Klotho is a prognostic indicator of oocytes qualities in polycystic ovary syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disease related to infertility in women. IVF-ET is the effective way to treat infertility in PCOS patients. But poor oocytes quality of PCOS patients results in low pregnancy rate and the underlying mechanism is unknown. Klotho, an anti-aging factor, was reported up-regulated in PCOS and related to excessive androgens. However, the role of Klotho in poor oocytes quality of PCOS has not been clarified.

Method: We enrolled 72 PCOS and 70 healthy participants who underwent IVF-ET and the follicular fluid was collected. In addition, granulosa cells from 15 patients underwent IVF-ET were collected. The level Klotho in follicular fluid was tested by ELISA. The mRNA level of Caspase-3, Caspase-9, IL-6, TNF, PCNA and GDF9 in granulosa cell were detected by qRT-PCR.

Results: Compared with the control group, the level of Klotho, Caspase-3, Caspase-9, IL-6 and TNF in PCOS group was significantly higher, especially in the group of PCOS with hyperandrogenism, while the level of PCNA and GDF9 was down-regulated. Correlation analysis shown that level of Klotho in follicular fluid of PCOS group was negatively correlated with the number of mature oocytes. Similarly, we divided the PCOS group into two groups according to the median of Klotho level and found that the number of mature oocytes is significantly lower in the group of high level of Klotho. In addition, the Klotho level in follicular fluid was also positively correlated with serum testosterone, LH, LH/FSH, menstrual cycle and number of total antral follicles in the group of PCOS.

Conclusions: These data suggested that Klotho may act as a new biomarker evaluating the quality of oocytes in patients with PCOS, indicating the severity of PCOS and the outcome of IVF in PCOS patients.

Background

Polycystic ovary syndrome (PCOS) is the most common type of endocrine disorder in women of reproductive age(1, 2). Nowadays up to 20% women of reproductive-age are suffering PCOS, which is mainly manifested by oligoovulation or anovulation, hyperandrogenism and polycystic ovarian morphology(1). There are many clinical treatments of PCOS, such as lifestyle management, medication, potentially surgery and assisted reproduction technology, but the high cost of treatments with low pregnancy rate has seriously reduced patients' quality of life(3-5). Hence, to find out a prognostic indicator in PCOS is of great value to provide early prevention and treatment to PCOS patients.

Follicular development disorder in PCOS is considered to be the main cause of anovulatory infertility(6). In vitro fertilization and embryo transfer (IVF-ET) is one of the most effective ways to treat infertility in PCOS patients. However, due to the narrow "treatment window" of follicular development, problems have also been revealed. Low dose of gonadotropin initiation may lead to follicular growth failure, while high dose may lead to ovarian overreaction, resulting in follicular dysplasia, and these may lead to few or too many oocytes harvested and poor-quality oocytes. Hence, how to obtain the oocytes with appropriate quantity and high quality from PCOS patients is a major challenge for clinicians. The

lack of a biomarker related to oocyte maturation may be a possible reason for the failure of PCOS treatment.

The microenvironment of follicular fluid and the normal function of granulosa cells are the two key factors for the development of oocytes(7), which affects the subsequent fertilization, early embryonic development and pregnancy outcome. Notably, evidences showed that hyperandrogenemia in PCOS is associated with ovarian granulosa cells and oocyte development disorder(8). Our previous study indicated that the high level of androgen in follicular fluid of patients with PCOS is closely related to granulosa cell apoptosis and inflammation(9). And in vitro experiments shown that testosterone could induce apoptosis of granulosa cells in a concentration-dependent manner(10). Follicular dysplasia was also confirmed in animal models with excessive androgen(11). Hence, excessive androgen in both peripheral circulation and ovary are major risks for abnormal follicular development in patients with PCOS, but further mechanism is not clear.

The Klotho gene is a senescence-related gene discovered in 1997(12). Klotho protein is involved in calcium and phosphorus homeostasis, inflammatory response, oxidative stress and apoptosis(13), so it has been taken as a good factor in protecting human body for a long time. For example, Klotho play a vital role in protecting renal function and inhibiting renal fibrosis through regulating the renin-angiotensin-aldosterone system and inhibiting cell apoptosis, oxidative stress and inflammation(14, 15). Decrease of Klotho may increase the risk of kidney disease, such as acute kidney injury, diabetic nephropathy, and chronic kidney disease(16). And exogenous administration of Klotho helps to slow diseases progression. In addition, Klotho helps to protect brain neurons and improve cognitive function, such as Alzheimer's disease(17, 18). At the same time, Klotho could also act as an effective tumor suppressor effect in malignant tumors such as breast cancer, kidney cancer, thyroid cancer, oral squamous cell carcinoma and ovarian cancer(19-24). However, interestingly, recent studies found that the expression of Klotho in serum and granulosa cells of PCOS patients was significantly higher(25, 26). They found that the expression of Klotho protein was significantly increased in ovarian granulosa cells of DHEA-induced PCOS rats, and these was related to granulosa cell apoptosis(25). And down-regulation of Klotho gene expression increased the pregnancy rate of DHEA-induced female rats, restored ovarian function and reduced the formation of cystic follicles(25). These results suggest that high expression of Klotho may be an unfavorable factor in the progression of PCOS disease. However, it seems that the role of Klotho in PCOS is contradictory to that of the other diseases, but there was no further study was found between Klotho and PCOS.

Herein, in this study, we conducted a retrospective study to explored the role of Klotho in follicular fluid of PCOS and its function on oocytes of PCOS patients. We report that Klotho in the follicular fluid of PCOS was associated with the decreased oocyte qualities. Furthermore, hyperandrogenism has a significantly positive correlation with follicular fluid Klotho of PCOS. We also show that a higher level of Klotho expression in the follicular fluid of PCOS patients was associated with cell apoptosis and inflammation in human granulosa cells (GCs). Thus, we conclude that follicular fluid Klotho is a promising marker of

oocyte maturation and highly associated with the serum testosterone in PCOS. It provides a new idea for improving the developmental potential of PCOS oocytes and the treatment of PCOS.

Materials And Methods

Patients

The study was approved by the Ethical Committee Faculty of Nanfang Hospital, Southern Medical University and informed consent was obtained from all participants. All participants were recruited from the center of reproduction medicine in Nanfang Hospital. All methods were performed in accordance with the guidelines and regulations. The recruited subjects included 72 infertile women with PCOS, and 70 women who served as healthy controls. PCOS was diagnosed according to the Rotterdam Criteria (2003), with at least two of the following symptoms: (1) oligomenorrhea/amenorrhea; (2) polycystic ovaries (PCO) on ultrasonography; (3) clinical and/or biochemical signs of hyperandrogenism (hirsutism or acne) and exclusion of other etiologies (such as Cushing's syndrome, congenital adrenal hyperplasia or testosterone secreting tumors). GCs were obtained from additional five PCOS patients with hyperandrogenism, five PCOS patients with normal serum testosterone and five control participants. Follicular fluid (FF) was collected from the PCOS patients and controls.

Collection of FF and human ovary GCs

An antagonist protocol for controlled ovarian hyperstimulation (COH) was used in the cycle of in IVF-ET. FF and human ovary GCs were collected and restored as previously described(9).

Enzyme-linked immunosorbent assay（ELISA）

ELISA technique was used to measure the concentration of Klotho in FF. The ELISA kit (Cloud-Clone Corp, Wuhan, China) is a sandwich enzyme immunoassay for in vitro quantitative measurement of Klotho. According to the protocol, the linear regression equation of the standard curve can be calculated. Then the sample concentration is calculated on the regression equation according to the absorbance of the sample.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The mRNA expression levels of Klotho, Caspase-3, Caspase-9, PCNA, GDF9. TNF and IL-6 in GCs were analyzed by qRT-PCR. Total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY) and cDNA was synthesized with the Reverse Transcription System kit (Promega, Madison, WI), qRT-PCR was performed by using SYBR Green PCR MasterMix (Applied Biosystems), according to the kit instructions by an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), The PCR reaction system contained 12.5μL SYBR Green PCR MasterMix (Applied Biosystems), 5μL diluted RT product (1:10), and 0.5 mmol/L sense and antisense primer sets. The expression levels of various genes were determined by the comparative CT method ($2^{-\triangle\triangle}$CT). Relative levels of mRNA were reported after normalization with GAPDH. The composition of the primers was as follows: Klotho：forward 5'-

CTGGTTGCCCACAACCTACT-3′reverse 5′-TCCGAAGGAGAGAGCAAAAA-3′Caspase-3forward 5′-TGCTATTGTGAGGCGGTTGT-3′reverse 5′-TCCAGAGTCCATTGATTCGCT-3′Caspase-9: forward 5′-CTCAGACCAGAGATTCGCAAAC-3′reverse 5′- GCATTTCCCCTCAAACTCTCAA-3′, PCNA: forward 5′-GCGTGAACCTCACCAGTATGT-3′reverse 5′- TCTTCGGCCCTTAGTGTAATGAT′„ GDF9: forward 5′-CAGAGCTTTGCACTACATGAAGA′reverse 5′- TGAAGAGCCGAACAGTGTTGT-3′, IL-6 forward 5′-ACTCACCTCTTCAGAACGAATTG-3′reverse 5′- CCATCTTTGGAAGGTTCAGGTTG-3′TNFforward 5′-CACAGTGAAGTGCTGGCAAC-3′reverse 5′-AGGAAGGCCTAAGGTCCACT-3′GAPDHforward 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′reverse 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Statistical analyses

Results were present as the means±SD and categorical variables were expressed as n (%). SPSS software, version 22.0 was used for statistical analysis. The student's t test or the Mann–Whitney U test were used to compare normally and non-normally distributed variables, respective. Correlations were conducted using Spearman's correlation coefficient for non-normally distributed data. Comparisons between different groups were determined by analysis of variance, followed by the LSD test. The value of $P < 0.05$ were determined statistically significant.

Results

Subject characteristics

A total of 142 subjects aged between 22 and 40 years were involved in the study. The detail data were shown in the supplemental Table 1. Among 72 patients with PCOS, 22 were hyperandrogenemia (serum testosterone > 0.481ng/ml is used as the diagnostic criterion in Nanfang hospital). Comparison between PCOS and control is shown as Table 1. In PCOS group, menstrual cycle, number of total antral follicles (No. of AFC-Total), basal LH, LH/FSH ratio, T, LH of HCG day, number of follicles above 14 mm (No.of follicles above 14 mm), and number of retrieved oocytes (No.of retrieved oocytes) showed significant higher ($P<0.05$), while the amount of Gn initiation dose and the total Gn dose were significant lower. ($P<0.05$)). There were no significant difference in BMI, mean arterial pressure (MAP), basal FSH, basal E2, basal P, basal PRL, Gn days, E2 of HCG day, P of HCG day, number of mature oocytes (No.of mature oocytes), mature oocyte rate, number of 2PN fertilization (No.of 2PN fertilization), number of abnormal fertilization (No.of abnormal fertilization), number of high-quality blastocysts on day 3 (No.of high quality embryos on D3) and number of high-quality blastocysts on day 5 (No.of high quality embryos on D5).

Table1. Clinical characteristics in participants

Characteristic	PCOS (N=72)	Control (N=70)	P value
Age (y)	30.28±3.06	31.39±3.72	0.055
BMI (kg/m^2)	21.64±3.03	21.21±2.76	0.375
Menstrual cycle (d)	54.46±24.72	30.96±2.76	0.000**
No.of AFC-Total	25.40±6.57	15.44±4.28	0.000**
MAP (mmHg)	85.83±8.37	86.60±8.51	0.589
Basal T (ng/ml)	0.41±0.27	0.22±0.09	0.000**
Basal FSH (mIU/ml)	6.23±1.40	6.65±1.37	0.077
Basal LH (mIU/ml)	10.49±6.43	5.48±3.38	0.000**
LH/FSH	1.67±0.89	0.86±0.64	0.000**
Basal E_2 (pg/ml)	39.31±23.64	37.27±21.06	0.590
Basal P (ng/ml)	0.44±1.04	0.28±0.23	0.209
Basal PRL (ng/ml)	23.79±34.21	17.87±6.77	0.154
Gn initiation dose	132.29±41.21	191.25±59.53	0.000**
Gn days	10.49±3.32	9.76±1.65	0.099
Total Gn dose	1466.08±621.19	1917.76±692.15	0.000**
E_2 of HCG day	3731.31±2043.41	3139.16±1775.64	0.068
LH of HCG day	3.42±3.38	2.25±1.25	0.007**
P of HCG day	0.63±0.38	0.63±0.31	0.933
No.of follicles above 14 mm	11.38±4.57	9.34±3.48	0.003**
No.of retrieved oocytes	17.76±8.56	14.73±6.91	0.022*
No.of mature oocytes	14.96±7.23	12.76±6.65	0.061
Mature oocyte rate (%)	84.5(1078/1276)	86.6(893/1031)	0.149
No.of 2PN fertilization	10.17±6.18	8.51±5.35	0.091
No.of abnormal fertilization	1.15±1.47	1.36±1.81	0.461
No.of high quality embryos on D3	3.25±3.18	3.96±2.89	0.168
No.of high quality embryos on D5	1.57±1.97	2.11±2.52	0.152

Continuous variables are expressed as the mean±SD; PCOS: polycystic ovary syndrome; BMI: body mass index; No.of AFC-Total：the number of antral follicles with a diameter from 2 to 9 mm both in the left and right ovary; MAP: mean arterial pressure; FSH: follicle-stimulating hormone; LH: luteinizing hormone; T: testosterone; E2: estradiol; P: progesterone; PRL: prolactin; Gn initiation dose: Initial dose of gonadotropin; Gn days: Number of days to use gonadotropin; Total Gn dose: The total dose of gonadotropin used; No.of follicles above 14mm: Number of follicles over 14mm in diameter; No.of retrieved oocytes: Number of retrieved oocytes; No.of mature oocytes: Number of mature oocytes; No.of 2PN fertilization: Number of 2PN fertilization; No.of abnormal fertilization: Number of abnormal fertilization; No.of high quality embryos on D3: number of high-quality embryos on day 3; No.of high quality embryos on D5: number of high-quality blastocysts on day 5. *P＜0.05, **P＜0.01.

Klotho in FF is correlated with the severity of PCOS

To identify the role of Klotho in PCOS, we used the ELISA kit to detect the Klotho protein in the follicular fluid of 142 subjects. The detail data were shown in Supplemental Table 2. As is shown in Figure 1A, the concentration of Klotho in the follicular fluid of the PCOS group significantly increased (331.88±190.03pg/ml vs 211.78±150.51pg/ml, P <0.01). This suggests abnormally increased expression of Klotho in the follicular fluid of PCOS. Next, we performed the correlation analysis with Klotho, age, BMI, menstrual cycle, and number of total antral follicles in PCOS group and control group. As is shown in Table 2 and Figure 1B-C, the level of Klotho in follicular fluid of the PCOS group was positively correlated with menstrual cycle and number of total antral follicles, while no similar correlation was observed in the control group. These suggested that the level of Klotho in follicular fluid is related to the severity of PCOS.

Table 2. Correlation analysis between Klotho in follicular fluid and the severity of PCOS.

Characteristic	Klotho (pg/ml)			
	PCOS (N=72)		Control (N=70)	
	r	P value	r	P value
Age (y)	-0.143	0.231	0.018	0.880
BMI (kg/m^2)	0.043	0.720	-0.084	0.491
Menstrual cycle (d)	0.376	0.001**	0.027	0.824
No.of AFC-Total	0.297	0.011*	0.082	0.501

BMI: body mass index; No.of AFC-Total：the number of antral follicles with a diameter from 2 to 9 mm both in the left and right ovary. *P＜0.05, **P＜0.01.

Klotho in FF is related to the quality of PCOS oocytes

Follicular fluid is the important microenvironment for follicular development. Disturbance of the microenvironment could lead to follicular arrested or follicular atresia. In order to further clarify the effect of high concentration of Klotho in follicular fluid on PCOS patients underwent IVF, we performed correlation analysis with the follicular fluid Klotho and IVF clinical parameters in the PCOS group and the control group. As is shown in Table 3 and Figure 2, Klotho in FF of PCOS group was negatively correlated with the number of mature oocytes, while there was no similar correlation in the control group (Figure 2A). At the same time, we divided the PCOS group into two groups according to the median of Klotho level, and found that the rate of mature oocytes was significantly reduced in the high level of Klotho group (Figure 2B). It is suggested that the high expression of Klotho in the follicular fluid of the PCOS group may affect the quality of oocytes. However, there was no correlation between Klotho and the number of retrieved oocytes, the number of 2PN fertilization, the number of abnormal fertilizations, the number of high-quality embryos on D3 and the number of high-quality embryos on D5 in the PCOS group and the control group.

Table 3. Correlation analysis between the level of Klotho in follicular fluid and IVF clinical parameters.

Characteristic	Klotho (pg/ml)			
	PCOS (N=72)		Control (N=70)	
	r	P value	r	P value
No.of retrieved oocytes	-0.232	0.050	0.086	0.479
No.of mature oocytes	-0.240	0.043*	0.073	0.550
No.of 2PN fertilization	0.029	0.811	0.069	0.569
No.of abnormal fertilization	0.065	0.587	0.089	0.462
No.of high quality embryos on D3	0.024	0.840	-0.055	0.651
No.of high quality embryos on D5	-0.002	0.989	-0.076	0.533

No.of retrieved oocytes: Number of retrieved oocytes; No.of mature oocytes: Number of mature oocytes; No.of 2PN fertilization: Number of 2PN fertilization; No.of abnormal fertilization: Number of abnormal fertilization; No.of high quality embryos on D3: number of high-quality embryos on day 3; No.of high quality embryos on D5: number of high-quality blastocysts on day 5. *P□0.05.

The high expression of Klotho in granulosa cells from PCOS patients was accompanied by increased apoptosis, inflammatory response.

To further confirm the effect of Klotho on the oocytes of PCOS patients, we used GCs collected from PCOS patients to tested the mRNA level of Klotho, Caspase-3, Caspase-9, PCNA, GDF9, IL-6 and TNF, respectively. As shown in Figure 3A, the mRNA level of Klotho in granulosa cells of PCOS group was significantly higher than the control group, and it was the highest in the group of PCOS with

hyperandrogenism. Next, we analyzed the mRNA levels of apoptosis-related factors Caspase-3, Caspase-9 inflammation-related factors IL-6 and TNF, cell proliferation related factors PCNA and folliculogenesis related factor GDF9 in granulosa cells. As shown in Figure 3B-F, the mRNA level of Caspase-3, Caspase-9, IL-6 and TNF in granulosa cells of the PCOS group, especially the expression of Caspase-3, IL-6 and TNF in the group of PCOS with hyperandrogenism, were significantly higher than that of the control group. Similarly, in Figure 3D, the mRNA level of PCNA is significantly down regulated in the group of PCOS. Although there was no statistical difference between the group of PCOS with hyperandrogenism and the group of PCOS with normal androgen level, there was a downward trend between the two groups. And these were consistent with the Klotho expression mentioned above, which suggested that the Klotho in the follicles is closely related to the apoptosis and inflammation of granulosa cells, and is increasing with the level of testosterone. In addition, we also tested the mRNA level of GDF9. As is shown in the in Figure 3G, the expression of GDF9 is significantly down regulated in the group of PCOS, especially in the group of PCOS with hyperandrogenism.

Klotho in the follicular fluid of PCOS was positively correlated with serum testosterone levels

As mentioned above, Klotho is more pronounced in the granulosa cells of patients with PCOS and hyperandrogenemia. In order to clarify the relationship between Klotho and androgen, we first analyzed the difference in the expression of Klotho in the follicular fluid between the group of PCOS with hyperandrogenism and the group of PCOS with non-hyperandrogenism. As shown in Figure 4A, the concentration of Klotho in group of PCOS with hyperandrogenism was much higher than the other group (439.44±207.67pg/ml vs 284.55±162.36pg/ml, $P < 0.01$). Then, to find out the relationship between Klotho in the FF of PCOS patients and their basic sex hormones, we carried out correlation analysis in PCOS group and control group. As shown in Table 4 and Figure 4B-D, the expression of follicular fluid Klotho from PCOS group was positively correlated with serum testosterone, LH, and LH/FSH levels ($P<0.05$), while there was no correlation in the control group. It is suggested that the Klotho level in follicular fluid in PCOS disease is closely related to high levels of serum androgen.

Table 4. Correlation analysis between the level of Klotho in follicular fluid and basal hormone levels.

Characteristic	Klotho (pg/ml)			
	PCOS (N=72)		Control (N=70)	
	r	P value	r	P value
Basal T (ng/ml)	0.410	0.000**	0.127	0.294
Basal FSH (mIU/ml)	0.089	0.456	0.135	0.266
Basal LH (mIU/ml)	0.266	0.024*	0.211	0.080
LH/FSH	0.270	0.022*	0.179	0.139
Basal E_2 (pg/ml)	0.068	0.569	-0.017	0.887
Basal P (ng/ml)	0.072	0.548	0.101	0.405
Basal PRL (ng/ml)	-0.010	0.930	0.052	0.668

T: testosterone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; LH/FSH: The ratio of luteinizing hormone to follicle-stimulating hormone; E2: estradiol; P: progesterone; PRL: prolactin. *P□0.05, **P□0.01.

Discussion

PCOS is one of the most common endocrine disease in women of reproductive age. Many studies have revealed that PCOS always accompanied with metabolic disorders such as obesity, dyslipidemia, insulin resistance, and predisposed to type 2 diabetes, cardiovascular disease and endometrial cancer(2, 5, 27), and these seriously affect their life qualities. Klotho, as an anti-aging gene, has been proved to play an important protecting role through inhibiting oxidative stress, regulating ion channel activity and cell apoptosis(13, 28). But recent studies in PCOS shown that Klotho in serum and granulosa cells is significantly increased, and these may induce granulosa cell apoptosis(25, 26). This suggested Klotho may have an intimate association with the development of PCOS, however, its underlying mechanism has not been clarified. Hence, in this study, we tested the concentration of Klotho in the FF of PCOS patients and analyzed its relationship with the clinical data.

Previous study has revealed that serum Klotho in PCOS is significantly increased(26). Our results confirmed that the concentration of Klotho in the follicular fluid of PCOS patients was much higher than the control group. These suggested abnormal increased expression of Klotho in the follicular fluid is closely related to PCOS disease. As we all known, prolonged menstrual cycle and increased number of antral follicles is the most important feature of PCOS, and these two symptoms reflect the severity of PCOS(9). Hence, we performed correlation analysis between Klotho and menstrual cycle and the number of total antral follicles. Not surprisingly, positive correlations were showed between the level of Klotho in follicular fluid of PCOS and the menstrual cycle and the number of total antral follicles. Therefore, these

indicated that the high concentration of Klotho may promote the development of PCOS disease, or at least, has a close relationship with the development of PCOS.

IVF-ET is one of the most effective ways to treat infertility in PCOS patients. The quality of oocytes is of great significance to the outcome of IVF-ET assisted pregnancy. Previous studies shown that poor quality of oocytes was observed in PCOS patients(29–31). As mentioned above, Klotho is closely related to the development of PCOS, we wondered whether Klotho affects oocytes quality in PCOS. Hence, correlation analysis was performed between Klotho in follicular fluid and IVF cycle clinical parameters, which were collected in Nanfang hospital. Results showed that Klotho was negatively correlated with the number of mature oocytes in PCOS, suggesting that an abnormal increase in Klotho can reduce the quality of oocytes. At the same time, we divided the PCOS group into two groups based on the median of Klotho level. Similarly, the rate of mature oocytes was significantly reduced in the group of higher level of Klotho, which further confirmed that the high expression of Klotho in the follicular fluid of the PCOS group can reduced the quality of oocytes.

The development of follicle and the oocyte quality are mainly regulated by hormones released by the hypothalamus-pituitary-ovarian axis. The abnormality release of hormones in PCOS result in follicular atresia or follicular arrested. In addition, microenvironment of follicular development is also another main factor. Granulosa cells and oocytes exist in the same microenvironment. At the same time, granulosa cells regulate the development and maturation of oocytes through gap junctions, therefore, the follicular fluid microenvironment disorder and granulosa cell dysfunction affects the developmental potential of oocytes(32–34). Tremendous studies showed that the mRNA level of Caspase-3, IL-6 and TNF in granulosa cells of PCOS patients were significantly increased(35), and these were consisted with our results. In addition, we also tested the mRNA level of Caspase-9, PCNA and Klotho in GCs, and found that Caspase-9 and Klotho were significantly increased in PCOS patients, especially in PCOS patients with hyperandrogenism, while PCNA was downregulated. These indicated that upregulation of Klotho may accompany with increased inflammatory and apoptotic response in granulosa cells. Furthermore, we also found that the mRNA level of GDF9 was downregulated in the PCOS group, especially in the group of PCOS with hyperandrogenism, this reflected the bad quality of oocytes in PCOS.

However, it seems contradictory, as Klotho has been considered as an inflammatory inhibitor for a long time(36). It can inhibit the NF-κB pathway, thereby reducing the expression of inflammatory factors such as IL-6 and TNF(37). And it is worth noting that a large number of studies believed that PCOS is in a chronic inflammatory state(38), accompanied with the activation of the NF-κB inflammatory pathway(39) and the secretion of a large number of pro-inflammatory factors(9, 40–43). Therefore, it is difficult to know whether the high expression of Klotho plays a protective or damaging role in inflammatory response of PCOS granulosa cells.

Previous studies have confirmed that hyperandrogenism is responsible for the chronic inflammatory state of PCOS(44, 45). Gonzalez et al. found that excessive androgen activated the NF-κB pathway in monocytes and induced the expression of inflammatory factors such as IL-6 and TNF(46). Another study

also confirmed that inflammation and apoptosis of granulosa cells in PCOS are closely related to high level of androgens(9). Although most studies believe that Klotho was an inflammation inhibitor, it does not seem to be able to inhibit the inflammation response in PCOS. Recent study showed that Klotho promoted the apoptosis of granulosa cells in PCOS(25). Our results also confirmed that the high expression of Klotho in the follicular fluid and granulosa cells of the PCOS group was accompanied by increased inflammation and apoptosis in granulosa cells. Therefore, we could reasonably speculate that there are two possible explanations for the role of Klotho in PCOS granular cells: the first one is that the high expression of Klotho along with high level of androgen may aggravate the inflammation state of PCOS, and the other is that although Klotho functions as an inflammation inhibitor in PCOS, excessive androgen can strongly increase the inflammation and apoptosis of granulosa cells, which exceeds the protective effect of Klotho to a certain extent, thereby covering up its protective role.

Studies found that the high expression of Klotho was accompanied by the up-regulation of androgen receptors in NRK-52e cells in testosterone-treated mice(47). And the overexpression of androgen receptors could in turn enhanced the expression of Klotho, suggesting that Klotho is closely related to the androgen(47). Similarly, our results in this study showed that the level of Klotho in the follicular fluid of PCOS with hyperandrogenism was the highest among the three groups, and it was consistent with the Klotho level in granulosa cells. In addition, we found a positive correlation between Klotho and serum testosterone, LH and LH/FSH. These indicated that the increasing level of Klotho in follicular fluid of PCOS was closely related to excessive androgen. As we all know, in female ovaries, androgens are synthesized and secreted by follicular membrane cells, and androgen receptors are rich expressed in oocytes, granulosa cells and follicular membrane cells. And androgens play a regulatory role in the development and maturation of follicles through their receptors. Hence, we proposed that the excessive androgen in PCOS patients may up-regulate the expression of Klotho in follicular fluid and granulosa cells through its receptors.

In this study, since the serum Klotho in PCOS patients was not tested, we cannot determine whether the Klotho in the plasma is responsible for the increase level of Klotho in the follicular fluid or the high level of Klotho is originally produced by granulosa cells. Because the follicular fluid derives mainly from plasma via the vascular compartment in the follicle wall and Klotho in the follicular fluid may be one of the components that derives from plasma. Future studies should be designed to analyze the level of Klotho both in the plasma and follicular fluid. Furthermore, the underlying mechanisms of Klotho in granulosa cell and its effects of oocytes should be further explored to verify our conclusions.

Conclusion

Although more studies are needed, these results provide proof of principle that Klotho plays a pivotal role in the onset and development of PCOS. It is of great importance to know the clinical significance of Klotho in PCOS, as it provides a new clue for clinicians to improve the oocytes quality of PCOS patients. Overall, Klotho may be regarded as a novel indicative marker for clinical prevention and treatment of PCOS and the outcome of IVF of PCOS patients.

Abbreviations

PCOS: polycystic ovary syndrome; IVF-ET: in vitro fertilization and embryo transfer; GCs: granulosa cells; PCO: polycystic ovaries; FF: follicular fluid, COH: controlled ovarian hyperstimulation; No. of AFC-Total: number of total antral follicles; No.of follicles above 14mm: number of follicles above 14mm; No.of retrieved oocytes: number of retrieved oocytes; No.of 2PN fertilization: mature oocyte rate, number of 2PN fertilization; No.of abnormal fertilization: number of abnormal fertilization; No.of high quality embryos on D3: number of high-quality blastocysts on day 3; No.of high quality embryos on D5: number of high-quality blastocysts on day 5;

Declarations

Ethical approval and consent to participate

This study was approved by the Ethical Committee Faculty of Nanfang Hospital, Southern Medical University, and informed consent was obtained from all of the participants recruited into this study and was carried out in accordance with the World Medical Association Declaration of Helsinki. All methods were performed in accordance with the relevant guidelines and regulations. The number of ethical approvals is NFEC-2018-154.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' Contributions

Y.S., W.Y. and T.X had full access to all the data in the study. W.Y. and T.X. contributed with the clinical interpretation of the results. Y.S. and W.Y. wrote the first version of the manuscript, which was thoroughly reviewed, edited, and approved by all other authors.

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Supplementary Tables

Supplementary Tables S1-S2 are not available with this version

Figures

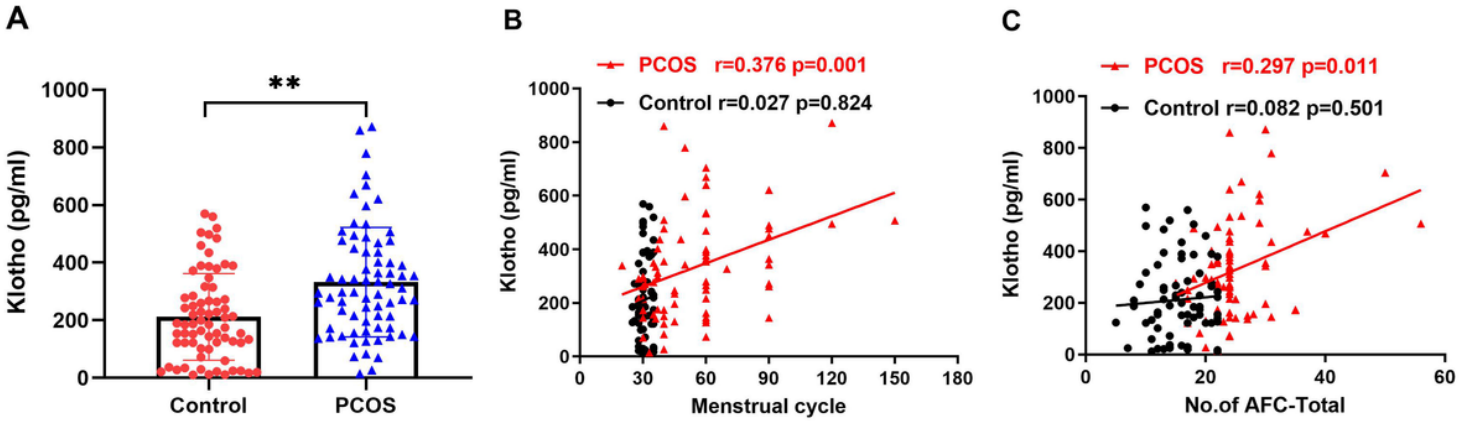

Figure 1

Figure 1

Comparison of Klotho level in follicular fluid between PCOS and control.

A. The concentration of klotho is significantly increased in PCOS group.

B. Correlations between the level of Klotho in follicular fluid and the menstrual cycle in the PCOS group and the control group.

C. Correlations between the level of Klotho in follicular fluid and No. of AFC-Total in the PCOS group and the control group.

**P□0.01. (n = 72 in PCOS group, n = 70 in control group).

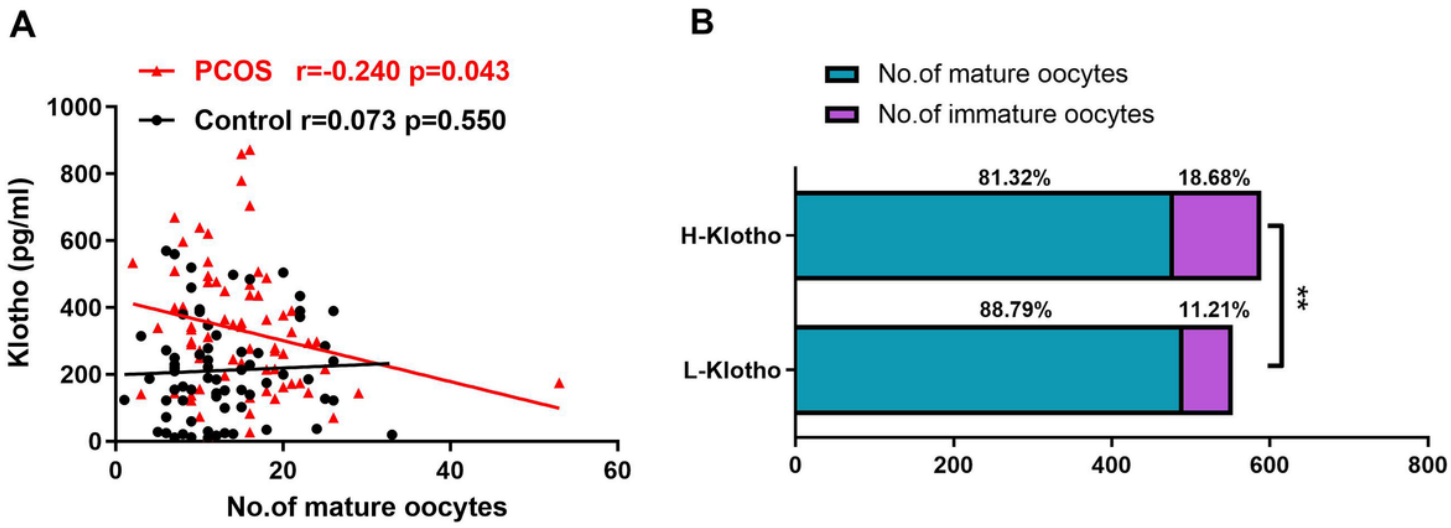

Figure 2

Figure 2

Correlations between the level of Klotho in follicular fluid and mature oocytes.

A: Correlations between the level of Klotho in follicular fluid and the number of mature oocytes (n=142). B: Comparison of mature oocyte rates between different level of Klotho in PCOS patients (n=72). PCOS: polycystic ovary syndrome; No.of mature oocytes: the number of mature oocytes; No.of immature oocytes: the number of immature oocytes; L-Klotho: the low level of Klotho group; H-Klotho: the high level of Klotho group. **P□0.01.

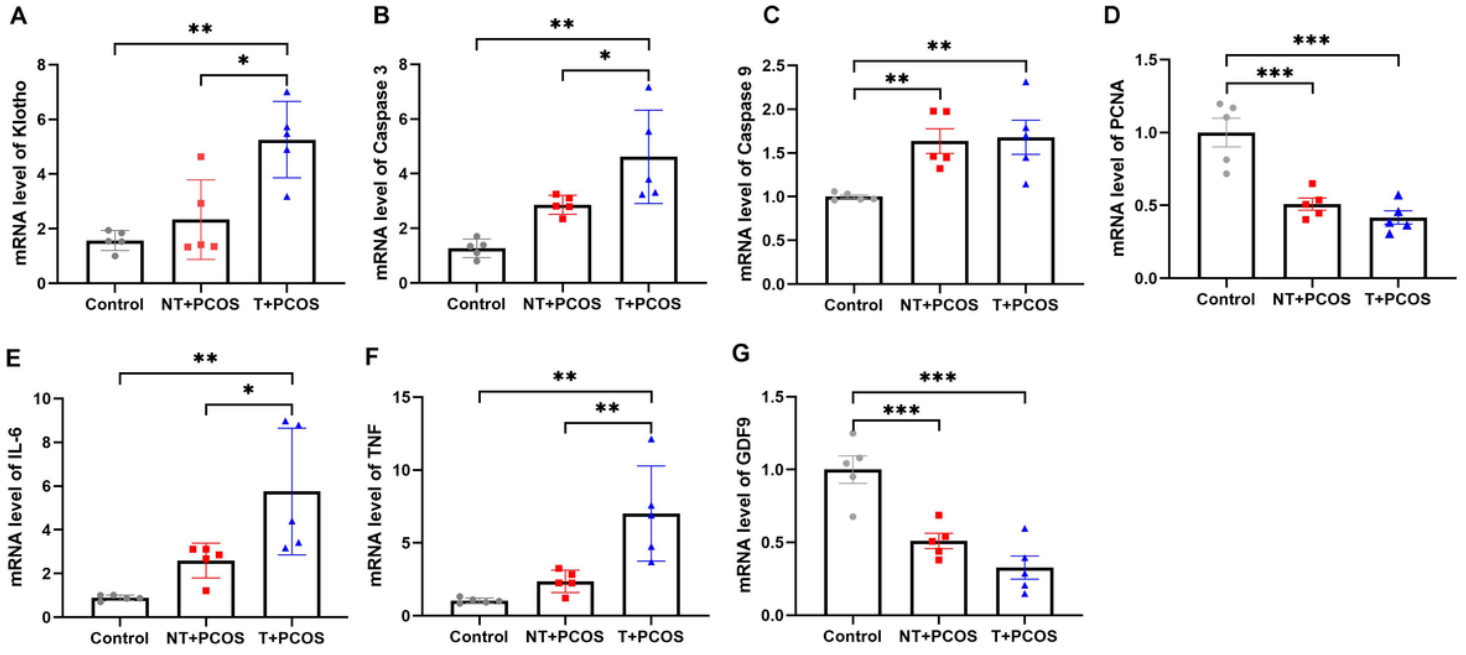

Figure 3

Figure 3

The mRNA expression levels of Klotho, caspase-3, Caspase-9, PCNA, IL-6, TNF and GDF9 in GCs of the high T+PCOS group, non-high T+PCOS group and the control group.

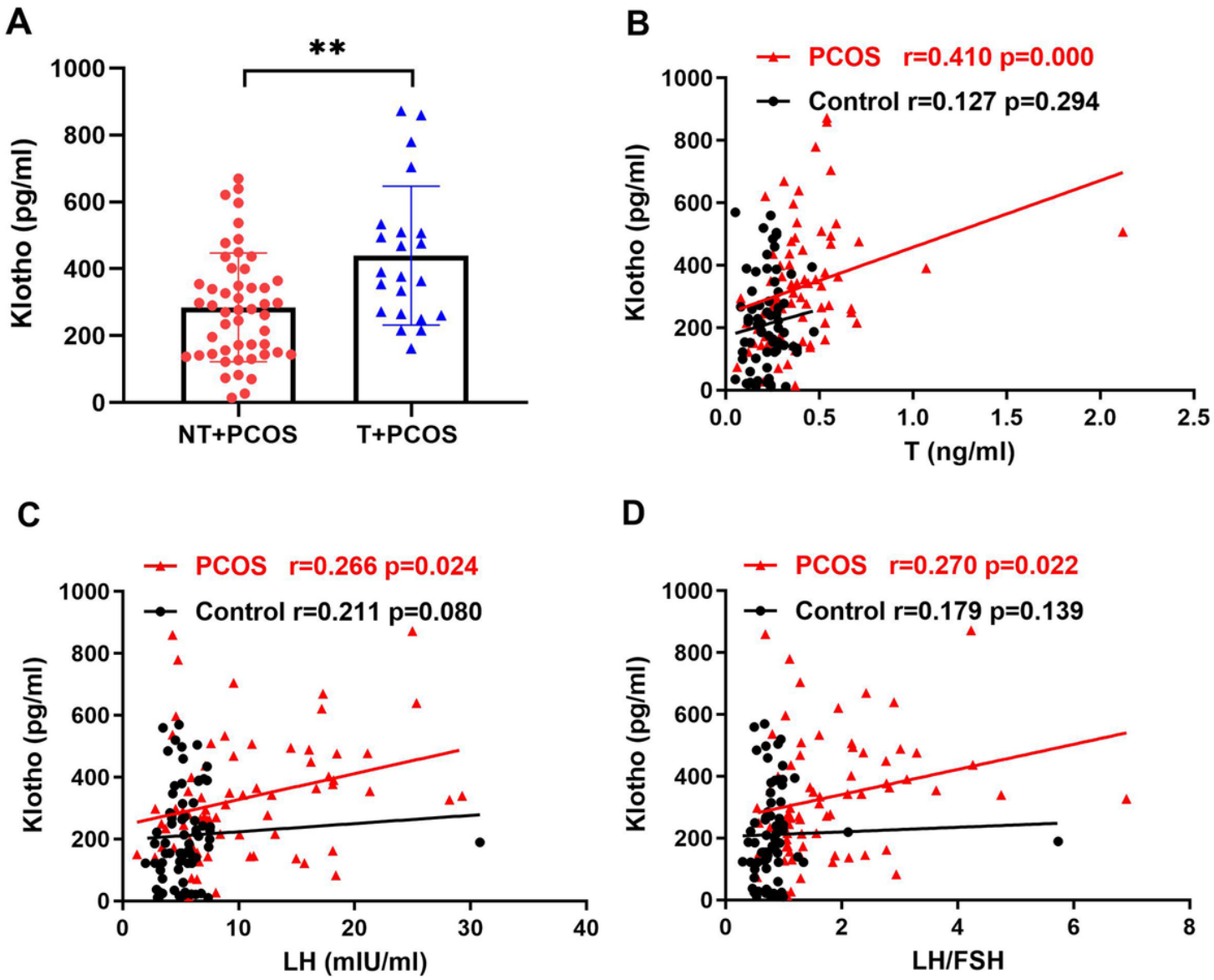

Figure 4

Correlation between follicular fluid klotho and androgen-related clinical parameters in PCOS group.

A: Comparison of Klotho expression in follicular fluid between T+PCOS and NT+PCOS. B: Correlation between Klotho and serum androgen in PCOS group. C: Correlation between Klotho and serum LH in PCOS group. D: Correlation between Klotho and LH/FSH in PCOS group.