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The expression of Nuclear Factor-κB in a rat model of polycystic ovary syndrome

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Summary

Objective: To discuss the effect of Nuclear Factor-κB (NF-κB) in polycystic ovary syndrome (PCOS). *Materials and Methods:* Thirty female SD rats were divided into a control group (n=15) and a PCOS group (n=15). The expression of NF-κB in ovaries of each group was monitored by way of immunohistochemistry and quantitative PCR. *Results:* The expression of NF-κB p65 in ovaries by immunohistochemical and fluorescence quantitative PCR shows no significant difference between the two groups ($p > 0.01$). *Conclusion:* The pathogenesis of this research model has no obvious association with NF-κB.

Key words: Polycystic ovary syndrome; NF-κB.

Introduction

Polycystic ovary syndrome (PCOS) is a type of endocrine metabolic disorder syndrome that occurs among women of reproductive-age. Its etiology is unclear, but in recent years studies have shown that PCOS is likely to be a low-grade chronic inflammatory disease [1-7]. Nuclear Factor-κB (NF-κB) is a transcription factor that has a very important function in the inflammatory response. Gonzalez *et al.* [8] reported that NF-κB is significantly activated in the mononuclear cells of women with PCOS following glucose stimulation. In vitro studies by Su *et al.* [9] and Zhu *et al.* [10] and have shown that testosterone can activate the NF-κB signaling pathways, and then produce inflammatory factors. The present authors are establishing an animal model using immunohistochemical and fluorescence quantitative PCR to study the expression of NF-κB p65 protein and mRNA in PCOS rat ovaries.

Materials and Methods

Establishment of the PCOS rat model

Six week old clean animal (CL) SD female rats having regular estrus cycles were selected. Animals were purchased from the Fujian Medical University Laboratory Animal Center, license Number SCXK (Fujian) 2012-0001. Animals were bred in the Fujian Medical University Animal Center, with the clean animals raising license SYXK (Fujian) 2008-0001. Thirty (30) rats were randomly divided into two groups: The control group (n=15) was allowed free access to water and a standard rat diet, and the PCOS group (n=15) had similar access to food and water and was given a daily oral gavage of 1 mg/kg letrozole for 21 days. The authors took vaginal smears everyday to determine the changes in estrus. Predominant cells associated with each phase of the estrus cycle: nucleated epithelial cells (proestrus, P), enucleated cornified cells (estrous, E), an equal mix of leukocytes, and epithelial cells (metaestrus, M), leukocytes (diestrous, D).

Detection of rat serum indicators after the last lavage

All the experimental rats fasted for 12 hours. Radioimmunoassay was used to assay the serum luteotropic hormone (LH), testosterone (T), and insulin (INS) (kit, assayed by the hospital immunology technician). The glucose oxidase method was used to detect fasting plasma glucose (assayed by our biochemical technician). Homeostasis model of assessment of insulin resistance (Homa-IR) = fasting plasma glucose (FPG) × fasting insulin (FINS)/22.5.

The expression of NF-κB p65 in the ovaries of two groups was monitored by immunohistochemistry

Tissue from one ovary was fixed in 10% methanol. The tissues were processed routinely for paraffin embedding, and 4-μm sections were cut for immunohistochemistry. According to the method of the SP immunohistochemical operation process, the primary antibody was monoclonal mouse anti-rat NF-κB p65 and the secondary antibody was sheep anti-mouse. NF-κB was visualized with DAB. Results were determined by evaluating the number of positive cells within the same cell population and by color intensity [11, 12]. Labeling was scored as; no specific staining = 0, light yellow = 1, tan = 2, and brown = 3. In each tissue the authors randomly counted five fields at high magnification (×400) to determine the positive rate of each field. Data are expressed as an average: 1-10% = 1 point, 11-50% = 2 points, 51-75% = 3 points, and 76% or higher = 4 points. The staining intensity was multiplied by positive cell percentage semi-quantitative analysis and the results scored as: 0 = negative, 1- 4 points = + weakly positive, 5-8 points = + + positive, 9-12 points = + + + strongly positive. Results were determined by two experienced Pathologists.

Real-Time PCR detection of NF-κB P65 mRNA in the ovaries of each group

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Ovarian tissue was frozen in liquid nitrogen. Trizol was used to extract total RNA from the ovarian tissue and the reverse transcription kit from TaKaRa (DRRR037S) was used to synthesize cDNA. The SYBR Premix EX Taq fluorescence quantitative PCR Kit was used for Real-Time PCR. Primers were synthesized. The NF-κB p65 primer (156 bp): upstream, 5'-ATG CGT TTC CGT TAC AAG-3' and downstream, 5'-GTG AGG TGG GTC TTT GGT-3'. The β-actin primer (185 bp): upstream, 5'-GAG GGA AAT CGT GCG TGA C-3' and downstream: 5'-GGA AGG AAG GCT GGA AGA G-3'. Amplification conditions: 95°C for one minute; 95°C for five seconds, and 60°C for 30 seconds for 40 cycles. Conditions for dissolution curve: 95°C for 15 seconds, 65°C for 30 seconds, and 95°C for 15 seconds. The calculation of relative gene expression: comparative threshold value was used to quantify the target gene expression in controls and in the PCOS group were shown as $2^{-\Delta\Delta Ct}$, ΔΔCt = ΔCt PCOS - ΔCt Controls, ΔCt = Ct NFκB - Ct actin. Each sample was evaluated twice and data is expressed as the average CT value. Each sample was evaluated twice and data was expressed as the average CT value.

Statistical analysis

All data are presented as mean ± standard ($\bar{x}\pm s$), using SPSS 17.0 statistical software package. A single-factor analysis of variance was carried out, and $p < 0.01$ indicates a significant difference.

Results

The change of estrus cycle

All the rats in the control group had a regular estrus cycle (Figure 1). By the ninth day of administration of letrozole, the rats in PCOS group were no longer cycling normally and continued in diestrus, displaying anovulation.

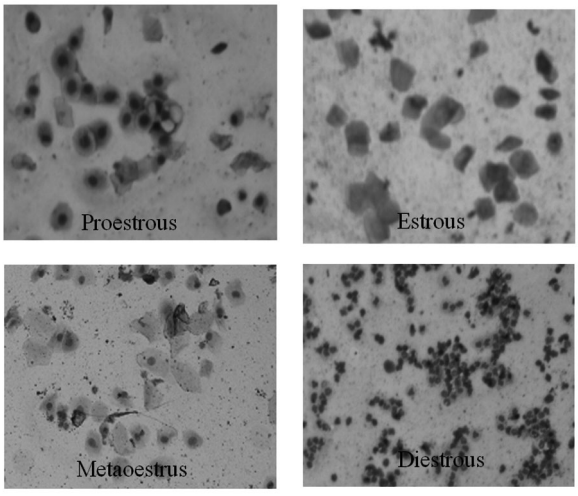

Figure 1. — Rats estrus cycle (Wright's staining).

Serum factors

Serum T, LH, FINS, and HOMA-IR levels in the PCOS group were significantly higher than in the control group ($p < 0.01$). There was no significant difference in the serum FPG levels between the two groups ($p > 0.01$) (Table 1).

Immunohistochemical expression of NF-κB P65 in the ovaries

Control group demonstrated visible ovarian follicles at different stages of development. PCOS group showed polycystic ovary, without different stages of follicle development. Immunohistochemistry results of the ovarian tissue showed that NF-κB P65 had consistent expression in the nucleus of ovarian granulosa cells. Therefore nuclear expression was used for the semi-quantitative analysis. There was no statistically significant difference between the two groups ($p > 0.01$) (Table 2, Figure 2).

Expression of NF-κB P65 mRNA in the ovaries

Amplification in both groups was carried out under the same conditions. The melting curves of NF-κB p65 and β-actin are only single peaks; the melting temperatures were 85.6°C and 86.6ºC, respectively. By quantitative PCR analysis there was no statistically significant difference between the two groups ($p > 0.01$) (Table 3, Figure 3).

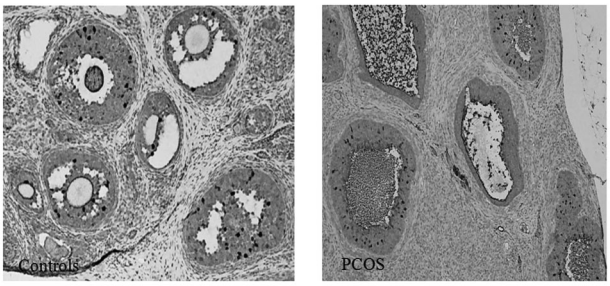

Figure 2. — NF-κB p65 on the ovaries in two groups

Table 2. — *Results of NF-κB p65 on the ovaries in two groups*

Grouping	Positive expression rate	Relative expression score
Control group	100%	2.633±0.516
PCOS group	100%	2.867±0.420
p		0.048

Table 1. — *Results of two groups in serum*

Grouping	T (ng/dl)	LH (mIU/ml)	FPG (mmol/L)	FINS (mIU/L)	HOMA-IR
Control group	21.609±16.246	1.215±1.094	5.111±0.202	11.671±1.836	2.585±0.387
PCOS group	213.304±68.113	3.147±1.301	5.213±0.296	34.801±19.313	9.475±4.229
p	0.000	0.002	0.277	0.001	0.001

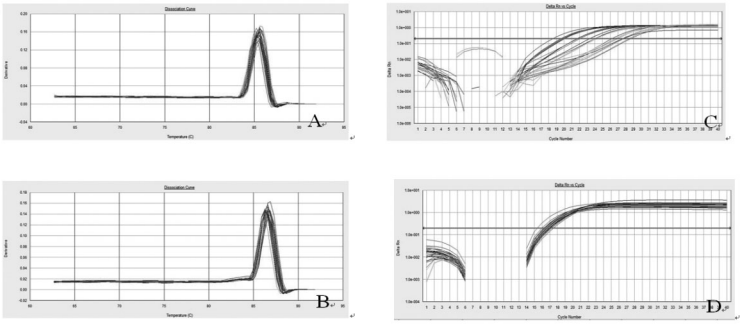

Figure 3. — A) Melting curve of NF-κB p65. B) Melting curve of β-actin. C) Amplification curve of NF-κB p65. D) Amplification curve of β-actin.

Table 3. — *Results of NF-κB p65 mRNA on the ovaries in two groups*

Grouping	$Ct_{\beta\text{-actin}}$	$Ct_{NF\text{-}\kappa B}$	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Control group	17.509±0.643	21.275±1.333	3.767±1.707	0	1
PCOS group	17.387±0.481	21.268±2.336	3.881±2.401	0.115±3.388	4.509±9.068
p					0.145

Discussion

This study used the establishment letrozole animal model with SD rats with anovulatory polycystic ovary that has the characteristics of hyperandrogenemia and insulin resistance. The model is similar to human PCOS.

PCOS is a one of the most common gynecologic endocrine diseases and the cause has not yet been clarified. Recent studies have shown that PCOS may be a low-grade chronic inflammatory disease, but this view has been controversial. NF-κB is a transcription factor that is prominent in the inflammatory reaction. It can induce the production and release of inflammatory factors, and under physiological conditions the NF-κB family members are usually present in the form of homologous or heterologous dimers, such as P50/P65. The monomer P65 has transcriptional activity is also one of the most common monomers. Gonzalez *et al.* [8] published prospective studies using glucose stimulation in 16 women with PCOS and 16 controls and they separated peripheral blood mononuclear cells. They found that in women with PCOS that intra-nuclear NF-κB was significantly increased. In vitro studies by Su *et al.* [9] and Zhu *et al.* [10] have shown that testosterone can activate macrophage and fat cell NF-κB signaling pathways, and promote the production of inflammatory factors.

Results of the present research included the immunohistochemical method which was used to detect NF-κB P65 in the ovaries in the two groups of rats. All positive expression was clearly differentiated. Positive signals were restricted to the nucleus of the granular cells. The nuclear expression of the NF-κB P65 has transcription activity, but the present results showed there was no statistically significant difference between the two groups. Fluorescence quantitative PCR to detect NF-κB P65 gene expression in the ovaries in the two groups of rats, also found that there was no significant difference between the two groups. Different results have been reported from other studies both at home and abroad. An analysis of the reason for the differences may be: 1) the research object, the research object of overseas research is human, domestic studies are *in vitro* experiments, and the present authors are using an animal model; 2) drugs to stimulate: Gonzalez *et al.* [8] used 75 grams of oral glucose to stimulate subjects, and domestic research used testosterone stimulation of cells; 3) PCOS may be a heterogenous condition, producing differences in expression. The pathogenesis of PCOS in this research model has no obvious association with NF-κB.

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