The Abnormal Expression of HSP70 Is Related to Treg / Th17 Imbalance in PCOS Patients

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Research Article

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a disease with chronic nonspecific low-grade inflammation. The imbalance of immune cells exists in PCOS. Several studies have found that heat shock protein 70 (HSP70) may be involved in the immunological pathogenesis of PCOS, but the relationship between HSP70 and Regulatory T cell (Treg)/ T helper cell 17(Th17) ratio remains unclear. This study aims to explore the correlation between HSP70 and Treg/Th17 ratio, and to find out the role of HSP70 in the immunological etiology of PCOS.

Results: There was no significant difference in age and body mass index (BMI) between two groups. The concentrations of basal estradiol (E2), basal follicle stimulating hormone (FSH) didn't show significant difference between two groups. The concentrations of basal luteinizing hormone (LH) ($P < 0.05$), testosterone (T) ($P < 0.01$), glucose ($P < 0.001$) and insulin ($P < 0.001$) in PCOS patients were significantly higher than those in control group. The expressions of HSP70 were significantly higher in serum in the PCOS group ($P < 0.001$). The percentage of Treg cells was significantly lower ($P < 0.05$), while the percentage of the Th17 cells of the PCOS group was significantly higher than that of the control group ($P < 0.05$). The ratio of Treg/Th17 in PCOS group was significantly lower ($P < 0.001$). The concentrations of Interleukin (IL)-6, IL-17 and IL-23 were significantly higher, while the levels of IL-10 and Transforming growth factor-β (TGF-β) were significantly lower in PCOS group ($P < 0.001$). Spearman rank correlation analysis showed strong negative correlation of serum HSP70 levels with Treg/Th17 ratio, IL-10 and TGF-β levels. In contrast, HSP70 levels were significantly positively correlated with IL-6, IL-17, IL-23, LH, T, insulin, and glucose levels.

Conclusion: The abnormal expression of HSP70 is correlated with Treg/Th17 imbalance and corresponding cytokines, which indicates that HSP70 may play an important role in PCOS immunologic pathogenesis.

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disorder, affecting approximately 6–15% of women worldwide[1]. The disease is characterized by irregular menstruation, hyperandrogenemia and polycystic ovary, with individual differences in clinical manifestations[2]. It has been reported that the risk of developing type 2 diabetes mellitus (2-DM) in PCOS women is 2–5 times higher than that in healthy women[3]. In addition, PCOS patients are at higher risk of metabolic and cardiovascular diseases[4].The pathogenesis of PCOS is still unclear. It is generally believed that PCOS is a disease affected by multiple factors such as heredity, endocrine, and environment. The current treatment of PCOS is still at the stage of controlling the disease progression and cannot achieve the goal of cure.

Heat shock proteins (HSPs) are a group of highly conserved protein molecules which can be produced by all prokaryotic cells and eukaryotic cells under physiological and pathological conditions including high

temperature, hypoxia, virus infection or stress [5]. HSPs participate in the regulation of cell function and play an important role in the maintenance of protein homeostasis[6]. Among these proteins, heat shock protein 70 (HSP70) is the most conserved protein, which has a variety of biological functions including molecular chaperone, regulation of immune response, anti-apoptosis and improvement of cell tolerance to stressors[7]. Jansen observed that the expression of 5 genes encoding HSP increased, of which HSP70 was the main increased protein[8]. According to several reports, the levels of heat shock protein 70 were elevated in preeclampsia[9], 2-DM [10], cancer[11] and PCOS patients[12].

Both T helper cell 17(Th17) and CD4 + CD25 + Foxp3 + Regulatory T cell (Treg) belong to CD4 + T lymphocytes, but are different from Th1 and Th2 cells. Th17 cells play a key role in the pathogenesis of allergic and autoimmune diseases. They produce interleukin 17 (IL-17) and interleukin 23 (IL-23), which can recruit neutrophils and promote inflammation in infected areas. Treg cells produce anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor-β (TGF- β). Treg cells can inhibit the activity of a variety of immune cells, thus inhibit the immune response, maintain immune tolerance and keep the immune system in a balanced state. Therefore, these two types of cells inhibit each other functionally and play an opposite role in inflammation and immune response[13].The imbalance of Treg/Th17 cells commonly exist in autoimmune diseases[14], and the balance between them plays an important role in maintaining the homoeostasis of the immune system.

PCOS is considered as a pro-inflammatory state. Chronic low-grade inflammation is considered to be a key factor in the pathogenesis of PCOS[15]. It has been reported that the imbalance of T cell subsets and the abnormal cytokine concentrations exist in the ovary of women with PCOS[16]. Th1 and Th17 bias, and the decrease of Treg and Th2 cells may be involved in the pathogenesis of PCOS[17]. HSP70 plays an important role in antigen presentation, activation of macrophages and lymphocytes, as well as activation and maturation of dendritic cells[7]. Studies have shown that HSP70 has the ability to transform Th17 cells immune response mode into Treg cells immune response mode[18]. However, whether HSP70 is related to the ratio of Treg / Th17 in PCOS patients has not yet been reported.

In this work, we measured the ratio of Treg / Th17 in peripheral blood of PCOS patients, as well as the levels of HSP70 and corresponding cytokines in serum, to further explore the possible role of HSP70 in the immunological etiology of PCOS.

Materials And Methods

1. Patient samples

Peripheral blood from10 women with PCOS and 10 healthy women were collected from December 2020 to March 2021. These PCOS patients were diagnosed according to the revised Rotterdam consensus[19]. The control subjects were healthy women with regular menstrual cycle and without abnormal reproduction diseases or metabolism. All these women were not treated with any medicine within 3 months. Any autoimmune disease, infection within 3 months, and other possible causes of anovulation or hyperandrogenemia were excluded. Informed consent of all participants was obtained. This

experiment was approved by the institutional ethics committee of people's Hospital of Wuhan University (Ethical approval No.: WDRY2018-K027).

2. Peripheral blood mononuclear cells isolation and flow cytometry analysis

3ml of peripheral venous blood from PCOS and healthy women was collected respectively, and then peripheral blood mononuclear cells (PBMCs) were separated from peripheral venous blood by density gradient on Ficoll-Paque (GE Life, Sweden). To detect Th17 cells, lymphocytes were stimulated with the Leukocyte Activation Cocktail (BD Pharmingen, USA) for 6 hours in a 37°C humidified CO_2 incubator. The cells were then stained with FITC conjugated anti-CD4 in the dark at 4°C for 30 minutes. Subsequently, intracellular cytokine staining was performed according to the manufacturer's protocol. The cells were fixed and permeabilized using Fix/Perm Buffer (BD Pharmingen, USA) in the dark at 4°C for 40 minutes, and then were washed with Perm/Wash Buffer (BD Pharmingen, USA) before labeling the cells with PE conjugated anti-IL-17 in the dark at 4°C for 30 minutes. Similarly, to access Treg cells, lymphocytes were stained with FITC conjugated anti-CD4 and PE-CY7 conjugated anti-CD25 in the dark at 4°C for 30 minutes. After fixation and permeabilization, the cells were stained intracellularly with APC conjugated anti-Foxp3 for 40 minutes. All antibodies involved in flow cytometry were purchased from BD Biosciences. Then the lymphocytes were washed in PBS, resuspended in 200ul buffer, and then analyzed using cytoflex (American Society of Biological Sciences). Data was analyzed using FlowJo VX V10.4 software.

3. Enzyme-linked immunosorbent assay (ELISA)

The serum concentrations of HSP70, follicle stimulating hormone (FSH), luteinizing hormone (LH), E_2, T, insulin, interleukin 6 (IL-6), IL-10, IL-17, IL-23 and TGF-β were measured by ELISA kits. All ELISA kits were purchased from Bioswamp (Wuhan, China). The ELISA was performed according to the manufacturer's instructions. The sample was diluted to 100μL (1:20), incubated with specific capture antibody and detection antibody. All samples were detected at 450 nm optical density.

4. Statistical analysis

Statistical analysis was performed using SPSS software V.19.0. The Shapiro-Wilk method was used to test whether the data were normally distributed. Comparisons between two groups were made via the unpaired two-tailed t-test. Data are shown as means ± standard. Regression assessment was made using the Spearman's rank correlation analysis. $P < 0.05$ was accepted to be statistically significant.

Results

1、Clinical and biochemical features of patients

A total of 20 patients (10 PCOS women, 10 healthy women) were included in this study. Table 1 summarized the clinical characteristics of the subjects. The results showed that there was no significant

difference in age and body mass index (BMI) between two groups. The AMH of PCOS patients was significantly higher than that of the control group ($P < 0.05$). In addition, the concentrations of basal luteinizing hormone (LH) ($P < 0.05$) (Fig. 1C), testosterone(T) ($P < 0.01$) (Fig. 1D), glucose ($P < 0.001$) (Fig. 1F) and insulin ($P < 0.001$) (Fig. 1E) in PCOS patients were significantly higher than those in control group. Besides, there was no significant difference between two groups in basal estrogen(E2) and basal follicle stimulating hormone (FSH) (Fig. 1A, B).

2、Treg/Th17 ratio in the peripheral blood of PCOS patients

Next, flow cytometry was used to detect the population of Th17 cells (CD4 + IL17+) and Treg cells (CD4 + CD25 + Foxp3+) in peripheral blood of PCOS and healthy women. As shown in Fig. 2, Treg cells population of the PCOS group was significantly lower ($P < 0.05$) (Fig. 2A, C), while the Th17 cells population was significantly higher than that of the control group ($P < 0.05$) (Fig. 2B, D). In addition, compared with the control group, the ratio of Treg/Th17 in PCOS group was significantly lower ($P < 0.001$) (Fig. 2E).

3、HSP70 expression and cytokines levels in PCOS patients

Furthermore, we detected the levels of HSP70 and cytokines corresponding to Th17 and Tregs cells including IL-6, IL-10, IL-17, IL-23 and TGF- β in both groups. As demonstrated in Fig. 3A, an obvious elevation of HSP70 level was shown in PCOS serum ($P < 0.001$). In addition, in PCOS women, the levels of IL-6 ($P < 0.001$), IL-17 ($P < 0.001$)and IL-23 ($P < 0.001$) were significantly higher (Fig. 3B, C, D), while the levels of TGF-β ($P < 0.001$) and IL-10 ($P < 0.001$) were significantly lower than those in control group (Fig. 3E, F).

4、The correlation of serum HSP70 with Th17/Treg ratio, cytokines, hormones, insulin and glucose

To explore whether HSP70 was involved in Treg/Th17 imbalance, the correlations between serum HSP70 and Th17/Treg ratio, hormones, insulin, glucose, as well as corresponding cytokines were also investigated (Table 2). Spearman rank correlation analysis showed that HSP70 levels were significantly negatively correlated with Treg/Th17 ratio ($P < 0.05$), IL-10 ($P < 0.001$) and TGF-β ($P < 0.01$) levels. In contrast, HSP70 levels were significantly positively correlated with IL-6 ($P < 0.01$), IL-17 ($P < 0.05$), IL-23 ($P < 0.05$), luteinizing hormone ($P < 0.05$), testosterone ($P < 0.01$), insulin ($P < 0.01$), and glucose ($P < 0.01$) levels. Besides, the results showed that the ratio of Treg/Th17 was significantly negatively correlated with insulin ($P < 0.01$) and glucose levels ($P < 0.001$).

Discussion

After being stimulated by different cytokines, CD4 + T cells differentiate into different types of effector T cells including Th1, Th2, Th17 and Treg. The initial differentiation of Th17 and Treg cells share a common signaling pathway mediated by TGF-β. However, terminally differentiated cells perform the opposite function. Th17 cells can lead to autoimmune response and inflammation, while Treg cells inhibit

these inflammatory phenomena and maintain immune homeostasis[20]. Th17 and Treg cells also maintain the balance of maternal-fetal interface immunity and play an important role in recurrent pregnancy loss[21] and preeclampsia[22]. In addition to genetic and environmental factors, the important role of immune system in PCOS has received widespread attention in recent years.

In our study, the results of flow cytometry showed that compared with control group, the proportion of Treg cells in PCOS patients decreased, while the proportion of Th17 cells increased, and the proportion of Treg / Th17 cells in peripheral blood of PCOS patients decreased significantly. A series of previous studies have shown that compared with healthy women, the number of activated T cells in ovarian follicular fluid of PCOS patients increased[23], while the number of Treg cells in peripheral blood of PCOS patients decreased[24]. The imbalance of Th1 / Th2 and Th17 / Treg was also observed in PCOS patients. The percentage of CD4 + CD25 + Foxp3 + T cells in PCOS patients decreased, and the proportion of Th17 subsets increased, although the difference was not statistically significant[17].These results were consistent with ours. It has been reported that a single injection of testosterone into female rodents can lead to the decrease of androgen in newborns and increase the relative and absolute number of CD4 + CD25 + Foxp3 + Treg cells in peripheral blood [25]. Androgen can preserve the number of male Treg cells directly or indirectly through its metabolites[26]. Our results showed that the androgen levels of PCOS patients were higher, but the ratio of Treg cells was lower than that of the control group. Spearman correlation analysis demonstrated that there was no correlation between androgen levels and Treg, Th17 cells. However, a significant positive correlation was observed between the ratio of Treg cells and luteinizing hormone levels. The reason may be that the serum hormones in PCOS patients, except LH, other hormones such as FSH, estradiol and androgen, will not affect the ratio of Treg cells. Congenital disability of peripheral Tregs may exist in PCOS women. According to previous study, the decrease of Tregs in peripheral blood of PCOS patients is due to the inherent low responsiveness of the body to IL-2, which lead to the abnormal activation of STAT5B and the reduction of Foxp3 expression[24].

In our previous study, elevated levels of inflammatory molecules in peripheral blood of PCOS rats were observed, including C-reactive protein, IL-6 and tumornecrosis factor-α[27]. In this study, we detected the levels of Treg and Th17 cell-related cytokines including TGF-β, IL-10, IL-6, IL-17 and IL-23 in the serum of PCOS patients. As expected, we found that Treg cell-related cytokines TGF-β and IL-10 decreased in PCOS patients, while the levels of Th17 cell-related cytokines IL-6, IL-17 and IL-23 increased. This result further confirms the view that chronic low-grade inflammation is involved in the pathogenesis of PCOS. Besides, we found that the levels of insulin and glucose were negatively correlated with the proportion of Treg cells, positively correlated with the proportion of Th17 cells, and negatively correlated with the proportion of Treg/Th17 cells. Insulin resistance (IR) may be one of the main factors in the development of PCOS. IR and hyperinsulinemia play an important role in pathophysiology of PCOS. They aggravate the disorder of the reproductive endocrine and metabolism of glucose and lipid in PCOS patients[28]. IR is one of the key factors affecting the efficacy of PCOS treatment. In recent years, more and more evidence showed that inflammation plays an important role in insulin resistance. Insulin resistance is related to immune factors such as adipocytokines and leptin. Chronic subclinical inflammation may be the initial cause of IR[29].

Relevant studies have shown that insulin resistance can lead to Inflammatory response and Th17/Treg imbalance, which can be rescued by IL-6[30].

As chaperone proteins, heat shock proteins participate in the assembly of proteins in cells and contribute to protein homeostasis[6]. Heat shock protein 70 has attracted more and more attention due to its important function in gametogenesis or pregnancy regulation[31]. In this study, ELISA results showed that the serum HSP70 levels of PCOS patients were increased, which were consistent with the previous research[32]. The increase of serum HSP70 levels were related to insulin resistance, oxidative stress and low-grade chronic inflammation in PCOS individuals. Elevated serum HSP70 levels were considered to indicate the ovarian damage of transgenic mice under oxidative/ischemic stress[33]. Spearman correlation analysis showed that HSP70 and testosterone were significantly positively correlated. It has been reported that testosterone is necessary for the enhancement of HSP70 expression. Physiological testosterone can enhance the expression of HSP70 induced by ischemic pretreatment, which may be due to the fact that testosterone can stimulate phosphorylation and upregulate the mRNA expression of HSP70[34].

In addition to the chaperone function, HSP70 can also stimulate and inhibit inflammation. The correlation between HSP70 and Treg/Th17 cells ratio was studied for the first time in our work. Spearman rank correlation analysis demonstrated that the levels of HSP70 in human serum was significantly negatively correlated with Treg/Th17 ratio. The ability of HSP70 to induce autoimmune response may be part of natural autoimmunity, or it may be related to pathological autoimmune diseases. Anti-HSP70 antibody has been confirmed in cord, and may play a role in normal immune system[35].

Heat shock protein is a stress-induced protein with immunomodulatory properties. It contains peptide binding domain, which can bind proteins and non-protein molecules with exposed hydrophobic residues. The function of antigen presentation and the ability of inducing cytokines in vitro are the results of the binding between HSPs and molecules or molecular chaperone role of HSPs[36]. In inflammatory disease models, T cells that respond to heat shock proteins inhibit the disease by producing anti-inflammatory cytokines. The anti-inflammatory activity of HSP-specific T cells depends on their recognition of endogenous HSP epitopes. It has been reported that these T cells can be induced by the conservative sequence of HSP of microorganisms. The upregulation of endogenous HSP expression induced by drugs can promote the production of anti-inflammatory T cells[37]. HSP70 can induce protective, anti-inflammatory regulatory T-cell response[35].

In our study, serum HSP70 levels were significantly negatively correlated with the Treg/Th17 ratio. The possible reason may be that in addition to regulating protein homeostasis, HSPs also participate in the enhancement of immune response under a variety of stress conditions, including fever, oxidative stress and inflammatory cytokine signaling activation during viral infection. In response to these stress signals, HSPs mediate the constitutive and inducible danger signals that activate the immune response[38].HSP70 has been reported to activate the innate immune system[39]. Studies have shown that the E3 ligase Stub1, which is expressed in response to danger signals during inflammation, is

responsible for the ubiquitination of Foxp3 with the help of HSP70 chaperone protein, which leads to the degradation of major Treg cell transcription factors[38].The high level of heat shock protein 70 directly promotes the expression of Th17 gene after TCR stimulation. This effect stems from the direct intracellular interaction of HSP70 with a RISC complex. The activity of heat shock protein 70 in Th17 promotion depends on the regulation of a set of specific miRNA expression. Selective inhibition of these microRNAs or directly blocking the function of heat shock protein 70 will downregulate the expression of Th17 gene[40].

In our study, Spearman rank correlation analysis showed that serum HSP70 levels were significantly negatively correlated with IL-10 and TGF-β levels, while significantly positively correlated with IL-6, IL-17, IL-23 and other inflammatory cytokines. Our previous study has shown that the levels of serum heat shock protein 70 in PCOS rats were decreased, and they were strongly negative correlated to testosterone, luteinizing hormone and inflammatory factors such as C-reactive protein, IL-6, IL-18, and tumor necrosis factor-α[27]。This is contrary to our results in PCOS patients. The possible reason is that the transient superphysiological testosterone characteristics caused by the construction of PCOS rat model down-regulated the expression of HSP70 in rat serum. HSP 72 is another member of the heat shock protein 70 family. According to the previous research, transient hyperphysiological testosterone can down-regulate the expression of heat shock protein 72. The regulation of HSP70 expression is mediated by heat shock transcription factor (HSF)-1. Testosterone can induce the inhibition of heat shock protein 72 expression or HSF1 activation, which may directly block the trimerization and phosphorylation of HSF1, or indirectly inhibit HSF1 activation[41], leading to the reduced expression of HSP70.

Insulin has the capacity to promote the occurrence and development of PCOS through PI3K and MAPK signaling pathways[42]. Here, our current investigation found that there was a significant positive correlation between serum insulin levels and testosterone levels. 17α- Hydroxylase was found to be decreased due to the inhibition of PI3K in follicular cells of PCOS patients, indicating that insulin may promote steroidogenesis through PI3K pathway[43].The existence of specific high affinity insulin receptor on human follicular membrane indicates that insulin can directly mediate the physiological effects induced by follicular membrane cells. Insulin can directly increase the secretion of androstenedione in theca cells[44]. Previous studies have shown that the interaction between insulin and luteinizing hormone upregulates the expression of StAR and CYP17A1 genes, and then increases androgen levels[45]. In addition, increased insulin can reduce SHBG synthesis, thereby reducing its binding with testosterone, leading to hyperandrogenemia[46]. Studies have shown that physiological concentration of testosterone can promote insulin secretion and Ca2 + uptake through membrane edema mechanism[47]. Graham et al. proposed a mathematical model of menstrual cycle. The model showed that with the increase of insulin-mediated testosterone production, ovulation interruption increased[48], indicating that the role of insulin in ovulation dysfunction is usually related to the increase of ovarian androgen production.

Our research also proved that insulin levels and anti-Mullerian hormone (AMH) levels were significantly positively correlated. The relationship between insulin levels and AMH levels in PCOS patients is controversial. A series of studies have found that compared with PCOS patients without insulin

resistance (IR), the concentration of AMH in PCOS patients with IR is significantly higher[49], The serum AMH is positively correlated with HOMA-IR[50], which is consistent with our results. However, other studies have not found a link between AMH and IR[51, 52]. It has also been reported that there is a negative correlation between AMH and HOMA-IR[53]. These contradictory data may be partly caused by the heterogeneity of the study population.

Our study has several limitations. Due to its cross-sectional design, the causal relationship between HSP70 and Treg/Th17 is unclear. In addition, due to the limitation of sample collection, the small number of patients limits the statistical power and the generalizability of our findings. Besides, both insulin and glucose are not tested under fasting conditions, thus the judgment of insulin resistance is not clear. The relationship between HSP70 and Treg/Th17 ratio still needs further research. Although this study confirmed that the upregulation of HSP70 expression was negatively correlated with Th17/Treg ratio, it is still necessary to study the specific regulatory mechanism of molecular biology to provide new targets for disease diagnosis and treatment.

Conclusion

Herein, the imbalance of Treg/Th17 exists in peripheral blood of PCOS patients, and the abnormally elevated heat shock protein 70 in serum of PCOS patients is related to Treg/Th17 imbalance and cytokines, suggesting that heat shock protein 70 may play an important role in the immunological etiology of PCOS.

Abbreviations

PCOS: Polycystic ovary syndrome; HSP70: Heat shock protein 70; IR: Insulin resistance; BMI: Body mass index; FSH: Follicle stimulating hormone; E2: Estradiol; LH: Luteinizing hormone; T: Testosterone; AMH: Anti-Mullerian hormone; IL-6: Interleukin 6; IL-10: Interleukin 10; IL-17: Interleukin 17; IL-23: Interleukin 23; TGF-β: Transforming growth factor-β; Th17: T helper cell 17; Treg: Regulatory T cell; 2-DM: Type 2 diabetes mellitus

Declarations

Ethics approval and consent to participate

This experiment was approved by the institutional ethics committee of people's Hospital of Wuhan University (Ethical approval No.: WDRY2018-K027).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The first author, Yiqing Yang did the experiments, collected all the data, analyzed the data, made all the figures in this manuscript and drafted the manuscript. Jing Xia and Zhe Yang help to collect patient samples. The corresponding author, Jing Yang and Gengxiang Wu have designed the research and guided writing. All authors read and approved the final manuscript.

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Tables

Table 1 Clinical and biochemical features of women included in this study

	Control	PCOS	P value
Age	30.33±3.14	30.13±4.85	0.929
BMI (Kg/m^2)	20.34±2.03	21.27±2.24	0.344
AMH (ng/ml)	3.14±1.02	6.34±2.55	0.027

Table 2 Spearman rank analysis for the correlation of HSP70, Th17/Treg ratio, hormones and cytokines

		HSP70	Treg	Th17	Treg/ Th17	IL-6	IL-10	IL-17	IL-23	TGF-β	BMI	AMH	E2	FSH	LH	T	Insulin
Treg	r	-0.305															
	p	0.19															
Th17	r	0.56	-0.354														
	p	**0.01**	0.125														
Treg/Th17	r	-0.484															
	p	**0.031**															
IL-6	r	0.689	-0.332	0.575	-0.546												
	p	**0.001**	0.152	**0.008**	**0.013**												
IL-10	r	-0.746	0.369	-0.479	0.508	-0.663											
	p	**0**	0.11	**0.033**	**0.022**	**0.001**											
IL-17	r	0.56	-0.571	0.614	-0.726	0.75	-0.63										
	p	**0.01**	**0.009**	**0.004**	**0**	**0**	**0.003**										
IL-23	r	0.498	-0.459	0.538	-0.591	0.759	-0.602	0.623									
	p	**0.026**	**0.036**	**0.014**	**0.006**	**0**	**0.005**	**0.003**									
TGF-β	r	-0.567	0.535	-0.314	0.55	-0.671	0.615	-0.764	-0.559								
	p	**0.009**	**0.015**	0.178	**0.012**	**0.001**	**0.004**	**0**	**0.01**								
BMI	r	0.126	-0.271	-0.201	-0.135	0.248	-0.387	0.081	0.165	-0.108							
	p	0.597	0.248	0.396	0.571	0.292	0.092	0.733	0.487	0.651							
AMH	r	0.25	-0.067	0.65	-0.533	0.833	-0.2	0.55	0.883	-0.383	0.067						
	p	0.516	0.865	0.058	0.139	**0.005**	0.606	0.125	**0.002**	0.308	0.865						
E2	r	0.03	-0.297	-0.018	-0.224	-0.188	-0.055	-0.018	0.236	-0.176	-0.172	-0.2					
	p	0.934	0.405	0.96	0.533	0.603	0.881	0.96	0.511	0.627	0.634	0.8					
FSH	r	-0.491	0.261	-0.042	0.273	-0.333	0.091	-0.503	0.588	0.552	0.32	-0.4	-0.152				
	p	0.15	0.467	0.907	0.446	0.347	0.803	0.138	0.074	0.098	0.367	0.6	0.676				
LH	r	0.758	-0.455	-0.006	-0.273	0.564	-0.455	0.273	0.503	-0.43	0.468	0.2	-0.212	-0.564			
	p	**0.011**	0.187	0.987	0.446	0.09	0.187	0.446	0.138	0.214	0.173	0.8	0.556	0.09			
T	r	0.709	-0.121	0.536	-0.286	0.659	-0.264	0.462	0.505	-0.566	-0.212	0.257	0.167	-0.571	0.357		
	p	**0.007**	0.694	0.059	0.344	**0.014**	0.384	0.112	0.078	**0.044**	0.487	0.623	0.693	0.139	0.385		
Insulin	r	0.623	-0.49	0.666	-0.671	0.674	-0.514	0.574	0.656	-0.561	-0.029	0.817	0.176	-0.345	0.442	0.731	
	p	**0.003**	**0.028**	**0.001**	**0.001**	**0.001**	**0.02**	**0.008**	**0.002**	**0.01**	0.905	**0.007**	0.627	0.328	0.2	**0.005**	
Glucose	r	0.609	-0.618	0.56	-0.746	0.69	-0.489	0.663	0.663	-0.65	0.127	0.45	0.224	-0.2	0.358	0.39	0.669
	p	**0.004**	**0.004**	**0.01**	**0**	**0.001**	**0.029**	**0.001**	**0.001**	**0.002**	0.593	0.224	0.533	0.58	0.31	0.188	**0.001**

Bold text means P<0.05

Figures

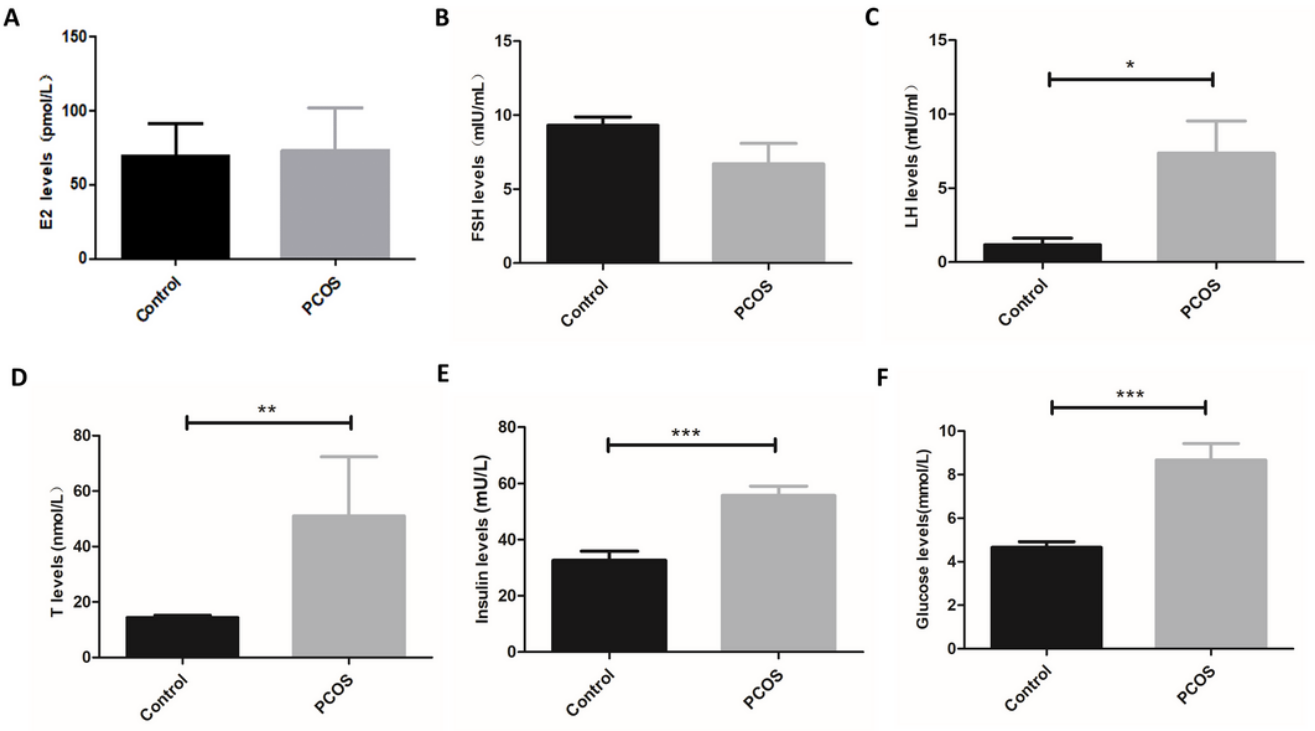

Figure 1

Serum concentrations of basal hormones, Insulin and glucose in PCOS and healthy women. A: Serum E2 levels; B: Serum FSH levels; C: Serum LH levels; D: Serum testosterone levels; E: Serum insulin levels; F: Serum glucose levels. (*P< 0.05, **P < 0.01, ***P < 0.001)

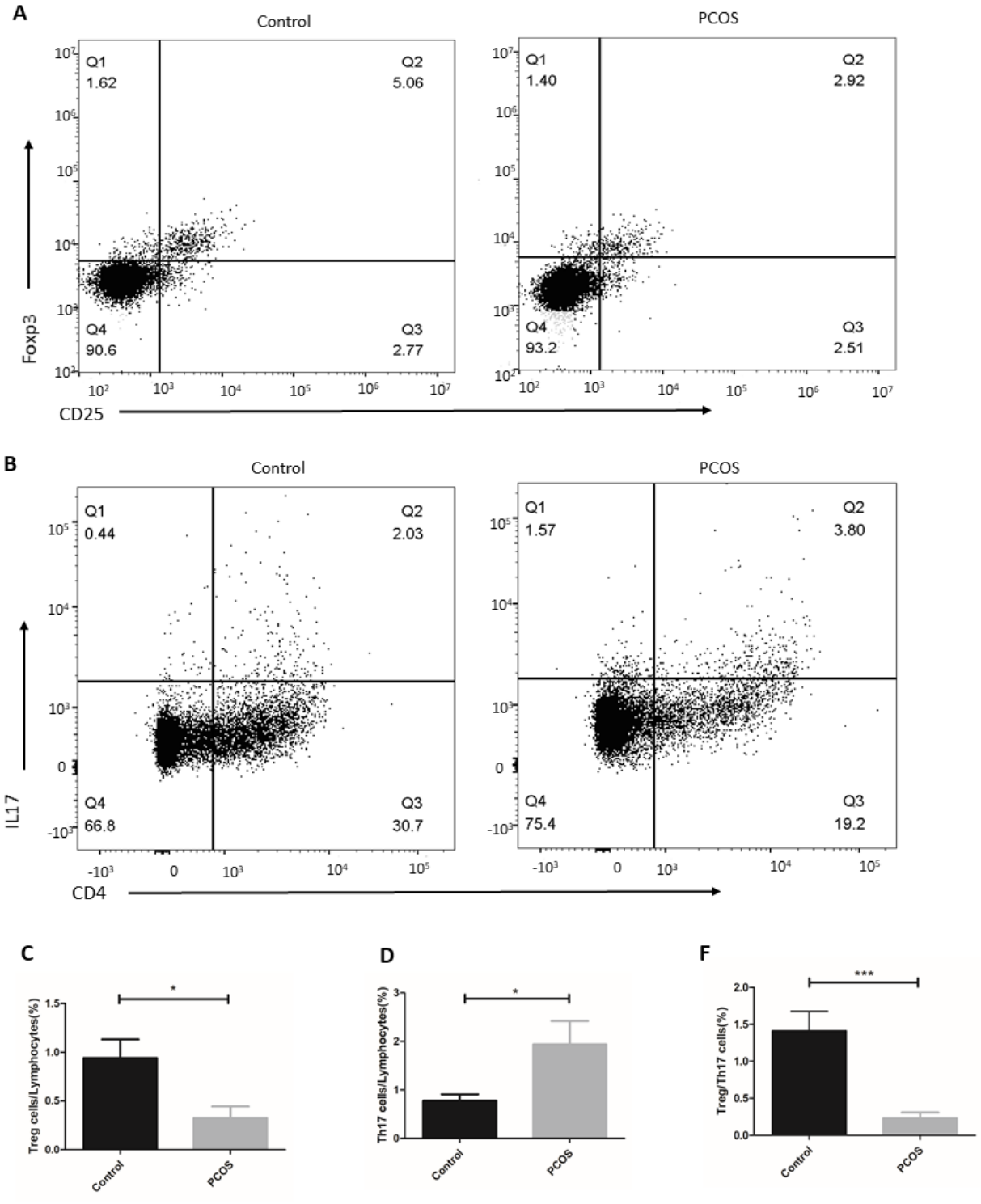

Figure 2

The ratio of Treg/Th17 cells in the peripheral blood of PCOS patients. A: FCS data for Tregs cells (CD4+CD25+Foxp3+) in the blood of subjects in two group; B: FCS data for Th17 cells (CD4+ IL17+) in

the blood of subjects in two group; C: Bar plot of the Treg cells/lymphocytes in the blood of subjects in two group; D: Bar plot of the Th17/lymphocytes cells in the blood of subjects in two group; E: Bar plot of the Th17/Treg ratio in the blood of subjects in two group. (*P< 0.05, ***P < 0.001)

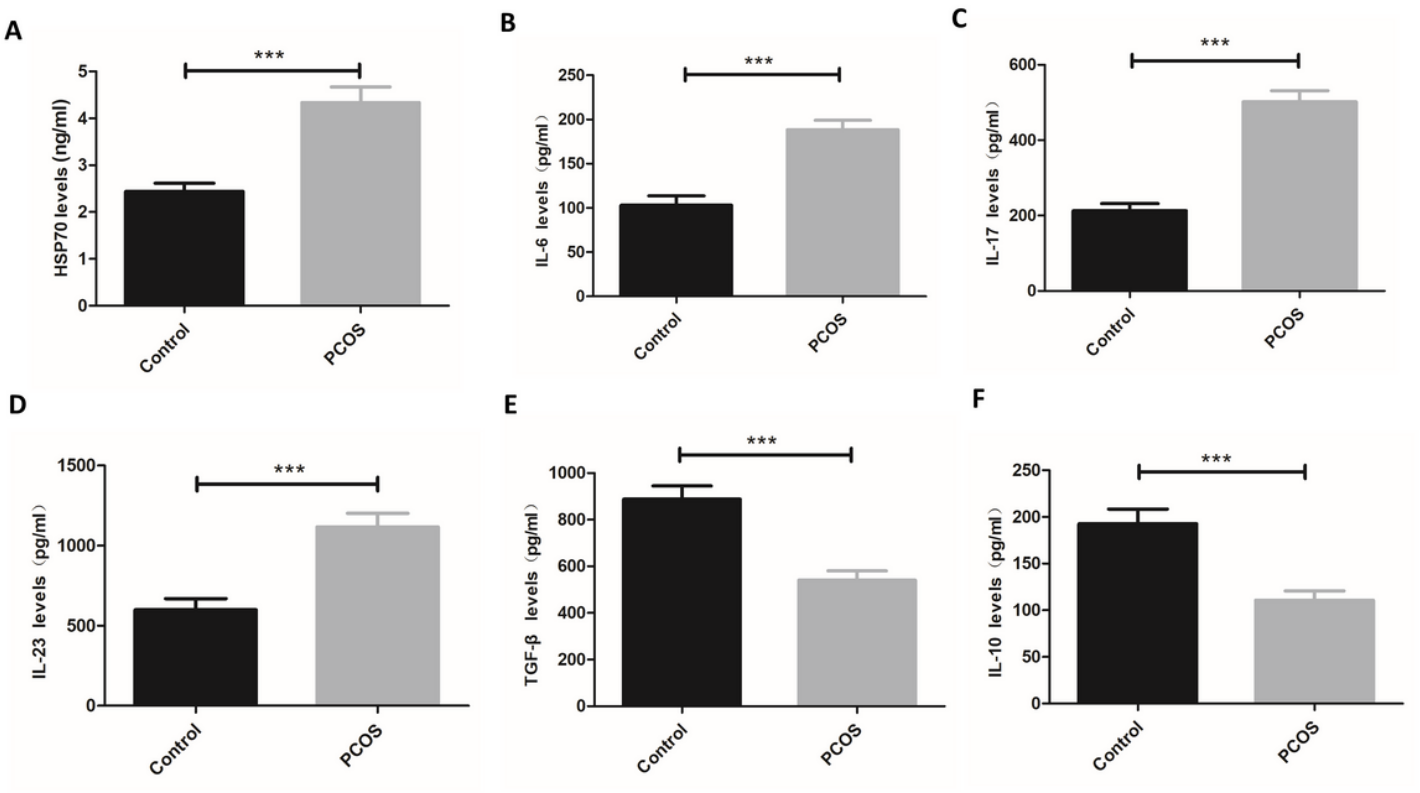

Figure 3

HSP70 expression and cytokines levels in PCOS and healthy women. A: Serum HSP70 levels; B: Serum IL-6 levels; C: Serum IL-17 levels; D: Serum IL-23 levels; E: Serum TGF-β levels; F: Serum IL-10 levels. (***P < 0.001)