



# Relationship of Estrogen Intestinal Microbiological Axis of Estrogen with Metabolic Defects in SD Rat Model of Polycystic Ovary Syndrome Induced by DHEA

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## ABSTRACT

The objective was to study the relationship between the intestinal microbiological axis of estrogen and metabolic defects in SD rat model of polycystic ovary syndrome (PCOS) induced by DHEA, and to explore the possible mechanism. Forty rats were randomly divided into four groups, the control group, the DHEA group (low dose, 15 µg/kg/d) and the DHEA group (high dose, 30 µg/kg/d). DHEA subcutaneous injection was used to construct the PCOS model of the polycystic ovary syndrome (PCOS) model of rats. The general condition of rats (body weight, ovary weight) and ovarian tissue pathology changes were observed. The changes in content of testosterone (T), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were detected. The fasting blood glucose, serum insulin and insulin sensitive index changes were analyzed. The RT-PCR method was used to detect ovarian tissue TNF-α and the level of IL-6 gene expression; Western blot was used to detect the changes in the expression levels of IKKβ and NF-κB protein expression. The results of fasting blood glucose and serum insulin test showed that DHEA could significantly reduce fasting blood glucose and insulin levels and improve insulin sensitivity index, and the effect was more obvious in the high-dose group. The above results indicated that DHEA could regulate the intestinal microbial level of rats, and further regulate the sugar metabolism process of rats by increasing the serum estrogen level through the estrogen-microbial axis. RT-PCR and Western Blot analysis showed that the expression of TNF-α, IL-6, IKKβ, NF-κB increased significantly after the establishment of the model; after the administration of DHEA, the levels of TNF-α, IL-6, IKKβ, NF-κB were down-regulated, and the difference was statistically significant compared with the model group ( $P < 0.05$ ). It is concluded that DHEA could regulate estrogen levels in rats through the estrogen-intestinal microbiological axis, thereby improving insulin resistance and reducing blood glucose levels. The mechanism of DHEA may be related to the inhibition of the expression of inflammatory factors and the inhibition of NF-κB nuclear transposition through the IKK/NF-κB signaling pathway.

## Article Information

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## Authors' Contribution

JP grouped SD rats. SH measured the changes of serum testosterone, follicle stimulating hormone and luteinizing hormone. QW analyzed the changes of fasting blood glucose, serum insulin and insulin sensitivity index. All authors conducted the experiments, analysed the results and wrote the manuscript.

## Key words

Polycystic ovary syndrome, Insulin resistance, Intestinal microbial axis, Metabolic defects

## INTRODUCTION

Estrogen, as a very important sex hormone in vertebrates, participates in the regulation of body system function, and plays an important role in regulating diabetes, obesity, cardiovascular diseases and osteoporosis of postmenopausal women (Smith *et al.*, 2010; Yue *et al.*, 2020;

Chang, 2019). Insulin resistance syndrome (IRS), as a typical abnormal pathological phenomenon caused by estrogen metabolism disorder, has aroused widespread concern in recent years (Iwanishi *et al.*, 2019; Adnan *et al.*, 2019; Wang *et al.*, 2020). Studies have shown that intestinal microbes can regulate the level of estrogen and their metabolites in organisms through the estrogen-microbial axis, and then have a better regulation on IRS and sugar metabolism (Zetterberg and Celojovic, 2015; Ohlsson *et al.*, 2014). DHEAS (dehydroepiandrosterone) is a steroid hormone with the highest concentration

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secreted by adrenal gland. It has weak androgen activity and is a prerequisite for the synthesis of adrenal steroid hormones such as testosterone and estrogen. DHEAS is closely related to aging, tumor, insulin resistance, obesity and coronary heart disease (Holton *et al.*, 2020; Peixoto *et al.*, 2020). At present, it has not been reported whether DHEA can participate in the pathogenesis of IRS through the intensive microbiological axis of estrogen, and its mechanism is unclear. In view of this, this experiment studied the effect of DHEA on diabetes resistance and glucose metabolism through estrogen microbial axis and discussed its possible mechanism.

## MATERIALS AND METHODS

### *Experimental animal*

Forty healthy 20 day old SPF SD female rats, weighing 60g-70g, were purchased from Chengdu Dashuo Institute of Biology and fed alternately at room temperature and in light and dark (12h:12h). They were fed adaptively for 3 days before the experiment and fed freely with water.

### *Establishment of model of polycystic ovary syndrome in rats and experimental grouping*

The model of polycystic ovary syndrome (PCOS) of SD rats was established by DHEA injection. Forty female rats were randomly divided into four groups, each of 10 rats: control group, model group, DHEA group (low dose, 15 $\mu$ g/kg/d) and DHEA group (high dose, 30 $\mu$ g/kg/d). The model group and the administration group were injected with DHEA solution subcutaneously in the neck every morning (DHEA was dissolved with tea oil for injection at a concentration of 30mg/ml with an injection dose of 0.2 mg/100 g/d), while the control group was injected with the same amount of tea oil subcutaneously for 20 days. After the model was successfully established, the administration group was given intragastric administration according to the predetermined dose, and the control group and the model group were given the same dose of saline every day (2:00-3:00 every afternoon) for 4 weeks. Before the experiment, the rats in each group were in good mental state, with shiny hair and flexible movement. After the experiment, the control group was as usual. The rats in the model group were depressed, puffy, with dry hair and slow movement, which was significantly different from the control group rats. Compared with model group, the mental state of rats after DHEA administration had been improved to a certain extent, with smoother hair and more flexible movement, and the situation of rats in high dose administration group was better. The general situation of rats indicated that DHEA induced PCOS model in SD rats

was initially established successfully. After HE staining, it could be seen that the ovary of control group rats was bright red, and follicles at all levels (primary follicles, primary follicles, secondary follicles, etc.) existed during development. Besides, there were few atresia follicles; after PCOS modeling, the follicles of model group rats showed polycystic dilatation. The number of atresia follicles and preantral follicles increased, and the number of corpus luteum decreased. The ovarian capsule thickened and the ovarian stroma proliferated; after DHEA administration, the polycystic dilatation and interstitial hyperplasia of follicles were improved to some extent, and the number of developing follicles at all levels increased.

### *Determination of general condition and ovarian weight of rats*

During the experiment, the hair color, spirit and activity of rats were observed. After administration, the rats were weighed and the changes of their body weight were observed. The rats were killed by decapitation. After that, the ovarian tissues were separated, and then the weight of the ovaries was measured three times to get the average value.

### *Determination of testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH) in rats*

After 4% chloral hydrate was used to anesthetize rats in abdominal cavity, the abdominal aorta blood was collected, centrifuged at 3000rpm for 10min, and the serum was stored at -20 $^{\circ}$ C. Serum T, FSH and LH were detected by ELISA.

### *Determination of fasting blood glucose(FBG), insulin and insulin sensitivity index (ISI) in rats*

After fasting for 12 h, rat tail venous blood was collected, and FBG was measured by Roche blood glucose meter using glucose oxidase method. Blood was taken from rat tail vein, and centrifuged for 10min at 3000 r/min. The supernatant was taken for measurement of insulin content in rat serum by radioimmunoassay and ISI was calculated according to the instructions of insulin kit.

### *Histological analysis of rat ovary*

After decapitation of rats anaesthetized with 4% chloral hydrate by abdominal cavity, the ovaries were washed with PBS until the exudate was clear. The bilateral ovaries were removed. After adipose tissue on the surface was removed, the ovaries were fixed with 4% paraformaldehyde for at least 24 h. After that, the tissues were soaked in 85% alcohol, 90% alcohol and absolute ethyl alcohol for 1.5h respectively and in xylene solution

for 20mi;After dehydration, paraffin embedding, and slicing, the tissues were baked at 60 °C for 3h. After dyeing with hematoxylin and washed with water for 3 min, they were immersed in hydrochloric acid (1%) and alcohol for 10s, washed with water and turned blue with ammonia water (0.1%), then dyed with eosin solution for 1.5 min, and put in 80% ethanol for 2 min, 95% ethanol for 5 min and absolute ethanol for 5 min, respectively. After being transparentized with xylene solution, they were sealed with neutral gum and dried. The pathological changes of ovarian tissues in each group were observed under microscope.

#### *Determination of TNF- $\alpha$ and IL-6 gene expression by RT-PCR*

Total RNA of rat ovary was extracted by Trizol method, and the concentration and integrity of total RNA were detected. Then, cDNA was amplified by reverse transcription reaction system with mRNA as template and stored at -20 °C. The expression of TNF- $\alpha$  and IL-6 gene was detected by RT-PCR. PCR amplification conditions were: pre-denaturation at 95 °C for 2 min, denaturation at 93 °C for 20s, annealing at 57 °C for 30s, extension at 70 °C for 20s, cycle for 40 times, and extension at 72 °C for 60s. 10 $\mu$ L of amplification product and 6 $\mu$ L of DGL-200 Maker were sampled at the same time. The samples were separated by 1.5% agarose gel electrophoresis. [Table I](#) shows the primers of TNF- $\alpha$ , IL-6 and GAPDH.

#### *Western blot analysis for assessing IKK $\beta$ and NF- $\kappa$ B expression*

The protein extracted from ovarian tissue cells of each group was run on 10% SDS-polycystic amide. The SDS-PAGE pattern was transferred to PVDF membrane. After adding 5% skimmed milk powder, they were sealed at room temperature for 1h. The membrane was washed and then soaked in diluted (1:1000) protien antibody at 4 °C overnight. The membrane was washed with TBST for 3 times, 5 min each time; and then, HRP labeled secondary antibody (1:500) was added. After reaction at 37 °C for 2h, they were washed with TBST for 3 times (5min each time). A negative control group was established with GAPDH monoclonal antibody as primary antibody and HRP labeled IgG as secondary antibody.

**Table I. Primers designed in PCR.**

Primer name	Forward primer	Reverse primer	Length (bp)
TNF- $\alpha$	5'-GGAACAGCTTGCACCAGCTG-3'	5'-GAGCCTTGTATGTTTACAG-3'	132
IL-6	5'-AACGCAGCTTAGCGCAGCTG-3'	5'-AAGCCTTGACGCGTTTACAG-3'	138
GAPDH	5'-CAGCTATGGAAGTGCATTGG-3'	5'-TAGGACGAGTATGCTAGACA-3'	136

#### *Statistical analysis*

This study adopted SPSS20.0 statistical software (IBM Company, USA); the measurement data was expressed by "mean standard deviation" ( $\bar{x} \pm s$ ), the comparison between groups was analyzed by one-way variance, and the pairwise comparison between groups was tested by LSD-t; the counting data was expressed by percentage (%), and the comparison between groups was analyzed by  $\chi^2$ ;  $P < 0.05$  indicated the statistically significant difference.

## RESULTS

[Table II](#) shows the, increased body weight and decreased weight of ovary in the model group compared with the control group. After DHEA administration, the body weight as well as the ovary weight increased. compared with the model group.

[Table II](#) shows increase in the level of testosterone (T) luteinizing hormone (LH) levels in serum of model group rats and decreased in follicle stimulating hormone (FSH) levels ( $p < 0.05$ ), which was consistent with PCOS characteristics. After administration of DHEA, T and LH levels decreased While the FSH level increased compared with the model group compared with the control group, the blood glucose and insulin level of rats in the model group increased. After DHEA administration, the level of fasting blood glucose and insulin in high-dose and low-dose groups decreased, and the effect of high-dose group was better ([Table II](#)). Compared with the model group, the glucose and insulin levels in serum of rats decreased and insulin sensitivity index increased after DHEA administration. In rat ovarian tissue under PCOS model, the expression of TNF- $\alpha$ , IL-6, IKK $\beta$ , NF- $\kappa$ B genes compared with control group ( $P < 0.05$ ), whereas after DHEA administration the expression of TNF- $\alpha$ , IL-6, IL-6, IKK $\beta$ , NF- $\kappa$ B decreased ([Table II](#)) and the effect of high dose administration group was better than that of model group ( $p < 0.05$ ) ([Table II](#)).

## DISCUSSION

Intrinsic microorganisms play an important role in host metabolism and nutrient absorption. Recent studies have shown that they also play an important role in

**Table II. Effect of DHEA on the rat body and ovary weights, levels of serum testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), fasting glucose, insulin level, insulin sensitivity index (ISI) and expression of TNF- $\alpha$  and IL-6, IKK $\beta$  and NF- $\kappa$ B protein in rat ovarian tissue under PCOS model.**

	Control group (n=10)	Model group (n=10)	DHEA group		F value	P value
			15 $\mu$ g/kg/d	30 $\mu$ g/kg/d		
Body weight (g)	152.12 $\pm$ 15.41	126.39 $\pm$ 18.02	179.64 $\pm$ 14.96	197.47 $\pm$ 16.23	1.682	0.259
Ovary weight (mg)	73.21 $\pm$ 9.74	45.45 $\pm$ 7.23	54.62 $\pm$ 7.80	62.94 $\pm$ 8.82	1.195	0.425
T (ng/mL)	12.72 $\pm$ 0.71	18.22 $\pm$ 0.95	16.08 $\pm$ 0.72	14.12 $\pm$ 0.96	25.397	0.001
FSH (IU/L)	3.67 $\pm$ 0.56	1.21 $\pm$ 0.21	1.88 $\pm$ 0.22	2.62 $\pm$ 0.26	19.501	0.001
LH (IU/L)	17.63 $\pm$ 2.71	38.49 $\pm$ 3.42	26.26 $\pm$ 2.41	23.17 $\pm$ 2.75	32.358	0.001
Fasting blood glucose (mmol/L)	4.43 $\pm$ 0.31	11.27 $\pm$ 1.46	9.31 $\pm$ 1.22	6.82 $\pm$ 1.21	31.349	0.001
Insulin ( $\mu$ IU/ml)	6.77 $\pm$ 1.49	35.12 $\pm$ 3.86	21.38 $\pm$ 2.43	11.81 $\pm$ 1.80	24.392	< 0.05
ISI	-3.22 $\pm$ 0.30	-5.98 $\pm$ 0.14	-5.25 $\pm$ 0.15	-4.34 $\pm$ 0.24	32.046	< 0.05
TNF- $\alpha$ (gray value)	1.06 $\pm$ 0.06	3.23 $\pm$ 0.32	2.66 $\pm$ 0.40	1.87 $\pm$ 0.35	33.397	0.001
IL-6 (gray value)	1.05 $\pm$ 0.13	3.40 $\pm$ 0.32	2.75 $\pm$ 0.50	1.29 $\pm$ 0.43	21.716	0.001
IKK $\beta$ (gray value)	1.08 $\pm$ 0.21	3.62 $\pm$ 0.26	2.23 $\pm$ 0.25	1.71 $\pm$ 0.23	25.738	0.001
NF- $\kappa$ B (gray value)	2.12 $\pm$ 0.04	4.86 $\pm$ 0.16	4.01 $\pm$ 0.21	3.26 $\pm$ 0.22	26.215	0.001

regulating estrogen and its metabolites and participate in the occurrence and development of a variety of estrogen metabolic disorders (Fuhrman *et al.*, 2014; Schwarzer *et al.*, 2016). Studies have shown that tumor necrosis factor TNF- $\alpha$  and interleukin IL-6, as important pro-inflammatory cytokines, can reduce insulin sensitivity and lead to insulin resistance (Khan *et al.*, 2017; Saiki *et al.*, 2018; Wang *et al.*, 2018; Jiang *et al.*, 2018), and the increase of their levels is an important reason for insulin resistance. IKK $\beta$ /NF- $\kappa$ B signaling pathway plays an important role in the production of inflammatory factors (Zhang *et al.*, 2017; Goldberg-Stern *et al.*, 2014). When cells are stimulated by the outside world, IKK $\beta$  is activated and NF- $\kappa$ B is activated and released, promoting the expression of various inflammatory genes to release a large number of inflammatory factors and promote the intrinsic resistance (Zhu *et al.*, 2017). At present, there are few reports about whether DHEA can regulate insulin resistance through estrogen-microbial axis to regulate glucose metabolism, and its mechanism has not been made unclear, which has great limitations on its clinical drug application.

In this study, the PCOS model of SD rats was induced by DHEA. After modeling, the rats were depressed, puffy, slow-moving, with gained weight and decreased ovary weight; their serum T and LH levels increased significantly, while FSH levels decreased significantly; after DHEA administration, their weight, serum T and LH levels decreased to a certain extent, while their ovarian weight and serum FSH levels increased to a certain extent, indicating that the rat PCOS model was successfully

established and DHEA had a certain inhibitory effect on PCOS in rats. The detection results of FBG and insulin in serum showed that the contents of FBG and insulin in serum increased significantly after PCOS modeling in rats, while the levels of FBG and insulin decreased significantly after DHEA administration. The ISI increased significantly, especially in high dose group. This indicated that DHEA could regulate the intrinsic microbes level of rats and then increase the level of serum estrogen through estrogen-microbial axis to further regulate the process of glucose metabolism in rats. The expression of TNF- $\alpha$ , IL-6, IKK $\beta$  and NF- $\kappa$ B increased significantly after the establishment of the model. The levels of TNF- $\alpha$ , IL-6, IKK $\beta$  and NF- $\kappa$ B were down-regulated after DHEA administration. This indicated that DHEA could regulate the level of estrogen in organisms through the estrogen-microbial axis, and the intrinsic microbiological axis of estrogen was closely related to insulin resistance. It could effectively inhibit the expression of inflammatory factors TNF- $\alpha$  and IL-6 through IKK $\beta$ /NF- $\kappa$ B signaling pathway, so as to improve insulin resistance and glucose metabolism.

By analyzing its mechanism, the author found that DHEA, synthesized in the adrenal reticular zone, is a steroid with weak androgen effect. It is mainly converted into T and estradiol (E2) in peripheral tissues, and then exerts indirect biological effects. DHEA supplementation can effectively improve ovarian reserve function and responsiveness and improve ovulation induction. Abnormally low DHEA leads to low E2 content. DHEA can increase the concentration of insulin-like growth factor

(IGF-1) in blood and can be used as the precursor hormone of estrogen; DHEA, as a ligand of androgen receptor (AR), can stimulate follicular differentiation and growth together with FSH, and promote follicular recruitment. DHEA is an essential substrate for steroid hormone synthesis. Almost all androgens and holmium hormones in the body come from DHEA. There is no negative feedback regulation mechanism of DHEA secretion in the body. When DHEA is lacking, it can be supplemented by exogenous sources, thus up-regulating E2. Estrogen can regulate vaginal microbial flora. It is found that the composition of vaginal flora is different among women before menopause, perimenopausal period and postmenopausal period. *Lactobacillus curvatus* and *Lactobacillus inermis* are the main species before menopause, *Lactobacillus gasseri* is the main species during perimenopausal period. After menopause, streptococcus and proteus are the main specie, and the number of *Lactobacillus* decreases obviously. This shows that the change of estrogen level in women will lead to the fluctuation of vaginal microorganisms. Vaginal microbes are not the only microbial community related to estrogen in women, and there is also a connection between intensive microbes and estrogen. Estrogen can be metabolized by the intensive microbes. At the same time, it affects the intestinal microecological structure, forming the regulatory axis of estrogen intestinal microbes. Intrinsic microbiological axis of estrogen is closely related to insulin resistance. DHEA can inhibit insulin resistance and glucose metabolism in PCOS rats, and its mechanism may be related to the inhibition of inflammatory factors. However, its specific mechanism is still unclear, and further in-depth study is needed. The current research results show that DHEA has a good inhibitory effect on insulin resistance and glucose metabolism, which is of great significance to the treatment of insulin resistance, and also provides some data and theoretical support for the clinical application of DHEA.

To sum up, DHEA can regulate estrogen level in rats through estrogen-intensive microbiological axis, thereby improving insulin resistance and lowering blood glucose level. Its mechanism may be related to inhibiting expression of inflammatory factors and inhibiting NF- $\kappa$ B nuclear transposition through IKK/NF- $\kappa$ B signaling pathway.

#### Statement of conflict of interest

The authors have declared no conflict of interest.

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