

Original Research Article

Mitigating effect of metformin on polycystic ovarian syndrome and insulin resistance in rats, and the mechanisms involved

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Abstract

Purpose: To study the effect of metformin on polycystic ovarian syndrome (PCOS) and insulin resistance (IR) in rats, and the mechanism involved.

Methods: Eighty healthy female SD rats, aged 6 weeks, were selected. Three groups of rats were used: model, metformin + PI3K inhibitor, and metformin groups, with 20/group. Testosterone, leutenizing hormone (LH), and follicle-stimulating hormone (FSH) were assayed by enzyme-linked assay (ELISA), while HOMA-IR was calculated from fasting blood sugar (FBG); the effect of metformin on the IR of PCOS rats was determined. The expressions of PI3K and AKT in ovaries and liver of rats in each group were assayed by Western blotting.

Results: Fasting blood glucose, fasting insulin, and insulin resistance index were markedly higher in model than in control rats, and also significantly higher in inhibitor-treated rats than in metformin rats ($p < 0.05$). Relative to control, FSH level was higher, while levels of LH, LH/FSH ratio and testosterone in the metformin group were significantly lower ($p < 0.05$). The expression levels of PI3K and AKT in the ovary and liver were reduced in the inhibitor group, relative to the levels in metformin-treated rats ($p < 0.05$).

Conclusion: Metformin mitigates PCOS-linked ovarian changes and IR in rats via PI3K/AKT route. These findings may be useful in the design of new drugs.

Keywords: Metformin, Polycystic ovary syndrome, Leutenizing hormone, Insulin resistance, Fasting blood sugar, Follicle-stimulating hormone

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a disease caused by abnormal endocrine metabolism which is characterized by ovulation dysfunction/loss, and hyperandrogenemia. It is mainly manifested as irregular menstrual cycle, infertility, insulin resistance (IR) and polycystic

ovarian changes. It is the most common endocrine disease in women [1]. At present, PCOS occurs in about 10 % of the total number of women in the world, and is increasing year by year. Thus, PCOS has become an important issue affecting women's health [2]. It has been found that the development of PCOS is closely related to IR. Insulin resistance (IR) not only

upsets the balance between glucose and lipid metabolism in PCOS patients, but also causes disorder in the regulation of reproductive endocrine hormones [3]. Research on pathogenesis of PCOS suggest that the development of IR in PCOS patients has an important relationship with the PI3K/AKT route [4]. At present, metformin is considered the first-line drug in the treatment of IR and glucose intolerance. It ameliorates reproductive endocrine disorders and metabolic abnormalities in patients, promotes intestinal absorption of glucose, inhibits output of liver glycogen, and reduces liver glycogenesis [5]. The purpose of the present investigation was to study the mitigating influence of metformin on polycystic ovarian changes and IR in PCOS rats, and the underlying mechanism.

EXPERIMENTAL

Animals

Eighty healthy female SD rats aged 6 weeks (mean weight = 195 ± 25 g) were obtained from Shanghai Ruitaimo Biotech. Co. Ltd {production license SCXK (Shanghai) 2016-0001}.

This study received approval from the Animal Ethical Committee of Tongde Hospital of Zhejiang Province, and it was implemented in line with the Principles of Laboratory Animal Care [6].

Main equipment and reagents

The instruments and reagents used, and their suppliers (in brackets) were: electronic balance (Shenyang Longteng Electronic Co. Ltd, Model: ESJ-4B); biological microscope (Shanghai Nuochai Trading Co. Ltd., model: FL-306A); autoclave (Shandong Boke Gas Industry Co. Ltd., model: BKQ-B50II); paraffin slicer (Hubei Taiwei Technology Co. Ltd., model: R139); constant temperature water bath box (Suzhou Peiying Experimental Equipment Co. Ltd., model: HWS-20); electronic blood glucose meter (Shenzhen zhuangshengtang Pharmaceutical Co. Ltd., model: GLM-76); low temperature refrigerator (Shanghai Kaizhen Instrument Equipment Co. Ltd., model: MDF-86V188E); centrifuge (Guangzhou Jidi Instrument Co. Ltd., model: JIDI-4D-WS); metformin (Shanghai Shiguibao Pharmaceutical Co. Ltd., production batch No.: 20173370, specification : $0.5 \text{ g} \times 20 \text{ s}$); 0.9 % NaCl injection (Sichuan Kelun Pharm. Co. Ltd., production batch No.: 20176626, specification: 100 mL), and glucose (Henan Kelun Pharmaceutical Co. Ltd., production batch No.: 41022311, specification: 100 mL).

Establishment of PCOS IT model and animal grouping

The rats were fed adaptively for one week at a temperature of 24 ± 2 °C and 52 ± 18 % humidity. They were allowed free access to feed and water. Three groups of rats were used, with 20 control rats and 40 rats in model group. Rats in the model group were given letrozole (1 mg/kg daily, in 1 mL 1 % sodium carboxymethylcellulose) via gavage for 30 days, and cytological changes of vaginal exfoliation were monitored using methylene blue staining. Vaginal epithelial cells of PCOS rats were keratinized for 10 days. After fasting for 10 h, FBG and insulin were measured with FBG meter, and HOMA-IR was calculated. Rats with established PCOS IR were assigned to model, metformin + PI3K inhibitor, and metformin groups, with 20 rats/group. Rats in the model group were fed high fat diet.

Treatment indices

Blood (5 mL) was taken from the abdominal aorta of each rat, and serum samples were obtained after centrifugation at the speed of 3000 rpm. The serum samples were then cryopreserved in the refrigerator at -80°C prior to assay of biochemical parameters. The level of HOMA-IR was calculated using Eq 1.

$$\text{HOMA-IR} = [(\text{FBG} \times \text{FINS})/22.5] \dots\dots\dots (1)$$

from which the effect of metformin on IR of PCOS rats was determined.

The complete bilateral ovaries and livers were taken out, weighed, and their appearance was examined. The ovaries were rinsed with phosphate buffer and stored in a low temperature refrigerator. A portion of ovary was placed in the test tube, fixed with 4 % formaldehyde, and processed into paraffin sections. The ovarian tissue sections were stained with H&E staining method, and their histological morphologies were examined under a light microscope. Luteinizing hormone (LH), FSH and testosterone (T) were assayed with ELISA, and LH/FSH ratio was determined. The kit and serum samples were placed at room temperature for 20min, diluted 1:5 with distilled water, dissolved using the doubling dilution method, and used for standard curve. Serum sample (100 μL) was added to the reaction well and the universal diluent was added to the blank well. The reaction well was sealed and placed in an incubator at 37°C for 90 min. Biotinylated antibody working solution was prepared 30min in advance, and the plate was cleaned. Then, 350 μL of washing solution was injected into each well. The interval between

injection and suction was 15 seconds. Subsequently, biotinylated antibody diluent was added to the blank well, and working solution was added to the other well. The wells were sealed again and incubated in the incubator at 37°C for 60 min. The microplate reader was open, the parameters were set, the absorbance was read at 450 nm.

The expressions of PI3K and AKT in ovaries and livers of rats in each group were assayed with Western blotting. The tissue blocks were rinsed 2 to 3 times with cold PBS buffer, placed in a mortar, and ground into powder. The powder was transferred to a 1.5mL centrifuge tube, and oscillated. The tissues were bathed in ice for 30 min, during which they were repeatedly blown with a pipette to ensure complete cell lysis. This was followed by centrifugation at 12000 g for 10 min, and the protein content of the supernatant was measured. An appropriate amount of cytoplasmic protein extraction reagent A and B was mixed in 40:1 ratio, and 200 μ L of BCA mixture was added. The mixture was incubated at 37°C for 30 min. The absorbance of the protein was read at 540 nm in a microplate reader, and the value obtained was multiplied by 10 to obtain the protein concentration of the original sample. Equal amounts of protein were subjected to SDS-PAGE, followed by transfer to PVDF, blocking and incubation with the appropriate primary antibodies overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 h. The relative expressions were determined using gel imaging system, with GDAPH as reference gene. The experiment was repeated more than 3 times to reduce error.

Statistical analysis

Statistical analysis was done with SPSS 21.0 software package. Two-group comparisons were done with independent sample *t*-test, while single factor multi-sample test was used to compare multiple groups. Count data were compared using χ^2 test. The comparison of grade data was performed with Ridit test. Values of $p < 0.05$ were taken as indicative of statistical significance.

RESULTS

Changes in FPG, FINS and HOMA-IR levels of rats

As shown in Table 1, fasting blood glucose, fasting insulin, and insulin resistance index were markedly higher in model than in control rats,

and were significantly higher in the inhibitor-treated rats than in metformin rats ($p < 0.05$).

Table 1: Changes in FPG, FINS and HOMA-IR levels (mean \pm SD)

Group	FINS (mU/L)	FPG (mmol/L)	HOMA-IR
Control	19.59 \pm 2.18	4.68 \pm 0.37	4.05 \pm 0.49
Model	37.94 \pm 4.07 ^a	6.85 \pm 0.18 ^a	11.46 \pm 1.84 ^a
Inhibitor	29.29 \pm 1.39 ^{ab}	5.62 \pm 0.21 ^{ab}	7.28 \pm 0.76 ^{ab}
Metformin	23.34 \pm 2.44 ^{abc}	5.25 \pm 0.29 ^{abc}	5.99 \pm 0.43 ^{abc}
<i>F</i>	175.382	226.941	179.512
<i>P</i> -value	< 0.001	< 0.001	< 0.001

^a $P < 0.05$, vs control rats; ^b $p < 0.05$, vs model rats; ^c $p < 0.05$, vs inhibitor-treated rats

Ovarian histomorphology of the rats

In the control group, the number of granulosa cells in the follicles was more than that in the model group, and the number of granulosa cells and the formation of lutein were significantly reduced (Figure 1). In the inhibitor group, the number of granulosa cells and the formation of lutein were significantly improved, but were markedly reduced, when compared with the metformin group. In the metformin group, the number of granulosa cells and the formation of lutein were markedly improved. Cystic expansion of follicles was either absent or rare, and the number of granular layers was significantly increased.

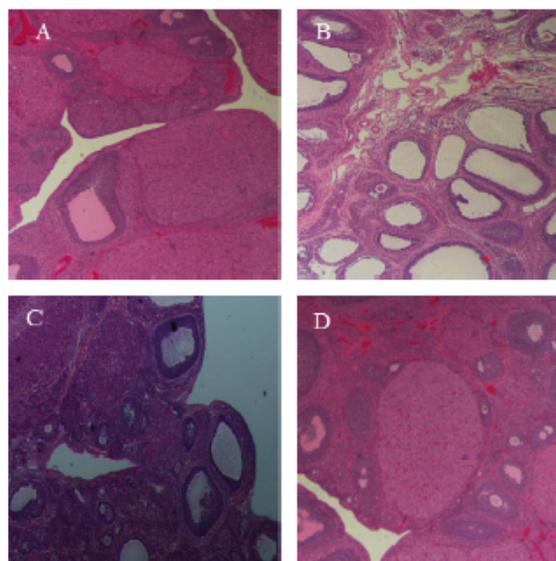


Figure 1: Comparison of ovarian histomorphology amongst the groups. A: control; B: model; C: + inhibitor; D: + metformin

LH, FSH, LH/FSH ratio and T levels

Table 2 shows that LH, LH/FSH ratio and T levels in the model group were significantly raised, while the level of FSH was significantly low, relative to control; LH, LH/FSH ratio and T levels in inhibitor and metformin groups were significantly decreased, while FSH level was significantly raised, relative to model rats. In addition, LH, LH/FSH ratio and T levels were markedly reduced in metformin rats, relative to model rats, while FSH level was significantly raised ($p < 0.05$).

Expression levels of PI3K and AKT in ovary and liver of rats

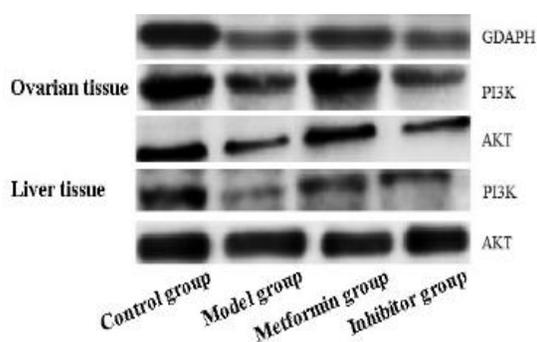


Figure 2: PI3K and AKT levels in ovaries and livers of rats in each group

The expression levels of PI3K and AKT in the ovary and liver were highest in the control group, but were lowest in the model group, while the corresponding expression levels in the inhibitor

group were significantly lower than those in metformin group ($p < 0.05$; Figure 2 and Table 3).

DISCUSSION

Polycystic ovarian syndrome (PCOS) is the most common reproductive endocrine disorder in women of child-bearing age. Its clinical manifestations are diverse, and its pathological mechanism is complex. It is believed that the etiology is associated with factors such as genetic susceptibility, abnormalities in gonadotropin synthesis and metabolic disorders, amongst other factors. Insulin resistance (IR) is involved in the pathogenesis of PCOS [7]. At present, drug therapy is still the main treatment strategy for PCOS. However, due to limited access to clinical specimens, the use of animal models has become the major route of PCOS-based investigations. Many studies have shown that the PCOS-IR model in rats is similar to PCOS in human patients in terms of ovarian morphology, abnormal sexual hormone secretion, and glucose metabolism, and it is accompanied with IR [8,9]. Thus, this research involved investigation of the mitigating effect of metformin on polycystic changes associated with IR in PCOS rats.

Metformin is a biguanide oral hypoglycemic drug which delays glucose uptake from the gastrointestinal tract, increases the utilization of peripheral glucose by improving insulin sensitivity, and inhibits excessive glycogenesis in liver and kidney [10].

Table 2: Comparison of LH, FSH, LH / FSH, T levels in each group (mean \pm SD)

Group	LH (mIU/mL)	FSH (mIU/mL)	LH/FSH ratio	T (ng/mL)
Control	0.89 \pm 0.36	5.47 \pm 1.66	0.18 \pm 0.08	0.59 \pm 0.15
Model	12.89 \pm 1.41 ^a	2.54 \pm 1.26 ^a	5.46 \pm 1.02 ^a	4.08 \pm 1.41 ^a
Inhibitor	5.48 \pm 1.46 ^{ab}	3.56 \pm 1.08 ^{ab}	1.61 \pm 0.44 ^{ab}	1.69 \pm 0.74 ^{ab}
Metformin	2.62 \pm 2.08 ^{abc}	4.94 \pm 1.02 ^{abc}	0.78 \pm 1.16 ^{abc}	1.14 \pm 0.29 ^{abc}
F	261.694	21.605	174.521	71.531
P-value	< 0.001	< 0.001	< 0.001	< 0.001

a, b, c $P < 0.05$ (a, b, c vs control, model and inhibitor-treated rats, respectively)

Table 3: PI3K and AKT levels in ovary and liver of rats (mean \pm SD)

Tissue	Group	PI3K	AKT
Ovary	Control	0.97 \pm 0.09	3.99 \pm 0.27
	Model	0.36 \pm 0.13 ^a	2.11 \pm 0.35 ^a
	Inhibitor	0.49 \pm 0.04 ^{abc}	2.51 \pm 0.16 ^{ab}
	Metformin	0.69 \pm 0.09 ^{abc}	2.98 \pm 0.11 ^{abc}
Liver	Control	1.09 \pm 0.38	2.71 \pm 0.12
	Model	0.38 \pm 0.02 ^a	1.41 \pm 0.14 ^a
	Inhibitor	0.63 \pm 0.26 ^{ab}	1.84 \pm 0.09 ^{ab}
	Metformin	0.83 \pm 0.29 ^{abc}	2.12 \pm 0.18 ^{abc}

a, b, c $P < 0.05$ (a, b, c vs control, model and inhibitor-treated rats, respectively)

Sturrock *et al.* have reported that metformin also reduced insulin levels, decreased IR, corrected disorders in reproductive endocrine levels and glucose metabolism in PCOS patients, and improved ovarian ovulation [11].

In IR, the efficiency of insulin in promoting glucose uptake and utilization is reduced for various reasons. In an attempt to maintain normal blood glucose levels, insulin is over-secreted in a compensatory way, resulting in hyperinsulinemia [12]. Insulin resistance occurs mostly in the skeletal muscle, myocardial muscle, liver, endometrium and other target organs. Studies in China and elsewhere have demonstrated that the etiology of PCOS-IR has an important relationship with the insulin signal pathway. The post-insulin receptor signal pathway comprises the PI3K/AKT signal route and the MAPK signal pathway. The PI3K/AKT signal pathway is key to post-insulin receptor signal transduction, and it is present in the islet of Langerhans [13,14]. It is implicated in the metabolic control of glucose in post-insulin receptor signal transduction [15,16]. Li *et al* [17] have reported that PI3K/AKT signaling route is closely associated with ovarian function. When the activity of PI3K/AKT signaling pathway is decreased, insulin receptor is activated, and it directly acts on the follicular membrane cells, causing excessive androgen secretion, resulting in pituitary gonadotropin secretion disorder, and ultimately in excessive ovarian androgen secretion.

In this study, values of FPG, FINS, HOMA-IR, LH, T, FSH and LH/FSH ratio were significantly higher in model rats than in control rats, while their corresponding levels were markedly lower in metformin rats than in model rats. However, after PI3K inhibitor was used, FPG, FINS, HOMA-IR, LH, T and LH/FSH ratio were markedly increased, while FSH level, and the expressions of PI3K and AKT in ovary and liver were significantly decreased. These results demonstrate that metformin significantly reduced FPG, FINS, HOMA-IR, serum LH, T and LH/FSH ratio; and decreased polycystic ovarian changes and IR in PCOS rats. Moreover, metformin increased the expressions of PI3K and AKT in ovarian and liver tissues. The therapeutic effect of metformin on PCOS was inhibited by PI3K inhibitor. This finding is consistent with the report of Jin *et al* [18].

CONCLUSION

LH and T levels, and LH/FSH ratio in PCOS rats are markedly increased, relative to normal rats, while expressions of PI3K and AKT in FSH, liver

and ovary tissues are lower than their respective levels in normal rats. Thus, metformin can potentially mitigate polycystic ovarian changes and IR in humans via a mechanism which may be related to PI3K/AKT insulin signaling pathway.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Baimiao Wang. Lanyawen Hu, Baimiao Wang, Yingli Tao collected and analyzed the data, while Lanyawen Hu and Baimiao Wang wrote the manuscript. All authors read and approved the manuscript for publication.

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