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Research Article
Association of Perforin and Granzyme-B Levels with Hyperandrogenism in Polycystic Ovary Syndrome: A Case-Control Study

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Abstract

Background and Objective: Polycystic ovary syndrome (PCOS) is shown to be associated with hyperandrogenemia and has some features such as cytotoxic T-cells activation and release of perforin and granzyme-B. Present work was aimed to investigate the relation of perforin and granzyme-B to androgenic state in PCOS. **Materials and Methods:** Forty three PCOS and 40 control women were recruited. After recording demographic data, sex hormone status and cardiovascular disease (CVD) risk factors were evaluated. Perforin and granzyme-B levels were measured using sandwich ELISA kits. **Results:** Sex hormone binding globulin (SHBG) levels were lower in patients. Luteinizing hormone (LH), free testosterone (FT), dehydroepiandrosterone sulfate (DHEA-S), free androgen index (FAI), perforin and granzyme-B values were higher in PCOS group. Perforin and granzyme-B were positively correlated with FT and FAI and with each other in PCOS group. In patients, granzyme-B and perforin were related with FT and FAI, respectively. **Conclusion:** The results of present study together with evidences about the release of pro-inflammatory cytokines in insulin resistance, CVD and PCOS suggest that perforin/granzyme-B may be involved in interactions of sex hormones system in PCOS patients.

Key words: Perforin, polycystic ovary syndrome, granzyme-B, androgen, sex hormone, hyperandrogenemia, cardiovascular disease, sex hormone binding globulin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is an endocrinopathy in reproductive age that is mentioned as the most common cause of chorionic anovulation and anovulatory infertility[1-3]. This syndrome is associated with reproductive dysfunction and metabolic disorders. It is said that 5-10% of women in reproductive age are affected by PCOS. Polycystic ovary syndrome is recognized as a lifelong condition which is originated from the postnatal period toward adolescence and adulthood[2-4].

Some studies have shown that PCOS is associated with insulin resistance, hyperinsulinemia and hyperandrogenemia. Also, increased levels of androgens in PCOS women has indicated previously[1,5]. Raised prevalence of cardiovascular disease (CVD) is showed in these patients and is attributed to known CVD risk factors which are also associated with PCOS, including insulin resistance (IR), obesity, dyslipidemia, inflammation, hyperandrogenism and type 2 diabetes (T2D)[6-8]. On the other hand, thyroid hormones play an important role in all aspects of metabolism including cardiovascular disorders and simultaneously, immune system can damage the thyroid gland with subsequent abnormalities in thyroid hormones production, whether the role for thyroid hormones in metabolism in PCOS in relation to perforin and granzyme-B[9-15].

Perforin is a pore-forming protein that is stored in secretory granules inside the natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) just like granzyme-B as a serine protease and both induce the activation of caspase-dependent apoptotic pathways in target cells together[4,16,17]. Recently, some studies have demonstrated the contribution of granzymes in inflammation and non-apoptotic extracellular activities[4,17,18]. Besides, it is recognized that granzyme-B and perforin could play important roles in diabetes and CVD[4,19]. Also, granzyme-K expression is showed to be regulated by testosterone in rat model testes[20]. Granzyme-B is indicated to be increased in PCOS and be related with insulin resistance in these patients[4,16].

Some studies evinced recently that there is an expansion of $CD4^+CD28^-$ T-lymphocytes in all phenotypes of PCOS independent from patients' weight and that the activation of these cells could cause inflammatory cytokines production and cytotoxic molecules expression such as perforin and granzyme-B[4,8,21-23]. It is said that these cells could participate in atherosclerosis occurrence and are associated with poor glycemic control as first CVD event and T2D adverse outcome[4,8,24,25]. These findings suggested that this type of T-lymphocytes is related to the CVD risk, IR and hyperandrogenemia among women with PCOS. Obesity- related IR was shown in obese mice with increased granzyme-B^+ adipose-resident T-cells[4,25]. It is demonstrated that PCOS share common features similar to diabetes, obesity and CVD, such as hyperandrogenemia, cytotoxic T-cells activation and the release of perforin and granzyme-B as cytotoxic enzymes[4].

Based on the results of previous works, present study was aimed to investigate serum levels of perforin and granzyme-B in PCOS and control women and to determine whether these variables are related to androgenic status.

MATERIALS AND METHODS

Study subjects: In present case-control study, 2 groups of women at a total sample size of 83 were selected. Forty three PCOS women were recruited consecutively from patients aged 15-37 years old referred to Sayyad-Shirazi and Dezyani educational hospitals from January, 2018-April, 2018 in Gorgan, Iran. According to the criteria revised in 2003 by American Society for Reproductive Medicine (ASRM) and European Society of Human Reproduction and Embryology (ESHRE) in Rotterdam, PCOS diagnosis in study patients was done based on the presence of all three of the following criteria in them[6,26]: (1) Clinical and/or biochemical hyperandrogenism, (2) Chronic anovulation or oligo-amenorrhea (less than 8 cycles in 12 months or cycles longer than 35 days), (3) Polycystic ovaries on trans-abdominal ultrasound (ovarian volume above 10 cm^3).

Exclusion criteria included pregnancy, infectious diseases, usage of particular medications, metabolic disease, smoking, family history of some endocrinopathies, current use of oral contraceptives and immune diseases.

Forty women were also recruited as control group who were matched with PCOS group for age and BMI. These women had normal ovulation cycles (28 ± 2 days in each cycle) and did not consume any drug that affects the hypothalamic-pituitary-gonadal axis in last 6 months before inclusion in the study. All of them were evaluated by a single experienced physician to exclude any possible systemic or endocrinological disorders.

Ethical considerations: All participants received written informed consent; for children aged <18, their parents have received informed consent. The study protocol was approved by the Ethics Committee of Golestan University of Medical Sciences. Approve number is ir.goums.rec.1395.120.

Laboratory measurements: Clinical examinations and anthropometric measurements were performed on volunteers in the early of follicular phase (on days 2nd-5th of a menstrual cycle) and the results were recorded in one checklist for each person. Blood samples were obtained from participants intravenously after overnight fasting.

Serum levels of total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C) and triglyceride (TG) were measured using enzymatic assays (1500010, Pars-Azmoon, Iran, 1150011, Pars-Azmoon, Iran and 1006110, Pars-Azmoon, Iran, respectively). Low density lipoprotein-cholesterol (LDL-C) concentration was calculated by Friedewald formula.

Dyslipidemia was defined as the presence of at least one of the followings: (1) HDL-C<35 mg dL^{-1}, (2) LDL-C$\geq$110 mg dL^{-1}, (3) TG>150 mg dL^{-1} and (4) Total cholesterol>170 mg dL^{-1} [27].

Following CVD risk factors were examined in this study: overweight or obese (BMI>25 kg m^{-2}) with abdominal obesity, insulin resistance and dyslipidemia. Increased CVD risk is defined as the presence of at least two out of the three mentioned risk factors in the present study[4].

All biochemical tests were done in the Biochemistry Laboratory of Medical Faculty, Golestan University of Medical Sciences. Blood levels of fasting blood sugar (FBS) were determined using the colorimetric method (1500017, Pars-Azmoon, Iran). Fasting insulin concentration was detected by sandwich ELISA kit (5825-300, Monobind, USA).

Concentrations of FSH (425-300, Monobind, USA), LH (625-300, Monobind, USA), estradiol (4925-300, Monobind, USA), DHEA-S (EDS127, IBL, Germany), prolactin (725-300, Monobind, USA), 17-α-OH-progesterone (EOH195, IBL, Germany), SHBG (302K026, IBL, Germany) and free-testosterone (ETE122, IBL, Germany) were measured using sandwich ELISA assay kits. Also, the concentrations of serum perforin and granzyme-B were determined using sandwich ELISA Kits (CK-E91666, Hangzhou Eastbiopharm, China; CK-E11083, Hangzhou Eastbiopharm, China, respectively).

Statistical analysis: Study data were analyzed using the SPSS-16 statistical software. The data normality was assessed using Shapiro-Wilk test and all study parameters showed normal distribution. T-test was used to compare the quantitative variables (mean±standard deviation) and chi-square test was used to compare qualitative variables. Pearson's correlation coefficient was used to assess the correlations between variables. Linear regression was used for finding linear relationship between perforin and FAI and between granzyme-B and free-testosterone. Statistical significance was defined as less than 0.05 of the corresponding p-value.

RESULTS

Demographic and laboratory findings: In present study, a total number of 83 women (43 PCOS patients and 40 controls) participated. The data showed that waist/hip ratio (WHR), waist circumference (WC), FBS and HOMA-IR were significantly higher in PCOS group than control group. Also, it was found that FSH and SHBG levels were significantly lower in PCOS than controls ($p<0.001$) whereas LH, free-testosterone (FT), 17-OH-progesterone, DHEA-S, estradiol, FAI and LH/FSH values were higher in PCOS group than controls that were statistically significant. Compared with control group, perforin and granzyme-B were significantly increased in PCOS women ($p = 0.002$ and $p = 0.003$, respectively) (Table 1).

Prevalence of CVD and its risk factors: As shown in Table 2, CVD was more prevalent among PCOS group than controls that was significant ($p = 0.001$). Also, the prevalence of CVD risk factors including dyslipidemia, abdominal obesity and insulin-resistance were significantly higher among PCOS women compared to controls ($p = 0.027$, $p = 0.001$ and $p = 0.007$, respectively).

Table 1: Comparison of study variables between PCOS and controls

Parameters	PCOS	Control	p-value
Metabolic features			
Age (years)	24.07	24.43	0.77
WHR	0.81	0.77	0.002
WC (cm)	90.65	75.10	<0.001
Fasting glucose (mg dL^{-1})	95.39	90.92	0.01
HOMA-IR	2.46	1.76	0.04
Total cholesterol (mg dL^{-1})	170.42	158.22	0.15
Triglyceride (mg dL^{-1})	142.21	89.38	0.001
HDL-C (mg dL^{-1})	52.08	53.11	0.73
LDL-C (mg dL^{-1})	89.90	87.23	0.70
Hormonal features			
FSH (mIU mL^{-1})	4.09	5.45	<0.001
LH (mIU mL^{-1})	10.63	5.76	<0.001
Prolactin (ng mL^{-1})	15.10	14.29	0.62
Free-testosterone (ng mL^{-1})	0.99	0.62	<0.001
17-OH-progesterone (ng mL^{-1})	1.13	0.90	0.001
DHEA-S (μg mL^{-1})	3.30	2.04	0.001
Estradiol (pg mL^{-1})	95.67	62.44	0.004
SHBG (nmol L^{-1})	11.77	26.12	<0.001
FAI	9.63	2.58	<0.001
LH/FSH	2.85	1.11	0.001
Granzyme-B (ng L^{-1})	74.62	23.10	0.003
Perforin (ng mL^{-1})	74.58	24.16	0.002

Table 2: Comparison of CVD prevalence and its related risk factors between PCOS and control groups

Variable	PCOS	Control	p-value
CVD (n%)	26 (60.5)	10 (25.0)	0.001
Dyslipidemia (n%)	24 (55.8)	13 (32.5)	0.027
Abdominal obesity (n%)	33 (76.7)	4 (10)	0.001
Insulin resistance (n%)	15 (34.9)	4 (10.0)	0.007

Table 3: Results of Pearson correlation analysis between granzyme-B, perforin and study variables

	Perforin				Granzyme-B			
	PCOS		Control		PCOS		Control	
Variables	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
FSH	0.23	0.005	-0.03	0.40	0.25	0.05	0.10	0.26
LH	0.39	0.005	0.05	0.37	0.40	0.003	0.05	0.36
Free-testosterone	0.30	0.02	-0.30	0.02	0.21	0.08	-0.26	0.05
DHEA-S	0.07	0.32	0.06	0.34	0.007	0.48	0.09	0.28
Estradiol	0.24	0.06	-0.30	0.02	0.21	0.08	0.003	0.49
SHBG	0.04	0.38	0.23	0.07	0.04	0.39	0.17	0.13
FAI	0.27	0.04	-0.34	0.01	0.20	0.09	-0.31	0.02
Granzyme-B	0.98	<0.001	0.68	<0.001	-	-	-	-

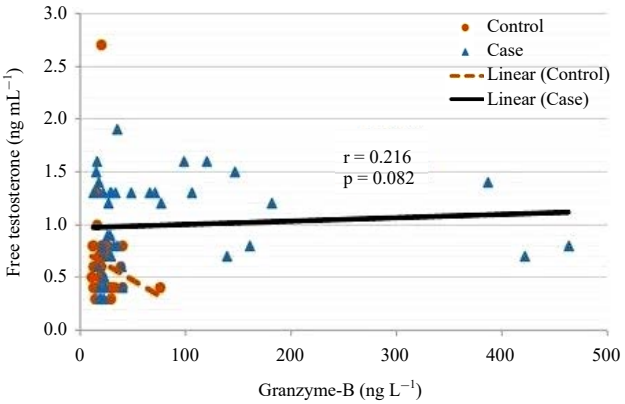

Fig. 1: Evaluation of the association between granzyme B and free testosterone levels

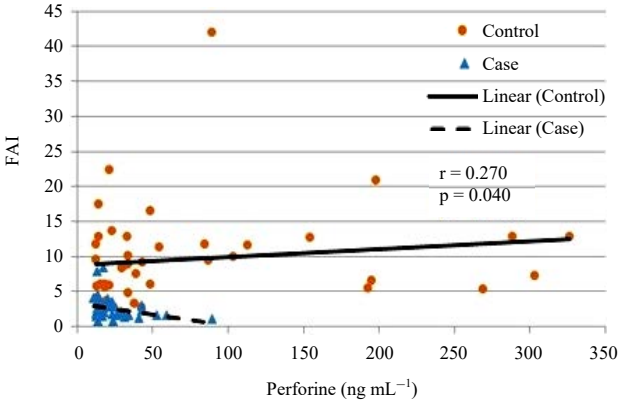

Fig. 2: Evaluation of the association between perforin and FAI values

Correlations between study variables: The correlation between serum granzyme-B and perforin concentrations with other study parameters in both PCOS and control groups was also evaluated. Significant positive correlations were shown between perforin levels with FSH, LH, FT and FAI and between granzyme-B with FAI values in PCOS group (Table 3).

Regression analysis: Regression analysis illustrated a positive association between perforin and free-testosterone concentrations (r = 0.216, p = 0.082) that was positive in PCOS group while it was negative in the control group as presented in Fig. 1. Also, a significant positive association was shown between perforin and FAI values (r = 0.270, p = 0.040) in PCOS group (Fig. 2); this was negative among controls.

DISCUSSION

It is demonstrated that PCOS is a state of inflammatory activation and has an autoimmune nature[8,28,29]. So, significantly higher levels of perforin and granzyme-B as two inflammatory cytotoxic molecules indicated in present and some other works in PCOS could be related to this inflammatory nature. One explanation to support these findings is the described expansion of $CD4^+CD28^-$ T-cells, perforin, granzyme-B and hs-CRP in the peripheral circulation of patients with PCOS and all its phenotypes, autoimmune diseases, chronic inflammatory diseases and immune deficiency[4,8,17,21,29,30]. Higher levels of $CD4^+CD28^-$ T-lymphocytes in PCOS may play a role in the complex pathogenic mechanism of this syndrome[21] because activation of these cells may cause the production of inflammatory cytokines and cytotoxic molecules such as perforin/granzyme-B which together can induce apoptotic activation in target cells[4,17,21].

Increased granzyme-B levels were demonstrated to be associated with increased WHR and IR in PCOS patients[16]. Also, a potential role is suggested for granzymes in the development of IR and type 2 diabetes and it is stated that $CD4^+CD28^-$ T-cells are expanded in T2D patients[4,8,24,31-33]. It was proposed that pancreatic b-cell dysfunction in PCOS patients may be due to systemic inflammation[4,34]. These findings suggested that in PCOS patients, increased levels of granzyme-B/perforin may be related with IR; current findings supported this hypothesis[4,16,35]. Perforin/granzyme-B were

postulated to be implicated in the pathogenesis of diabetes and CVD as conditions which are related with PCOS, inflammation and IR, as shown in present work[17,19,21]. In addition, increased risk of obesity and diabetes are shown to be implicated with granzyme-B levels in PCOS patients[16].

Significantly higher levels of free androgens and FAI and lower levels of SHBG in PCOS women were demonstrated in present work and previous studies[8,21]. These findings together with the significant positive correlation between perforin with granzyme-B, FT levels and FAI index in present work can reflect existence of a relationship between higher androgen levels and perforin/granzyme-B concentrations in PCOS. This may occur through expansion of $CD4^+CD28^-$ T-cells in PCOS women as evidences show higher levels of these cells in PCOS patients, especially in the hyperandrogenic state and also show significant correlation between $CD4^+CD28^-$ frequency and FAI in these patients[21,29]. Another suggestion comes through indicated IR and high levels of LH in PCOS patients (as shown in present work) because IR is demonstrated to be correlated with hyperandrogenemia in PCOS through its effect on theca cells, just as the effect of LH on these cells[36-38]. Since IR is shown to be related to inflammation, as mentioned above, it could be concluded that hyperandrogenemia state may be correlated with higher levels of perforin/granzyme-B in PCOS.

Perforin and granzyme-B are two cytotoxic molecules which induce apoptosis in cytotoxic lymphocytes to eliminate virus-infected or transformed cells; this is the main pathway used by these cells to eliminate these agents[17]. Some findings suggested that the activation of innate immunity, as well as dysregulation of adaptive immunity, may play pathogenic roles in PCOS as one complex syndrome[21]. Recent recognition of granzymes has demonstrated that these molecules have additional extracellular, perforin-independent, non-apoptotic proteolysis activities and are contributed to inflammation[35]. Recent studies suggested a primary mechanism for atherosclerotic plaque formation as one inflammatory state through activation of perforin/granzyme pathway. Also, significant and separate roles for granzyme-B and perforin in the pathogenesis of atherosclerosis rather than the traditional apoptotic pathway has been shown in previous studies[4,19,39]. Similar evidence was found previously in pancreatic islet cells among people with IR and T2D[4]. Altogether, perforin and granzyme-B are involved in complex interactions of the immune system and may affect the endocrine system. Limitations of this work included a relatively small sample size and a single measurement of baseline granzyme-B/perforin levels in time of menstrual cycle. Evaluation of RNA levels of these molecules and assessing the immune status in time of sampling is suggested.

CONCLUSION

The PCOS patients had higher perforin and granzyme-B levels than controls that were positively correlated with LH and androgen status. Also, significant relationships were shown between androgenic state and the levels of these two cytotoxic molecules. It is suggested that perforin/granzyme-B may be involved in complex interactions of the sex hormones endocrine system in PCOS patients. Further studies are warranted to clarify these relations.

SIGNIFICANCE STATEMENT

This study discovers the positive relation of perforin and granzyme-B to the androgenic state in PCOS. This study will help the researchers to uncover the critical areas of both endocrinology and immunology that many researchers were not able to explore. Thus a new theory on endocrinology/immunology may be arrived at.

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