

Evaluation on the characteristics of gut microbiome in polycystic ovary syndrome rats induced by dihydrotestosterone or letrozole

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Research

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Abstract

Background: Etiology of polycystic ovary syndrome (PCOS) is unclear. Recent reports indicated that gut microbiota regulates metabolism and plays a major role in the development of PCOS. The present study aimed to investigate and compare the effects of dihydrotestosterone or letrozole on the gut microbiome in rats.

Methods: We used dihydrotestosterone (DHT) or letrozole (LET) to induce PCOS model rats. At the end of the experiment, ovarian morphology, hormonal and metabolic status were investigated in all rats. We analyzed the gut microbiome of rats by deep sequencing the 16S rDNA V3-V4 hypervariable regions. Predictive metagenomics profiling using PICRUST revealed functional alternation of the KEGG pathways in rats.

Result: Rats induced by letrozole exhibited endocrine and reproductive characteristics, such as hyperandrogenism, abnormal oestrus cycles or complete acyclic, polycystic ovaries, and obesity. DHT-induced rats were obesity, irregular oestrus cycles, polycystic ovaries, with lower level of HDL-C and lower activity of SOD than controls. The bacterial diversity (Chao1 and Ace) of DHT group was decreased compared with the control group. Furthermore, principal coordinates analysis revealed that DHT and control groups could be distinguished from each other. The DHT group exhibited increased numbers of *Firmicutes*, *Cyanobacteria*, *Actinobacteria* and *Saccharibacteria*, and decreased *Bacteroidetes* compared with the control group at the phylum level. Moreover, PICRUST-predicted KEGG pathways related to Carbohydrate metabolism, Amino acid metabolism, Metabolism of terpenoids and polyketides, Metabolism of other amino acids, Replication and repair, Lipid metabolism and Energy metabolism, Translation, Folding, sorting and degradation and Xenobiotics biodegradation and metabolism were significantly elevated in the DHT group. And pathways related to Metabolism of terpenoids and polyketides, Replication and repair, Xenobiotics biodegradation and metabolism, Nucleotide metabolism, Infectious diseases and Excretory system were significantly elevated in the LET group.

Conclusion: The findings of the present study indicated that DHT affected the composition and diversity of gut microbial community, and leads to the gut dysbiosis. KEGG function profiles calculated by PICRUST suggested that DHT may affect metabolism of the gut microbiota more directly than letrozole.

Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial, heterogeneous and complex genetic, endocrine and metabolic disease, which is characterized by hyperandrogenism, hyperinsulinemia, insulin resistance (IR) and chronic anovulation. [1-3]. Hyperandrogenism may play an important role in altering the gut microbiome in women with PCOS [4]. Some studies have reported that compared with healthy women, the overall bacterial species richness (α diversity) of the gut microbial community of PCOS women decreased, and several bacterial taxa changed [5,6]. Zhang et al. [7] confirmed that gut microbiome was involved in the development of IR and menstrual disorders in PCOS patients by affecting intestinal wall permeability. Some researchers believed that dysbiosis of gut microbiota can increase the production of ovarian

androgen, and then interfere with the development of normal follicle by triggering a chronic inflammatory reaction and IR [8]. The gut microbiome is closely related to PCOS, but there is no consensus on which phylum or genera in gut microbiome is related to PCOS. The heterogeneity of PCOS is frequently reflected in many animal models. Therefore, if a rat model not only shows the characteristics of ovarian and metabolic syndrome, but also shows the dysbiosis of gut microbiota, it would be valuable for further study of new PCOS therapy. Testosterone has been proved to be related to the composition change of the gut microbiota [9]. Letrozole is a non-steroidal aromatase inhibitor, which can block the conversion of testosterone to estradiol [10], and increase the testosterone level by increasing the production of ovarian androgen [11]. Continuous exposure to Dihydrotestosterone (DHT) can establish a PCOS rat model with typical hormonal disorders and ovarian morphological changes [12]. Excessive androgen secretion in early life may lead to hormone secretion disorder, which may lead to PCOS in adulthood [13-15]. In this study, female rats took DHT or letrozole continuously from before puberty, which led to adult PCOS rats.

Materials And Methods

Animals

Fifteen specific-pathogen free (SPF) level female Sprague Dawley (SD) weighting $50\text{g} \pm 5\text{g}$, were aged 21 days at the onset of experiments. They were from the Experimental Animal Science Department of Guangzhou University of Chinese Medicine, Guangzhou, China (License number SCXK-2016-0168). The experimental protocols were performed after approval and in accordance with the guidelines set by the Experimental Committee of Guangzhou Medical University (Protocol number: GY2018-021). All rats were housed in a temperature-controlled environment at $22\text{ }^{\circ}\text{C}$ – $24\text{ }^{\circ}\text{C}$ and 60% humidity, with a 12-h light/dark cycle and free access to diet and water.

Study procedure

Fifteen rats were randomly divided into three groups on average. According to previous studies [16,17], from 3w age, the rats in DHT group were subcutaneously injected daily with $83\mu\text{g}$ 5α -DHT which dissolved in 0.2ml tea oil for 6 weeks to mimic the hyperandrogenic state in women with PCOS. The rats in other two groups were injected daily with the same volume of tea oil alone. Follow the experiment of Kafali H et al [10], from 6week age, the rats in letrozole (LET) group were oral administration with letrozole ($1\text{mg}/\text{kg}$ body weight of letrozole dissolved in 1 ml of 1% carboxymethyl cellulose (CMC) for 3 weeks. At the same time, the rats in control (C) group and DHT group were daily oral administration with 1 ml of 1% CMC. All the rats were fed commercial chow (Research Diets GB 14924.3-2010) [67% carbohydrate, 21% protein, 12% fat, and total 3.45kcal/g], was provided by Guangdong Medical Laboratory Animal Center.

Vaginal smears

The major cell type in vaginal smears obtained every day from 6 weeks old to the end of the experiment were analyzed by microscope to determine the periodic stage. Rats showing regular cycles of 4–5 days complete with the proestrus, estrus, metestrus and diestrus stages were defined as normal cyclic rats,

whereas animals in which the estrus cycle was found arrested in any one of the stages for 4 consecutive days were termed as acyclic rats [18].

Blood and tissue collection

The whole blood was collected from the orbit, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30mg/ kg) for blood collection and the pinch reflex was monitored to ensure full anesthesia. After blood collection, the rats were sacrificed by excessive anesthesia. Blood was separated by 10-min centrifugation (3,000 revolutions/min) at 4°C. Supernatant containing serum was separated and immediately stored at -20°C until analyzed for biochemical and hormonal analysis.

Blood sampling for lipid profile, sex steroids and other biochemical parameters

Fasting blood glucose (FBG) was analyzed by GOD-PAP. Testosterone (T), Free testosterone (FT), Fasting insulin (INS), Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Superoxide dismutase (SOD), Malondialdehyde (MDA), Interleukin-22(IL-22), Lipopolysaccharide (LPS), Toll-like receptors 4(TLR4) and Tumor necrosis factor- α (TNF- α) were determined using enzyme-linked immunosorbent assay (ELISA) kit (mlbio, Shanghai, China). Sensitivity of methods are 0.1 ng/ml (FT, TLR4), 1.0 U/ml (SOD), 1.0 pg/ml (TNF- α), 0.1mU/L (INS), 0.1 pg/ml (IL-22), 0.1 mmol/ml (MDA), 1.0 pg/ml (T), 1.0 EU/L(LPS), and 0.1 mIU/ml (LH, FSH) at 95% confidence limit. Assays were performed according to manufacturers' protocols. The optical density values were read at 450 nm using a microplate reader. HDL-C, LDL-C, total cholesterol and triglyceride levels were quantified by following the protocols provided with kits on UniCel DxC 600800 Synchron Clinical System. IR was appraised with the homeostasis model assessment of insulin resistance (HOMA-IR) method. HOMA-IR was calculated using the following formula:

$$\text{HOMA-IR} = \text{FBG (mmol/L)} * \text{FINS (mU/L)} / 22.5 [19].$$

Ovarian Histology

Rats were sacrificed by excessive anesthesia, and ovaries were rapidly isolated. The left ovary of rats was fixed in 4% paraformaldehyde and embedded in paraffin. 5 μ m thick sections were prepared and stained with Hematoxylin-Eosin and histo-anatomical changes were observed and photographed under light microscope (BX-51, Olympus, Tokyo, Japan at X40 magnification).

16s rDNA sequencing data analysis

The fecal microbiome for 15 fecal samples collected from 15 rats in the three groups. The 16S rDNA high through put sequencing (V3-V4 region) was performed using an Illumina MiSeq platform. After assembly, quality filtering and the random extraction of sequences at 97% similarity, the operational taxonomic units (OTUs) for species classification were obtained. The abundance of bacteria was calculated using the method of Ace and Chao 1, and the diversity of bacteria was estimated using the method of Shannon and Simpson. In the aspect of β diversity, based on Bray-Curtis dissimilarity, permutational ANOVA (PERMANOVA) was used to assess bacterial community compositional difference. Using PICRUST

(Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to predict metagenomics profiling. Metabolic pathway analysis was performed on the basis of KEGG database.

Statistical analyses

Most statistical evaluations were performed with SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, United States). All data were presented as mean \pm SEM. One-way ANOVA was used to determine the significance, $p \leq 0.05$ was considered significant. When the ANOVA revealed significant differences among three groups, post hoc analysis was performed by a Tukey honest significant difference test. Kruskal–Wallis test was used for not normally distributed values.

Results

Animal characteristics

The weight of DHT and LET rats increased more than that of the C group. The concentration of HLD-C and the activity of SOD decreased in DHT group, compared with the C group. And the level of MDA in DHT group was higher than in LET group ($p \leq 0.05$). Level of T was significantly elevated in LET rats ($p \leq 0.05$).

Vaginal smears

The vaginas in all rats were mostly opened at the age of 40 days. The normal oestrus cycle of all control rats is 4~5 days, including proestrus, estrus, metestrus and diestrus. Vaginal smears of DHT and LET groups showed predominantly leukocytes, which indicated that they appeared continuously.

Ovarian morphological changes

A cystic follicle was considered to be a large fluid-filled cyst with an attenuated granulosa cell layer. Cross-sectional area of ovary was smaller in DHT rats and bigger in LET rats than in the controls. Light microscope observation showed that enlarged ovaries with multiple cysts in LET rats, while the ovaries of rats in DHT group had a tendency of follicle enlargement. The number of cystic follicles was increased with a relatively thin granulosa cell layer in DHT and LET rats compared with controls (Fig. 1).

The biodiversity in the gut microbiome

The indices of Shannon and Simpson (calculated for the diversity of bacteria), Chao 1 and Ace (calculated for the abundance of bacteria), together with the total tags and OTUs of each group are presented in were shown in Fig.2A and B. Compared with the other two groups, DHT group had lowest Chao1 and Ace indices ($p \leq 0.01$). The Shannon and Simpson indices in DHT group was significantly higher than the LET group ($p \leq 0.05$), and no significant differences were observed between DHT and C group (Fig. 2A). The Venn diagram demonstrated that there were 475 common OTUs identified among groups, and there were 573, 513 and 416 OTUs specific to the C, LET and DHT group, respectively (Fig.2B). Differences in microbial community structure between groups (β -diversity) were examined with PERMANOVA and visualized with PCoA plots (Fig. 3). At the taxonomic level of OTU, significant differences in microbial communities were

observed between C and DHT rats ($p = 0.006$) and between LET and DHT rats ($p = 0.008$). No significant difference was observed between C and LET group ($p = 0.054$). (Table.2)

The microbial community structures at the phylum and genera levels

We evaluate the microbial composition by comparing the relative abundances of taxa at the phylum and genera levels. The taxonomic compositions of the bacterial phylum of the groups are presented in Fig 4A and B. The majority of the samples exhibited high percentages of *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Verrucomicrobia* on the phylum level. As shown in the Fig 4B, the DHT group exhibited increased numbers of *Firmicutes*, *Cyanobacteria*, *Actinobacteria* and *Saccharibacteria* species, and decreased *Bacteroidetes* species, compared with the C group at the phylum level. As shown in the Fig 5, the DHT group exhibited increased numbers of *Lachnospiraceae_NK4A136_group*, *Ruminococcus_1*, *Alistipes*, *Clostridium_sensu_stricto_1* and *Anaerotruncus* species, and LET group exhibited increased numbers of *Romboutsia* species, compared with the C group at genus level. KEGG signaling pathway enrichment analysis demonstrated that the following signaling pathways were upregulated in the DHT group (Fig 6 A and B). PICRUST-predicted KEGG pathways related to Carbohydrate metabolism, Amino acid metabolism, Metabolism of terpenoids and polyketides, Metabolism of other amino acids, Replication and repair, Lipid metabolism and Energy metabolism, Translation, Folding, sorting and degradation and Xenobiotics biodegradation and metabolism were significantly elevated in the DHT group. And pathways related to Metabolism of terpenoids and polyketides, Replication and repair, Xenobiotics biodegradation and metabolism, Nucleotide metabolism, Infectious diseases and Excretory system were significantly elevated in the LET group.

Discussion

PCOS is a complex endocrine and metabolic disorder, which is related to ovulation dysfunction, hyperandrogenism, obesity and IR. Hyperandrogenism is a key feature of PCOS, and androgen may promote early follicular differentiation [20]. In present study, rats letrozole group exhibited endocrine and reproductive characteristics, such as hyperandrogenism, abnormal oestrus cycles or complete acyclic, polycystic ovaries, and obesity. DHT induced rats were showed obesity, irregular oestrus cycles, polycystic ovaries, lower level of HDL-C and lower activity of SOD than controls. Abnormal lipid metabolism is often present in women with PCOS, and its incidence rate is reported as high as 50% [21]. In PCOS patients, hyperandrogenemia is related to higher concentrations of plasma TG and lower HDL-C [22,23]. In our study, the DHT rats had lower HDL-C concentration than controls, while letrozole did not reduce HDL-C. Oxidative stress (OS) is defined as an imbalance in normal cellular environment, which is considered to be the cause of the development of many pathological diseases or deterioration of symptoms (such as inflammation) [24-26]. OS can also contribute to PCOS [27], the activity of SOD which related to the antioxidant capacity in DHT group was lower than in C group. We suspected that DHT may have a stronger and more direct role in regulating HDL-C and SOD activity than letrozole.

PCOS is a kind of complex heterogeneous disease [28,29], altered gut microbiome may be associated with the occurrence of PCOS [30]. Sex hormones play an important role on the gut microbiome of the host

[31,32]. A study demonstrated that a diversity has a negative correlation with hyperandrogenism, total testosterone, and the other primary reproductive and metabolic characteristics of PCOS [10]. The Chao1 and Ace indices which accounted for richness of a community, DHT group owned the lowest richness of the gut microbiome in three groups. The Shannon and Simpson indices are commonly used to describe the species diversity in a community. No significant difference in Shannon index was observed between C and DHT rats, and between C and LET rats. But Shannon and Simpson indices were significantly higher in DHT group than in LET group. Alpha diversity analysis showed the gut microbiota abundance and diversity in DHT group changed significantly. And the results demonstrated that the effects of DHT on gut microbial community may be complicated. With respect to beta diversity, PCoA demonstrated significant differences between DHT group and the other two groups on the first axis, suggesting that the microbial community composition from LET group was more similar to C group and DHT influenced the structure and composition of gut microbiota. Changes in gut microbial composition may be unfavorable, and lead to some diseases. In the field of ecology, species richness is considered to be related to ecosystem health, because diverse communities may improve the stability and productivity of an ecosystem [33]. According to several recent meta-analyses, compared with healthy individuals, it was also observed that a diversity of the gut microbiome reduced in human with metabolic diseases, which is related to human obesity [34-36].

The microbial structure in DHT rats was noticeably differed from that in control rats, as indicated by altered the abundance of *Bacteroidetes*, *Firmicutes*, *Cyanobacteria*, *Actinobacteria* and *Saccharibacteria*. *Bacteroides* is involved in the metabolic process, which could absorb and metabolize polysaccharides, bile acid and steroids in foods that cannot be metabolized by the host. [37]. Turnbaugh et al. found that obese mice had more *Firmicutes* and less *Bacteroides* than lean mice, and that this distribution of gut microbiome can better absorb energy [38]. *Bacteroidetes* and *Firmicutes* are significantly related to metabolic syndrome, and the abundance of *Bacteroides* increased while that of *Firmicutes* decreased in exercise rats [39]. So, it showed that gut microbiome disorders may accelerate the process of DHT induced PCOS by affecting energy absorption, and appear obese. In addition, compared with C groups, DHT significantly increased the abundance of *Actinobacteria*, *Cyanobacteria* and *Saccharibacteria*. And the relative abundance of *Actinobacteria* and *Saccharibacteria* were significantly high in DHT group than LET group. The changes in the gut microbiota are related with specific metabolic states, such as obesity [40]. *Actinobacteria* was positively correlated with obesity [41], the high level of *Actinobacteria* aggravated the disorder of the intestinal flora and contributed to the progression from obesity to the related diseases [42]. *Saccharibacteria* are prominent in the oral cavity, and increased abundance of *Saccharibacteria* is correlated with the development of periodontitis [43] and inflammatory bowel disease [44]. However, the role of increased abundance of *Saccharibacteria* in DHT model rats are still unclear, which may be related to the inflammatory conditions.

The results showed that DHT group exhibited increased numbers of *Lachnospiraceae_NK4A136_group*, *Ruminococcus_1*, *Alistipes*, *Clostridium_sensu_stricto_1* and *Anaerotruncus* species, and LET group exhibited increased numbers of *Romboutsia* species, compared with the C group at genus level.

The *Lachnospiraceae NK4A136 group* is an indicator of gut dysbiosis, because when present in great abundance, gut dysbiosis is more severe [45]. *Ruminiclostridium* has a significantly positive relationship with hyperglycemia [46], and was richness in DHT group. A study showed that the distribution and structure of the intestinal microflora (including *Anaerotruncus*) changed after ovariectomy in rats [47]. *Anaerotruncus* was significantly positively correlated with ovary weight [48], which might be in line with rats' ovaries enlarged in our study. In addition, an increased relative abundance of *Anaerotruncus* species is related to aging and age-associated inflammation with elevated pro-inflammatory chemokines in a mouse model [49]. Interestingly, as a taxa of the family *Ruminococcaceae*, genus *Ruminococcus_1* is one of the dominant butyrate-producing bacteria [50]. Butyrate is a short-chain fatty acid, which is helpful for maintaining cellular health by inhibiting the expression of pro-inflammatory cytokines in the intestine through mechanisms including nuclear factor kappa B signaling [51]. *Clostridium_sensu_stricto_1* was reported positively associated with amino acid metabolism (alanine, phenylalanine and glutamine) and energy metabolism (citrate, pyruvate, creatine and lactate) in serum [52]. Gut microbiota dysbiosis correlates with host metabolic changes, KEGG analysis identified several altered metabolic pathways in DHT group, mainly involving Carbohydrate metabolism, Amino acid metabolism, Metabolism of terpenoids and polyketides, Metabolism of other amino acids, Replication and repair, Lipid metabolism and Energy metabolism, Translation, Folding, sorting and degradation and Xenobiotics biodegradation and metabolism.

In a word, our findings indicated that DHT affect the composition and diversity of gut microbial community, and leads to the gut dysbiosis. Letrozole may influence the gut microbiome in some aspects, however DHT may affect metabolism of the gut microbiota more directly than letrozole.

Declarations

List of abbreviations

Polycystic ovary syndrome (PCOS), androgen dihydrotestosterone (DHT), letrozole (LET), Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Testosterone (T), Free testosterone (FT), Fasting insulin (INS), Fasting blood glucose (FBG), Homeostasis model of assessment for insulin resistance index (HOMA-IR), High-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C), Total triglyceride (TG), Total cholesterol (TC), Toll-like receptors 4 (TLR4), Lipopolysaccharide (LPS), Superoxide dismutase (SOD), Malondialdehyde (MDA), Interleukin-22 (IL-22), Tumor necrosis factor- α (TNF- α), insulin resistance (IR), principal co-ordinate analysis (PCoA).

Ethics approval: Animal studies were performed in compliance with the Experimental Committee of Guangzhou Medical University.

No conflicts of interest, financial or otherwise, are declared by the authors.

Consent for publication: Not Applicable

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Authors Contributions:

Conceived and designed the experiments: YHZ, YX and HXM. Performed the experiments: CJL and TY. Analyzed the data: YHZ and HXM. Wrote the paper: YHZ and YX.

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Tables

Table 1. Comparison of biochemical parameters among groups.

	C	DHT	LET	ANOVA	Tukey HSD (Adjusted for Multiple Comparisons)		
	N=5	N=5	N=5	P value	P1	P2	P3
Weight(g)	232.3±9.05	260.2±21.21	269.83±15.9	0.01	0.045	0.01	0.674
LH(mIU/ml)	5.31±0.82	6.91±1.46	5.02±0.66	0.053	0.098	0.909	0.063
FSH(mIU/ml)	7.36±1.13	7.44±1.17	5.95±0.91	0.138	0.993	0.183	0.181
T(pg/mL)	28.46±4.54	38.99±10.21	42.38±4.36	0.02	0.08	0.02	0.746
FT(ng/mL)	2.07±0.53	2.32±0.42	2.78±0.83	0.075	0.63	0.064	0.26
INS(mU/L)	2.49±0.46	2.79±0.89	2.17±0.40	0.325	0.730	0.706	0.294
FBG (mmol/L)	5.46±0.82	6.12±1.14	5.50±1.37	0.604	0.641	0.998	0.675
HOMA-IR	0.59±0.07	0.76±0.29	0.49±0.06	0.088	0.324	0.637	0.076
HDL(mmol/L)	1.15±0.22	0.78±0.0.9	1.04±0.12	0.011	0.009	0.502	0.072
LDL(mmol/L)	0.41±0.13	0.35±0.04	0.45±0.08	0.304	0.577	0.833	0.282
TG(mmol/L)	0.90±0.46	1.30±0.31	0.88±0.44	0.221	0.296	0.997	0.268
TC(mmol/L)	1.80±0.41	1.36±0.16	1.67±0.22	0.084	0.079	0.765	0.244
TLR4(ng/mL)	3.23±0.40	3.00±0.70	3.09±0.38	0.783	0.767	0.910	0.956
LPS(EU/L)	100.99±18.68	113.35±19.91	98.40±17.30	0.452	0.568	0.977	0.485
SOD(U/ml)	24.96±2.95	19.70±8.93	23.65±3.38	0.046	0.026	0.737	0.097
MDA (nmol/ml)	0.26±0.06	0.37±0.11	0.17±0.06	0.011	0.138	0.291	0.009
IL-22(pg/ml)	3.80±0.57	4.50±0.27	3.83±0.49	0.059	0.084	0.996	0.098
TNF-α(pg/ml)	50.16±5.39	52.08±13.01	55.04±8.62	0.723	0.949	0.704	0.873

Abbreviation:

C: control group; DHT: dihydrotestosterone group; LET: letrozole group ; Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Testosterone (T), Free testosterone (FT), Fasting insulin (INS), Fasting blood glucose (FBG), Homeostasis model of assessment for insulin resistance index (HOMA-IR), High-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C) ,Total triglyceride(TG), Total cholesterol (TC), Toll-like receptors 4(TLR4), Lipopolysaccharide (LPS), Superoxide dismutase (SOD),

Malondialdehyde (MDA), Lnterleukin-22(IL-22), and Tumor necrosis factor- α (TNF- α).Data are presented as mean \pm standard deviation, analyzed by one-way analysis of variance followed by the Tukey HSD test. P1:C group vs DHT group; P2: C group vs LET group; P3: DHT group vs LET group.

Table 2. Adonis analysis between Groups.

Compare	F_value	R2	P_value
C vs DHT	4.2934	0.3492	0.006
C vs LET	1.8852	0.1907	0.054
LET vs DHT	5.6578	0.4143	0.008

C: control group; DHT: dihydrotestosterone group; LET: letrozole group.