Journal of **R**eproduction & **I**nfertility
Volume 27, Issue no. 1
https://doi.org/10.18502/jri.v27i1.22122

Original Article

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The Effect of Serine Proteases and Their Inhibitors on In Vitro Fertilization Outcomes

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Received: 20, Fab. 2026
Accepted: 14, Jul. 2026

Abstract

Background: Proteolysis imbalance can result in infertility. The purpose of the current study was to investigate the function of kininogen, neutrophil elastase, trypsin-like proteases, α1-protease inhibitor (α1-PI), and α2-macroglobulin (α2-MG) in blood, saliva, and follicular fluid as an indicator of the success of in vitro fertilization (IVF).

Methods: In general, twenty-one women undergoing an IVF cycle, as well as a control group consisting of ten healthy women, were included. The proteinase activity was measured by the rate of hydrolysis of specific substrates. The proteinase inhibitor activity was determined by the intensity of trypsin inhibition. The analysis was conducted using R software (version 4.4.1). The significance level was set at $p<0.05$.

Results: In women with infertility, blood activities of neutrophil elastase, α1-PI, and α2-MG were elevated, whereas kallikrein and prekallikrein activities were decreased, compared with controls. In follicular fluid, neutrophil elastase activity increased 3.4-fold, α1-PI 1.8-fold, and α2-MG 5.6-fold, whereas prekallikrein decreased by 85%, compared with women who had been infertile for fewer than five years ($p<0.05$). During pregnancy, kallikrein and α1- PI levels in follicular fluid were low, and unsuccessful IVF was associated with higher trypsin-like protease activity and an increased kallikrein/α1- PI ratio.

Conclusion: High elastase- and trypsin-like proteinase activity, along with elevated α1- PI and α2- MG levels in blood was associated with IVF benefits. Salivary kininogenase and α2-MG can be early prognostic indicators for IVF outcomes.

Keywords: Follicular fluid, In vitro fertilization, Saliva, Serine proteases, α_1-protease inhibitor, α_2-macroglobulin.
To cite this article: Spirina LV, Akbasheva OE, Dyakov DA, Kudisova KD, Romanova EA, Sidorenkova KA, et al. The Effect of Serine Proteases and Their Inhibitors on In Vitro Fertilization Outcomes. J Reprod Infertil. 2026;27(1):57-69. https://doi.org/10.18502/jri.v27i1.22122.

Introduction

Infertility is a reproductive disorder defined by the inability to achieve pregnancy following at least 12 months of consistent, unprotected sexual activity. This problem affects approximately 48 million couples and 186 million people worldwide, based on prevalence data compiled from studies conducted between 1990 and 2021 (1). Over the decade from 2011 to 2021, the rate of infertility among Russian women increased by about one-third, and the incidence among men nearly doubled during the same period. This notable demographic change has led to a corresponding rise in the use of in vitro fertilization (IVF) procedures. To maximize the success of these assisted reproductive methods, it is crucial to have a thorough grasp of the various factors that affect

IVF outcomes (2). IVF success depends on multiple factors including immune status, female age, embryo quality, thrombophilia, hormonal imbalances, endothelial dysfunction, endometrial abnormalities, microbiome disturbances, and chronic reproductive conditions or comorbidities (3).

Proteolytic enzymes, their inactive precursors (proenzymes), and activators are fundamental to the reproductive system's functioning (4). Systemic protease inhibitors promote embryonic and placental growth, angiogenesis, cell migration, and tissue morphogenesis. Trypsin-like proteases (TLPs) are essential for fertilization and facilitating the blastocyst's migration to the endometrium (4, 5). Trophoblast metalloproteases, such as gelatinases and neutrophil elastase, degrade the extracellular matrix (5). They prepare the matrix for oocyte implantation, regulate uterine trophoblast activity, and support angiogenesis (4-6). The kinin system's enzymes (kallikrein and prekallikrein) maintain the balance between vasoconstrictors and vasodilators, ensuring proper endothelial cell function during gestation. This is crucial for successful embryo implantation and pregnancy development (6). Proteolytic enzyme activity is controlled by α1-protease inhibitor (α1-PI) and α2-macroglobulin (α2-MG) (7). These proteins have antiproteolytic, anti-inflammatory, antiviral, immunomodulatory, anti-apoptotic, and cytoprotective properties (7-9). An imbalance between proteolytic enzymes and their inhibitors can lead to excessive protease activity, causing cellular and tissue damage (7). This disruption is the primary driver of acute and chronic inflammation, which can negatively impact the quality of oocytes and spermatozoa as well as hinder the successful implantation of embryos (8, 9).

This problem underscores the importance of studying the complex relationship between inflammation, proteolytic activity, and reproductive health to devise innovative solutions for infertility. The aim of the study was to investigate the activity of neutrophil elastase and trypsin-like proteases, kininogenesis, α1-PI, and α2-MG in blood serum, saliva, and follicular fluid, depending on IVF effectiveness.

Methods

Twenty-one women were recruited from the Assisted Reproductive Technologies clinics at Siberian State Medical University (SibSMU), the Ministry of Healthcare of the Russian Federation. The average age of the women was 35.5±4.7 years. Of the 21 women, 33% had tubal factor infertility, 38% had male-factor infertility, 5% had anovulatory infertility, and 10% had other unspecified forms of female infertility.

Design of the investigation: This prospective study included women of reproductive age who were candidates for IVF. The infertile group comprised women with a clinical diagnosis of infertility (no pregnancy after ≥1 year of regular unprotected intercourse). The control group included women with no female factor infertility (*i.e.*, normal ovulation, patent fallopian tubes, and no uterine pathology), whose infertility was attributable solely to a male factor. Exclusion criteria for all participants included the presence of acute inflammatory or infectious diseases, any serious chronic systemic illness, and failure to provide informed consent. No randomization was applied in this observational study; instead, all eligible patients during the study period were enrolled sequentially to avoid selection bias. An additional examination, apart from the standard procedures, was carried out on 21 women undergoing IVF/ICSI at SibSMU ART center.

Based on the duration of infertility, two subgroups were identified: subgroup 1 with infertility lasting less than 5 years (n=10), and subgroup 2 with infertility exceeding 5 years in duration (n= 11). Among women with infertility duration of <5 years (n=10), 90% (9/10) underwent IVF for the first time, whereas 10% (1/10) had a history of previous unsuccessful IVF attempts (up to five attempts). Among women with infertility duration of ≥ 5 years (n=11), 45.4 % (5/11) underwent IVF for the first time, 18% (2/11) had two to three previous attempts, and 36.4 % (4/11) had four or more previous attempts. A history of pelvic surgery, such as salpingectomy, polypectomy, or myomectomy was found in half of the women with infertility lasting less than 6 years, and in 80% of those with infertility lasting more than 6 years. Additionally, six patients had grade one obesity, three had grade two obesity, and four were underweight.

In most cases (80%), patients were prescribed Utrogestan (Besins Healthcare SA (Belgium) at a dose of 800 *mg*. The remaining women received Kraynon (Dendron Brands Limited, United Kingdom) at a dose of 1125 *mg*. Due to the results of pre-implantation genetic testing, embryo transfer was cancelled for 4 patients. Embryo transfer was performed for 17 individuals, resulting in pregnancy for 6 (35%) of the patients. Five women

had spontaneous miscarriages within 12 weeks, and one delivered at term. The control group consisted of 10 women aged 34.6±3.5 years who underwent IVF due to male-factor infertility; the infertility diagnosis was related to the male partner, and the women had no identified female factor infertility.

Control group consisted of ten healthy women. The inclusion criteria for the group of healthy women without fertility issues are as follows: female participants aged 18–35 years; regular menstrual cycles (24–35 days) for at least 12 consecutive months prior to enrollment; no history of infertility or subfertility; no known structural uterine or ovarian abnormalities (confirmed by ultrasound within the past 12 months); no prior diagnosis of polycystic ovary syndrome (PCOS), endometriosis, or other reproductive disorders; no use of hormonal contraception or fertility medications within 6 months prior to screening; body mass index (BMI) within the normal range (18.5–24.9 kg/m^2); no chronic medical conditions (*e.g.*, diabetes, thyroid disorders, autoimmune diseases) that could affect reproductive health; no history of pelvic surgery or radiation/chemotherapy; negative screening for sexually transmitted infections (STIs) within the past 3 months; and willingness to abstain from assisted reproductive technologies (ARTs) during the study period.

The exclusion criteria comprised current pregnancy or breastfeeding; history of recurrent pregnancy loss (≥2 miscarriages); known male factor infertility; use of intrauterine devices (IUDs) or other long-acting reversible contraceptives at screening; clinically significant abnormalities in reproductive hormone levels (*e.g.*, follicle-stimulating hormone [FSH], luteinizing hormone [LH], anti-Müllerian hormone [AMH], prolactin, and thyroid-stimulating hormone [TSH]); presence of ovarian cysts or fibroids ≥3 cm in diameter; active smoking or substance abuse within the past year; excessive alcohol consumption (>14 standard drinks per week); participation in another clinical trial within 30 days prior to screening; any contraindication to transvaginal ultrasound; and inability to provide informed consent or comply with study procedures.

Randomization method: Block randomization, a constrained randomization technique, guarantees balanced group sizes by distributing participants equally across comparison groups within predetermined blocks. The approach maintains group equivalence until the completion of each block.

Venous blood was collected and centrifuged at 3,000 *rpm* to separate the serum, which was then aliquoted and stored at -20°*C* until analysis. Follicular fluid was obtained during transvaginal follicular puncture; after retrieval of oocytes, the remaining fluid was collected for further testing. Mixed saliva was collected after rinsing the mouth with distilled water and 1-3 *ml* of saliva was obtained in a polyethylene tube over 5-10 *min* and centrifuged at 3,000 *rpm*. All biological samples (serum, follicular fluid, and saliva) were stored at -20°*C* prior to the assays. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Siberian State Medical University (Protocol No. 8, 18.01. 2023).

Determination of the neutrophil elastase and TLPs activity: The activity of elastase-like proteases (ELPs) and TLPs was measured using the method of esterolytic assay (10). To determine the activity of neutrophil elastase, 0.1 *ml* of serum, saliva, or follicular fluid diluted 10-fold was mixed with 2.7 *ml* of a 0.05 *M* sodium phosphate buffer solution (pH=6.5) and 0.1 *ml* of a 1 *mM* N-butyl nitrophenyl ether (Sigma-Aldrich, USA), in acetonitrile. After mixing, the increase in absorbance was measured for 5 *min* with readings taken every minute at 347.5 *nm*. Activity was expressed as *nmol* of N-butyl-4-nitrophenyl ether (BNPE) hydrolysate per minute per milliliter of biological sample (*nmol/min/ml*).

To measure TLP activity, 0.1 *ml* of serum (saliva) was diluted 10-fold in 1.9 *ml* of a 0.05 *M* Tris-HCl buffer (pH=7.8), followed by the addition of 1 *ml* of a 1.5 *mM* N-benzoyl-L-arginine-ethyl ester (BAEE) (Sigma-Aldrich, USA). Optical density was measured at 253 *nm* for 6 *min*, and activity was expressed in *nmol* BAEE/*min*×*ml*.

Determination of kallikrein and prekallikrein activity: Kallikrein activity was determined by esterolytic assay (11). Isolation of kallikrein and prekallikrein from biological material was performed by gel filtration on a DEAE-Sephadex A-50 column (Sigma-Aldrich, USA). To do this, 0.25 *ml* of serum (saliva and follicular fluid) was mixed with 0.75 *ml* of 0.02 *M* phosphate buffer solution (pH=7.0) and applied to a polystyrene column filled with Sephadex and balanced with the buffer. They were eluted with 0.02 *M* phosphate buffer containing 0.5 *M* NaCl (pH=7.0). The eluate was used to determine the activity of the kallikrein and

prekallikrein.

To measure kallikrein activity, 1 *ml* of eluate was added to 1 *ml* of 0.1 *M* phosphate buffer (pH= 8.0), followed by 1 *ml* of 1.5 *mM* BAEE solution. The optical density was measured at 253 *nm* for 9 *min* against a control sample.

To determine kallikrein activity, 1 *ml* of the eluate was added to 0.8 *ml* of 0.1 *M* phosphate buffer (pH=8.0) and 0.1 *ml* of a 0.01% trypsin solution. The mixture was incubated at 20°*C* for 2 *min* to activate kallikrein. After that, 0.2 *ml* of soybean trypsin inhibitor (prepared in 0.4 *M* phosphate buffer at pH=8) was added to inactivate excess trypsin. After 15 *min*, 1 *ml* of BAEE (1.5 *mM*) was added and the optical density was measured every minute at 253 *nm* against the control. The activity of kallikrein and kallikreinogen was expressed as *IU/ml*.

Determination of α1-PI and α2-MG activity: Inhibitory activity was measured by the method introduced by Nartikova and Paskhina (12). To determine α1-PI activity, two experimental samples were prepared. The first contained 1.9 *ml* of 0.05 *M* Tris-HCl buffer (pH=8.0) and 0.1 *ml* of 0.01% trypsin solution. The second contained 1.8 *ml* of 0.05 *M* Tris-HCl buffer, 0.1 *ml* of 0.01% trypsin solution, and 0.1 *ml* of 50-fold diluted serum (saliva or follicular fluid). Both samples were incubated for 5 *min* at 25°*C*, after which 1 *ml* of 1.5 *mM* BAEE solution was added to each. The optical density at 253 *nm* was measured for 3 *min*. To determine α2-MG activity, 1.75 *ml* of 0.05 *M* Tris-HCl buffer (pH=8.0) was mixed with 0.05 *ml* of 0.1% trypsin solution and 0.1 *ml* of 10-fold diluted biological fluid (serum, saliva, or follicular fluid). Then, 0.1 *ml* of a 3% trypsin inhibitor solution (Sigma-Aldrich, USA) in 0.05 *M* Tris-HCl buffer (pH=8.0) was added for 5 *min* at 25°*C*. The optical density was measured at 253 for 10 *min* against the control sample (2 *ml* of Tris-HCl buffer and 1 *ml* of BAEE). The activity of inhibitors was expressed in conditional inhibitory units per 1 *ml* of biological fluid (*IU/ml*).

All spectrophotometric measurements were carried out using a UNICO 2800 UV–VIS spectrophotometer (United Products and Instruments, USA). Enzyme activities were calculated from the absorbance data using known molar extinction coefficients or standard curves for the respective substrates, and are reported in *nmol/min/ml* for proteolytic enzymes and *IU/ml* for inhibitors. Each assay was performed in duplicate for each sample, and the mean value was used in the analysis. The intra-assay coefficient of variation (CV) for these measurements did not exceed 10%.

Statistical analysis: Statistical data analysis was performed using the R programming language (version 4.4.1) in the RStudio environment (version 2023.12.0+369) (13). The Shapiro–Wilk test with Royston correction was used to assess normality of quantitative variables. Welch's t-test with Satterthwaite approximation was used to compare the mean values of the normally distributed variables. For the quantitative characteristics that did not follow a normal distribution, the Brunner–Munzel test was used. To evaluate the relationship between the indicators and predict the success of IVF, Spearman's correlation analysis and multinomial logistic regression were employed. Results are presented as mean±SD for normally distributed variables, and as median (Q1, Q3) for non-normally distributed variables. A two-tailed $p<0.05$ was considered statistically significant.

Results

Serum and saliva activities of neutrophil elastase, trypsin-like proteases, kallikrein, prekallikrein, and their inhibitors in infertile women.

The results of testing for the neutrophil elastase and trypsin-like protease activity, kallikrein, prekallikrein, and α1-P and α2-MG activity in the serum and saliva of infertile women, are presented in table 1. All indicators were investigated in follicular fluid: neutrophil elastase activity was 198.0 (90.3; 305.8) *nmol* N-butyl nitrophenyl ether/*min/ml*; trypsin-like protease activity was 41.9 (20.5; 82.5) *nmol* N-butyl nitrophenyl ether /*min/ml*; kallikrein activity was 17.2 (8.1; 24.2) *mU/ml*; prekallikrein activity was 149.3 (39.6; 303.1) *mU/ml*; α1-PI activity was 38.4 (22.4; 72.6) *IU/ml*; and α2-MG activity was 16.1 (4.9; 27.2) *IU/ml*.

The activity of neutrophil elastase and trypsin-like proteases in the blood serum and saliva of infertile women demonstrated a 3-fold and 1.5-fold increase, respectively, compared with the control group. Nevertheless, trypsin-like protease levels in blood serum dropped by 44% (from 67.9 (45.5; 90.3) to 38.2 (30.3; 60.1)), with no significant change in saliva. Kallikrein activity was reduced by 47% (from 18.4 (18.3; 18.6) to 9.7 (7.7; 18.4)) in serum and 93% (from 122.5 (94.3; 130.0) to 8.5 (7.7; 15.8)) in saliva, compared to healthy individuals. Furthermore, prekallikrein

Table 1. The activity of neutrophil elastase, trypsin-like protease, kallikrein, prekallikrein, and their inhibitors in serum and saliva of infertile women compared to the control group. Values are presented as median (Q1; Q3)

Indicators	Material	Control group, n=10	Infertile women, n=21	R
Neutrophil elastase, *nmol* N-butyl nitrophenyl ether */min×ml*	Serum	103.2 (90.6; 109.5)	316.7 (152.9; 327.6)	p<0.001
	Saliva	82.5 (79.3; 86.3)	120,1 (98.3; 142.0 *cm*)	p<0.001
Trypsin-like proteases, *nmol* N-butyl nitrophenyl ether */min×ml*	Serum	67.9 (45.5; 90.3)	38.2 (30.3; 60.1)	p=0.009
	Saliva	29.00 (22.8; 29.8)	27.3 (10.9; 32.8)	p=0.32
Kallikrein, *mU/ml*	Serum	18.4 (18.3; 18.6)	9.7 (7.7; 18.4)	p=0.035
	Saliva	122.5 (94.3; 130.0)	8.5 (7.7; 15.8)	p<0.001
Prekallikrein, *mU/ml*	Serum	159,0 (142.5; 173.3)	42.8 (40.8; 46.5)	p<0.001
	Saliva	269.3±39.3	35.1±13.8	p<0.001
α_1-PI, *IU/ml*	Serum	27.3 (20.7; 32.4)	55.7 (46.4; 60.1)	p<0.001
α_2-Macroglobulin (α_2-MG), IE/*ml*	Saliva	0.2 (0.2; 0.2)	26.5 (18.6; 30.6)	p<0.001
α_2-MG, *IU/ml*	Serum	4.6 ± 0.5	13.9±5.0	p<0.001
	Saliva	0.10 (0.00; 0.10)	15.3 (12.6; 19.5)	p<0.001

Note: α2- MG activity in serum and prekallikrein activity in saliva are presented as M ± SD; p-values are for comparison with the control group

activity declined by an additional 73% (159.0 (142.5; 173.3) to 42.8 (40.8; 46.5)) in serum and 87% (269.3±39.3 to 35.1±13.8) in saliva.

Protease inhibitors exhibited a marked enhancement of anti-proteolytic activity in the serum and saliva of women facing fertility issues. In their blood, α1-PI activity doubled (27.3 (20.7; 32.4) to 55.7 (46.4; 60.1)), and α2-MG activity tripled (from 4.6±0.5 to 13.9±5.0) compared to healthy women. This augmentation in inhibitory capacity was particularly notable in saliva. The activity of α1-PI demonstrated a 132-fold increase (0.2 (0.2; 0.2) to 26.5 (18.6; 30.6)) and activity of α2-MG a 153-fold rise compared with the control group (from 0.10 (0.00; 0.10) to 15.3 (12.6; 19.5)). Spearman's correlation analysis disclosed significant associations between α1-PI and α2-MG activities, with correlation coefficients (r_s)=0.55 in serum (p<0.05) and r_s=0.88 in saliva (p<0.001). As a result, in the blood serum of women with infertility, the trypsin-like proteases, kallikrein, and prekallikrein activity was reduced. Conversely, the neutrophil elastase, α1-PI, and α2-MG activity was increased. Similar alterations were found in the saliva of infertile women, except for the trypsin-like proteases activity.

Activity of proteases and their inhibitors in follicular fluid based on infertility duration: Additionally, the proteolysis levels in follicular fluid were assessed alongside those in blood serum and saliva. These levels were then compared according to infertility duration (Table 2). The trypsin-like proteases and kallikrein activity in follicular fluid remained unchanged regardless of the length of infertility. Nevertheless, in women with infertility lasting more than five years, prekallikrein activity decreased by 85% (from 258.9 [250.5; 303.1] to 39.6 [10.1; 45.7]). In contrast, neutrophil elastase activity increased 3.4-fold (from 90.3 [90.3; 109.6] to 305.8 [229.3; 327.6]), α1-PI activity rose 1.8-fold (from 27.3 [22.4; 27.3] to 49.4 [40.4; 72.6]), and α2-MG activity increased 5.6-fold (from 4.9±0.9 to 27.2±6.5), compared with women whose infertility lasted less than five years. The activity levels of proteases and inhibitors in saliva reflected those in follicular fluid. The correlation coefficients for α1-PI activity in saliva and follicular fluid were 0.75; for α2-MG activity,

Table 2. The activity of neutrophil elastase, trypsin-like protease, kallikrein, prekallikrein, and their inhibitors in follicular fluid based on infertility duration; values are presented as medians (Q1; Q3)

Indicators	Infertility <5 years n=10	Infertility 5 years n=11	r
Neutrophil elastase, *nmol* N-butyl nitrophenyl ether /*min*×*ml*	90.3 (90.3; 109.6)	305.8 (229.3; 327.6)	<0.001
Trypsin-like proteases, *nmol* N-butyl nitrophenyl ether /*min*×*ml*	45.5 (45.5; 82.6)	38.2 (20.5; 66.8)	0.083
Kallikrein, *mU/ml*	18.5 (18.4; 18.7)	15.8 (8.1; 24.2)	0.090
Prekallikrein, *mU/ml*	258.9 (250.5; 303.1)	39.6 (10.1; 45.7)	<0.001
α_1-PI, *IU/ml*	27.3 (22.4; 27.3)	49.4 (40.4; 72.6)	<0.001
α_2-MG, *IU/ml*	4.9 ± 0.9	27.2 ± 6.5	<0.001

Note: α2- MG activity is presented as M±SD; p<0.05 was considered statistically significant for between-group differences

0.78; and for kallikrein and prekallikrein activities, 0.61 and 0.70, respectively (p<0.05).

Furthermore, the activity of α1-PI and α2-MG in saliva was directly correlated with the activity of neutrophil elastase in the follicular fluid, as confirmed by positive correlation with α1-PI (r_s= 0.74; p<0.05) and α2-MG (r_s=0.81; p<0.05). As infertility duration increases, the activity of neutrophil elastase and its inhibitors rose in the follicular fluid. A positive correlation emerged between the α1-PI, α2-MG, kallikrein, and prekallikrein in both the follicular fluid and saliva of infertile women.

Trypsin-like protease activity and α1-PI/ prekallikrein and prekallikrein/kallikrein ratios in follicular fluid according to IVF outcome: In the subsequent phase, the findings were analyzed in relation to IVF success and pregnancy outcomes in infertile women (Table 3). It was revealed that trypsin-like protease activity in the follicular fluid of infertile women was 4.3 times higher compared to those who conceived successfully (ranging from 16.4 (10.9; 21.8) to 70.9 (65.5; 92.8)). Furthermore, a correlation was established between the elevated activity of trypsin-like proteases in the follicular fluid and increased neutrophil elastase activity in the blood (r_s=0.87). To evaluate the proteolytic imbalance, the ratios of α1-PI/prekallikrein and prekallikrein/kallikrein were calculated in the follicular fluid samples of IVF cycles from both pregnant and non-pregnant women.

An unsuccessful IVF cycle, defined as the absence of pregnancy, correlated with several alterations in specific blood protein levels. In 50% of the women, the prekallikrein/kallikrein ratio increased 1.4-fold, and in 19%, it rose 4.4-fold. Overall, 69% of the women who experienced an unsuccessful IVF had a 2.9-fold increase in this ratio compared to women who became pregnant after IVF. Another crucial indicator was the α1-PI/prekallikrein ratio, which is linked to the control of proteolysis. In 50% of the cases, this ratio

Table 3. Trypsin-like protease activity and the α1-PI/ prekallikrein and prekallikrein/kallikrein ratios in follicular fluid based on IVF outcome; values are presented as medians (Q1; Q3)

Indicators	Presence of pregnancy after IVF, n=5	No pregnancy after IVF, n=16		r
Trypsin-like proteases, *nmol* N-butyl nitrophenyl ether /*min*×*ml*	16.4 (10.9; 21.8)	n=9 (56%)	70.9 (65.5; 92.8)	0.006
Prekallikrein/kallikrein	1.7 (1.4; 1.9)	n=8 (50%)	2.4 (0.9; 4.0)	0.019
		n=3 (19%)	7.6 (5.7; 7.6)	0.018
α1-PI/ prekallikrein	1.8 (1.2; 4.0)	n=4 (25%)	6.0 (3.3; 8.7)	0.011
		n=8 (50%)	0.9 (0.7; 1.4)	0.011

Table 4. The coefficients of the logistic regression model for predicting pregnancy after IVF

Indicators	B	r	Exp (B)
Salivatory kallikrein activity	0.602	0.006	1.827
α_2-MG in the blood	-0.258	0.047	0.773
Constant	-5.234	0.043	0.005

was halved, suggesting that protease activity surpassed inhibitor activity. An increase in the α1-PI/prekallikrein ratio signified high inhibitor activity, but in 25% of the women who did not conceive, this ratio increased 3-fold.

The logistic regression model for predicting pregnancy after IVF: A multinomial logistic regression was used to determine the most precise proteolytic indicators of pregnancy during IVF in infertile women cohort. The study identified two statistically significant markers: salivary kallikrein activity and α2-MG activity in blood serum (Table 4). Notably, α2-MG activity in the serum made the largest contribution (OR=1.827). The model demonstrated a sensitivity of 0.75 and a specificity of 1.0.

An increase in trypsin-like protease activity, kininogenesis activation in the follicular fluid, and a breakdown in the proteolytic system are linked to unsuccessful IVF outcomes. Salivary kallikrein and serum α2-MG levels can serve as predictors of successful pregnancy following IVF.

Discussion

This study reveals a significant association between the activity of proteolytic enzymes including neutrophil elastase, trypsin-like proteases, and components of the kinin system and reproductive outcomes in women undergoing IVF. Special emphasis is placed on non-invasive diagnostics. For the first time, a strong correlation has been demonstrated between proteolytic markers in saliva and follicular fluid, opening new perspectives for using saliva as a biomarker matrix in assessing reproductive potential.

Proteolysis and reproductive processes: Proteolytic enzymes are involved in key stages of reproduction, including oogenesis, fertilization, blastocyst implantation, and placental formation. For example, trypsin-like proteases are essential for sperm penetration through the zona pellucida and subsequent embryo implantation into the endometrium (4). Trophoblast cells secrete matrix metalloproteinases (MMPs), which remodel the extracellular matrix to prepare the tissue for implantation. It is critically important that blastocyst maturation and the opening of the "implantation window" occur simultaneously; otherwise, early reproductive loss may result (4).

Placental angiogenesis and vascularization also depend on proteolysis; enzymes degrade the extracellular matrix and regulate trophoblast invasion (5, 14). Dysregulation of neutrophil elastase, and trypsin-like protease activity, accompanied by reduced levels of α1-PI, has been associated with placental insufficiency (15). This aligns with our findings that in infertile women, neutrophil elastase activity was increased 3-fold in serum, 1.5-fold in saliva, and 3.4-fold in follicular fluid (Tables 1 and 2). This systemic activation of proteolysis is likely driven by chronic ovarian inflammation, prior pelvic surgery (reported in 67% of women), comorbidities (*e.g.*, obesity in 14% of patients), or mechanical trauma during follicular puncture.

Inflammation, α1-PI, and α2- MG as regulators of proteolysis and immunity: Neutrophil elastases are biochemical markers of a generalized inflammatory response. These include neutrophil elastase, pancreatic elastase, cathepsin G, protease 3, and bacterial elastase. Neutrophil elastase (EC 3.4.21. 37), which is found in the azurophilic granules of neutrophils, is released during degranulation. This release is essential for inflammation and aids in resolving the inflammatory process (16).

In women with infertility, the level of neutrophil elastase activity in blood increased three-fold, while salivary activity rose 1.5-fold compared with controls (Table 1). In follicular fluid, the activity of this enzyme increased 3.4-fold in cases of infertility that had persisted for more than five years (Table 2). The heightened neutrophil elastase activity observed in all examined biological fluids is likely attributable to an initial activation of proteolysis within ovarian tissue, subsequently leading to an increase in protease activity in both blood and saliva of these women. This augmentation may be associated with a history of chronic

inflammation, previous pelvic surgical interventions (experienced by 67% of women), the presence of comorbidities such as obesity (identified in 14% of women), or the detrimental effects of microsurgery utilized for oocyte retrieval.

Neutrophil elastase in the ovaries can promote thrombin inflammation by aiding in the formation of neutrophil extracellular traps (NETs). These NETs, intricate structures comprising chromatin, proteins, and neutrophil serine proteases, are generated in response to immune complexes and cytokines like IL-8, IFN-γ, and TNF-α in chronic inflammation of the reproductive system (17). Consequently, increased α1-PI activity in serum, saliva, and follicular fluid can be a dependable marker of tissue and plasma proteolysis in women with reproductive disorders.

In infertile women, α1-PI activity was increased 2-fold in serum, 132-fold in saliva, and 1.8-fold in follicular fluid (Tables 1 and 2). The high concentration in saliva reflects easy transudation from plasma, whereas penetration into the ovary is limited. Importantly, α1-PI possesses not only inhibitory but also broad biological effects; they can be categorized as anti-inflammatory, immunomodulatory, antiviral, anti-apoptotic, and tissue-protective properties (7, 8). These functions are mediated through mitochondrial membrane stabilization, NF-κB activation, caspase suppression, MMP inhibition, enhanced secretion of the anti-inflammatory cytokine IL-10, and induction of immune tolerance (18, 19).

Signaling pathways and TLPs: TLPs are the members of the protease-activated receptors (PARs), triggering signaling cascades involving phospholipase C, protein kinase C, Raf, Src, MAPK, NF-κB, STAT2/3, intracellular Ca^{2+} elevation, and prostaglandin synthesis (35). These pathways regulate cell proliferation, differentiation, and inflammation, the processes critical for implantation and placentation. Thus, our finding of elevated trypsin-like protease activity in follicular fluid among women with failed IVF (Table 3) suggests dysregulation of these signaling cascades, potentially impairing angiogenesis and stromal remodeling.

α2-MG performs a similar but broader regulatory role. It inhibits proteases acting on large substrates of serine, cysteine, aspartate, and metalloproteases while preserving their ability to cleave oligopeptides and peptides. Additionally, α2-MG binds inflammatory mediators (*e.g.*, IL-1, IL-6, TNF-α) and growth factors, contributing to innate immunity (20, 21). α2-MG is synthesized not only in hepatocytes but also in fibroblasts, macrophages, and ovarian granulosa cells (9, 25).

Via the low-density lipoprotein receptor-related protein-1 (LRP-1), expressed in granulosa and Leydig cells, α2-MG facilitates protease clearance and inflammation regulation (24). Through the GRP78 receptor, it exerts proliferative and anti-apoptotic effects (20, 25). In the reproductive system, α2-MG modulates FSH and LH actions, promoting follicular growth and embryonic development (27, 28). A four-fold increase in α2-MG concentration is associated with inflammation and unsuccessful IVF attempts (31).

α2-MG is a key component of the innate immune system and serves as the primary hemostatic regulator in the cardiovascular system. It exhibits anticoagulant, procoagulant, and antifibrinolytic activities, thus controlling thromboinflammation (9, 24). α2-MG's function in regulating inflammation involves the elimination of proteases from the bloodstream via binding to low-density lipoprotein receptor-1 (LDLR-1) receptors. These receptors are subsequently endocytosed and degraded within liver endosomes (24). LDLR-1 is expressed in various cell types, including ovarian granulosa cells and Leydig cells in the testes, as well as monocytes, macrophages, fibroblasts, epithelial cells, smooth muscle cells, and neuronal cells such as astrocytes and interstitial dendritic cells (IDC) in the kidneys. Additionally, α2-MG interacts with the GRP78 receptor, stimulating cell proliferation and inhibiting apoptosis (20, 25). α2-MG plays a crucial role in regulating the function of the gonads (2, 6). It modulates the action of follicle-stimulating and luteinizing hormones (27), promoting cell proliferation and fetal development (28). It inhibits inflammatory proteases such as neutrophil elastase, cathepsin G, mast cell chymase, and ADAMTS proteases, whose activation is responsible for fetal development. This makes it a potential factor in reproductive dysfunction and the development of pregnancy complications (29). In the first trimester of pregnancy, the concentration of α 2-MG in the blood increases two-fold (30). After delivery, it decreases in 30% of women (30). However, a four-fold increase in the concentration of α 2-MG is an indicator of inflammation and is often associated with unsuccessful pregnancy attempts (31).

α1-PI and α2-MG exhibit an inhibitory effect by

decreasing TLP activity in the serum of infertile women (Table 1). TLPs belong to the serine protease family, characterized by mixed nucleophilic activities, and are part of superfamily A (32). This family includes enzymes such as trypsin, renin, thrombin, plasmin, their activators (tissue and urokinase types), protein C, and factors VIIa, IXa, Xa, and XIIa, as well as components of the complement system (C1r, C1s, C3 convertase, C5, and factor D) (33). Within the body, serine proteases generated by mast cells are crucial for clot formation, tissue remodeling, and wound healing (34). Upon binding to PAR proteins, TLPs activate numerous signal transduction pathways, including phospholipase C, protein kinase C, Raf, SCR, MAPK, NF-kB, STAT2, STAT3, inositol trisphosphate (InsP3), calcium (Ca^{2+}), diacylglycerol (DAG), prostaglandin E2 (PGE2), and prostaglandin Fα (PGFa), which stimulate cell proliferation and differentiation (35). TLPs are essential for the proper functioning of the reproductive, cardiovascular, respiratory, and nervous systems (32).

Kinin system and vascular tone regulation: The kallikrein-kinin system also belongs to the family of trypsin-like enzymes. Plasma kallikrein (synthesized in the liver) participates in coagulation, fibrinolysis, and complement activation (36–38). Tissue kallikreins (KLK1–KLK15) are expressed in reproductive organs and regulate local blood flow via bradykinin, a potent vasodilator that dilates uterine and placental vessels (41).

In our study, kallikrein and kininogen activity was reduced in serum and saliva in infertile women (Table 1), possibly reflecting exhaustion of the kinin system due to chronic inflammation. Bradykinin deficiency impairs vasodilation, contributing to placental insufficiency and fetal growth restriction (4). Among 65% of women who failed to achieve pregnancy after embryo transfer, low kallikrein activity coincided with high neutrophil elastase activity in follicular fluid (Table 2), confirming the inflammatory nature of implantation failure.

In this study, low levels of kallikrein and prekallikrein activity were likely insufficient to sustain vascular resistance and were associated with failure of pregnancy following embryo implantation in 65% of cases. This may be due to increased activity of neutrophil elastase, which is an indicator of inflammation in ovarian tissue (Table 2). Additionally, higher levels of trypsin-like protease activity were observed in follicular fluid from women who failed to conceive after IVF treatment (Table 3).

The high activity of trypsin-like proteases is often seen in inflammatory conditions and is associated with the breakdown of the extracellular matrix, impaired blood supply, and angiogenesis (42). These processes are all necessary for successful development of a dominant follicle and successful oocyte implantation (43). The results of our research suggest that the activity of certain enzymes in the follicular fluid is associated with the breakdown of ovarian tissue and impaired angiogenesis during embryo implantation. This can lead to unsuccessful outcomes in IVF.

Correlations across biological fluids and predictive modeling: In our research, the potential of non-invasive diagnostics and the effectiveness of IVF were the main focus. Specifically, the markers in saliva were explored and their relationship with indicators in the blood was examined. Our study has revealed a strong correlation between the activity of trypsin-like proteases in the follicular fluid and the activity of neutrophil elastase in the blood. This suggests that measuring the activity of elastase-like enzymes in the blood serum could be a promising indicator for predicting low IVF success rates and providing a basis for additional rehabilitation and anti-inflammatory treatment prior to IVF.

The chemical composition of saliva is closely related to that of blood and can be linked to gynecological conditions and fertility issues (44, 45). Key indicators in follicular fluid are associated with follicle development and oocyte maturation. It is hypothesized that the follicular fluid proteome plays a critical role in regulating follicle growth and oocyte maturation in women with infertility (46). Research has shown that α1-PI and α2-MG activity in saliva is associated with neutrophil elastase activity in follicular fluid in infertile cases. This relationship was validated by positive Spearman correlation coefficients. Measuring α1-PI and α2-MG activity in saliva could provide a non-invasive indicator for assessing the follicular fluid proteomics. Specifically, an increase in α2-MG activity requires close monitoring as it might signal potential issues with IVF success (47).

The unique proteolytic profile associated with successful IVF cycles is highlighted by the low activity levels of trypsin-like proteases in follicu-

lar fluid, compared with levels in women who did not achieve pregnancy following the same procedure. The ratio of prekallikrein /kallikrein in follicular fluid was 1.7 in pregnant women. However, in unsuccessful IVF attempts, this ratio elevated, indicating potential prekallikrein activation during the depletion phase, which could contribute to the procedure's failure. These findings align with the established correlation between elevated protease levels and placental insufficiency and fetal growth restriction.

However, the activity of inhibitors and proteases exhibited similar directional changes in both pregnant and non-pregnant women after IVF. The α1-PI/prekallikrein ratio varied significantly between the groups. In women who achieved pregnancy following IVF, the mean α1-PI/prekallikrein activity ratio was 1.8, whereas in unsuccessful cycles, this ratio varied bidirectionally, either increasing or decreasing relative to that value. A decrease in the α1-PI/ prekallikrein ratio likely results from an increase in prekallikrein activity, while an increase may be attributed to an excessive elevation in α1-PI activity in the follicular fluid. Given the high activity of TLP, an increase in the α1-PI/prekallikrein ratio proved ineffective. Generally, a deviation in the α1-PI/prekallikrein ratio is linked to a disruption in proteolysis regulation during unsuccessful IVF attempts.

Our results align with the findings of Franasiak et al., which emphasizes the role of an inflammatory endometrial immune profile, characterized by M1 macrophage predominance and natural killer (NK) cell dysfunction in implantation failure (3). In 55% of unsuccessful cases, the failure was attributed to a pro-inflammatory shift, characterized by elevated neutrophilic elastase and trypsin-like protease activity, along with increased acute-phase inhibitors. This supports the concept of inflammation as a key pathogenic mechanism underlying reproductive loss.

For the first time, a strong positive correlation was observed between trypsin-like protease activity in follicular fluid and neutrophil elastase activity in serum (r_s=0.87). This suggests that serum neutrophil elastase activity may serve as a predictive marker for IVF failure.

Even more significant is the correlation between salivary levels of α1-PI and α2-MG and protease activity in follicular fluid (confirmed by Spearman's correlation). This enables non-invasive assessment of the proteomic status of the follicular microenvironment. Notably, elevated salivary α2-MG levels may predict IVF failure (47).

Based on these findings, a predictive model was developed using multinomial logistic regression: Y=–5.234+0.602×KK (saliva)–0.258×α2-MG (serum).

Pregnancy is predicted when Y ≥0.69. The model demonstrates high specificity (1.0) but moderate sensitivity (0.75), likely due to the influence of additional factors such as maternal age, endometrial immune profile, embryonic genetics, and progesterone receptor functionality (3, 49).

These findings have direct clinical relevance. Measuring α1-PI, α2-MG, and kallikrein activity in saliva prior to IVF could serve as a basis for personalized patient preparation. When an inflammatory profile is detected, anti-inflammatory therapy, antioxidants, or immunomodulators may be warranted, approaches consistent with recent meta-analyses on adjuvant therapies in reproductive medicine.

It has been determined that the number of previous unsuccessful IVF attempts can significantly influence the probability of achieving a clinical pregnancy and the risk of early spontaneous abortion. Studies have shown that patients who have undergone three or more unsuccessful attempts have significantly lower rates of embryo implantation, clinical pregnancies, and live births.

In our study, the pregnant and non-pregnant groups were comparable with respect to the number of prior IVF cycles, with the majority of participants undergoing their first cycle. Cases of pregnancy termination were observed following the first attempt (in three patients), as well as the third and fifth attempts. Only one woman successfully delivered at the appropriate gestational age; she was 38 years old and underwent her second IVF attempt. The lowest levels of proteolysis were detected during the preimplantation period. Specifically, the neutrophil elastase activity in blood was 142 *nmol/min×ml*, compared to 316.7 *nmol/min×ml* in the control group. Additionally, the α1-PI/prekallikrein ratio was 1.12, compared with 1.8 in the control group, and the prekallikrein/kallikrein ratio was 1.66, compared with 1.7 in the controls. α2-MG activity was 9.9 *IU/ml* in blood versus 13.9 *IU/ml* in controls and 9.9 *IU/ml* in saliva versus 15.3 *IU/ml* in controls. It is plausible that the examined indicators of proteolysis and inflammation may serve as more precise predictors of IVF success.

In summary, our study demonstrates that proteolytic imbalance, particularly when coupled with

chronic inflammation, is a key factor in IVF failure. Established mechanisms were not only confirmed, but robust correlations between saliva and follicular fluid were also established for the first time, and a clinically meaningful predictive model was provided. This contributes to the global literature on non-invasive diagnostics and personalized management of infertility.

Conclusion

The activation of neutrophil elastase and trypsin-like proteases in conjunction with a concomitant decline in the activity of protease inhibitors may serve as early predictive biomarkers for IVF outcomes. The methodology exhibits considerable promise for enhancing the IVF benefits by facilitating embryo transfer evaluations. The state of proteolysis, as indicated by the presence of neutrophil elastase and trypsin-like protease activity in saliva, parallels the changes detected in follicular fluid. Elevated levels of neutrophil elastase and trypsin-like proteases, coupled with increased concentrations of α1-PI and α2-MG, are indicative of inflammatory processes and are associated with unsuccessful IVF outcomes. Additionally, salivary kallikrein activity and α2-MG levels in blood serum may further enhance the predictive capacity of these biomarkers, offering a comprehensive assessment of the reproductive environment.

Conflict of Interest

The authors declare no conflicts of interest.

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