Original Article

Testosterone Induced Wistar Rat Model for Gut Microbiota Dysbiosis of Polycystic Ovarian Syndrome Research

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Abstract

Introduction: *Gut microbiota modification based on dysbiosis of gut microbiota dysbiosis (DOGMA) theory may provide a new therapy approach in polycystic ovarian syndrome (PCOS). Research of this new therapy needs a suitable animal model thus this study was aimed to investigate whether Wistar rats that were injected by testosterone propionate (TP) could induce both PCOS and gut microbiota dysbiosis condition.*
Methods: *Design of this study was post-test only control group design randomized control trial. Wistar rats were divided into two groups: control and TP. Blood, faecal and ovarian tissue sampling also vaginal smear were obtained after 28 days of TP injection.*
Results: *TP group had testosterone concentration, preantral follicle and fasting blood glucose concentration higher than control group ($p=0.047$, $p=0.018$, $p=0.032$). Fasting insulin, HOMA-IR value, serum zonulin level, TNF-α concentration and gut microbiota diversity were not significantly different*
Conclusion: *TP injection intramuscularly (10 mg/kgBW) for 28 days succeeded to induce PCOS and hyperglycaemia in Wistar rat but was failed to induce insulin resistance, low grade inflammation, impaired gut permeability, and gut microbiota dysbiosis thus it's not suitable as animal model for gut microbiota dysbiosis research in PCOS.*

Keywords: *Animal model, Dysbiosis, Hyperglycaemia, Polycystic ovarian syndrome*

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Tikus Wistar yang Diinjeksi Testosterone Propionate Sebagai Hewan Coba Penelitian Mikrobiota Usus pada Sindrom Ovarium Polikistik

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Abstrak

Pendahuluan: *Modifikasi mikrobiota usus berdasar teori dysbiosis of gut microbiota (DOGMA) dapat memberikan pendekatan terapi baru pada sindrom ovarium polikistik (SOPK) dan penelitiannya memerlukan model hewan yang sesuai sehingga penelitian ini bertujuan untuk mengetahui apakah tikus Wistar yang disuntik testosteron propionat (TP) dapat memicu manifestasi klinis SOPK dan disbiosis mikrobiota usus.*
Metode: *Desain penelitian ini adalah post-test only control group design dengan randomized control trial. Tikus wistar dibagi menjadi dua kelompok yaitu kontrol dan TP. Pengambilan sampel darah, feses dan jaringan ovarium serta apusan vagina diperoleh setelah 28 hari injeksi TP.*
Hasil: *Kelompok TP mempunyai kadar testoteron, jumlah folikel preantral dan kadar gula darah puasa lebih tinggi daripada kelompok kontrol (p=0,047, p=0.018, p=0.032). Kadar zonulin serum, TNF-α, insulin puasa, HOMA-IR dan diversitas mikrobiota usus antara kelompok kontrol dan kelompok TP berbeda tidak bermakna.*
Kesimpulan: *Pemberian TP secara intramuskular dengan dosis 10 mg/kg BB selama 28 hari berhasil memicu manifestasi klinis SOPK dan hiperglikemia pada tikus Wistar namun gagal menginduksi resistensi insulin, inflamasi derajat rendah, gangguan permeabilitas usus dan disbiosis mikrobiota usus sehingga tidak dapat digunakan sebagai model hewan untuk penelitian disbiosis mikrobiota usus pada SOPK.*

Kata kunci: *Hewan coba, Disbiosis, Hiperglikemia, Sindrom ovarium polikistik*

Introduction

Polycystic ovarian syndrome (PCOS) is an endocrinopathy with three major clinical manifestations such as anovulation, hyperandrogenism and polycystic ovary morphology that is occurred in 15-20% women in reproductive age.[1] Rotterdam consensus in 2003 stated that PCOS diagnosis require two of three clinical manifestation presents in patients. This syndrome leads to female reproductive dysfunction, metabolic dysfunction, and even endometrial cancer. PCOS etiopathogenesis may comprise genetic, epigenetic, and abnormal maternal environment hormonally (hyperandrogenism) and metabolically (hyperinsulinism). Environment factor including unhealthy lifestyle such as high fat, high sugar and low fibre diet may leads to PCOS. New theory involving in this condition is called dysbiosis of gut microbiota (DOGMA) theory.[2]

This theory highlighted that PCOS patients tend to have obesity and high fat intake, high sugar diet, low fibre diet that favour the growth of harmful gram negative bacteria and reducing beneficial bacteria growth such as

Bifidobacteria and Lactobaccilus. This condition also increase gut permeability (regulated by zonulin) due to decreasing tight junction function (leaky gut) Lipopolysaccharide (LPS) contained gram negative bacteria could enter to blood circulation because of leaky gut and induce natural immune system in gut and systemic circulation. It's also mediated an increase of serum tumor necorisis factor α (TNF-α) and interleukin-6 (IL-6) that associated with insulin resistance especially TNF-α that can activate c-Jun N-terminal kinase 1 (JNK1) and nuclear factor κB (NF-κB) which leads to serine phosphorylation on the insulin receptor substrate-1 (IRS-1) protein. This will prevents IRS-1 interaction with beta subunit insulin receptor and causing insulin resistance and hyperinsulinemia and leads to PCOS.[2,3] This new DOGMA theory give a new insight about PCOS pathogenesis that usually heavily weighted on hyperandrogenism condition and provide an alternate approach in PCOS therapy beside lifestyle modification therefore a suitable research model needed.[4]

Research in human has limitations such as difficulty to obtain faecal sample in ileo-caecal region and standardized diet in each participants and assessment of gut microbiota in the ileo-caecal region can only be obtained by performing surgery on experimental animals and it's difficult to obtain in human because its needs an invasive procedure such as endoscopy.[5] There are large similarities between the rodents and humans at metabolic level thus rodents are suitable models to investigate gut microbiota dysbiosis and other metabolic parameters in PCOS because animal models will have similar in food intake that difficult to control in human.[6] Han, et al[7] found that dehydroepiandrosterone (DHEA) treatment injections for 35 days succeed to induce the PCOS major clinical manifestations and gut microbiota dysbiosis in Sprague Dawley rats. Animal models of PCOS may has limitations but the use of appropriate animal models may leads to discovery, validation, and optimization of novel biomarkers and treatments for women with PCOS thus this study was aimed to investigate whether Wistar rats injected by testosterone propionate (TP) with lesser dose and period could induce gut microbiota dysbiosis measured with Firmicutes/Bacteroidetes ratio and alpha diversity, increasing gut permeability measured by zonulin level, inflammation state measured by TNF-α level, insulin resistance measured by HOMA-IR value and clinical manifestations of PCOS (oligo-anovulation, hyperandrogenism and polycystic ovaries).

Methods

Experimental Design

This was a post-test only control group design randomized control trial involving two groups: Control and TP (testosterone group). Androgen excess is proven to induce PCOS traits, previous research used TP successfully induce PCOS clinical manifestations such as multiple cystic follicles, increased serum testosterone level, anovulation and increased fasting glucose and insulin concentration.[8] Wistar rats (*Rattus norvegicus*) aged 8 weeks with mean weight of 100 grams were housed under-regulated lighting (12-hour light/dark cycle) and given standard meal (10.3% fat, 65.5% carbohydrate, and 24.2% protein) ad libitum. Control group is given standard meal and aquades ad libitum without any injection. TP group is given standard meal, aquades and daily testosterone propionate (TP) intramuscularly injection (Wonderindo Pharmatama, Jakarta, Indonesia) with dosage of 10 mg/kg BW for 28 days which begin at estrous phase. Experiments were conducted according to the ethics, guidelines and animal care by Health Research Ethics Committee of Faculty of Medicine, Diponegoro University, Indonesia (No. 138/EC/H/FK-UNDIP/XII/2021).

Collection of samples

Blood sampling for serum zonulin concentration, TNF-α concentration, fasting blood glucose, fasting insulin, and serum total testosterone concentration were obtained and measured using enzyme-linked immunosorbent assay (ELISA) kit. Ovarian tissue samples were obtained after rats were sacrificed and then were fixed in 10% neutral buffered formalin and stained with haematoxylin and eosin (HE) then were analysed by microscope. Ovarium morphological changes were defined by preantral follicles. Vaginal smears were obtained at the start and the end of experiment and evaluated with microscope using Giemsa staining for estrous cycle determination.

At the end of the experiment, the entire intestinal tract was removed from mice, and faecal contents from the ileo-caecal region were collected aseptically. All samples were snapped frozen in liquid nitrogen and stored at - 80°C immediately after collection. Faecal samples were homogenized, then bacterial DNA was extracted using the Favor PrepTM Stool DNA isolation Kit (Favorgen Biotech, Ping-Tung, Taiwan, China). DNA concentration was calculated by Maestrogen nanodrop machine

and RT-PCR amplification and detection were performed with the CFX96 Real Time System. A triplicate PCR reaction was performed on 20 µL (total volume) mixture of 10µL Thunderbird SYBR Green Real Time PCR Master Mix (TOYOBO Co. Ltd., Osaka, Japan), 2µL each of the specific primers at a concentration 0.3µM, DNA template and nuclease free water (NFW) were adjusted to a volume of 20µl. The temperature settings used for amplification were 50°C for 2 minutes for 1 cycle, 95°C for 10 minutes for 1 cycle, 40 cycles at 95°C for 15 seconds, annealing temperature was 60°C for 30 seconds, and then were followed by 45 seconds at 60°C then 1 cycle at 72°C for 5 minutes.

Automated ribosomal intergenic spacer analysis(ARISA) method was used to measure the length heterogeneity of the bacterial rRNA operon 16S–23S intergenic spacer to estimate microbial richness and diversity or in this study, Firmicutes/Bacteroidetes ratio. This procedure was begun with diluting PCR master mix reagents, primers, and DNA isolate then vortexed the mixture for 15 seconds and prepared the thermocycler machine. The cycle and temperature settings were set as follows: 1 cycle at 98C for 45 seconds, 30 cycles at 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 1 cycle at 72°C for 5 minutes. PCR results were read on capillary gel electrophoresis to check fragments using electrophoresis using agarose gel with a concentration of 1.5% and sent for further analysis.

Statistical Analysis

Statistical analysis was conducted using Fisher Exact, T-test and Mann-Whitney U to determine the significance of the differences between each parameter. Significant difference determined by p-value less than 0.05. SPSS Version 26 was used to analyse research data.

Result

PCOS Clinical Manifestations In Animal Model

PCOS was defined by Rotterdam criteria that required two of three manifestations including anovulation, hyperandrogenism, and polycystic ovary morphology. Serum total testosterone concentration in study groups were significantly difference as shown in table 1. Control group had significantly lower serum total testosterone concentration compared to TP group (p=0.037), this showed that the PCO model had hyperandrogenism. Estrous cycle was obtained by vaginal smear at day one and in the end of experiment showed that in TP group, and all Wistar rats had anovulation cycle. Fisher Exact test results showed that the anovulation status between research groups were not significantly different and thus the ovulation status between study groups were found to be similar. Preantral follicles count was used to determine ovarian morphological abnormalities and the result was there's significant difference between study groups. Control group had significantly lower preantral follicles count compared to TP group (p=0.009) and this showed that TP group had polycystic ovary.

Table 1. PCOS Clinical Manifestations in Research Groups

	Control	TP	*p*-value
TT (ng/ml)	2.9± 2.01	7.1±4.07	0.047*
PA	8.7±5	17.3±5.6	0.018**
ANOV	4	6	0.455***

Data were presented as mean ± standard deviation except for ANOV, it was presented as number of rats that had an anovulation cycle. TT: serum total testosterone; PA: preantral follicles; ANOV: anovulation cycle. *Mann-Whitney U; **T-test; ***Fisher Exact; statistically significant if p-value less than 0.05.

Metabolic Parameters in Animal Model

Serum zonulin concentration used for measuring gut permeability and in this study was no different between control and TP group (p=0.847) as shown in table 2. TNF-α concentration in control group was lower than TP group but was not significantly different (p=0.938). HOMA-IR value was resulted of multiplying fasting glucose concentration (mg/dl) and fasting insulin concentration (mIU/L) then divided by 405. Fasting blood glucose concentration in control group as lower than TP group (p=0.032). Fasting insulin concentration and HOMA-IR value in control group were lower than TP but the difference was not significant (p=0.332 and p=0.107 respectively).

Gut Dysbiosis Microbiota in Animal Model

Firmicutes abundance in control group was lower than TP group but there's no difference between control and TP group (p=0.423). Bacteriodetes abundance in control group was

Table 2. Metabolic Parameter in Research Group

	Control	TP	*p* value
BW pre injection (gram)	88±16.7	101.5±15.41	0.176*
BW post 28-day injection (gram)	125.2±12.4	124.5±12.88	0.929*
Serum zonulin (pg/mL)	303±120.36	288.9± 127.30	0.847*
TNF-α (pg/mL)	78.6± 7.52	79.4± 4.11	0.938*
FBG (mg/dL)	122.5±9.46	134.7±7.31	0.032*
FINS (mU/L)	3.1± 0.51	3.4± 0.65	0.332*
HOMA-IR	0.9± 0.19	1.1± 0.23	0.107*

Data were presented as mean ± standard deviation. BW: body weight; TNF-α: tumour necrosis factor alpha; FBG: fasting blood glucose; FINS: fasting insulin; HOMA-IR: homeostasis model assessment for insulin resistance index. *T-test; Statistically significant if p-value less than 0.05

similar with TP group but it was not significantly different (p=0.990). Firmicutes/Bacteroidetes Ratio between two groups were similar (p=0.482). Alpha diversity could be seen with four parameters, namely Observed, Chao1, Shannon, and Simpson. Species richness was analysed by observed species and Chao 1 value. Observed species value in the control group was higher than TP group but the difference was not significant (p=0.072). Chao1 value in the control group was higher than TP group but there's no significant different. (p=0.075). Shannon index and Simpson index were used to define diversity. Shannon index in in the control group was higher than TP group although the differences among groups were not significantly different (p=0.093). Simpson index closer to 0 defined that there were abundant species but there's no difference in this index among groups (p=0.423) that meant the abundant species in all groups was the same (Table 3).

Discussion

The animals model used in PCOS research usually required characteristics such as easy/clearly identified reproductive cycle, anatomical and physiological appearance were similar to human phenotype, and must be in accordance with the objectives and hypothesis. Rodents are the most used animal models in PCOS research because of their small size, short lifespan, high reproductive index, and varied genetic strains. PCOS research recently emphasize in gut microbiota dysbiosis modification which involving daily intake and probi-

Table 3. Gut Microbiota in Research Group

	Control	TP	*p*-value
Firmicutes (log CFU)	4.882.69	6.51±1.34	0.423*
Bacteroidetes (log CFU)	5.050.94	5.04±0.64	0.990**
Firmicutes/ Bacteroidetes Ratio	1.06±0.71	1.28±0.14	0.482**
Alpha diversity			
Observed	10.8±1.94	7.7±3.33	0.072**
Chao1	10.3±0.50	8.5±3.15	0.075*
Shannon	1.6±0.32	1.1±0.61	0.093**
Simpson	0.8±0.06	0.7±0.22	0.423*

Data were presented as mean ± standard deviation. log CFU: log colony forming unit. *Mann-Whitney U; **T-test; Statistically significant if p-value less than 0.05

otics, prebiotics and synbiotics. The advantage of using animal models in dysbiosis of PCOS is that we can standardized animal models food intake compared to human. Examining gut microbiota dysbiosis needs ileo-caecal faecal sampling which is easier to obtain in animal models than in human.[8,9]

PCOS can be induced in rodent through drug administration, constant light exposure, or with transgenic technology. This study used daily testosterone propionate (TP) intramuscular injection with 10 mg/kg BW for 28 days at estrous phase to induce PCOS clinical manifestations and Wistar rats as animal models of PCOS. The assessment of successful PCOS model is determined based on whether two of the three Rotterdam criteria found including ovulation status, number of follicles, and serum testosterone level.[1] Number of preantral follicles and testosterone level in TP group was significantly higher than control group but ovulation status cycle was similar between groups. This condition possibly was because examination of vaginal smear was only conducted in day one and last day of experiments. Nevertheless, the Wistar rat model which was injected with testosterone 10mg/kg BW was successful as PCOS model because it met the requirements for a PCOS diagnosis, namely two of the three criteria were met according to the Rotterdam criteria.

This study result was in accordance with a study where 21 days old female rats induced by testosterone propionate 10mg/kg BW dissolved in propylene glycol for 35 days. The PCOS induced mice had polycystic ovarian morphology (cystic follicles, hyperthecosis, thickening of the tunica capsula, and the proportion of preantral follicles increases), testosterone concentration was increased, and had anovulation cycle.[8] Wang, et al[10,11] showed that female Wistar rats that received intraperitoneal injection of testosterone propionate 10 mg/kg BW for 6 weeks had an increase of cystic follicles count, a decrease of corpus luteum, and an elevated level of LH but level of total testosterone was not increasing. Oktanella, et al[12] reported white rats (*Rattus norvegicus*) aged 6–8 months injected by 100 mg/kg BW testosterone propionate intraperitonially for 12 days found irregularity in estrous cycle and high testosterone level but they did not assess ovarium morphology. Every research had its own criteria to define PCOS and usually they used only two from three clinical manifestations of PCOS to validate the animal models.

Gut microbiota dysbiosis usually defined by bacterial abundance and diversity, in this study we used Firmicutes/Bacteroidetes ratio and alpha diversity. Firmicutes and Bacteroidetes are the largest proportion of phylum in gut microbiota. Firmicutes has a capacity to extract and absorb energy from food and thus contribute to weight gain that leads to obesity meanwhile Bacteroidetes produce short chain fatty acid (SCFA) such as propionate and acetate that leads to increased fat storage and appetite that contribute to obesity. Obesity leads to insulin resistance that result in PCOS. Alpha diversity analysed using four indexes including Chao1 and observed indexes to assess number of each bacterium in one individual or sample, while the Shannon and Simpson indexes are used to define diversity. A decreasing Shannon index value indicates reduced diversity, while a Simpson index value close to 1 represent the dominance of one or two bacteria.[13]

Testosterone propionate injection slightly decreased diversity measured by Observed and Chao1 index but not differed significantly with control group thus was failed to provide suitable animal model for gut microbiota dysbiosis. Paris et al. used letrozole induced PCOS rodent model on modified diet and reported that group given diet with P:C:F ratio 33:20:47 and P:C:F ratio 23:38:38 exhibited the highest α-diversity measurements of evenness and Shannon's diversity index although richness of gut microbiota (Chao1) did not differ.[14] Li, et al[15] reported that there was no difference in alpha diversity and an increase in Bacteroidetes abundance in response to 60 mg/kg BW dehydroepiandrosterone (DHEA) daily subcutaneously in 3-week-old female Sprague Dawley. Zheng, et al[16] found that letrozole induced female Sprague Dawley rats with high fat diet demonstrated an increased in Firmicutes and Bacteroidetes ratio (F/B ratio) and decreased alpha diversity indexes (Chao1 and Abundance-based Coverage Estimator (ACE)) compared to letrozole with standard meal. This results emphasized that diet has a stronger influence over gut microbiota dysbiosis than androgen exposure only.

Serum zonulin concentration used for measuring gut permeability and in this study had the result that there was no difference between control and TP group. This result is in accordance with no alteration in gut microbiota conditions in both groups. Zonulin is a haptoglobin precursor that has a role to modulate the permeability of tight junction (TJ) between cells of the digestive tract. An increase of gut permeability usually associated with high serum zonulin level. The publication about serum zonulin concentration of PCOS rats model in

PubMed was found no result but there's limited publication in zonulin concentration of PCOS patients.[17] Cetin, et al[18] reported that serum zonulin level was similar between PCOS and control group and concluded that it would not increase in PCOS without metabolic syndrome. Lingaiah, et al[19] found that in premenopausal PCOS women, serum zonulin level was not increasing compared to women without PCOS with matching body mass index.

Leaky gut (increased gut permeability) leads to endotoxin entering circulation and induce pro-inflammation cytokine such as TNFα that caused insulin resistance via JNK1) and nuclear factor (NF-κB) activation in insulin receptor substrate-1 (IRS-1).[20] TNF-α concentration in control group was lower than TP group but it's not significantly different. This condition happened as a result of undetermined dysbiosis and no leaky gut in both groups. However, this condition found in other study such as research by Siahaan, et al[21], who used three-month-old white Wistar rats injected by 100 mg/kg BW testosterone propionate for 21 days and found an increase expression of TNF-α in the ovaries compared to healthy rats group. TNF-α level in plasma was also increased by another study using letrozole-induced PCOS group compared with control.[22] Kirici, et al[23] reported that TNF-α level was higher in PCOS rat model using letrozole oral daily for 21 days on Sprague Dawley rats. This opposite result was expected because of difference dose and method to induce PCOS and because there was no evidence of increasing gut microbiota in this study.

Insulin resistance measured in TP group was similar to the control group thus Wistar rats injected by 10 mg/kg BW testosterone propionate for 28 days at estrous phase could not utilized as a model for insulin resistance in PCOS. This result was opposite with Stener-Victorin, et al[24,25] research using continuous low-dose DHT in adult mice. PCOS induced mice had impaired glucose tolerance, insulin resistance and pancreatic B-cell defects. Beelosesky et al. found that in rats induced by testosterone propionate for 35 days with same dose, fasting glucose concentration would be normal or decreased and insulin would increase, which would result in a decrease in the glucose/insulin ratio.[7] Jiang, et al[26] reported that 8 weeks female rats fed by a high-fat diet (HFD) and injected with 1.0 mg/kg letrozole for 21 days intraperitoneally had increasing fasting blood glucose level, fasting insulin level, and HOMA-IR value compared to the control group.[26] Testosterone propionate injection with 10 mg/kg BW intramuscularly for 28 days may induce hyperglycaemia but not insulin resistance in this rats model.

There are limitations in this research such as estrous cycle was measured only twice in the beginning and the end of treatment and caused the difficulty to define anovulation cycle from this data only therefore vaginal smear should be conduct daily in future research. PCOS diagnosis defined by hyperandrogenism examined by high serum total testosterone concentration and polycystic ovarian morphology represented by abundant preantral follicles, nevertheless, this model provides a validate animal model for major clinical manifestations of PCOS. A longer administration and higher dose may have a different favourable result to provide more suitable model for assessing gut microbiota dysbiosis.

Conclusion

Daily TP intramuscular injection with the dosage of 10 mg/kg BW for 28 days at estrous phase in rodent model succeed to induce PCOS manifestation according to the Rotterdam criteria especially testosterone serum concentration and preantral follicles that represent polycystic ovarian syndrome. Vaginal smear daily is needed to clearly define ovulation status. Fasting blood glucose was significantly higher in PCOS animal models despite that standard meal although insulin resistance is not found. Parameters that related to DOGMA theory such as gut microbiota dysbiosis, gut permeability and also inflammation in TP group was not different from control group so this model is not suitable for research based on DOGMA. Further research with difference dose, onset of injection, and longer treatment duration of TP needed to provide a suitable model for investigating dysbiosis of PCOS patients or using non-homornal approach to provide other alternative model for PCOS research on dysbiosis gut microbiota.

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Conflict of Interest

We declare that there were no conflicts of interest in this study.

Author Contribution

All of the authors equally contributed to the study.

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