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Plasminogen activator inhibitor 1 is a novel predictor in human serum/follicular fluid for diminished ovarian reserve

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Abstract

Background Diminished ovarian reserve (DOR) is a common female reproductive aging disease, which showed significant impacts on the quality of life and fertility in women. Plasminogen activator inhibitor 1 (PAI-1) is considered to be a major profibrotic factor. The development of DOR is closely related to ovarian fibrosis. The aim of the study was to investigate the expression of PAI-1,which is clinically relevant to DOR.

Methods This case-control study included 40 infertile women with DOR and 40 infertile women with normal ovarian function. PAI-1 and reproductive hormones in serum and follicle fluid were determined in all subjects. Receiver operating characteristic curve (ROC) was applied to evaluate PAI-1 in prediction and diagnosis of DOR. The mRNA and protein expression of PAI-1 in KGN cells induced by cyclophosphamide (CTX) were observed by Western blot (WB) and quantitative real-time PCR (qRT-PCR).

Results The sensitivity and specificity of PAI-1 levels in serum/follicular fluid for predicting DOR were 90%/97.5% and 70%/82.5%, respectively. The AUC of PAI-1 in follicular fluid was 0.955(95% CI 0.913–0.997), which cutoff level and Youden index were 68.58 ng/mL and 0.825 for DOR. PAI-1 in serum and follicle fluid showed negative association with Anti-Müllerian hormone (AMH) and antral follicle count (AFC) (serum r= -0.391,r= -0.453;follicle fluid r= -0.486,r= -0.534;$p < 0.01$), however, they were positively correlated with follicle-stimulating hormone (FSH) and follicle-stimulating hormone/luteinizing hormone (FSH/LH) (serum $r = 0.307$,$r = 0.388$;follicle fluid $r = 0.300$,$r = 0.384$;$p < 0.05$). The ROC analysis indicated that serum PAI-1 has great prediction of DOR, with an AUC of 0.841, a sensitivity of 90%, and a specificity of 70%. Additionally, the qRT-PCR results demonstrated that the mRNA levels of PAI-1 increased in the CTX-induced cells ($P < 0.05$). The western blot results were consistent with qRT-PCR results.

Conclusion Our study reveals that the expression of PAI-1 is higher in serum and follicular fluid of DOR patients. And it is positively correlated with FSH, FSH/LH and negatively correlated with AMH/AFC, which is necessary to investigate the role of PAI-1 in regulating the growth and development of follicles and the pathogenesis of DOR in future.

Highlights

- PAI-1 in serum and follicle fluid was significantly higher in DOR than control.
- PAI-1 is positively correlated with FSH, FSH/LH and negatively with AMH and AFC.

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- PAI-1 in follicular fluid was negatively correlated with endometrial thickness.
- CTX-induced cells had increasing mRNA and protein levels of PAI-1 than control.

Keywords Plasminogen activator inhibitor 1, Diminished ovarian reserve, Marker, Infertility

Background

Diminished ovarian reserve (DOR), characterized by reductions in oocyte quantity and quality with advanced age, typically occurs in the mid-40s as a normal physiologic aging process, but some women may experience pathologic DOR at earlier age and become prematurely infertile [1]. DOR increases a large range of risks including infertility, perimenopausal syndrome, coronary disease and osteoporosis [2]. According to the latest statistics, the prevalence of DOR in the Society for Assisted Reproductive Technology (SART) registration is estimated at 26% [3]. In vitro fertilization (IVF) treatment, patients with DOR are more likely to have poor ovarian stimulation response, high cancellation rates, and significantly lower pregnancy rates [4]. Currently, the main DOR indicators mainly used in clinics include serum hormone levels (follicle-stimulating hormone (FSH), luteinizing hormone (LH), Anti-Müllerian hormone (AMH)) and antral follicle count (AFC). However, when these indicators were abnormal, patients were already diagnosed with DOR. This study will develop a better marker to predict the occurrence of DOR in the early stage.

Oocytes are stagnated in meiotic period in the ovary, surrounded by extracellular matrix in follicles and interstitial tissue. Besides, increased collagen deposition, indicative of tissue fibrosis, has been demonstrated in the ovaries of postmenopausal women [5] and animal models of reproductive aging [6]. The pathological change of ovarian fibrosis destroys the normal physiological structure of ovarian tissue, and further the ovarian function of reproduction and endocrine regulation will decline or even fail as the functional tissue is gradually replaced by the fibrotic tissue. Therefore, ovarian fibrosis plays a critical role in the physiological pathogenesis of DOR [7, 8]. In addition, ovarian stroma involves in maintaining the dormancy of primordial follicles and nourishing growing follicles. Michio Kitajima et al. [9] believed that fibrosis is an early hallmark of ovarian stroma aging and alterations in this microenvironment may lead to age-related decreases in oocytes quality. Linlu Cui et al. [7] reported that the human umbilical cord mesenchymal stem cell (hUMSC) transplantation restores ovarian function and significantly increases the number of follicles and fertility at all stages of development in rats by reducing the ovarian fibrosis. However, the molecular mechanism that cause ovarian fibrosis are not well elaborated, and whether this interesting change in matrix and tissue leading to DOR is not clear.

Jeon, Y. J et al. [10] firstly demonstrated an association between premature ovarian insufficiency (POI) and Plasminogen activator inhibitor 1 (PAI-1) polymorphisms. PAI-1, a type of serine protease inhibitors, is considered as the characteristic of aging cells. PAI-1 is a major physiological inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), both of which can activate plasminogen to plasmin, thus promote fibrinolysis. In addition to its known effects to promote thrombosis by inhibiting t-PA and u-PA, PAI-1 has important potential effects on matrix degradation, since plasmin and t-PA and u-PA with proteolytic function can activate matrix metalloproteinases (MMPs) from latent to active state. It was recently reported that PAI-1 is expressed in granulosa cells and follicular membrane cells, indicating its potential role in human ovulation [11]. In addition, Zhai J et al. [12] also suggest that PAI-1 plays different roles in follicular development, ovulation, and embryo implantation. These studies provide scientific evidence for the important role of PAI-1 in ovarian function. However, whether it is expressed in the tissues/blood/follicular fluid of DOR and clinically relevant to DOR, there is little research on this topic.

The occurrence and development of DOR are both closely related to ovarian fibrosis, however, the expression of PAI-1 in DOR and its association with clinical characteristics remain to be elucidated. In this study, the PAI-1 levels were detected in DOR patients and investigated correlation analysis and ROC curve to predict the risk of DOR.

Materials and methods

Patients

Women, with or without DOR, aged 19–39 years were included from the Outpatient of Reproductive medicine center of General Hospital of Ningxia Medical University. The study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University (KYLL-2022-1107). Each patient with DOR met the recruited DOR criteria [13]. Inclusion criteria: Women with high FSH level (10IU/L ≤ FSH ≤ 25 IU/L), and/or AMH < 1.1ng/mL, and/or AFC < 5–7 were considered as DOR in our study. Besides, the control group included 40 infertile women who had serum 1.1 ≤ AMH ≤ 6.8ng/mL, FSH < 10 IU/L and AFC > 7. Exclusion criteria: (1) Congenital reproductive dysfunction and reserve dysfunction caused by unilateral ovarian resection; (2) Suffering from other endocrine disorders, such as hyperprolactinemia, polycystic ovary syndrome, and hyperandrogenism; (3)

Ovarian malignant tumor and systemic infectious diseases; (4) Long term use of hormone replacement therapy; Clinical pregnancy was defined as the presence of a fetal heart beat on ultrasound examination at 7 weeks of pregnancy.

Hormone measurement and ultrasonography

Peripheral blood samples were collected on days 1–3 of the menstrual cycle. The levels of endocrine hormones (FSH, LH, E2 and AMH) were detected through enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Company, Beijing, China), according to the manufacturer's instructions. The absorbance was measured at 450 nm by multi-functional enzyme analyzer (EnVision, PerkinElmer, MA, USA). Transvaginal ultrasonography was routinely conducted. AFC was defined as the number of bilateral follicles (2–10 mm in diameter) in early follicular stage.

Ovarian stimulation, oocyte retrieval, embryo culture and transfer

GnRH-a long protocol: Patients underwent pituitary down-regulation with GnRH agonist (Triptorelin Acetate, Ipsen Pharma Biotech, France) at the mid-luteal phase of the menstrual cycle (days 18–20). When the concentration of serum E2 was less than 50 ng/L and the endometrial thickness was less than 5 mm with an absence of 10 mm large follicles in both ovaries by transvaginal ultrasound, FSH/HMG was initiated at a dose of 225–300 IU/day until the trigger day.

GnRH-anta protocol: Patients were commenced on FSH/HMG at 225–300 IU/day from day 2 of the menstrual cycle and the antagonist was administered at a dose of 0.25 mg/day from the 6th day of Gn stimulation (fixed protocol) or when the diameter of follicles was larger than 14 mm (flexible protocol) until the trigger day.

GnRH-a short protocol: patients were given a subcutaneous injection of 0.1 mg /d of short-acting GnRH-a, triptorelin acetate injection, German Fering Company) from the 2nd day of the menstrual cycle, and a gonadotropin (Gn, urinary gonadotropin for injection, Lizon Pharmaceutical) from the 3rd to 5th day of the menstrual cycle, 150–300 IU/d intramuscular injection. Monitor follicle size and hormone levels regularly and adjust Gn dose to HCG (human chorionic gonadotropin) day.

When the diameter of dominant follicles reached 18 mm, an initial individualized dose (6000–10,000 IU) of human chorionic gonadotrophin (Pregnyl, Organon, Oss, Netherlands) or an additional 0.1 mg GnRH-a (dual trigger) was administered to trigger follicle maturation. Then, oocyte retrieval was performed 34–36 h later, guided by a transvaginal ultrasound scan. IVF or ICSI (Intracytoplasmic Sperm Injection) was performed according to the laboratory's routine insemination procedures. Fresh embryo transfer (ET) was performed with 3-day cleaved embryos or 5-day blastocysts as the priority. The luteal phase was supported with daily administration of vaginal progesterone for 17 days starting on the day of oocyte retrieval, and if pregnancy occurred, the progesterone was continued for another 8 weeks. Serum levels of β-hCG and progesterone were measured 14 ± 3 days after embryo transformation. Clinical pregnancy was confirmed through ultrasonic observation of the intrauterine gestation sac at 3 weeks after a positive serum hCG test. Additionally, pregnancy and delivery status were followed up.

Follicular fluid collection

Follicular fluid from dominant follicles containing oocytes (diameter > 1.4 cm) was collected during oocyte retrieval. After the follicular fluid was centrifuged (3000 rpm,10 min), the supernatant was retained and stored at -80℃.

Serum and follicular fluid measurements

Serum and follicular fluid were measured by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Elabscience Biotechnology Company, Beijing, China). Measurements were performed in duplicate and averaged. All readings were performed on the Thermo Scientific Multiskan GO Reader at 450 nm.

Cell culture and treatment

KGN cell, a Human ovarian granulosa cell line, was obtained Procell Life Technology Co. Ltd Wuhan, China. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) (Invitrogen, Carlsbad, CA) at 37℃. For experiments, KGN cells were seeded into 6-well plates at approximately 80% confluence. Then, the cells were induced with cyclophosphamide (CTX,250μM) in 48 h.

Enzyme-linked immunosorbent assay (ELISA)

The cell culture medium was collected and centrifuged to obtain the supernatant and the levels of anti-Mullerian hormone (AMH) and PAI-1 were measured with ELISA kits (Shanghai Jianglai Biotech, Shanghai, China). According to the manufacturer's instructions, add the sample, standard, biotinylated detection antibody and HRP enzyme conjugate in sequence into the micropores coated with anti-Mullerian hormone antibodies. After incubation and washing, the substrate TMB was used for coloration. TMB was converted to blue under the catalysis of HRP (Horseradish Peroxidase) and to the final yellow under the action of acid. All readings

were performed on the Thermo Scientific Multiskan GO Reader at 450 nm.

Western blot assay (WB)

The cells were lysed and collected to obtain total proteins following WB assay. The protein samples separated through SDS-PAGE were transferred to the polyvinylidene fluoride (PVDF) membrane and added with TBST containing skimmed milk (5%) for 2 h. Primary antibodies were then added overnight (4℃), after which being replaced with the secondary antibody at 37℃ for 2 h. After that, wash it for 3 times with TBST and visualized by the BIO-RAD ChemiDoc XRS chemiluminescence system.

Quantitative real-time PCR (qRT-PCR)

We extracted total RNA from control and CTX-induced KGN cells using TRIzol method. RNA was reversely transcribed into cDNA (42 °C at 15 min and 95 °C at 3 min) by FastKing gDNA Dispelling RT SuperMix kit (TIANGEN, KR118-02, China). PAI-1 expression in control and CTX-induced KGN cells were assessed utilizing SuperReal PreMix Plus (SYBR Green)(TIANGEN, China) according to the manufacturer's instructions. And then, qRT-PCR analysis was done on 7500 Real-time PCR System (Applied Biosystems, USA) by the following two-step PCR amplification procedure: 1 cycle of pre-degradation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s. Beta-actin was utilized as an internal reference to normalize gene expression. Primers were purchased from Sangon Biotech (Shanghai) Co., Ltd. Relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method. Primers were as follows:

PAI-1: Forward primer 5'-TGGTTCTGCCCAAGTTC TCC-3'.

Reverse primer 5'-CACCGTGCCACTCTCGTTC – 3'.

Beta-actin: Forward primer 5'-CCTGGCACCCAGCA CAAT – 3'.

Reverse primer: 5'-GGGCCGGACTCGTCATAC – 3'.

Statistical analysis

The SPSS 23.0 statistical software was used for data analyses. Continuous variables are presented as mean ± standard deviation and categorical variables are described as numbers (percentages). The Student's t-test was applied for comparisons of the mean between the two groups when the variables were normally distributed, and the Pearson correlation was used for the correlation analyses. The F-test was utilized for the equality of variance test. Fisher's exact test was applied when the number of categorical variables was less than 5 in each group. Statistical significance was set at $P < 0.05$ and all tests were two-tailed.

Results

Analysis of clinical characteristics of patients

As shown in Table 1, compared with control group, FSH and FSH/LH levels were significantly higher in the DOR group(6.64 ± 1.43 vs. 9.84 ± 3.82; 1.89 ± 0.66 vs. 3.63 ± 1.76;

Table 1 Baseline characteristics of participants enrolled in this study. Values are shown as mean ± sd or n(%)

Variable		Control($n=40$)	DOR($n=40$)	*P*-value
Age(years)		31.55 ± 1.83	32.55 ± 3.60	0.121
BMI(kg/m^2)		22.99 ± 3.63	22.84 ± 3.07	0.988
Gravidity				0.779
0		22(55.0%)	22(55.0%)	
1		13(32.5%)	11(27.5%)	
≥ 2		5(12.5%)	7(17.5%)	
Parity				0.402
0		34(85.0%)	30(75.0%)	
1		6(15.0%)	10(25.0%)	
Type of infertility				1.000
Primary		22(55.0%)	21(52.5%)	
Secondary		18(45.0%)	19(47.5%)	
Duration of infertility(years)		3.99 ± 3.40	3.88 ± 3.63	0.671
Serum basal levels	FSH(mIU/mL)	6.64 ± 1.43	9.84 ± 3.82	< 0.01**
	LH(mIU/mL)	3.71 ± 0.96	3.25 ± 1.56	0.368
	FSH/LH	1.89 ± 0.66	3.63 ± 1.76	< 0.01**
	E_2(pg/mL)	42.06 ± 16.41	44.13 ± 18.69	0.601
	AMH(ng/ml)	4.08 ± 1.84	0.51 ± 0.37	< 0.01**
B-US	AFC	9.98 ± 4.95	4.58 ± 3.14	< 0.01**
	EMT (mm)	12.39 ± 2.36	10.75 ± 2.75	0.006**

Note: Body mass index (BMI), follicle-stimulating hormone(FSH), luteinizing hormone (LH), estradiol (E2), anti-Mullerian hormone (AMH), B-model Ultrasound Scanning(B-US), antral follicle count (AFC), Endometrial thickness(EMT). *$P < 0.05$; **$P < 0.01$

$P<0.01$). However, AMH, AFC and EMT levels were significantly lower in the DOR group(0.51 ± 0.37;4.58 ± 3 .14;10.75 ± 2.75;$P<0.01$). There were not statistically different in age, body mass index, gravidity, parity, LH and E_2 in the two groups ($P>0.05$). As shown in Table 2, although the number of retrieved oocytes were fewer in DOR group(4.43 ± 2.51) than the control (11.56 ± 4.67), the number of embryos transferred were higher in DOR group ($p=0.009$), compared with control group. However, there were not statistically different in cycle cancellation rate, transplantation methods and sequential treatment conditions. Similarly, as for maternal perinatal outcomes of all participants, the rates of pregnancy, miscarriage and live births were not significantly different between the two groups(45% vs. 35%, 61.1% vs. 28.6%, 50% vs. 64.3% respectively, $P>0.05$).

Correlation analysis between PAI-1 expression and clinical features

In order to verify whether there was a correlation between PAI-1 expression in serum and follicular fluid and clinical features in DOR. We detected PAI-1 expression in serum and follicular fluid. The results confirmed that PAI-1 expression levels in serum and follicular fluid were significantly higher in DOR group (32.56 ± 7.88 and 166.40 ± 75.45) than control group (22.20 ± 6.15 and 43.13 ± 35.72, all $p<0.01$) (Fig. 1). Meanwhile, Pearson's correlation in women with DOR showed that the levels of PAI-1 in serum and follicular fluid were positively correlated with FSH and FSH/LH (serum $r=0.307$ and 0.388;follicular fluid $r=0.300$ and 0.384; respectively; all $P<0.01$) and negatively with AMH and AFC (serum r=-0.391 and–0.453;follicular fluid r=-0.486 and-0.534;respectively; all $P<0.01$). This suggested that high PAI-1 levels in follicular fluid were correlated with the decline of ovarian function. Finally, to investigate the predictive value of serum and follicular fluid PAI-1 as a diagnostic marker for DOR, the specificity and sensitivity of PAI-1 was analyzed by the ROC curve. As shown in Fig. 2, the area under curve (AUC) of PAI-1 in serum was 0.841 (95% CI 0.752–0.930), which cutoff level and Youden index were 25.63 ng/mL and 0.6 in DOR group.

Table 2 IVF cycle and maternal perinatal outcomes of participants enrolled in this study. Values are shown as mean ± sd or N (%)

Variable	Control ($n=40$)	DOR($n=40$)	*P*-value
IVF cycle outcomes			
Cycle canceled			0.139
Yes	4(10.0%)	10(25.0%)	
No	36(90.0%)	30(75.0%)	
Number. of oocytes retrieved	11.56 ± 4.67	4.43 ± 2.51	0.075
Induced ovulation protocol			
Short protocol	1	9	
Long protocol	31	7	
natural cycle	0	3	
Antagonist protocol	8	21	
Transplantation methods			0.114
Fresh embryo transfer	27(67.5%)	34(85.0%)	
Frozen-thawed embryo transfer	13(32.5%)	6(15.0%)	
Sequential transplantation			1.000
Yes	11(27.5%)	10(25.0%)	
No	29(72.5%)	30(75.0%)	
Number embryo(s) transferred			0.009**
0	2(5.0%)	10(25.0%)	
1	21(52.5%)	10(25.0%)	
2	17(42.5%)	20(50.0%)	
Maternal perinatal outcomes			
Pregnancies			0.494
Yes	18(45.0%)	14(35.0%)	
No	22(55.0%)	26(65.0%)	
Miscarriage			0.087
Yes	11(61.1%)	4(28.6%)	
No	7(38.9%)	10(71.4%)	
Live births			0.490
Yes	9(50.0%)	9(64.3%)	
No	9(50.0%)	5(35.7%)	

Note: In vitro fertilization (IVF). *$P<0.05$; **$P<0.01$

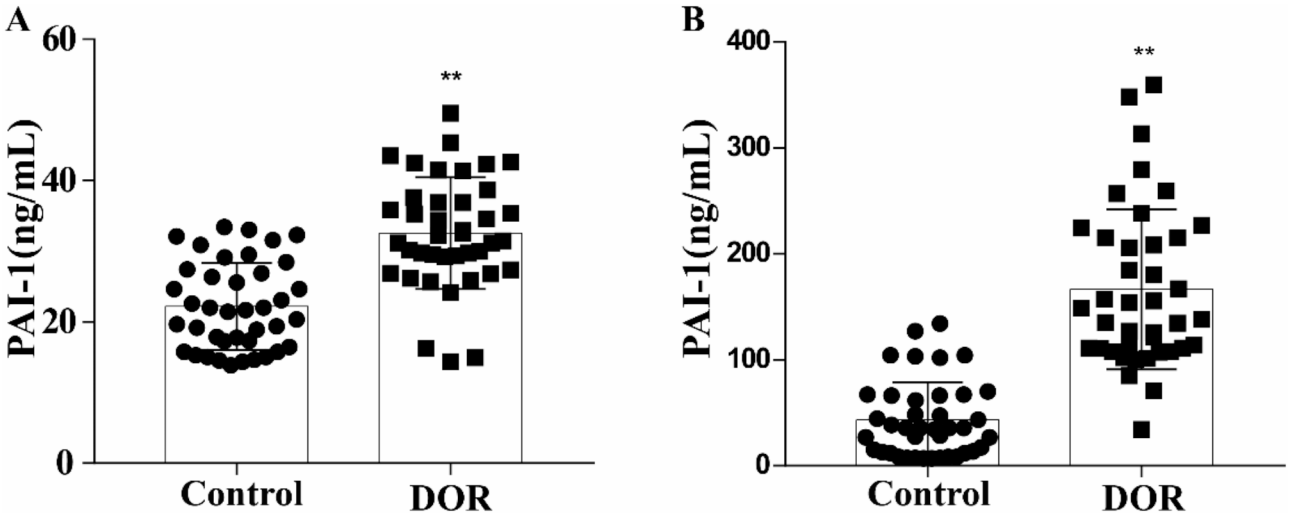

Fig. 1 The expression of PAI-1 in (**A**) serum and (**B**) follicle fluid (Control vs. DOR) $^{**}P < 0.01$

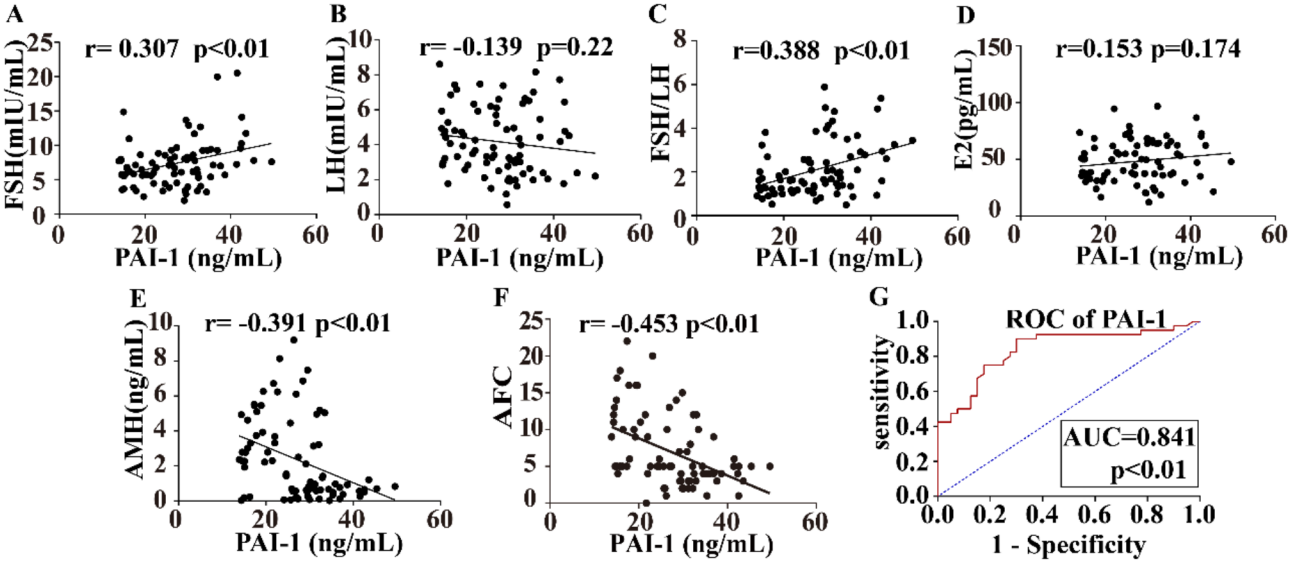

Fig. 2 Correlation analysis of serum plasminogen activator inhibitor 1(PAI-1) and ovarian reserve markers. (**A**) Follicle-stimulating hormone (FSH) ($r = 0.307$; $P < 0.01$). (**B**) Luteinizing hormone (LH) ($r = -0.139$; $P = 0.22$). (**C**) FSH/LH ($r = 0.388$; $P < 0.01$). (**D**) Estradiol (E2) ($r = 0.153$; $P = 0.174$). (**E**) Anti-Müllerian hormone (AMH) ($r = -0.391$; $P < 0.01$). (**F**) Antral follicle count (AFC) ($r = -0.453$; $P < 0.01$)**. (G)** The receiver operating characteristic (ROC) curve analysis for diminished ovarian reserve(DOR) prediction

The sensitivity and specificity of PAI-1 levels in serum for predicting DOR were 90% and 70%, respectively. Similarly, as shown in Fig. 3, the AUC of PAI-1 in follicular fluid was 0.955 (95%CI 0.913–0.997), which cutoff level and Youden index were 68.58ng/mL and 0.825 for DOR. The sensitivity and specificity of PAI-1 levels in follicular fluid for predicting DOR were 97.5% and 82.5%, respectively.

Differential expression of PAI-1 in CTX-induced DOR model

The formation of ovarian fibrosis may result from degeneration of granulosa cells leading to fibrinogen, collagen deposition and proliferation of interstitial fibroblast [14]. To further validate the differential expression of PAI-1 between normal and DOR patients, we established a DOR model using KGN cells induced with cyclophosphamide. Firstly, after KGN cells were induced with cyclophosphamide(CTX,250 μM) in 48 h, the levels of AMH were measured with an ELISA kit in cell culture medium. The results showed that the levels of AMH in CTX- induced KGN cells were significantly lower than the control, which was indicated the success of the model construction (Fig. 4A). Secondly, the relative levels of PAI-1 secretion after 48 h of induction are shown in Fig. 4B. The concentrations of PAI-1 in the culture supernatant were significantly higher in the

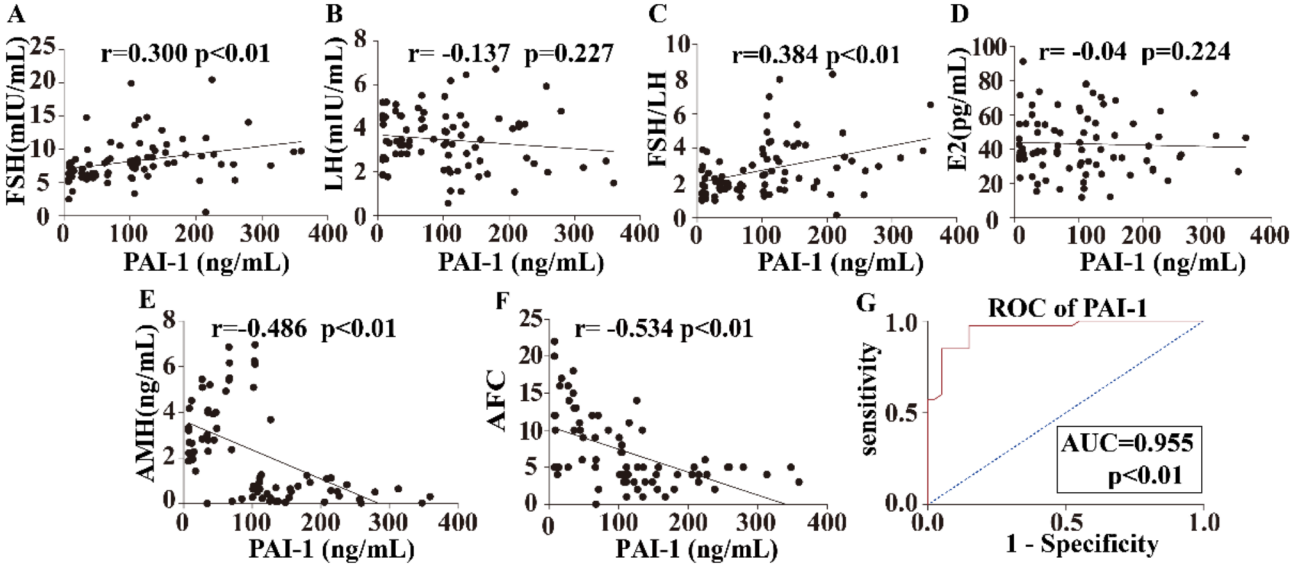

Fig. 3 Correlation analysis of follicle fluid plasminogen activator inhibitor 1(PAI-1) and ovarian reserve markers. (**A**) FSH ($r = 0.300$; $P < 0.01$). (**B**) LH (r=-0.137; $P = 0.227$). (**C**) FSH/LH ($r = 0.384$; $P < 0.01$). (**D**) E2 (r =-0.04; $P = 0.224$). (**E**) AMH (r = -0.486; $P < 0.01$). (**F**) AFC(r=-0.534; $P < 0.01$). (**G**) The ROC curve analysis for DOR prediction

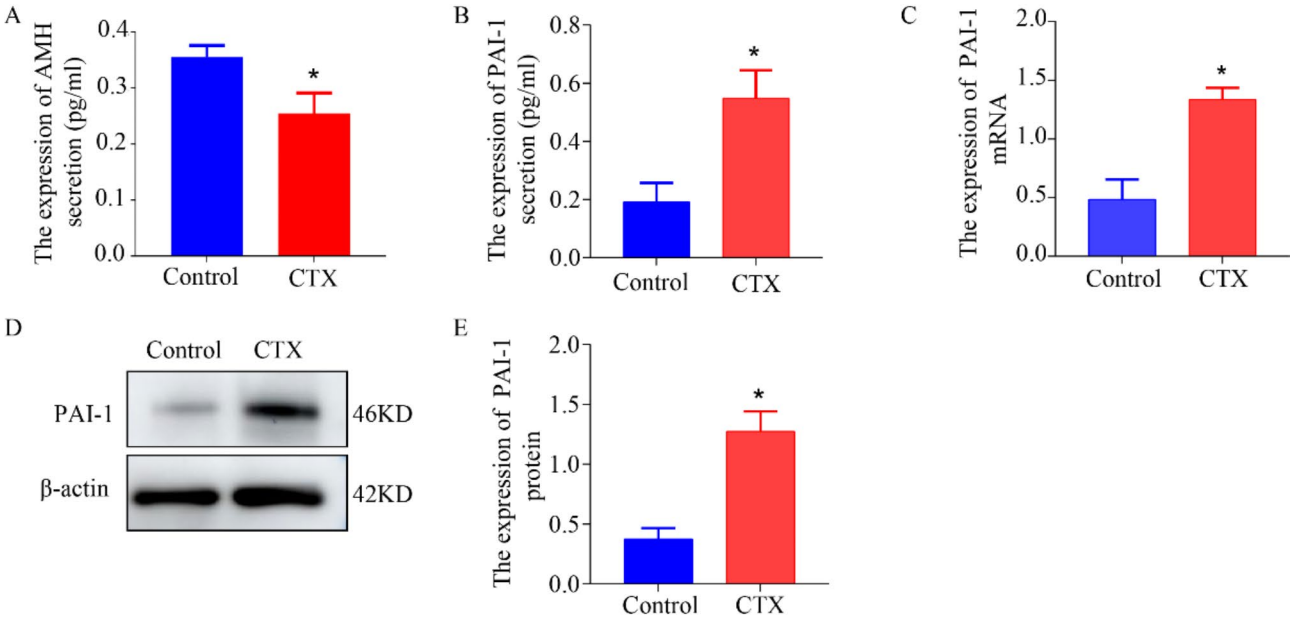

Fig. 4 The relative expression of PAI-1 after 48 h induction of CTX. (**A**) The relative levels of AMH secretion in KGN. (**B**) The relative levels of PAI-1 secretion in KGN. (**C**) The expression of PAI-1 in transcription level. (**D**, **E**) The expression of PAI-1 in protein level. *$P < 0.05$

CTX-induced cell (0.582 ± 0.106816) than in the control cells (0.192 ± 0.06676; $P < 0.05$). Finally, we further analyzed the relative protein expression levels of PAI-1-related transcription factors at 48 h in KGN cells (Fig. 4C to E). The results showed that CTX-induced cells had significantly increasing mRNA and protein levels of PAI-1, compared with control cells ($P < 0.05$).

Discussion

The extracellular matrix (ECM) promotes and/or inhibits many cellular processes, including but not limited to proliferation, differentiation, and survival, which must occur for follicle growth and oocyte maturation [15]. Oocytes are held in the meiotic arrest in the ovary, surrounded by extracellular matrix in follicles and embedded within stromal interstitial tissue. Fibrosis of tissues is characterized by excessive deposition of ECM. PAI-1 regulates the activity of u-PA/t-PA and plasmin dependent

matrix metalloproteinases (MMP), decreases fibrinolysis, induces ECM accumulation, and promotes fibrosis associated with inflammatory cells, macrophages, and fibroblast [16]. Ovarian fibrosis is characterized by excessive proliferation of ovarian fibroblasts and deposition of ECM and it is one of the principal reasons for ovarian dysfunction [17]. Meanwhile, Ovarian fibrosis is a pathological condition associated with aging and is responsible for a variety of ovarian dysfunctions [18], which plays an important role in the pathophysiological process of ovarian, which has not been received much attention from researchers worldwide. Takashi Umehara [19] showed that anti-fibrosis drugs restored ovulation in old and obese mice by eliminating fibrotic collagen. Remove excess collagen from the ovary facilitate the release of eggs and extend female fertility. DOR is the most common disorder of early ovarian aging. As an indicator of impaired ovarian function, ovarian fibrosis is closely related to various gynecological diseases, which seriously decreased or absent follicles to affect the endocrine function of the ovary.

The overexpression of PAI-1 contributed to the accumulation of ECM and the secretion of fibrosis factors. As a potent profibrotic factors, the role of PAI-1 in ovarian fibrosis of DOR disease has not been reported. Thus, in the present study, we found that the expression of PAI-1 in serum and follicle fluid derived from women with DOR were higher(32.56 ± 7.88; 166.40 ± 75.45) than the control group (22.20 ± 6.15; 43.13 ± 35.72), which suggested the significant role of PAI-1 in the DOR. Furthermore, Pearson's correlation in DOR showed that the level of PAI-1 in serum and follicular fluid were positively correlated with FSH and FSH/LH ($P < 0.01$) and negatively with AMH and AFC ($P < 0.01$). Finally, ROC curve was used to evaluate the predictive value of PAI-1 in serum and follicular fluid as a diagnostic marker for DOR. The area under the curve (AUC) of PAI-1 in serum was 0.841, and the sensitivity and specificity of PAI-1 in serum to predict DOR were 90% and 70%, respectively. Similarly, the AUC of PAI-1 in follicular fluid was 0.955, and the sensitivity and specificity of PAI-1 in follicular fluid to predict DOR were 97.5% and 82.5%, respectively. In addition, research has shown that PAI-1 is rarely expressed in granulosa cells of normal follicles, but exhibits different levels of expression in granulosa cells of atretic follicles [20]. Follicular atresia is the pathophysiological mechanism that accelerates DOR. Yi-Xun Liu et al. [21] found that PAI-1 and tissue inhibitor of metalloproteinases-1 (TIMP-1) can control the ovarian protein hydrolysis activity associated with ovulation, and increasing PAI-1 synthesis can inhibit ovulation. Therefore, we have reason to believe that the elevated levels of PAI-1 in the serum and follicular fluid of DOR patients can accelerate the depletion of ovarian reserve and impair ovarian function. However, its specific involvement in the development of DOR is still under investigation. Interestingly, studies have shown that multiple upstream transforming growth factor beta (TGF - β) response elements can highly induce the expression of PAI-1 during fibrosis [22–24]. Kaori Yamada Nomoto [20] found that in HGrC1 cells, a human GC cell line, addition of TGF-β can induce PAI-1 mRNA expression. Therefore, our laboratory will continue to investigate the role of TGF-β-induced PAI-1 expression in ovarian fibrosis in DOR patients.

The primary pathological features of ovarian fibrosis are a thick capsule, increased mesenchymal connective tissue, and decreased or absent follicles. As a potent profibrotic factors, is the role of PAI-1 in follicular fluid associated with DOR? Follicular fluid contains secretions from surrounding granulosa cells and serum diffused from local capillaries. Follicular fluid is a fluid used for material exchange and metabolism between oocytes and surrounding cells, forming a microenvironment for oocyte growth. Some biochemical properties in follicular fluid play a key role in oocyte and embryo development [25]. PAI-1 is not only secreted from vascular endothelial cells into the blood, but also accumulated in follicular fluid. In our study, we found that the PAI-1 concentration in follicular fluid was very high compared with its levels in serum. This suggests that the high expression of PAI-1 in follicular fluid is closely related to the adverse pregnancy outcome of DOR. Additionally, we also found that the endometrial thickness of patients with DOR seems to be lower than those for the controls (Table 1). Endometrial thickness, an important indicator of endometrial receptivity, is positively correlated with the pregnancy rate of IVF-ET women receiving embryo transfer [26–29]. This study is only a preliminary discovery, and further research is needed to verify the relationship between DOR and endometrial thickness, as well as its impact on pregnancy outcomes.

Our research also has some limitations. First, this study includes a small sample and requires further researches including a larger study population to confirm this finding. Second, the cross-sectional nature of this study may have limitations in the exploration of changes of PAI-1 expression as ovarian function gradually decreases, thus, longitudinal studies examining disease progression are needed to carry out. Besides, we only conduct clinical studies and in vitro experiments, we need to conduct in vivo experiments to further validate our observations.

Conclusion

Our study reveals that the expression of PAI-1 is higher in serum and follicular fluid of DOR patients. And it is positively correlated with FSH, FSH/LH and negatively correlated with AMH/AFC, which is necessary to investigate the role of PAI-1 in regulating the growth and

development of follicles and the pathogenesis of DOR in future.

Abbreviations

IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
PAI-1	Plasminogen activator inhibitor
DOR	Diminished ovarian reserve
ROC	Receiver operating characteristic curve
CTX	Cyclophosphamide
WB	Western blot
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
AMH	Anti-müllerian hormone
AFC	Antral follicle count
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
hUMSC	Human umbilical cord mesenchymal stem cell
t-PA	Tissue-type plasminogen activator
u-PA	Urokinase-type plasminogen activator
PCOS	Polycystic ovary syndrome
BMI	Body mass index
E2	Estradiol
B-US	B-model ultrasound scanning
EMT	Endometrial thickness

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Author contributions
XS L was responsible for experimental operations and manuscript writing. X C collected, analyzed and interpreted the data and made the figures and tables, H G have drafted the research and substantively revised it. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All included patients gave their oral and written informed consent. The study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University(KYLL-2022-1107). All methods are reported in accordance with ARRIVE guidelines.

Competing interests
The authors declare no competing interests.

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