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Original Research

Patatin-like phospholipase domain containing 3-gene (adiponutrin), preptin, kisspeptin and amylin regulates oocyte developmental capacity in PCOS

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Abstract: This study was planned to test whether follicular fluid (FF) levels of patatin-like phospholipase domain containing 3-gene (PNPLA3:adiponutrin), preptin, kisspeptin, and amylin change in polycystic ovarian syndrome (PCOS). A total of 40 infertile volunteers undergoing IVF/ICSI were included in the study. They were divided into two groups as PCOS (n=20) and control group without PCOS (n=20). The controls were recruited from subjects with a poor ovarian response. The PCOS and control participants were matched according to their body mass index (BMI). Each group of participants underwent ovarian stimulation with GnRH antagonist protocol. Blood and FF samples of one dominant follicle were obtained from each subject during the oocyte pick-up. FF and serum levels of PNPLA3, preptin, kisspeptin and amylin were measured through ELISA. Amylin and adiponutrin median values were not different according to study groups (p>0.05). FF-preptin median values in the control group were similar to the serum preptin values of control and PCOS groups (Z=0.970, *p*=1.000 and Z=2.631, *p*=0.051, respectively). Medians of the serum preptin in control and PCOS groups were the same (Z=1.649; *p*=0.595). FF-preptin median values of PCOS group were significantly lower than the preptin median values of the control group. Serum preptin levels were positively correlated with HOMA-IR, but not with pregnancy rates and the number of retrieved oocytes. Serum kisspeptin levels were negatively correlated with the number of retrieved oocytes and pregnancy rates. While amylin and adiponutrin have no role in the folliculogenesis, kisspeptin and preptin work together for regulating follicle developmental capacity in PCOS.

Key words: PCOS; Follicular fluid; IVF/ICSI; PNPLA3; Preptin; Kisspeptin; Amylin.

Introduction

The metabolic consequences of polycystic ovarian syndrome (PCOS) are not only limited to endocrine organs but they also may affect the cumulus oocyte complex and oocyte developmental potential. Follicular fluid (FF) is a product of oocyte retrieval. It reflects the metabolism of oocyte and granulosa cells. FF contains regulatory molecules for growing follicle. This fluid has both protector and nutritive ability for oocyte and cumulus cells. Correspondingly, FF content of some mammalian species changes depending on the growing potential of the follicle (1,2). These changes in the FF might be due to metabolic changes in the peripheral tissue or circulation (3). Investigation of the human and animal FF in terms of peptides and lipids has increased in the last decade, but little is known about its effect on follicle growing (1,4-8). Many characteristic futures of FF make it an ideal candidate for using as a noninvasive biomarker for oocyte well-being (9). Studies reported that biochemical, hormonal or spectroscopy analysis of FF could be a useful screening method for assessing fertility outcome (9-12). In a recent review, the relationship between the peripheral/central peptides and reproductive events was comprehensively discussed by our team (13). We have also demonstrated that there is a strong relationship between FF-irisin, cerebellin, betatrophin and oocyte quality and fertilization rates in PCOS subjects.

The interaction between peptides, adipose tissue, gonads, and central nervous system may be disrupted in PCOS (14-16). In addition to insulin resistance and hyperandrogenism, failed expression of peptides may be the main culprit of subfertility due to PCOS (17). Fluctuation in circulating levels of any peptide may lead to the disrupted release of GnRH and gonadotropins (18,19). This may result in lower implantation rates observed in women with PCOS.Many studies have sought to investigate the impact of circulating levels of peptides on metabolic and reproductive events (4,5). However, it is not clear whether subfertility in PCOS subjects is due to an excessive or low peptide production or action. Despite several studies have investigated circulating levels of preptin, kisspeptin, amy-

lin, and adiponutrin in metabolic disorders neither FF concentration nor the possible effect of these peptides on follicle developmental capacity and fertility outcome was investigated in PCOS (13,20-24). In the present study, in addition to three peripheral peptides including adiponutrin, amylin, and preptin, the possible impact of a central peptide, kisspeptin, on follicle dynamics was analysed in the FF of PCOS subjects undergoing IVF/ICSI. Correlation between these peptides and fertility outcome was also analysed.

Patients and Methods

We recruited 40 infertile women who were undergoing IVF treatment at the Medipol University Hospital. They were divided into two groups as PCOS (n=20) and control group without PCOS (n=20). Sample size calculation was provided before the study. We used an alpha cut-off of 5%. Follicular fluid or serum PNPLA3, preptin, kisspeptin, and amylinconcentrations were considered to be significant at type I error of 5%. The PCOS patients were recruited from infertility clinic and the patients without PCOS were recruited from poor responders (POR). The PCOS and control participants without PCOS were matched according to their body mass index (BMI). Participants were diagnosed as a PCOS if she had two of the following three findings: 1- oligo-anovulation, 2- either clinical or biochemical hyperandrogenism, and 3- ultrasonographic diagnosis of polycystic ovaries (25). Participants in the control group were diagnosed as a POR when at least two of the following three manifestations were present: (i) women with age ≥40 years; (ii) a previous history of POR (oocyte retrieval less than 3 with a conventional induction method); (iii) decline in ovarian reserve (the number of antral follicle at ultrasonography <5-7; serum AMH levels <0.5 to 1.1 ng/ml). A blood test was performed on the 2^{nd}–5^{th} day of the progesterone-induced bleeding of anovulatory PCOS and on the day 3 of ovulatory PCOS subjects and POR. Fasting blood samples were used for biochemical and hormonal evaluation. The homeostasis model assessment of insulin resistance index (HOMA-IR) was used for insulin resitance=Fasting serum insulin (mU/mL)×Fasting glucose (mg/dL)/405 (26).

Both serum and FF samples used in this study were of the same specimens collected for our earlier study that was approved by the local investigation and ethics committee (4). At the time of oocyte retrieval, the FF from one dominant follicle between the sizes of 16 and 18 mm was collected by transvaginal ultrasound-guided needle aspiration. To avoid the collection of blood contaminated FF, a midstream follicle content was collected. Samples were centrifuged at 3.000 x g for 10 minutes, and the supernatant was aliquoted and stored at -80 ^{0}C until analysis. On the day following oocyte retrieval, each oocyte was examined for evidence of fertilization. Patients underwent elective single or double-embryo transfer. Quantitative ELISA technique and kits properties used for the measurement of the peptides are shown in Table 1.

Statistical Analysis

The laboratory results obtained from experimental groups were transferred to the computer and necessary error checks and corrections were made. Normal distribution of amylin, kisspeptin, preptin, and adiponutrin values was graphically evaluated by using the Shapiro-Wilk test. Since none of the variables were normally

Table 1: Quantitative ELISA technique and kits properties used for the measurement of the peptides.

	Catalog number	Intra-inter Assay	Minimum detection limit	Assay range	Mikroplate Reader	Sensitivity
Adiponutrin	Human Adiponutrin (ADPN/PNPLA3) ELISA KIT SUNRED BIOSCIENCE Catalogno:201-12-7346 Shanghai, CHINA	Intra–assay:CV<10 % Inter assay: CV< 12 %	0.08 ng/mL	0.08 -20 ng/mL	ChroMate, Microplate Reader P4300 (AwarenessTechnology Instruments, USA)	0.072 ng/mL
Kisspeptin	Human (KISS1) ELISA KIT SUNRED BIOSCIENCE Catalogno: 201-12-4106 Shanghai, CHINA	Intraa-ssay: CV< 10% Inter assay: CV<12 %	5 pg/mL	5 - 1500 pg/mL	ChroMate, Microplate Reader P4300 (AwarenessTechnology Instruments, USA)	4.776 pg/mL
Preptin	Human PREPTIN ELISA KIT BIOABB Wuhan BIOABB Biological Engineering Co., Ltd. Catalog Number: B810196HU	Intra-assay:CV<8% Inter assay: CV<12%	25 pg/mL	57 -1000 pg/mL	ChroMate, Microplate Reader P4300 (AwarenessTechnology Instruments, USA)	25 pg/mL
Amylin	Human AMYLIN ELISA KIT (EASTBIOPHARM CO., LTD. Catalognumber CK-E10377 Hangzhou, CHINA)	Intra-assay:CV< 10% Interassay: CV< 15%	5 pg/mL	5 -2000 pg/mL	ChroMate, Microplate Reader P4300 (AwarenessTechnology Instruments, USA)	5 pg/mL

CV: coefficient of variation.

distributed, the median (IQR-Inter Quartile Range) was used for descriptive statistics. The differences between the laboratory results according to the experimental groups were investigated by the Kruskal-Wallis test. The Bonferroni corrected post-hoc Mann-Whitney test was used for the comparison of the preptin and kisspeptin variables which showed differences between the groups. Correlations between the serum and/or FF peptide levels and the number of retrieved oocytes, clinical pregnancy rates, and other parameters were evaluated with Spearman's correlation method. For statistical analysis, calculations, and graphical illustrations MS-Excel 2007 and IBM SPSS Statistics 22.0 were used (IBM Corp. Released 2013 and IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Statistical significance was accepted if *p*-value was less than 0.05 ($p<0.05$).

Results

The age of PCOS subjects was smaller than that of the controls. Infertility duration of the control group was longer than the PCOS group. Demographic, biochemical and clinical characteristics of PCOS and control groups are mentionedin Table 2. Although there was an upward increasing trend in BMI of PCOS patients, this did not reach statistical significance. PCOS subjects showed high serum levels of insulin, total testosterone, estradiol and HOMA-IR as compared to those of the controls. Basal FSH levels of the control group were significantly higher than that of the PCOS group. The number of retrieved oocyte and clinical pregnancy rates were significantly higher in PCOS group than that of the controls. A total of 80 samples were collected from 40 participants. Forty of these samples were serum and the remaining 40 were follicular fluid. Descriptive statistics of amylin, kisspeptin, preptin, and PNPLA3 values obtained from volunteers according to study groups are given in Table 3 and Figure 1. Amylin and PNPLA3 median values were not different according to study groups ($p>0.05$). A positive but insignificant correlation was detected between serum PNPLA3 and HO-

Table 2: Demographic, biochemical and clinical characteristics of PCOS and control groups.

Parameters	PCOS (n:20)	Control (n:20)	*P value
Age	31.0 ± 3.68	35.3±3.10	0.001
Body mass index (kg/m^2)	27.3±4.90	24.5±4.96	0.078
Infertility duration (yr)	6.80±0.23	13.8±1.4	0.000
Day 3 FSH (mU/ml)	5.82 ± 1.34	9.87 ± 5.17	0.045
Day 3 LH (mU/ml)	7.98±5.47	6.00±2.31	0.137
Day3 E2 (pg/ml)	41.6±29.9	51.8±42.1	0.368
Total testosterone (ng/dl)	74.4 ±3.32	34.1 ±6.72	0.001
E2 on the day of HCG (pg/ml)	4153.9±1835.7	332.2±126.6	0.001
HOMA-IR	4.22 ± 3.40	2.12 ± 1.52	0.005
Fasting insulin (mU/ml)	19.1 ± 1.43	12.1 ± 1.13	0.006
Fasting glucose (mg/dl)	93.4 ± 3.41	87.9 ± 6.41	0.340
Duration of rhFSH (day)	8.95±1.11	8.38±2.03	0.266
Total oocyte number retrieved	19.8±1.90	3.43±0.20	0.001
Fertilization rates (%)	78	67	0.001
Implantation rates (%)	40	22.8	0.001
Clinical pregnancy rates (%)	75	35	0.001

Data are presented as mean±SD, *$p<0.05$ is accepted statistically significant.

Table 3: Descriptive statistics of measured peptides according to study groups.

Variables	Group	Min – Max	Median (IQR)	χ^2	P
Amylin (pg/ml)	Control-FF	45.86 - 227.74	90.00 (28.20)	5.570	0.135
	PCOS-FF	33.75 - 195.62	78.17 (47.45)		
	PCOS-Serum	17.31 - 344.22	94.61 (50.05)		
	Control-Serum	32.88 - 315.47	111.78 (84.63)		
Kisspeptin (pg/ml)	Control-FF	112.77-1510.02	320.55 (358.01)	3.843	0.0279
	PCOS-FF	99.49-2107.86	241.10 (234.35)		
	PCOS-Serum	81.89-2026.50	221.14 (213.50)		
	Control-Serum	101.93-1706.01	206.02 (297.92)		
Preptin (pg/ml)	Control-FF	0.00-485.56	21.49 (119.18)	31.465	<0.001
	PCOS-FF	0.00-72.00	0.00 (7.48)		
	PCOS-Serum	0.00-1389.55	283.09 (377.93)		
	Control-Serum	0.00-2875.39	44.39 (248.56)		
PNPLA3 (ng/ml)	Control-FF	0.41-43.95	2.23 (1.85)	1.518	0.678
	PCOS-FF	0.25-129.80	2.07 (2.69)		
	PCOS-Serum	0.41-106.90	1.82 (1.61)		
	Control-Serum	0.00-51.89	1.75 (2.75)		

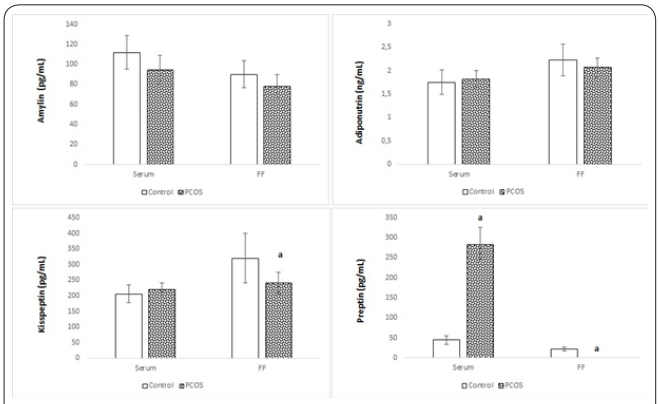

Figure 1. Comparison of Amylin, kisspeptin, preptin, and PNPLA3 values in polycystic ovarian syndrome. FF: Follicular fluid; PCOS: Polycystic ovarian syndrome; a: p< 0.05.

MA-IR (r=0.39, p<0.69). No correlation was detected between the amylin levels and other clinical and laboratory parameters. There is a statistically significant difference between the PCOS and control groups in terms of preptin (χ^2=31.465; p<0.001) and kisspeptin median values (χ^2=3.843; p<0.02). Spearman's correlation analysis showed no significant association between the FF and serum samples in terms of measured peptides. On the other hand, serum kisspeptin levels are negatively correlated with the number of retrieved oocytes (r=-0.420, p<0.02) and pregnancy rates (r=-0.501, p<0.04). No correlation was detected between FF-kisspeptin and other measured parameters. When it was investigated which group led to the difference in the preptin median values, we identified that preptin median values obtained from the follicular fluid in the control group were similar to the serum preptin values of control and PCOS groups (Z=0.970, p=1.000 and Z=2.631, p=0.051, respectively). It was also observed that the medians of the serum preptin in control and PCOS groups were the same (Z=1.649; p=0.595). Preptin medians obtained from the follicular fluid of PCOS group were significantly lower than the preptin median values of all the other groups (Z=2.770; p=0.034 for PCOS-follicle vs Control-follicle, Z=3.752; p=0.001 for PCOS-follicle vs Control-serum and Z=5.468; p<0.001 for PCOS-follicle vs PCOS-serum). Serum preptin levels were positively correlated with HOMA-IR (r=0.45, p<0.03), but not with pregnancy rates and the number of retrieved oocytes. Any correlation was not detected between the FF-preptin and other parameters.

Discussion

Metabolic disturbance in PCOS may adversely affect the production or release of some peptides that may have a role in follicle growing. Altered concentrations of follicular fluid irisin, cerebellin or betatrophin in PCOS subjects undergoing IVF/ICSI support this idea (4,5). Despite the fact that PCOS subjects have high or low circulating levels of some peptides, the impact of FF peptide levels on developing follicle remained elusive. In the current study, we showed for the first time that FF concentrations of kisspeptin, preptin, PNPLA3, and amylin can provide additional information about the oocyte developmental capacity, implantation and pregnancy rates.

Kisspeptin is a central peptide that stimulates GnRH release from the arcuate neurons and regulates the LH secretion (21,22). Stimulation of kisspeptin neurons increases LH secretion and causes PCOS-like ovarian morphology (13,21,22). Kisspeptin also mediates the functions of some peripheral and central peptides on the arcuate nucleus. Significantly increased kisspeptin levels in the serum of PCOS subjects have been reported (16). Nevertheless, FF-kisspeptin in PCOS has not been reported. In the present study, we found that serum levels of kisspeptin in PCOS and control subjects were same. However, FF-kisspeptin values of PCOS subjects were significantly lower than that of control subjects. On reviewing the literature, conflicting results have been reported regarding kisspeptin levels in PCOS. It has been reported that normal weight and obese PCOS women had higher kisspeptin levels compared to obese and overweight non-PCOS women (16). Authors also reported a negative correlation between kisspeptin, BMI, free androgen index, and insulin resistance (16). We showed that kisspeptin level is negatively correlated with the number of retrieved oocytes and pregnancy rates, suggesting a possible role of kisspeptin in subfertility due to PCOS. Similar to our results, Emekci et al. reported that serum kisspeptin levels of PCOSand non-PCOS subjects were not different (27). In the light of our findings, we propose that FF-kisspeptin has a role in the regulation of folliculogenesis in PCOS.

Preptin is a proinsulin-like growth factor II E-peptide present in islet b-cells and is co-secreted with insulin in response to glucose. It enhances insulin secretion in rats (28) and there is a potential link between preptin levels and insulin resistance (29). To date, few published trials have been conducted focusing on the significance of preptin in PCOS. In a previous study conducted by our team, we showed for the first time that plasma preptin levels of women with PCOS were higher than that of non-PCOS controls (30). In the present study, there was a statistically significant difference between the PCOS and non-PCOS subjects in terms of preptin median values. Preptin values obtained from the follicular fluid of PCOS women were significantly lower than the preptin median values of all the other groups. Serum preptin levels are positively correlated with HOMA-IR, but not with pregnancy rates and the number of retrieved oocytes indicating that there is a potential link between IR and preptin. Similar circulating and FF-preptin levels in non-PCOS subjects led us to think that there was a saturable transport system between serum and FF. On the other hand, despite high levels of serum preptin decreased FF-preptin levels in PCOS women, the transfer of the preptin from serum to FF was not allowed. If preptin was transported from serum to FF by the receptor-mediated system, the transport of preptin into FF should be facilitated in the presence of high levels of serum preptin. Due to the difference in circulating and FF-preptin levels in PCOS women, it is logical to think that there is a preptin resistance between two compartments. In view of these findings, we can propose that main production site of preptin is circulation. Follicular fluid preptin is most likely local production from oocyte or follicle cells excluding transport of preptin from circulation into the FF. Preptin resistance in developing follicle wall and decreased levels of FF-preptin may contribute to either ovulatory dysfunction

or subfertility due to PCOS.

PNPLA3 gene is a nutritionally regulated lysophosphatidic-acyltransferase expressed in the liver and adipose tissues. It is known that PNPLA3 as a lipase is associated with retinyl-palmitate hydrolysis in the hepatic cells (31). Dysfunctional PNPLA3 expression induces the accumulation of lipotoxic substrates (23,24) and is characterised with insulin resistance and increased risk for T2DM (32,33). Adipose tissue dysfunction has a critical role in PCOS and contributes to systemic IR (34). Similar to women having metabolic syndrome, most PCOS subjects display obesity suggesting metabolic dysfunction. Because synthesis and secretion of adipokines are altered in PCOS (35), FF-PNPLA3 may contribute to ovulatory dysfunction or subfertility due to PCOS. To our knowledge, circulating and FF-PNAPLA3 levels have not been reported previously in PCOS. This study has demonstrated for the first time that FF of PCOS subjects express PNPLA3. However, neither FF nor serum PNAPL3 levels of the PCOS and control subjects reached statistical significance. We have also shown that FF-PNPLA3 is not associated with the number of retrieved oocyte and pregnancy rates. If the PCOS cases were separated as obese and nonobese, the results might be different. For this reason, extensive studies are needed to draw a clear conclusion on the effect of FF-PNPLA3 on follicle development.

Amylin is a neuropeptide which is co-secreted with insulin. This peptide contributes to the homeostasis of energy consumption and glucose regulates its secretion (36). When reviewing the literature significantly increased amylin levels were noted in the serum of PCOS subjects. Moreover, a positive and significant correlation between insulin and amylin levels has been reported in PCOS (37). However, no other studies have addressed the role of amylin on follicle developmental potential in PCOS. In the present study, although trends toward decreased FF-amylin levels were noted in the PCOS subjects compared to controls, the differences failed to show statistical significance. We did not find any correlation between FF-amylin levels and other parameters including IR, oocyte quality and pregnancy rates.

Follicular fluid comprised of the plasma and follicle wall derived secretions (38). It contains many peptides which can provide supplies of nourishment to the oocyte and the somatic cells (39). Adequate cross-talk between peptides, central nervous system and oocyte may regulate fulfillment of normal follicle development in PCOS (13). Measurement of FF peptide composition is a non-invasive method by which the clinician can provide additional information about embryo development and selection.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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