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A Rat Model of Maternal Polycystic Ovary Syndrome Shows that Exposure to Androgens *In Utero* Results in Dysbiosis of the Intestinal Microbiota and Metabolic Disorders of the Newborn Rat

Authors' Contribution:
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Intestinal dysbiosis, or dysbacteriosis, is an abnormal interaction between the intestinal microbiota and the host cells due to altered microbial diversity**.** This study aimed to investigate the metabolic effects and changes in the intestinal microbiota in newborn rats following exposure to increased levels of maternal androgens in a rat model of maternal polycystic ovary syndrome (PCOS).

Material/Methods: The administration of androgen developed the rat maternal PCOS model during pregnancy. Maternal rat ovarian follicles were counting and assessed by histology. The metabolic phenotype of newborn rats was evaluated and included an insulin tolerance test, a glucose tolerance test, and measurement of serum levels of triglyceride, insulin, cholesterol, adiponectin, and leptin. Expression of pro-inflammatory cytokines was detected using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), serum levels were measured by enzyme-linked immunosorbent assay (ELISA), and proteins associated with adipose tissue remodeling and adipocyte differentiation were measured by Western blot.

Results: Markers of systemic inflammation were significantly increased in the female offspring but not in the male offspring born to rat in the PCOS model. Following birth, newborn rats that received antibiotics showed an improved metabolic phenotype, with reduced serum lipid levels, insulin resistance, body weight, inflammation of adipose tissue, and serum levels of inflammatory cytokines compared with controls. Probiotics had no significant effects on these parameters in newborn rats.

Conclusions: In a rat model of maternal PCOS, exposure to androgens *in utero* resulted in dysbiosis of the intestinal microbiota and metabolic disorders of the newborn female rats**.**

MeSH Keywords: 46, XY Disorders of Sex Development • Androgens • Fetal Diseases Microbiota • Polycystic Ovary Syndrome

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Background

Polycystic ovary syndrome (PCOS) is a common clinical condition that results in endocrine and metabolic dysfunction in women, characterized mainly by hyperandrogenism and insulin resistance. Fetal exposure to high concentrations of androgen *in utero* leads to an increased risk of metabolic disorders and a predisposition for PCOS in female offspring [1,2]. Several possible mechanisms have been proposed for the association between high androgen exposure *in utero* and the development of metabolic disorders, which may be due to alteration in the intrauterine environment, epigenetic change, and survival advantage associated with fetal malnutrition [3–5].

Intestinal dysbiosis, or dysbacteriosis, is the term used for the abnormal interaction between the host intestinal microbiota and the host cells due to altered microbial diversity. Recently, dysbiosis of the gut microbiota has been reported to be associated with fetal exposure to high androgen levels [6,7]. Dysbiosis of the gut microbiota exists in neonates who have undergone fetal exposure to high levels of androgens. The fetal microbiota profiles of postnatal androgenized animal models have shown relatively increased levels of bacteria associated with steroid hormone synthesis, including *Nocardiaceae* and *Clostridiaceae*, and relatively lower levels of *Akkermansia*, *Bacteroides*, *Lactobacillus*, and *Clostridium* compared with animals without fetal exposure to androgens [7,8]. A relative reduction in *Akkermansia* can lead to the disruption of the gut barrier, facilitating the passage of bacterial metabolites into the circulation and liver [8], inducing innate immune responses and low-grade inflammation.

Previous studies have shown that systemic inflammation occurs in women with PCOS, as shown by increased serum levels of inflammatory cytokines and biomarkers, including C-reactive protein (CRP), interleukin (IL)-6, IL-1β, IL-7, and IL-18 when compared with healthy controls [9,10]. Increased levels of matrix metallopeptidase 9 (MMP-9) and the calcium-binding and zinc-binding protein, S100A8, were also found in the children of mothers with PCOS [10]. These preliminary study findings have suggested that maternal PCOS increased the degree of circulating inflammatory cytokines in the offspring of women with PCOS, even in childhood, which might be partly attributed to systemic inflammation from dysbiosis of gut microbiota [10]. In non-obese diabetic mice, female mice have different gut microbiota and have a higher risk of developing pancreatic islet cell inflammation and hyperglycemia when compared with male mice [11,12]. These previously published findings are consistent with metabolic disorders reported in the female offspring of mothers with PCOS [11,12].

The effects of fetal exposure to androgens on systemic inflammation and possible autoimmune responses are linked to gender and might be dependent on the gut microbiota. A previous study showed that the transfer of gut microbiota from adult males to immature young females resulted in protection from type 1 diabetes mellitus and reduced pancreatic islet cell inflammation [13]. However, the effects of testosterone exposure in female fetuses and newborns, and the influence of the gut microbiota on systemic inflammation in female animals remain unknown.

Therefore, this study aimed to investigate the metabolic effects and changes in the intestinal microbiota in newborn rats following exposure *in utero* to increased levels of maternal androgens in a rat model of maternal PCOS.

Material and Methods

The rat model of polycystic ovary syndrome (PCOS) and the study design

The Animal Ethics Committee of the Xinjiang Uygur Autonomous Region People's Hospital approved the animal experimental procedures. The study protocol and the development of the prenatal rat model of polycystic ovary syndrome (PCOS) were as previously described [6]. Adult female Sprague–Dawley (SD) rats (n=20), aged between 75–95 days, and body weight between 170–190 gm, were obtained from the Experimental Animal Center of Nanjing University, China. One pair of male and female rats was kept in standard animal housing conditions with 12-hour light and dark cycle, in a controlled temperature of 22±3°C, and relative humidity of 45–55%. Observation of the rat vaginal plug after mating was considered as the first day of pregnancy. Pregnant rats were randomly divided into two groups, the study group and the control group, with 10 rats in each group. The offspring of the maternal rats in the PCOS model included two groups. One group of rat offspring was given a high-fat diet that consisted of 3.6 kcal/g with a nutritional composition of 45% kcal of fat, 35% kcal of carbohydrate, and 20% kcal of protein. The other group of rat offspring was fed with standard rat chow, consisting of 2.87 kcal/g with a nutritional composition of 15% kcal of fat.

Hormonal treatment, measurement of serum hormone levels, and ovarian histology to confirm the establishment of the rat model of PCOS

Pregnant rats in the PCOS model in the experimental group (n=15) received 0.5 mg/g/day of free testosterone (T1500) (Sigma-Aldrich, Steinheim, Germany) dissolved in a 500 µL cocktail containing sesame oil (S3547) (Sigma-Aldrich, Steinheim, Germany) and benzyl benzoate (B6630) (Sigma-Aldrich, Steinheim, Germany) in a ratio of 4: 1 by subcutaneous injection every day after gestational day 15 until delivery. Pregnant rats in the control group (n=15) received only 500 µL of solvent.

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Male and female offspring of the PCOS model (n=12) and the control rats (n=12) were provided *ad libitum* with food and water. The metabolic phenotype of the offspring and the expression of proteins in white adipose tissue were investigated between 100 and 110 days of age (in adulthood). Histological examination of the ovarian tissue and measurement of serum levels of testosterone in the pregnant rats in the PCOS model were used to confirm the establishment of the PCOS model. Hyperandrogenism was defined as a serum concentration of testosterone <100 ng/ml. Measurement of testosterone was performed according to a standard enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Calbiotech, El Cajon, CA, USA). The pregnant rats that showed no ovarian morphological change or hyperandrogenism were excluded from the study.

Antibiotic and probiotic treatment

Female rats in the antibiotic-treated group (n=10) were treated continually for four weeks with antibiotics from two weeks after birth. A mixture of vancomycin (0.25 mg/mL) and imipenem (0.25 mg/mL) was added to the sterile drinking water. Solutions and bottles were changed between twice and three times per week. The probiotic group (n=10) received 2 mL/day of Peifeikang probiotics (Shanghai XinYi Pharmaceutical, Shanghai, China) dissolved in distilled water for four weeks after birth. Peifeikang probiotic consisted of 2.0×10^8 colony-forming units (CFU)/mL of a combination of live *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*. To exclude the influence of a high-fat diet on the intestinal microbiota, the administration of antibiotics or probiotics ceased once the rat offspring received began the high-fat diet to investigate the metabolic phenotype in the offspring of PCOS rats.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from visceral white adipose tissue for quantitative RT-PCR analysis using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to standard protocols. Approximately 200–400 ng of eluted RNA from visceral white adipose tissue was obtained. Total RNA was reverse-transcribed using hexamer primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for analysis of the transcription levels. The cDNAs were used for qRT-PCR to analyze gene expression using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The transcription levels were normalized against GAPDH expression. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and the results were expressed as the fold-change over the control samples.

Western blot

Proteins from rat visceral white adipose tissue were prepared using lysis buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked using 5% dried skimmed milk powder in TBS for 2 h at 37°C. The membranes were incubated overnight at 4°C with primary antibodies to: uncoupling protein-1 (UCP-1) (1: 500) (ab2433; Abcam, Cambridge, UK), hormone-sensitive lipase (HSL) (1: 5,000) (ab45422; Abcam, Cambridge, UK), adipose triglyceride lipase (ATGL) (1: 2,000) (ab109251; Abcam, Cambridge, UK), and GAPDH (1: 1,000) (sc-47778; Santa Cruz Biotechnology Inc., Dallas, TX, USA). Membranes were washed and incubated with the secondary antibody for 1 h at 25°C. Protein bands were detected using an electrochemiluminescence (ECL) microplate reader and FUSION Fx software (Vilber Lourmat, Marne-la-Vallée, France). The relative protein expression was calculated with GAPDH used as the control protein.

Assessment of the metabolic phenotype of the newborn rats

Assessment of the metabolic phenotype of the newborn rats was performed, as previously described [14]. Glucose tolerance tests were performed after the mice fasted for 16 h. The rats received an intraperitoneal injection of glucose at 1 g/kg body weight. For the insulin tolerance test, rats were preconditioned by fasting for four hours before insulin injection, and insulin (Roche, Basel, Switzerland) was intravenously injected at 0.75 U/kg body weight for each rat. The blood glucose levels were measured after injection (as the baseline value), and measurements of blood glucose levels were repeated after 15, 30, 60, or 90 min after intravenous injection, Then measurement of serum triglyceride levels was performed using a serum triglyceride detection kit that included triglyceride reagent, T2449, and free glycerol reagent, F6428 (Sigma-Aldrich, Steinheim, Germany). Insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (EZRMI-13K) (Merck Millipore, Burlington, MA, USA). Cholesterol levels were measured using a cholesterol quantitation kit (MAK403, (Sigma-Aldrich, Steinheim, Germany), according to the manufacturers' instructions. Adiponectin and leptin were detected using an adiponectin quantitative detection INS ELISA kit (Wuhan Fine Biotech. Co., Wuhan, China) and rat leptin ELISA kit (LE-06887) (Beijing Laier Bio Pharmaceutical Co Ltd., Beijing, China), according to the manufacturer's instructions.

ELISA of pro-inflammatory cytokines

The serum concentrations of pro-inflammatory cytokines in the rats were measured by ELISA, according to the

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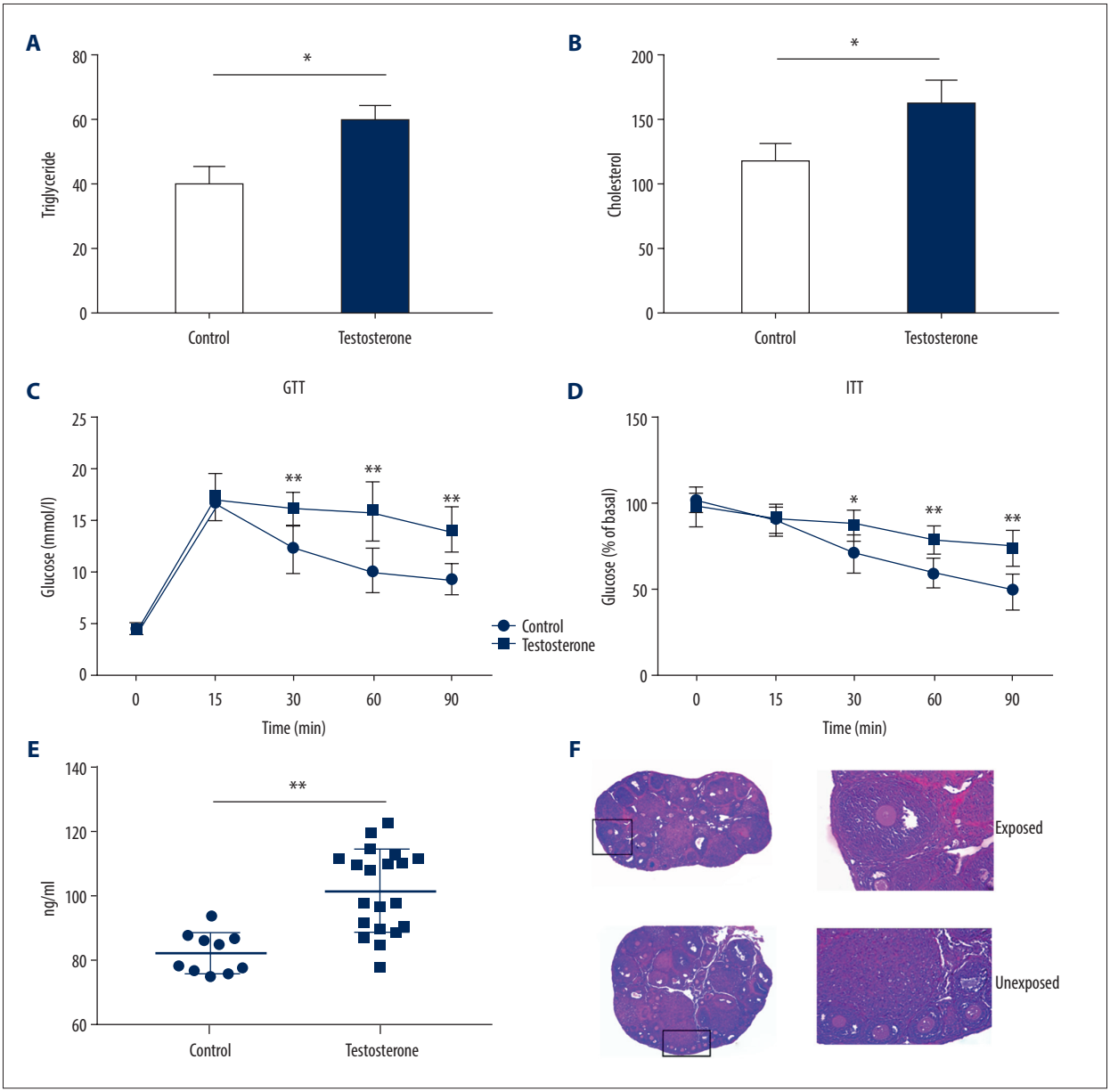
manufacturer's instructions. The ELISA kits used for detection of serum levels of cytokines included a rat interleukin (IL)-1β ELISA kit (Abcam, Cambridge, UK), a rat IL-6 ELISA kit (Abcam, Cambridge, UK), a rat IL-18 ELISA kit (Abcam, Cambridge, UK), a rat JE/CCL2/MCP-1 Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA), and a rat tumor necrosis factor-α (TNF-α) ELISA kit (Abcam, Cambridge, UK).

Ovarian histology and follicle counts

After blood collection and metabolic evaluation in female offspring, ovaries from offspring were removed, weighed, and fixed in Bouin's solution. After tissue fixation for up to six hours, the tissue samples were dehydrated and embedded in paraffin wax, and tissue sections were cut at 5 μm. Every fifth tissue section was stained with hematoxylin and eosin (H&E). Follicle counting and classification was performed using histology, as previously described [30]. Only follicles containing an oocyte nucleus were counted.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software (IBM, Chicago, IL, USA). Data were expressed as the mean±standard deviation (SD) of three independent experiments. The differences between the two groups were analyzed using an unpaired t-test. Comparisons of more than two groups or repeat data were performed with a one-way or



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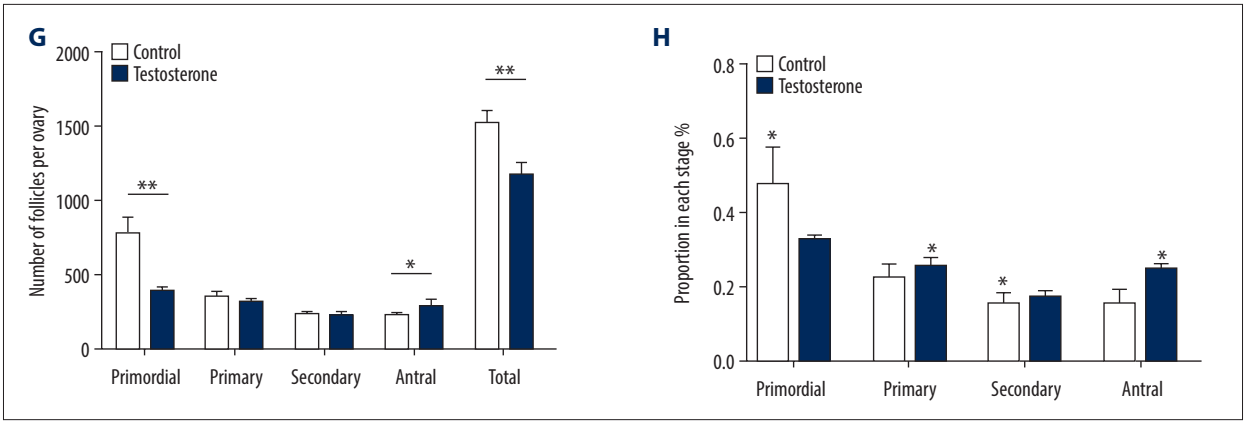

Figure 1. Changes in maternal metabolism and changes in phenotype in the offspring of the rat model of polycystic ovary syndrome (PCOS). Increased serum levels of triglyceride (**A**) and cholesterol (**B**) in rats treated with androgen. (**C, D**) Results of the insulin tolerance tests, and (**C**) the glucose tolerance tests (**D**) of maternal rats treated with androgen. (**E**) The representative ovarian histology is shown in in the offspring with fetal exposure to androgen and the controls. Fewer primordial follicles and more antral follicles are present compared with the controls. (**F**) The number of ovarian follicles at each stage in the female offspring. (**G**) The proportion of ovarian follicles at each stage in the female offspring. A lower percentage of primordial follicles and a higher percentage of other follicular stages in the female offspring with fetal exposure to androgen compared with the controls. (**H**) The serum concentration of testosterone in the maternal rats administrated by androgen or not. Data are presented as the mean±SD (n=10). * $p<0.05$ and ** $p<0.01$.

two-way analysis of variance (ANOVA). P-values <0.05 were considered to be statistically significant.

Results

Change in the metabolism of the maternal rat model of polycystic ovary syndrome (PCOS) and the responses to androgens in the rat offspring

After persistent exposure to androgen in pregnant rats, blood was sampled from pregnant rats in the polycystic ovary syndrome (PCOS) rat model immediately after delivery for measurement of hormone levels. Significantly increased serum levels of triglyceride ($p=0.012$) and cholesterol ($p=0.017$) were present (Figure 1A, 1B) in rats in response to androgen. The results of the insulin tolerance tests and glucose tolerance tests of maternal rats with androgen exposure are shown in Figure 1C and 1D. The delay in the decline in levels of blood glucose and insulin resistance in the maternal rats treated with androgens was compared with the controls. The serum concentrations of testosterone in the maternal rats are shown in Figure 1E. Significantly increased serum concentrations of testosterone were found in the maternal rats following subcutaneous injection of testosterone ($p=0.003$).

In female offspring with fetal exposure to androgen, the representative ovarian morphology is shown in Figure 1F. Follicle counts showed that offspring with fetal exposure to androgen had significantly fewer primordial follicles ($p=0.005$) and more antral follicles ($p=0.024$) compared with the controls. There was also a significant increase in the number of total follicles in rats that had fetal exposure to androgens ($p=0.004$) (Figure 1G). However, analysis of the proportion of follicles at each stage showed that rats with fetal exposure to androgen had a significantly lower percentage of primordial follicles ($p=0.014$) and a significantly higher percentage of the other two stages of follicle development compared with the controls, including primary follicles ($p=0.034$) and antral follicles ($p=0.018$) (Figure 1H). These findings suggested that follicles were activated in rats with fetal exposure to androgen (Figure 1H).

Fetal exposure to high levels of androgen resulted in increased serum levels of hormones and pro-inflammatory cytokines in rat offspring on a high-fat diet

The influence of fetal exposure to high levels of androgen was determined on hormone secretion and endocrine homeostasis in the offspring of PCOS rats. The hormone levels of adult female rats (6–8 weeks) were examined after fetal exposure to high levels of androgen in the groups fed normal chow and a high-fat diet. In the high-fat diet group, the serum levels of testosterone ($p<0.001$), insulin ($p=0.002$), and leptin ($p=0.004$) significantly increased, but adiponectin levels ($p=0.005$) level significantly reduced in the female offspring with fetal exposure to androgen when compared with the controls (Figure 2A–2C). These data showed that fetal exposure to high levels of androgen resulted in changes in endocrine and metabolic homeostasis.

Serum levels of pro-inflammatory cytokines were measured, including interleukin (IL)-1β, IL-18, IL-6, MCP-1, and tumor

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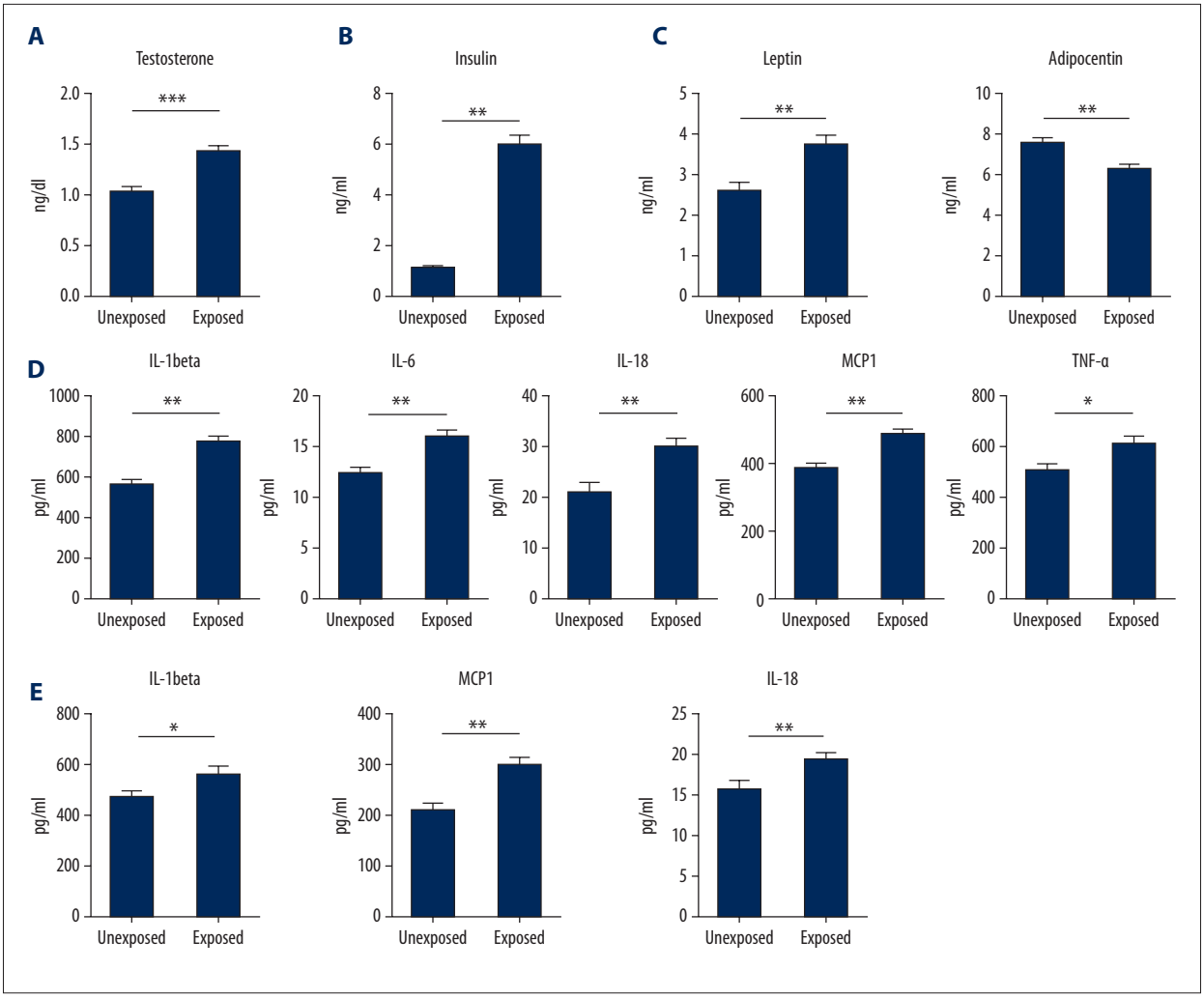

Figure 2. Increased levels of hormones and inflammatory cytokines in serum samples from the offspring of the rat model of polycystic ovary syndrome (PCOS) and controls. (**A–C**) Increased levels of hormones related to steroid and glycolipid metabolism. Increased levels of testosterone (**A**), insulin (**B**), and leptin and adiponectin (**C**) in the offspring of the rats in the PCOS model compared with the control. (**D**) Increased levels of inflammatory cytokines, including IL-1β, IL-18, IL-6, TNF-α, and MCP-1, in the offspring of the rats in the PCOS model on a high-fat diet compared with the control. (**E**) Increased levels of inflammatory cytokines, including IL-1β, IL-18, and MCP-1, in the offspring of the rats in the PCOS model with normal chow feeding compared with the control. Data are presented as mean±SD; * $p<0.05$ and ** $p<0.01$ by Student's t-test (n=10.) All experiments were performed in triplicate.

necrosis factor-α (TNF-α), using enzyme-linked immunosorbent assay (ELISA) (Figure 2D). Significantly increased levels of IL-1β (p=0.001), IL-6 (p=0.003), IL-18 (p=0.031), TNF-α (p=0.008), and MCP-1 (p=0.005) were found in the rats exposed to androgens *in utero* and fed a high-fat diet. In the normal diet group, increased serum cytokine levels, including IL-1β (p=0.041), MCP-1 (p=0.001), and IL-18 (p=0.002), were still found in the androgen-exposed offspring (Figure 2E). These results were consistent with the findings from a previous pilot clinical study that showed increased inflammatory cytokine levels in the offspring of women suffering from PCOS [10]. However, no difference was found in serum levels of hormones and inflammatory cytokines between the offspring exposed to increased androgen levels *in utero* and the controls in adult male rats (Tables 1, 2). These data suggested that adult female rats with fetal exposure to androgen had higher serum levels of inflammatory cytokines and hormonal abnormalities related to metabolic disorders when compared with controls.

Fetal exposure to high levels of androgen *in utero* resulted in metabolic disorders and obesity in rat offspring on a high-fat diet

To evaluate the metabolic phenotype in rats on a high-fat diet exposed to increased androgen levels *in utero*, the measurement of serum lipids, an insulin tolerance test, and an

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Table 1. Serum inflammatory cytokines in adult male offspring of the rat model of polycystic ovary syndrome (PCOS) on a high-fat diet that underwent *in utero* androgenization compared with the controls.

Cytokines	Androgenized offspring	Not-androgenized offspring	*P*-value
IL-1β (pg/ml)	423.25±62.36	421.56±72.30	0.31
IL-6 (pg/ml)	12.57±3.73	14.23±3.98	0.12
IL-18 (pg/ml)	18.75±4.23	19.32±3.89	0.27
TNF-α (pg/ml)	398.03±26.63	393.36±36.69	0.07
MCP-1 (pg/ml)	323.36±19.80	320.69±17.63	0.11

Serum cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) cytokine kit. The data are presented as the mean±standard deviation (SD). TNF-α – tumor necrosis factor-α; MCP-1 – monocyte chemotactic protein-1. There is no significant difference in the serum levels of cytokines between the two groups.

Table 2. Serum hormones levels in adult male offspring of the rat model of polycystic ovary syndrome (PCOS) on a high-fat diet that underwent *in utero* androgenization compared with the controls.

Cytokines	Androgenized offspring	Not-androgenized offspring	*P*-value
Insulin	0.56±0.16	0.61±0.14	0.12
Adiponectin	7.57±1.73	7.23±2.98	0.22
Leptin	2.36±2.23	2.68±1.89	0.09
Testosterone	86.69±16.63	89.36±20.63	0.17

Serum hormones levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit. The data are presented as the mean±SD. There is no significant difference in the serum levels of hormones between the two groups.

oral glucose tolerance test were performed in adult rats on a high-fat diet in the androgen exposed and non-exposed group. Impaired glucose tolerance and insulin resistance were found in female rats on a high-fat diet exposed to high fetal concentration of androgen (Figure 3A, 3B). Higher levels of triglyceride (p=0.035) and cholesterol (p=0.007) were found in the androgen-exposed female rats compared with the non-exposed rats (Figures 3C, 3D). The rat body weight was also increased in the androgen-exposed rats compared with the non-exposed rats (Figure 2E). There was no significant difference in serum levels of triglyceride (p=0.34) and cholesterol (p=0.023), insulin resistance, and body weight in the androgen-exposed and non-exposed male offspring (Figure 3A–3E).

These data illustrated that fetal exposure to androgen *in utero* only influenced the metabolic phenotype in female offspring of the PCOS model, and did not lead to the alteration of metabolic phenotype characterized by insulin resistance and hyperlipemia. In the rat group fed normal chow, there was a significant increase in body weight, impaired insulin resistance, and significantly increased serum levels of cholesterol (p=0.027) in the androgen-exposed female offspring compared with the non-exposed group (Figure 4A–4D). Consistent with the gender difference observed in hormone abnormalities and low-grade systemic inflammation in the PCOS model offspring on a high-fat diet, metabolic disorders also occurred only in female rats on a high-fat diet (Figure 4A–4D). These results showed that metabolic disorders predominantly occurred in female rats on a high-fat diet but not in male rats after exposure to high levels androgen *in utero*. Also, fetal exposure to high levels of androgen resulted in gender-associated metabolic disorders as shown by hormone abnormalities and increased systemic inflammation that was only significant in female rats.

Fetal exposure to high levels of androgen resulted in increased inflammation of adipose tissue and inhibited adipose tissue remodeling in female rat offspring

Because of the findings of significantly increased levels of pro-inflammatory cytokines and metabolic disorders observed in female rat offspring of the PCOS rat model, inflammation in the adipose tissue of the rats was investigated. The expression of pro-inflammatory cytokines in female and male rats showed that the expression of inflammatory cytokines in the androgen-exposed female offspring were significantly increased compared with the androgen-exposed male offspring and non-exposed female rat controls. These findings supported that exposure to androgens *in utero* led to aberrant inflammation in white adipose tissue (Figure 5A, 5B).

Androgens have previously been shown to promote lipolysis, reduce visceral fat accumulation, and adipocyte differentiation in adult male rats [15,16]. In the present study, the expression of uncoupling protein-1 (UCP-1), hormone-sensitive lipase (HSL), and adipose triglyceride lipase (ATGL), marker proteins of brown fat and key enzymes associated with lipolysis, were measured in the adipose tissue of fetal androgenized rats [17,18]. The findings showed that there was no difference in the expression of UCP-1 in androgen-exposed male rats compared with non-exposed rats, but the expression of HSL and ATGL significantly increased in androgen-exposed male rats (Figure 5C, 5D). However, in female offspring, the expression of HSL and ATGL showed no difference between the androgen-exposed and the non-exposed groups. These findings supported that androgen exposure had a positive effect on the lipolysis of adipose tissue in male rats,

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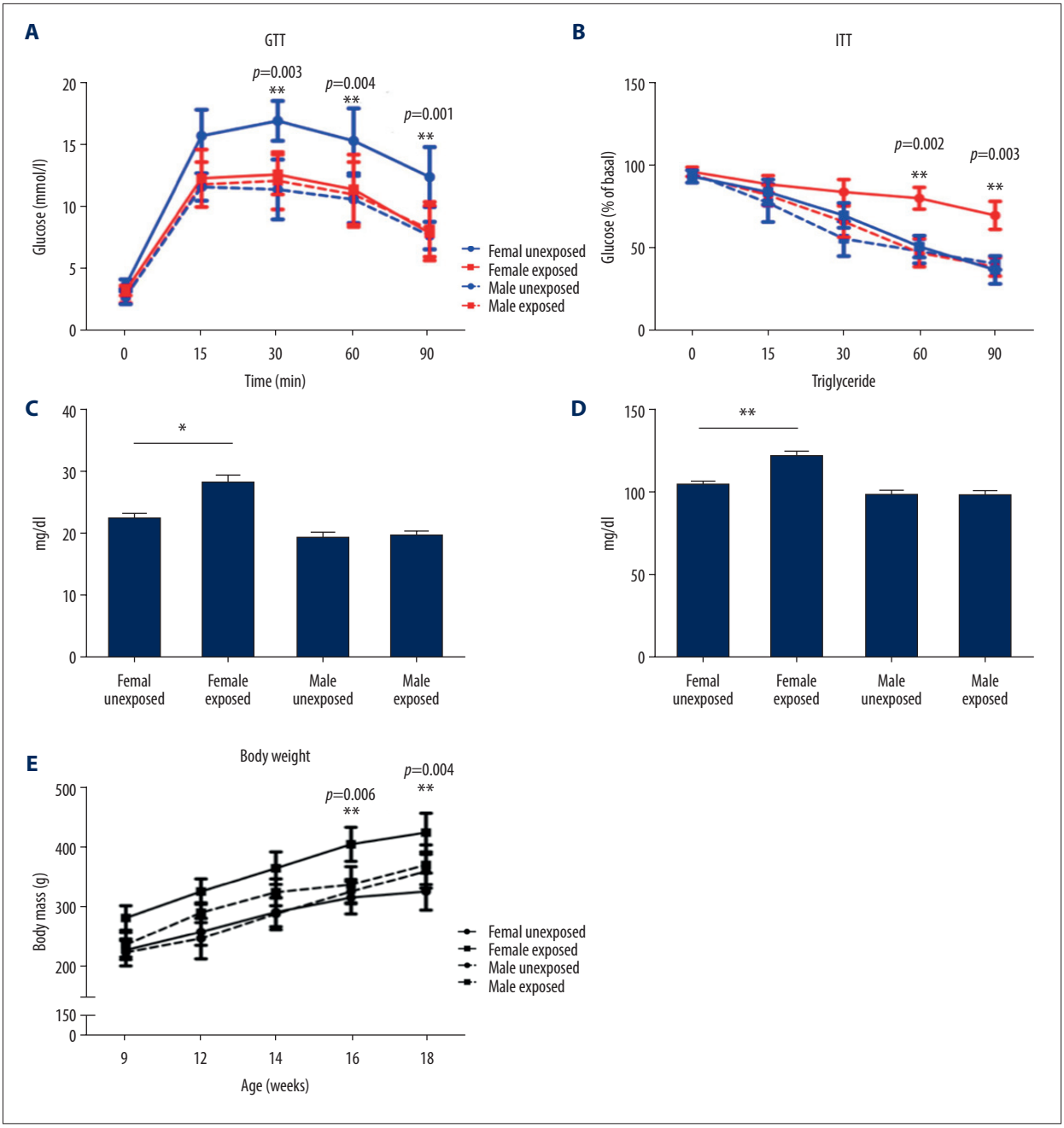

Figure 3. Insulin resistance, obesity, and hyperlipidemia induced by a high-fat diet in the offspring of the rat model of polycystic ovary syndrome (PCOS). The results of the glucose tolerance tests (**A**) and insulin tolerance tests (**B**) used for the assessment of insulin sensitivity in the offspring of the rats in the PCOS model with fetal exposure to androgen (blue) and the non-exposed control (red). Serum levels of cholesterol (**C**) and triglyceride (**D**) in the exposed and non-exposed group. There are significantly increased levels of lipid in the offspring of the rats in the PCOS model compared with the controls. (**E**) Increased body weight in the offspring of the rats in the PCOS model compared with the controls. The body weight was measured at 9, 12, 14, 16, 18, and 20 weeks. Data are presented as the mean±SD; * $p<0.05$ and ** $p<0.01$. Statistical analysis was performed using unpaired two-tailed Student's t-test or one-way or two-way analysis of variance (ANOVA) (n=10). All experiments were performed in triplicate.

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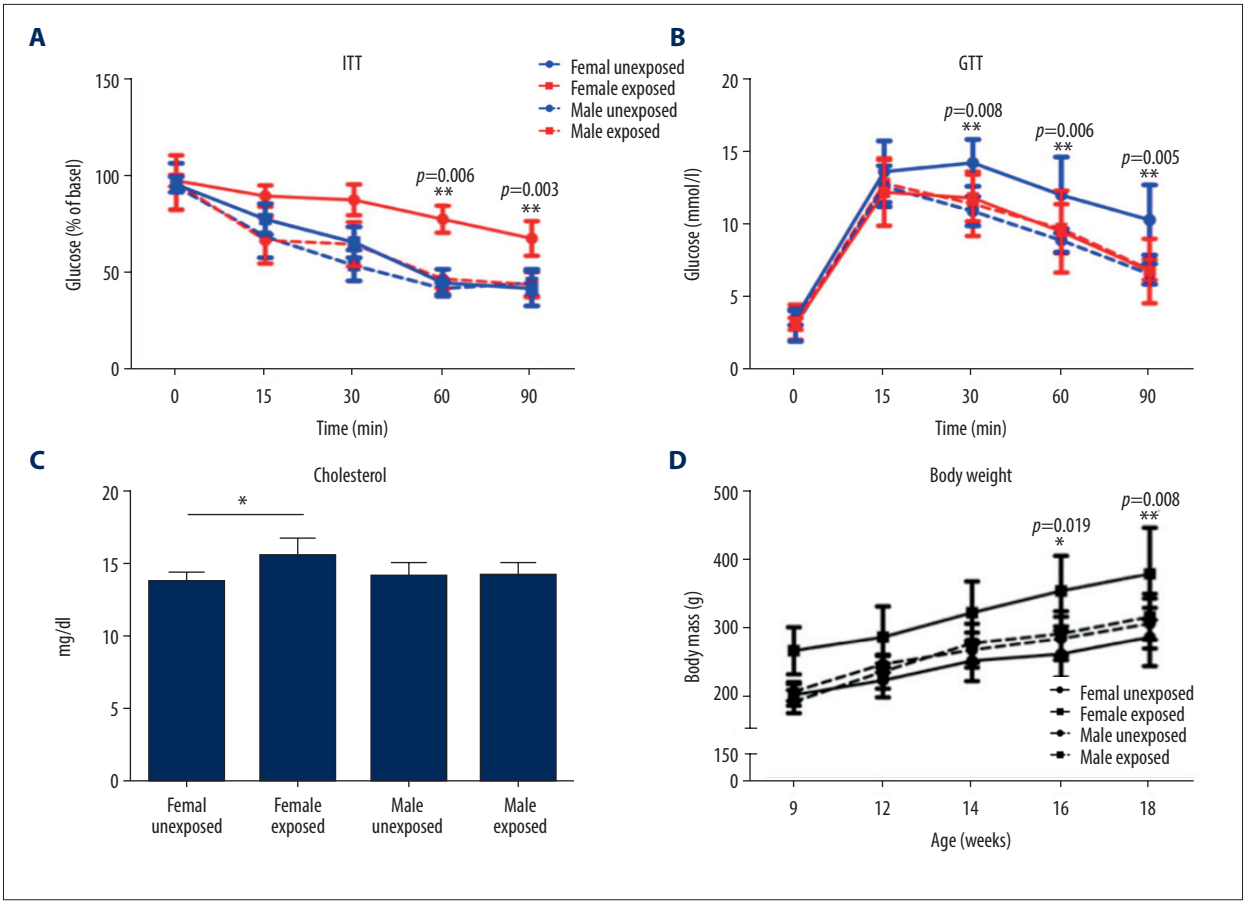

Figure 4. Insulin resistance, obesity, and hyperlipidemia on a normocaloric diet in the offspring of the rat model of polycystic ovary syndrome (PCOS). The results of the glucose tolerance tests (**A**) and insulin tolerance tests (**B**) used for the assessment of insulin sensitivity in the offspring of the rats in the PCOS model for the rats on a normocaloric diet with *in utero* exposure to androgen (blue) and the non-exposed control group (red). (**C**) The serum levels of cholesterol in the exposed and non-exposed group. The significantly increased levels of serum lipids in the offspring of female rats in the PCOS model compared with the controls. (**D**) Increased body weight in the offspring of female rats in the PCOS model compared with the controls. Data are presented as the mean±SD (n=10). **p* <0.05 and ***p* <0.01.

and this effect was reduced in female rats. In female rats, exposure to high levels of androgen *in utero* might be associated with the tendency to low-grade adipose tissue inflammation rather than the lipolysis of adipose tissue.

Treatment with antibiotics restored the metabolic phenotype in female rat offspring born to the PCOS rats

Previous studies have shown that dysbiosis of gut microbiota in female offspring of rats exposed to androgen in the late stage of pregnancy might be involved in the incidence of hypertension, increased body weight, and insulin resistance [7,8]. In the present study, persistent androgen exposure occurred in the whole fetal period due to androgenization of the pregnant PCOS rats. The administration of testosterone in the pregnant rats was not limited to the late stage of gestation. Broad-spectrum antibiotics and probiotics were used in female offspring for four weeks, commencing at two weeks following birth. Compared with the control group, antibiotics and probiotics reversed insulin resistance and reduced hyperlipidemia in the offspring with metabolic dysregulation induced by a high-fat diet (Figure 6A–6C).

The serum levels of pro-inflammatory cytokines and the expression of these cytokines in adipose tissue were significantly reduced after treatment with broad-spectrum antibiotics in adult rats with metabolic dysregulation induced by a high-fat diet following fetal exposure to androgen (Figure 6D). However, treatment with probiotics did not reduce the expression of cytokines in rat adipose tissue (Figure 6E). However, probiotics did not reduce insulin resistance and hyperlipidemia (Figure 7A–7C). The difference between control and probiotics group in body weight was insignificant either (Figure 7D). These data suggest that antibiotics may reduce low-grade systemic

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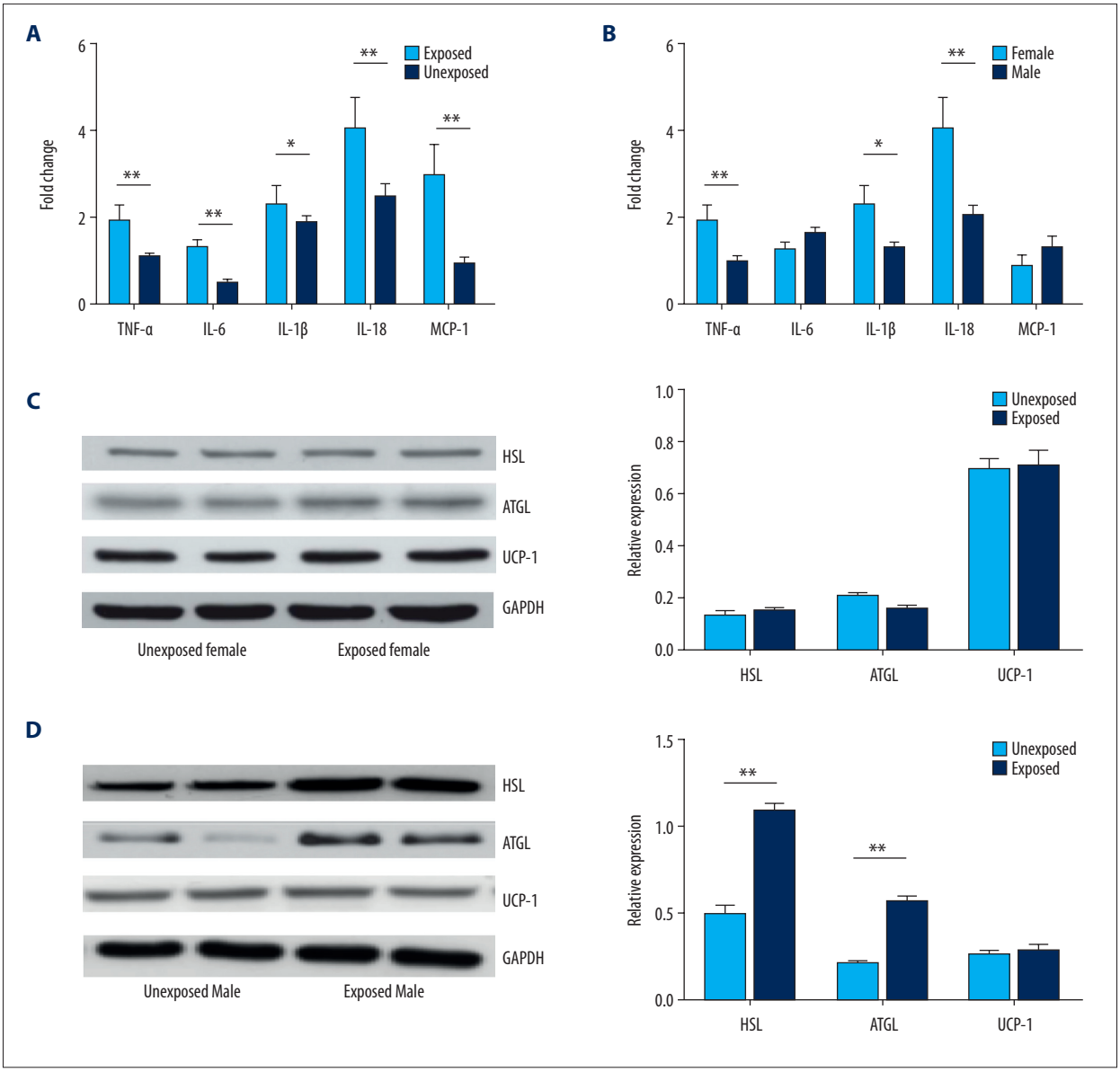

Figure 5. Expression of inflammatory cytokines, thermogenesis genes, and enzymes related to lipolysis in white adipose tissue from the offspring of the rat model of polycystic ovary syndrome (PCOS). (**A**) Increased expression of inflammatory cytokines measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in visceral white adipose tissue from the offspring of the rats in the PCOS model compared with female controls. (**B**) Increased expression of inflammatory cytokines measured by qRT-PCR in visceral white adipose tissue from the female offspring compared with the male offspring from the maternal PCOS rat model. (**C**) Unaltered expression of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and uncoupling protein-1 (UCP-1) in the offspring of the rats in the PCOS model and controls measured by Western blot. (**D**) Increased expression of HSL and ATGL in the offspring of female rats in the PCOS model compared with controls. Data are presented as the mean±SD; * $p<0.05$ and ** $p<0.01$. Statistical analysis was performed using unpaired two-tailed Student's t-test (n=5). All experiments were performed in triplicate.

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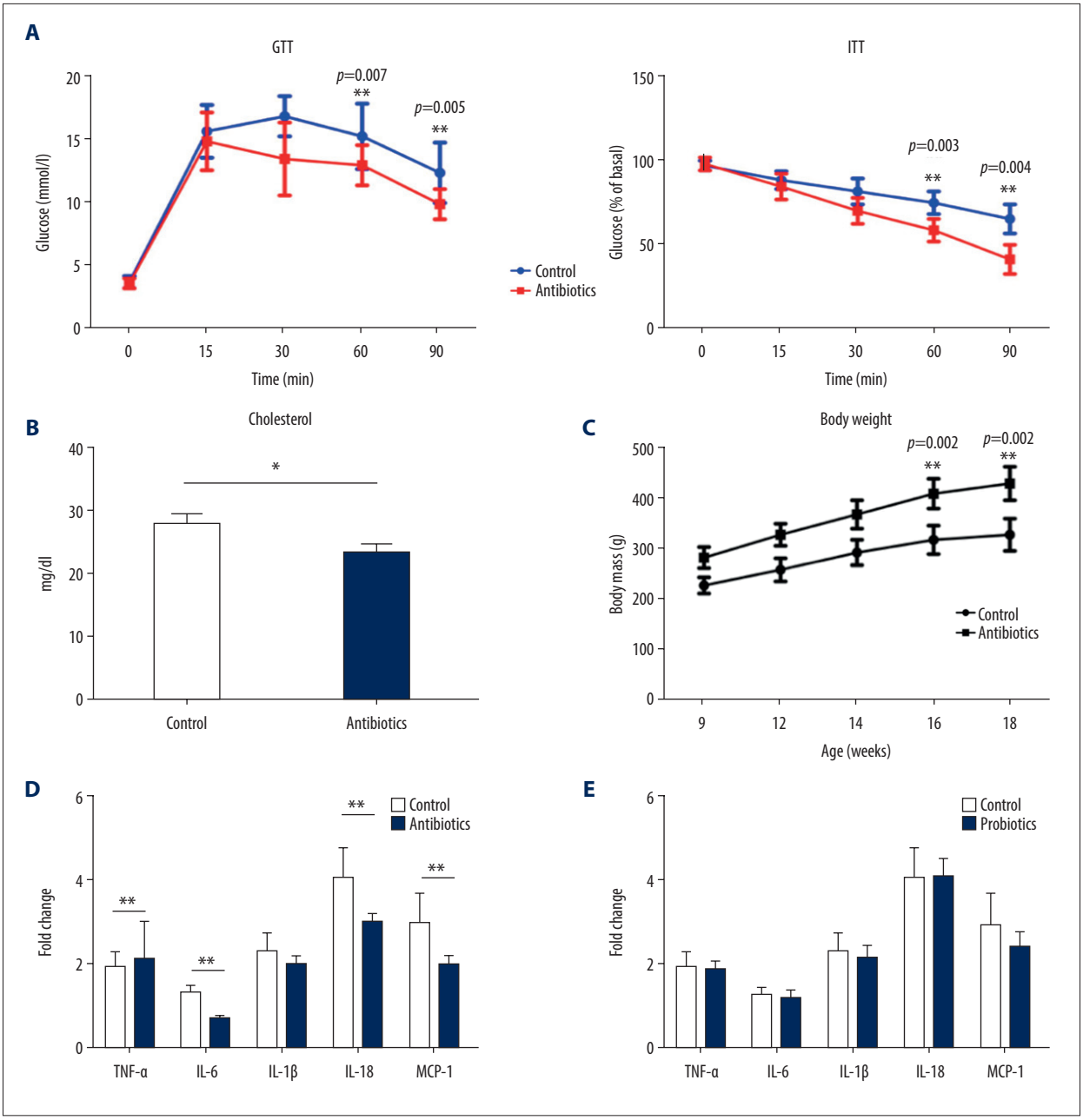

Figure 6. Antibiotics improved the metabolic disorders and reduced inflammation of adipose tissue in the offspring of the rat model of polycystic ovary syndrome (PCOS). (**A**) The administration of antibiotics improved insulin resistance. Glucose tolerance tests (**left**) and insulin tolerance tests (**right**) used for the assessment of insulin sensitivity in the offspring of the rats in the PCOS model treated with antibiotics (red) and controls (blue). (**B**) Reduced cholesterol levels in the offspring of the rats in the PCOS model treated with antibiotics compared with the controls. (**C**) Reduced body weight in the offspring of the rats in the PCOS model treated with antibiotics compared with the controls. Data are presented as the mean±SD; * $p<0.05$ and ** $p<0.01$. (**D, E**) Expression of inflammatory cytokines measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in visceral white adipose tissue from offspring of female rats in the PCOS model that received antibiotics and probiotics. Statistical analysis was performed using unpaired two-tailed Student's t-test or one-way or two-way analysis of variance (ANOVA) (n=10). All experiments were performed in triplicate.

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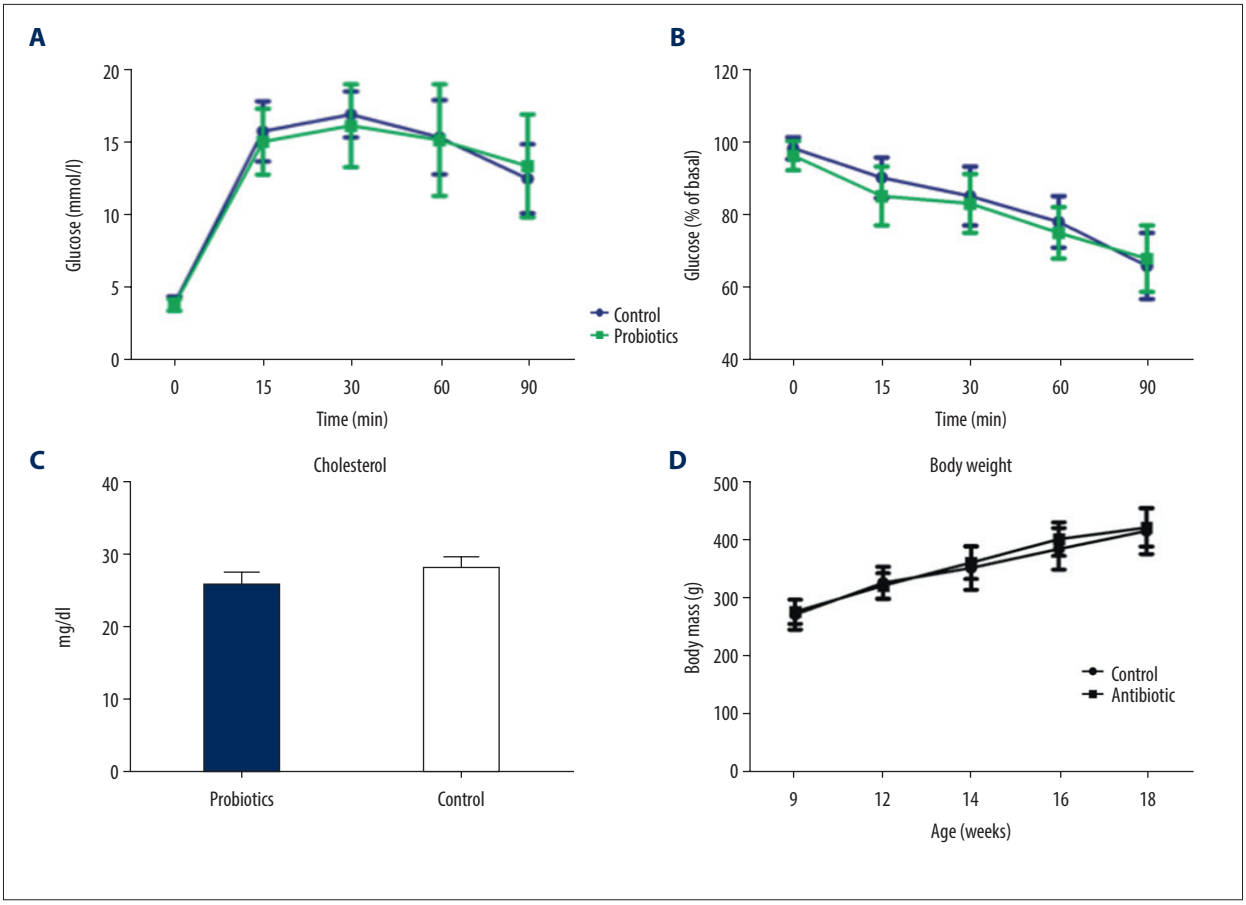

Figure 7. The administration of probiotics did not affect insulin resistance, obesity, and hyperlipidemia in the female offspring of the rat model of polycystic ovary syndrome (PCOS) on a normocaloric diet. The results of the glucose tolerance tests (**A**) and insulin tolerance tests (**B**) used for the assessment of insulin sensitivity in the offspring of the rats in the PCOS model treated with probiotics (green) and the control group (blue). (**C**) The serum levels of cholesterol. (**D**) The body weights. Data are presented as the mean±SD (n=10). * $p<0.05$ and ** $p<0.01$.

inflammation and metabolic disorders in female offspring, probably because of the effects of antibiotics on pro-inflammatory gut bacteria. Probiotics did not improve the phenotype in female offspring, indicating that beneficial gut bacteria might not be able to function in female offspring with persistent fetal exposure to high androgen levels.

Discussion

Polycystic ovary syndrome (PCOS) is a common condition in women of childbearing age that results in maternal and child health problems. Hyperandrogenism, hypertension, and insulin resistance usually manifest in pregnant women suffering from PCOS, resulting in an increased incidence of complications, but also in increased fetal morbidity and mortality [19–21]. Also, the offspring of women with PCOS have an increased risk of metabolic and endocrine disorders, compared with the offspring of healthy mothers, including insulin resistance and hyperandrogenism, which supports current theories on the fetal origin of some adult diseases [21,22].

Previous studies have proposed that fetal programming of the PCOS phenotype may be associated with the effects on target organ development, future reproduction, and the metabolic effects of aberrant epigenetic changes following exposure *in utero* to high levels of androgens [23,24]. For example, early exposure to male hormones shifts the balance of androgen and estrogen, altering the fetal differentiation of target organs toward a male phenotype. Following fetal exposure to androgens, ovarian structure and functional changes with ovarian theca cells that morphologically resemble Leydig cells [23]. Also, fetuses with early exposure to androgens show changes in global methylation and glucose homeostasis [23]. However, the mechanism underlying the associations between fetal exposure to androgen and metabolic disorders remains unclear. The changes in non-ovarian target organs and tissues involved in glucose and lipid metabolism also remain poorly understood.

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Recent studies have shown that the offspring of mothers with PCOS have systemic changes of inflammation and also undergo dysbiosis of gut microbiota that results in metabolic changes [6,7,25,26]. Dysbiosis, or dysbacteriosis, is an abnormal interaction between the intestinal microbiota and the host cells due to altered microbial diversity. In a previously published pilot clinical study, increased serum levels of inflammatory cytokines were observed, not only in women with PCOS but also in their offspring [10]. The findings from the present study also showed that an increase in inflammation was associated with metabolic disorders in the offspring of female rats in the PCOS model. Therefore, serum levels of pro-inflammatory cytokines were measured in the offspring of the PCOS rats to investigate the findings described previously. The findings from the present study showed increased levels of pro-inflammatory cytokines in the female offspring of the PCOS rat compared with the controls, regardless of feeding with a high-fat diet or normal rat chow. Increased expression of inflammatory cytokines was found in adipose tissue, and fetal exposure to androgen significantly increased the expression of cytokines in adult female rats. These data demonstrated that fetal exposure to androgen-induced inflammation in the adipose tissue of adult female rats, which might lead to adverse effects on the metabolic phenotype.

In the present study, the cause of increased systemic and adipose tissue inflammation was investigated. Prenatal (late trimester) and neonatal exposure to androgen led to dysbiosis of gut microbiota, as previously reported [6,7]. Previous studies have shown that the administration of dehydroepiandrosterone upregulated the expression of proteins associated with lipolysis and thermogenesis, including adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), in adult male rats, indicating that androgens influenced lipolysis and fat accumulation [16]. Therefore, in this study, the expression of ATGL and HSL in white adipose tissue from rats after fetal exposure to androgen were measured. Fetal androgen exposure in male offspring of PCOS rats did not influence on brown adipose tissue but could potentially promote lipolysis. These results are consistent with a previous study that showed androgen treatment in male rats upregulated the expression of enzymes related to lipolysis [16]. However, in the present study, exposure to androgens occurred *in utero* in fetal male rats and not in adult male rats. The findings in the female rat offspring differed from the male offspring, which was a finding supported by the increase in visceral fat in the offspring of the PCOS rat model, as the fetal exposure to androgen in female rats did not influence fat lipolysis.

Dysbiosis of the gut microbiota was detected in the offspring of PCOS rat models in two recent studies [6,7]. Dysbiosis of the gut microbiota might be related to hypertension and metabolic disorders observed in adulthood [6]. Clinically, previous studies have shown an increased proportion of *Firmicutes* to *Bacteroidetes* and reduced bacterial diversity in neonatal androgenized groups, resulting in obesity and metabolic syndrome [27,28]. Small-molecule metabolites and pathogen-associated molecular patterns (PAMPs) originate from Gram-negative bacteria that are resident in the gut and included lipopolysaccharide and ethanol, which lead to disruption of the gut barrier [27,28]. Following inflammation and damage to the gut, bacterial translocation and persistent low-grade inflammation in the liver and adipose tissue, accompanied by subclinical systemic inflammation may occur, as shown by increased serum levels of pro-inflammatory cytokines [27,28]. Therefore, the dysbiosis of gut microbiota observed in female rats in the present study may explain the findings of systemic inflammation and metabolic disorders in the offspring of rats in the PCOS model. This study also investigated whether changes in gut bacteria had an effect on the metabolic phenotype in the offspring of female rats in the PCOS model by the administration of broad-spectrum antibiotics. To exclude the influence of a high-fat diet on gut microbiota, the rats used to evaluate the therapeutic effect of antibiotics or probiotics were fed with a normal chow diet. The administration of antibiotics rather than probiotics reversed metabolic changes and reduced the changes of systemic inflammation. It may be proposed that the effects of beneficial gut bacteria are dependent on sex hormones as specific bacteria have different effects in male and female animals. This hypothesis is supported by the findings from previous studies that have shown a protective effect of segmented filamentous bacteria against the development of type 1 diabetes in female mice, but not in male mice [29].

A previous study showed that the effect of the gut microbiota on autoimmune and inflammatory responses was different in male and female mice in a model of type 1 diabetes, and these differences were associated with gender differences in the microbial profile of the gut [29]. The transfer of feces from male donors to female recipients was shown to protects non-obese diabetic female mice from type 1 diabetes, as shown by reduced pancreatic islet inflammation and reduced levels of autoantibodies [13]. Sex hormones may influence the gut microbiota indirectly by inflammatory mechanisms or directly by regulation of metabolites and maintenance of the integrity of the barrier function of the gut. Also, the findings from the present study showed that fetal androgenization promoted lipolysis in adult male rats but did not have the same effect on lipid metabolism, which could not reduce the visceral fat accumulation and metabolic disorders that were present in female rat offspring. Therefore, the difference in gut microbiota between males and females might explain why male rat offspring did not suffer from metabolic disorders in this study. However, further studies are needed to investigate the effects of gender on gut microbiota and metabolism and systemic inflammation.

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The findings from this study showed that the intestinal dysbiosis was associated with systemic inflammation and the development of a PCOS-like metabolic phenotype in the female offspring of maternal PCOS rats. However, this study had several limitations. The findings in animal models of human disease should be interpreted with caution and should be validated with further studies, including clinical studies. In the rat model of PCOS, the rat fetus *in utero* may have been exposed to other factors that altered the intrauterine microenvironment, which might have affected embryonic development instead of or in addition to intestinal dysbiosis. These other adverse factors that may have been inherent in the animal model were not studied or excluded. Also, in this study, the difference in the gut microbes between male and female offspring was not investigated. Because the administration of antibiotics improved the PCOS-like phenotype in the female rat offspring, indirect evidence supported that the dysbiosis of gut microbes might be correlated with the PCOS-related systemic inflammation and metabolic abnormalities. Therefore, future studies are needed to investigate the mechanisms involved in the relationship between specific gut bacteria and the development of metabolic and inflammatory changes in the offspring of mothers with PCOS, particularly in female offspring.

Conclusions

This study aimed to investigate the metabolic effects of dysbiosis of the gut microbiota in the newborn rat following exposure *in utero* to increased levels of maternal androgens in a rat model of maternal polycystic ovary syndrome (PCOS). The findings showed that alteration in the gut microbiota and the increased systemic inflammatory response were associated with metabolic disorders in the offspring of female rats in the PCOS model. Metabolic dysfunction in the female offspring of rats in the PCOS model was reversed using antibiotic treatment, which also reduced the degree of inflammation in rat adipose tissue. Because antibiotics improved the metabolic phenotype in female offspring of the PCOS rat model, therapeutic modification of the gut microbiota may be a potential therapeutic target for metabolic diseases of fetal origin.

Conflict of interest

None.

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