19.0(IBM, Armonk, NY, USA) was used for statistical analyses. Figures were generated by Graph Pad Prism 8 for Windows (GraphPad, San Diego, CA, USA). $P<0.05$ was considered to be statistically significant.

Results

Estrous cycle and ovaries

All mice in the control group had a normal estrous cyclicity. Most of the mice in PCOS and LDVD groups had estrous cycles, whereas exhibited an abnormal pattern (Fig. 1A). The mice that were cycling had a prolonged estrous or diestrus stage duration in the PCOS

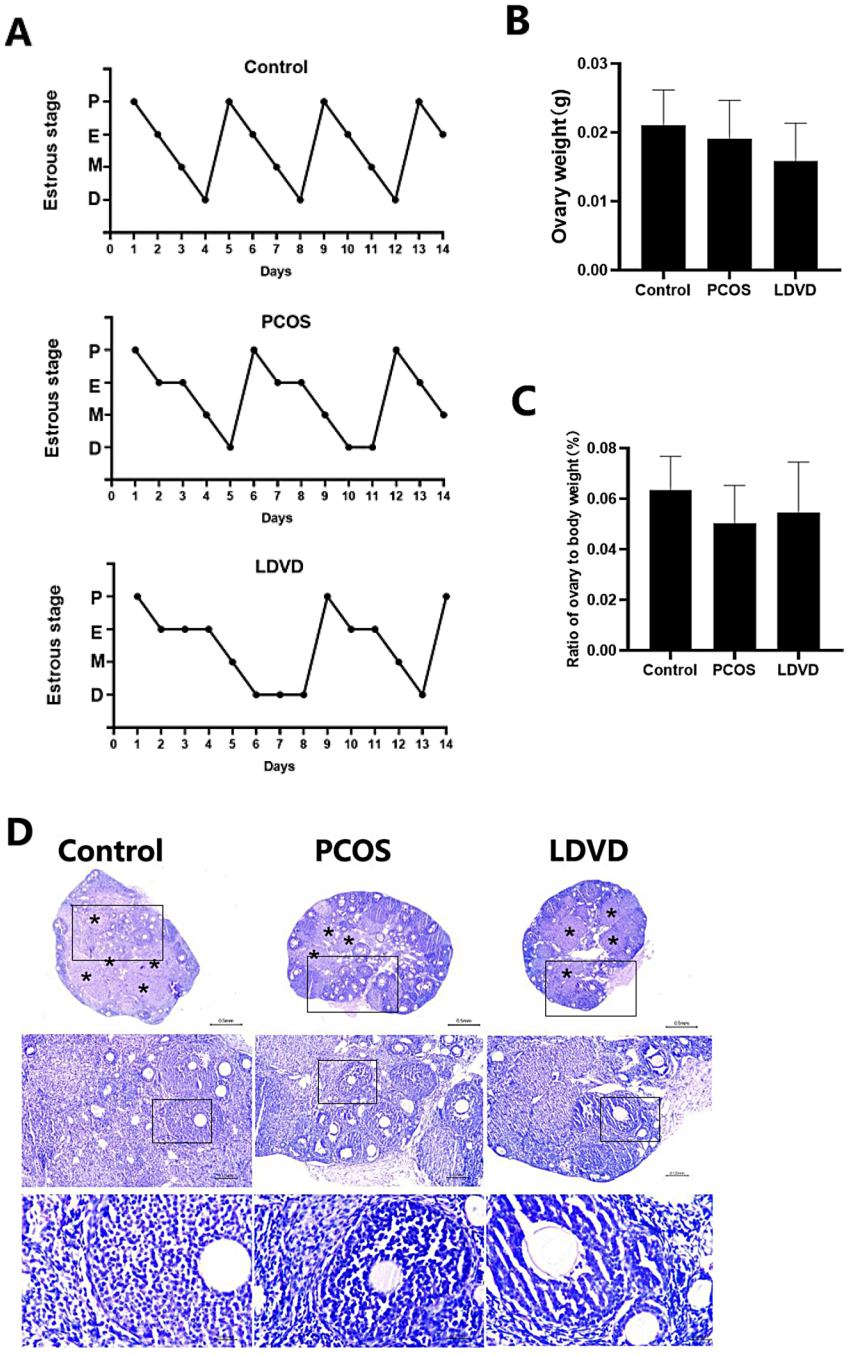

Fig. 1 Estrous cycle and ovaries of different groups of mice. (**A**) Representative estrous cycle pattern from each group. P, proestrus; E, estrus; M, metestrus; D, diestrus. (**B**) Ovary weight. (**C**) Ratio of ovary to body weight. (**D**) Representative H&E staining of ovarian sections. Micrographs were taken at magnifications ×25, ×100, and ×400, and bars = 500, 125, and 31.3 μm, respectively. The boxed areas are shown at higher magnifications. Black asterisk, corpora lutea

and LDVD groups compared with the control group. Ovary weight and the ratio of ovary to body weight did not differ among the groups (Fig. 1B and C). Representative micrographs of ovarian sections are shown in Fig. 1D. Healthy follicles at various developmental stages and corpora lutea were seen in the histomorphological inspection of ovaries in the control group. Corpora lutea and follicles at various developmental stages were also seen in the PCOS and LDVD groups. No significant difference was observed in theca cell layer or granulosa cell layer thickness among the three groups. The ovaries in PCOS group featured more follicles than in the control and LDVD groups, but no typical cystic follicles were observed.

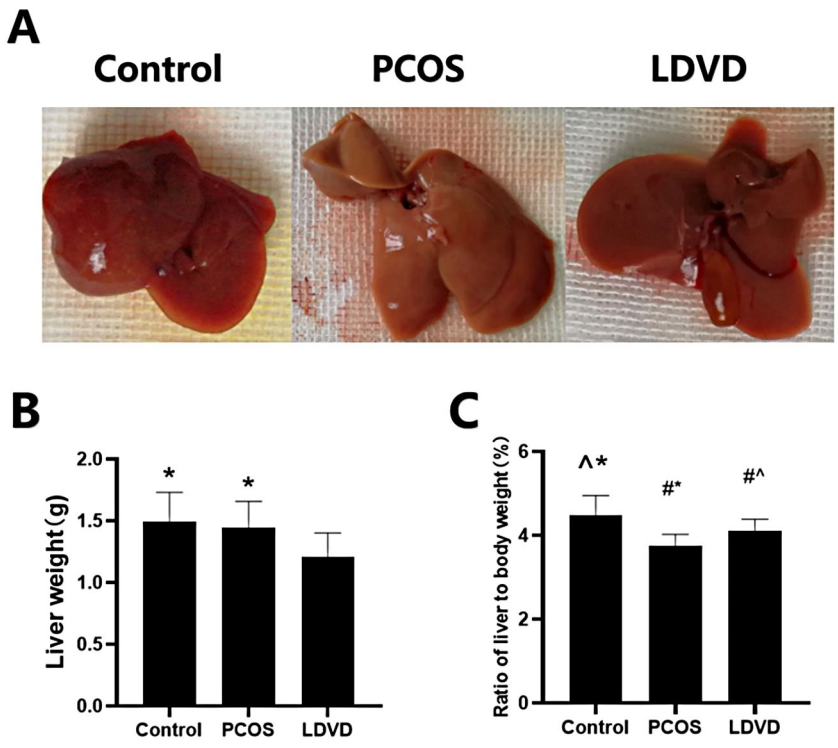

Fig. 2 Livers of different groups of mice. (**A**) Gross morphology. (**B**) Liver weight. (**C**) Ratio of liver to body weight. # $P<0.05$ vs. control group; ^ $P<0.05$ vs. PCOS group. * $P<0.05$ vs. LDVD group

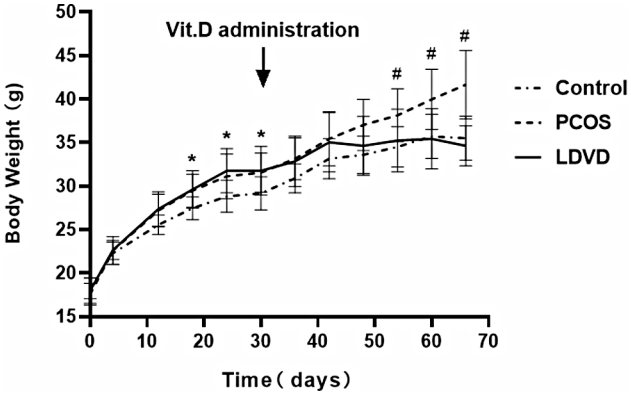

Fig. 3 Body weight of different groups of mice. * $P<0.05$ vs. control; # $P<0.05$ vs. PCOS

Livers

Compared with livers of mice in the control group, the livers in PCOS group appeared yellow and greasy, and the livers in LDVD group had an appearance that was between the two conditions (Fig. 2A). Moreover, the liver weight in LDVD group was lower than those of the control and PCOS groups significantly (1.21±0.19 VS 1.49±0.24, 1.44±0.21, LDVD VS control, PCOS, $P<0.05$) (Fig. 2B). Furthermore, the ratio of liver to body weight was different among the three groups (0.045±0.0046 VS 0.038±0.0027 VS 0.041±0.0027, control VS PCOS VS LDVD, $P<0.001$) (Fig. 2C).

Body weight

The initial body weight of the mice was similar among groups. Before Vit.D administration, the weight of mice in the control group was significantly lower than those in the other groups which were all PCOS mice. The mice in HDVD group appeared to be fatigue and anorexic after the Vit.D injections, then all died within two weeks. The body weight of mice in PCOS group was higher than those in the control and LDVD groups significantly at the end of the study (41.66±3.94 VS 35.50±2.51, 34.64±2.33 g, $P=0.000$) (Fig. 3; Table 1).

Serum biochemical analysis

The serum levels of 25(OH)D in LDVD group were significantly higher than those in the control and PCOS groups (93.80±14.83 VS 19.55±4.10,16.10±1.16 ng/ml, $P<0.001$). The testosterone levels in PCOS group were significantly higher than those in the control and LDVD groups (1.30±0.27 VS 0.93±0.16,0.89±0.18 ng/ml, $P<0.001$). The total cholesterol levels were lower in the control group than those in PCOS and LDVD groups (3.06±0.34 VS 4.43±0.33, 4.39±0.59 mmol/L, $P<0.001$). However, serum levels of triglyceride and glucose were not different among groups (Table 1).

Table 1 Measured values in different groups of mice

	Control	PCOS	LDVD	*P*
Body weight before study (g)	18.24 ± 1.19	17.57 ± 1.25	18.01 ± 1.48	0.520
Body weight before Vit.D administration (g)	29.17 ± 1.91*	31.55 ± 2.23	31.76 ± 2.78	0.022
Body weight at the end(g)	35.50 ± 2.51	41.66 ± 3.94***	34.64 ± 2.33	0.000
25(OH)D (ng/ml)	19.55 ± 4.10	16.10 ± 1.16	93.80 ± 14.83***	0.000
Testosterone (ng/ml)	0.93 ± 0.16	1.30 ± 0.27***	0.89 ± 0.18	0.000
Cholesterol (mmol/L)	3.06 ± 0.34***	4.43 ± 0.33	4.39 ± 0.59	0.000
Triglyceride(mmol/L)	2.18 ± 0.46	2.13 ± 0.52	2.21 ± 0.39	0.929
Glucose(mmol/L)	9.32 ± 2.58	8.72 ± 2.26	9.25 ± 1.63	0.799

Data are presented as mean ± standard deviation (SD)

* $P<0.05$ vs. the other two groups, *** $P<0.001$ vs. the other two groups

Discussion

PCOS is the most common cause of anovulatory infertility and the most common reproductive endocrine disease in women of reproductive age. Rodent models have been used as a versatile and valuable tool to investigate PCOS. Among them, far fewer models have been developed in mice than rats. In our study, we select mice to investigate the effects of Vit.D administration in PCOS because mice are more sensitive to certain effects.

Caldwell et al. compared the reproductive, endocrine, and metabolic traits comprehensively in PCOS mice models induced by hyperandrogenism, including prenatal dihydrotestosterone (DHT) treatment, or prepubertal long-term treatment with DHT, DHEA, or letrozole, and found that DHEA did not generate PCOS features in mice [23]. A high-fat diet led to an increase in anovulation and a decrease in fertilization rates in mice [10]. Lai et al.. found that a combination of DHEA and high-fat diet caused both reproductive and metabolic features of PCOS [24]. Poojary et al.. also confirmed that DHEA administration combined with high-fat diet feeding replicates PCOS conditions in mice more effectively than using DHEA alone or letrozole (with or without high-fat diet) [25]. Even though it was believed to be a more reliable PCOS model, the administration of DHEA resulted in a more than ten times increase in serum testosterone level compared to the control group [25]. Different from the very high serum testosterone levels in other studies, in the present study, with one tenth of their DHEA dose, the serum testosterone levels in PCOS mice were close to the physiological levels in patients with PCOS, which make our PCOS mouse model more reasonable. This may be a perfect explanation why the ovaries of mice in our PCOS group exhibited more follicles than control, which is similar to ovarian pathology in patients with PCOS, instead of cystic follicles reported in previous PCOS mouse model [23, 24]. Lai et al. found that high-fat diet induced fat accumulation, DHEA treatment alone downregulated fat mass, and when they combined, the fat to body weight ratio was similar to controls [24]. In our study, the combination of DHEA and high-fat diet was administered for 70 days, much longer than their 20 days, we found that low dose DHEA and high-fat diet led to pronounced obesity (17.3% higher body weight than the controls). This study is the first to develop a PCOS mouse model with combination of low dose DHEA and high-fat diet, and investigate long term effects of Vit.D administration in this model.

Obesity is common in women with PCOS. Furthermore, it has been an increasingly prevalent health problem worldwide. It is well-known that fertility is reduced in obese women. One study demonstrated that after bariatric surgery, 58% of infertile women became pregnant spontaneously [8]. More than one meta-analysis found that remarkable weight loss restores ovulation and fertility, thus bariatric surgery is recommended for obesity-related infertility patients who have failed to lose weight through behavioral and nutritional treatment [8, 9]. However, bariatric surgery is scary for most people, and comes with surgical risks and the possibility of complications, such as peritonitis due to anastomotic fistula formation, and post-operative malnutrition [26]. Meanwhile, it is extremely difficult to lose adequate weight by exercise and dietary modification for some people. Thus, it is meaningful to find conservative treatments for obesity.

It is now widely accepted that Vit.D deficiency is associated with obesity, not only in women with PCOS [27–29], but in adults with obesity [30–33]. Nevertheless, the weight and body mass index(BMI) of the patients with PCOS did not differ after taking Vit.D 3200 IU/d for 3-month [19]. Vit.D supplementation of 2000 IU/d for 12 months during weight loss did not increase weight loss in postmenopausal women [34, 35]. In a randomized controlled trial, healthy overweight and obese women took 1000 IU/d Vit.D for 12 weeks, although body weight did not change significantly, the body fat mass showed a significant decrease [36]. A systematic review and meta-analysis of randomized controlled trials demonstrate that Vit.D supplementation in adults with metabolic syndrome did not affect waist circumference, BMI and body fat percentage, but decreased waist-to-hip

ratio [37]. In the present study, we found that the body weight of mice in PCOS group was 17.3% higher than that in the control group(41.66±3.94 VS 35.50±2.51 g), whereas Vit.D administration reversed this increase completely(34.64±2.33 g). Our results provide evidence to support the notion that Vit.D administration may be a novel approach for inducing non-surgical weight loss.

Hyperandrogenism is the most consistent characteristic of PCOS, which could induce other characteristic such as insulin resistance, anovulation and polycystic ovaries. Previous investigations showed that there is no significant correlation between 25(OH)D and testosterone in reproductive age women with oligomenorrhea or PCOS [38, 39]. Irani et al. found Vit.D did not alter the free testosterone level in PCOS with Vit.D deficiency [40]. However, a meta-analysis of clinical trials revealed that Vit.D reduces total testosterone significantly in patients with PCOS [41]. In this study, we also found that Vit.D administration could eliminate the hyperandrogenemia completely. The testosterone levels in PCOS group were significantly higher than those in the control and LDVD groups (1.30±0.27 VS 0.93±0.16,0.89±0.18 ng/ml, $P<0.001$).

Consistent with the results of Lai et al., we found that a combined treatment of DHEA and high-fat diet induced apparent hepatic steatosis, and elevated serum cholesterol levels markedly, but did not affect triglyceride and fasting glucose levels in the same way [24]. Silvia et al.. found that Vit.D deficiency was observed in most liver diseases, and Vit.D showed therapeutic potential in these liver diseases [42]. Vit.D deficiency was speculated to induce disrupted autophagy malfunction in the liver [12]. Bozic et al.. demonstrated that Vit.D receptor expression was up-regulated in nonalcoholic fatty liver disease hepatocytes and was essential for high-fat diet induced hepatic steatosis [43]. Vit.D supplementation could attenuate the hepatic steatosis induced by fructose-rich diet or high-fat diet [44, 45]. Li et al.. found that Vit.D attenuated hepatic damage and steatosis by inducing autophagy in high-fat diet mice [46]. Ning et al.. found that Vit.D diminished hepatic damage, reduced both liver weight and the ratio of liver to body weight in diabetic rats [47]. Yin et al.. also demonstrated that Vit.D administration led to a significant reduction in both liver weight and the ratio of liver to body weight, mitigated liver injury, and attenuated hepatic steatosis in high-fat diet rats in a dose-dependent manner [45]. In the present study, there was an apparent hepatic lipid accumulation in the mice of PCOS group, and a mild increase in the mice of LDVD group, as revealed by the appearance of the livers. In contrast with other studies, liver weight of the mice in our PCOS group did not increase, and the ratio of liver to body weight decreased notably, which may be due to hyperandrogenemia. Impressively, Vit.D attenuated the reduction of ratio of liver to body weight, revealing that Vit.D has protective effect on the liver, which is consistent with other studies.

However, the mice in HDVD group appeared to be fatigue and anorexic after the Vit.D injections, then all died within two weeks. It revealed that over dose of Vit.D is toxic. Meanwhile, we couldn't exclude that the mice in LDVD group may also be exposed to a toxic but not lethal dose of Vit.D. A systematic review demonstrated that Vit.D mega-dose therapy is effective in normalizing serum vitamin levels in human, and the toxicity assessed through adverse effects was low, with no expressive clinical significance [48]. In the 1940s, massive doses of Vit.D (200,000–300,000IU/day) were considered an effective treatment strategy for chronic illnesses as diverse as tuberculosis and rheumatoid arthritis. In the 1950s, several cases of infants with facial abnormalities, supravalvular aortic stenosis, mental retardation, and hypercalcemia were reported mainly in the United Kingdom. Our study suggested that although Vit.D has therapeutic effects on obesity and hyperandrogenemia in PCOS mouse model, the appropriate dosage needs to be carefully considered. The IOM-recommended maximum daily intake dosage is 4,000 IU, and the recommended daily intake dosage in our country is 400 IU. Our original assumption was to simulate 400/4000 IU/d Vit.D in humans, using the body weight proportionality, in the more sensitive mouse model. Unexpectedly, we found that the serum 25(OH)D level was 93.8 ng/mL in LDVD group, which was much higher than the 13.6–36.2 ng/mL detected in humans supplementing Vit.D [19, 34–36]. Serum 25(OH)D concentrations > 150 ng/ml (> 375 nmol/L) would likely result in acute toxicity [49]. The features of Vit.D toxicity are mediated through hypercalcemia, and symptoms range from thirst and polyuria, to seizures, coma and death [50]. Kyeri et al.. administrated Vit.D by intraperitoneal injection in mice as in our study, with a dosage of 1 mg/kg/d, much higher than 1.3 and 13 μg/kg/week used in our study, and they did not report any toxic effects [51].

There are several limitations that we should acknowledge in the present study. First, we did not evaluate anti-Mullerian hormone and indicators of insulin resistance because of the small serum volume of mice, which may be the reason why previous studies did not choose mice as their optimal experiment animal. Further, it would be better to count the corpora lutea and follicles in the whole ovaries, examine hepatic histology and analyze liver composition. Additionally, the underlying mechanisms of the findings highlighted in this study require further investigation. However, these experiments cannot be done until we find a suitable dosage of Vit.D. Finally, we only performed animal studies; however, animal models cannot completely represent a complex human

disease. Thus, it is important to perform clinical studies to verify the therapeutic effect of Vit.D in patients with PCOS.

Conclusion

Our data illustrate that Vit.D has therapeutic effects on obesity and hyperandrogenemia in PCOS mouse model induced by low dose DHEA and high-fat diet. However, over dose of Vit.D is toxic. Further researches are needed to elucidate the mechanisms, figure out the optimal dosage of Vit.D, which is effective but not toxic, and perform clinical studies to verify the therapeutic effect of Vit.D in patients with PCOS.

Abbreviations

PCOS	Polycystic ovary syndrome
Vit.D	Vitamin D
LDVD	PCOS with low dose Vit.D
HDVD	PCOS with high dose Vit.D
DHEA	Dehydroepiandrosterone

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Author contributions

HX and SQ conceived and planned the study, collected and analyzed the data, and were major contributors in writing the manuscript. PL, XL, YL participated in the animal keeping and handling，and analyzed the serum samples. BZ and YS supervised the study and participated in writing the manuscript. All authors reviewed the manuscript.

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Data availability

Data sets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (approval number: 2018－232). All experiments were performed in accordance with relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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