Relation of Pregnancy Associated Plasma Protein-A (PAPP-A) Levels in Serum and Follicular Fluid with Embryo Development and Early Pregnancy Results

Serumda ve Foliküler Sıvıdaki Gebelikle İlişkili Plazma Protein-A (PAPP-A) Değerlerinin Embriyo Gelişimi ve Erken Gebelik Sonuçları ile İlişkisi

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Abstract

The objective of this study is to measure Pregnancy Associated Plasma Protein-A (PAPP-A) in serum and follicular fluid during oocyte retrieval, and to investigate the association of PAPP-A with oocyte maturation, embryo development and early pregnancy results. This prospective cohort study was conducted in single center including fifty-five patients to whom intracytoplasmic sperm injection (ICSI) and embryo transfer (ET) were applied. Long agonist protocol was applied for ovarian stimulation. Follicular fluid was aspirated from two separate follicules of each patient for oocyte retrieval and PAPP-A levels of follicular fluids and serum were measured via Fluorescence immunoassay (FIA). Marked oocytes from these follicles were evaluated for their maturation and fertilization and embryo development were followed. Association of PAPP-A levels with embryo quality and pregnancy rates were assessed. There was not statistically significant association between the follicular fluid PAPP-A levels and the embryo qualities obtained from the oocyte of the same follicle. Also, PAPP-A levels from follicles were not significantly different when compared between high and low quality embryos. In contrast, serum PAPP-A levels at the time of oocyte retrieval were significantly lower in patients who achieved pregnancy when compared to the patients at whom pregnancy was not achieved ($p<0.05$). PAPP-A level in follicular fluid was not significantly related to embryo quality and pregnancy rates while serum PAPP-A values were significantly different between patients who achieved pregnancy and the patients who could not achieve pregnancy. However, further studies with large number of patients are needed.

Keywords: Embryo Quality, Follicular Fluid, Oocyte Maturation, PAPP-A

Öz

Bu çalışmada, oosit toplanması sırasında elde edilen folikül sıvısında ve serumda Gebelikle İlişkili Plazma Protein-A (PAPP-A) bakılması, bunun oosit maturasyonu, embriyo gelişimi ve erken gebelik sonuçları ile ilişkisinin değerlendirilmesi amaçlanmıştır. Tek merkezde yürütülmüş olan bu prospektif kohort çalışmasına, intrasitoplazmik sperm enjeksiyonu (ICSI) ve embriyo transferi (ET) uygulanan 55 hasta dâhil edilmiştir. Tüm hastalara uzun agonist protokol kullanılarak ovulasyon indüksiyonu yapılmıştır. Her hastanın iki ayrı folikülünden elde edilen folikül sıvılarından ve oosit toplanması sırasında alınan kanlarından Floresan immunoassay (FIA) yöntemi ile PAPP-A seviyeleri ölçülmüştür. Bu foliküllerden elde edilen işaretli oositlerin matürasyon ve fertilizasyonları değerlendirilmiş ve bunlardan oluşan embriyoların gelişimi takip edilmiştir. PAPP-A düzeylerinin embriyo kaliteleri ve gebelik oranları ile ilişkisi değerlendirilmiştir. Folikül sıvılarında bakılan PAPP-A değerleri ile aynı foliküllerden elde edilen embriyo kaliteleri arasında anlamlı bir ilişki saptanmamıştır. Embriyolar kalitelerine göre ayrıldığında, elde edildikleri foliküllerin PAPP-A değerleri arasındaki fark da anlamlı bulunmamıştır. Ancak, gebelik oluşan hastalarda oosit toplanması sırasında serumda bakılan PAPP-A değerleri gebelik oluşmayan hastalardakine göre anlamlı olarak düşük saptanmıştır ($p<0.05$). Folikül sıvısındaki PAPP-A seviyeleri embriyo kalitesi ve gebelik oranları ile anlamlı olarak ilişkili bulunmadı. Serum PAPP-A değerleri ise gebelik oluşan ve oluşmayan hastalar arasında anlamlı olarak farklı bulundu. Ancak, daha geniş hasta popülasyonu ile çalışmalar yapılmalıdır.

Anahtar Kelimeler: Embriyo Kalitesi, Folikül Sıvısı, Oosit Matürasyonu, PAPP-A

Introduction

Oocyte maturation and embryo quality have a marked influence on in vitro fertilization (IVF) success. Besides, many molecules such as cytokines and growth factors in follicular fluid have been proven to have significant effects on follicular maturation, ovulation and follicular atresia. One of these molecules which has been shown to be produced by granulosa cells and present in follicular fluid is Pregnancy Associated Plasma Protein-A (PAPP-A), a component of Insuline Like Growth Factor (IGF) system. IGF system is primarily composed of IGF-I and IGF-II peptides, their binding proteins IGFBP-1 – IGFBP-6, and Type I and Type II IGF receptors on target cells. IGFs and IGF receptors are thought to play a role in follicular recruitment, oocyte maturation and potentially embryo development through autocrine and paracrine effects (1-4).

IGFs were shown to stimulate proliferation in granulosa cells, but also steroidogenesis in theca

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cells, and IGF-II is the main component that works synergistically with FSH, increases estradiol (E2) in antral follicles (1,5). IGF activity is controlled by catalytic proteins and IGFBPs, which protect IGFs from being destroyed and act as a carrier and storage for IGFs. Ovarian follicular growth is characterized by decrease in intrafollicular levels of low molecular weight IGFBPs (IGFBP-2,-4,-5), and consequently increase in bioavailability of IGF-II and increase in FSH sensitivity of granulosa cells (6,7). Decrement in binding proteins depends on increment in intrafollicular proteolytic activity (8,9) and PAPP-A, which is also shown to play an important role in destruction of IGFBP-5 in preovulatory follicles, is the specific protease for IGFBP-4 (7,10). Therefore, PAPP-A affects IGF activity.

Studies which evaluated the effect of IGF system on oocyte maturation and embryo development are limited. Since the role of PAPP-A on follicle recruitment was demonstrated before (1,5,11,12), the authors, in this study, aimed to examine the effect of serum and follicular fluid levels of PAPP-A on oocyte maturation and embryo development.

Material and Method

This cross sectional cohort study was conducted at a university hospital. Fifty-five infertile patients who had controlled ovarian hyperstimulation for intracytoplasmic sperm injection (ICSI) and embryo transfer (ET) within a 6-month period, were recruited in the study. Follicular fluid samples obtained from the largest two follicles separately were collected along with the serum obtained from venous blood sample at one ICSI-ET cycle of each patient. Written informed consent was acquired from all patients. The study was approved by Institutional Review Board (Project number: 08H3330009, Ankara-2010) and supported by University Research Fund.

Indications for ICSI and ET were male factor infertility, tubal occlusion/absence, and unexplained infertility. All patients who were <40 years old, and who did not have polycystic ovarian syndrome or any other systemic illnesses were included in the study. Patients older than 40 years of age and patients with polycystic ovarian syndrome were excluded as dysregulation in insulin/IGF system has been shown to disrupt folliculogenesis in these patients (13).

All patients were treated with long agonist protocol. Leuprolide Acetate (Lucrin, Abbott Laboratories, France) which is a Gonadotrophin Releasing Hormone (GnRH) agonist was applied subcutaneously starting from 21th day of the previous cycle until the day of hCG administration. Starting from the 3rd day of the next menstruel cycle 150-300 IU recombinant human follicle stimulating hormone (rFSH; Gonal-F, Serono Laboratories, England or Puregon, Organon, Holland) and/or human menopausal gonadotrophin (hMG; Menogon, Er-Kim) was/were given. Dosage was adjusted due to response of the patient. Follicular development was monitored by regular transvaginal ultrasound screening and serum estradiol levels. When at least two follicles reached a diameter of 18 mm, then, 10000 IU Human Chorionic Gonadotropin (hCG; Pregnyl, Organon, Holland) was administered and oocyte retrieval was performed 35 hours later.

Follicular fluid of the largest two follicles from which oocytes were obtained were collected and processed together with the blood samples drawn just before follicular aspiration for each patient. Follicles were not irrigated during oocyte retrieval. All samples were centrifuged at 4000 g for 10 minutes, supernatants were aspirated and collected in Eppendorf tubes. Samples were kept at -80ºC until the day of PAPP-A measurement. Any follicular fluids which were contaminated with blood were excluded from the study.

As for classification of oocyte quality, mature oocytes were assessed as metaphase II (M II), while development of other oocytes paused at metaphase I (M I) and germinal vesicle (GV) levels. Fertilization was defined as formation of a zygote with two pronucleus (2 PN) 16-18 hours after ICSI. After fertilization, quality of developing embryos were evaluated according to their morphological features; grade A (high quality embryo), grade B, grade C, and grade D (poor quality embryo). Embryos which could be transferred were chosen due to this classification. Embryo transfer was performed under ultrasound guidance, and Wallace catheter was used for the procedure. Implantation was assessed by measurement of β-hCG 11 days after embryo transfer, and clinical pregnancy was shown by detection of fetal heart beat on ultrasound. PAPP-A measurement was performed via Fluorescent Immunoassay (FIA) method, using Autodelphia device and Perkin Elmer (USA) kits.

SPSS 15.0 programme was used for statistical analysis. Eligibility of data for normal distribution was evaluated by Shapiro-Wilk test. Relation between variables were inspected by using Pearson correlation coefficient when data were suitable with normal distribution, and Spearman Correlation Coefficient when normal distribution did not occure. Mean/median values in two groups were compared by Student T test in case of normal distribution, and Mann-Whitney U test when normal distribution did not occur. In more than two groups, mean/medians were compared by ANOVA in the presence of normal distribution and Kruskal-Wallis variance analysis when there was not normal distribution. Wilcoxon test was used to compare median values in dependent groups. To determine utility of serum PAPP-A as a diagnostic test in prediction of pregnancy, ROC curve was drawn and area under curve was calculated. Youden's Index was used to

find optimum intersection point. P<0.05 was considered statistically significant.

Results

Median infertility time of the patients was 5 (1-17) years. Of the 55 cycles, 27 (49.1%) were first ICSI cycle, while other cycles were 2nd-4th cycles. Cause of infertility was male factor in 45.5% of patients, other causes of infertility were unexplained infertility (40.0%), ovulatory disorders (10.9%), and tubal factor (3.6%) infertility. Follow up characteristics of the patients are shown in Table 1.

Table 1. Follow up characteristics of the patients

	Range	Mean±SD/Median
Age (years)	19-37	27
BMI (kg/m^2)	16.4-39.1	24.9±4.7
bFSH (mIU/mL)	2.6-12	6.4±1.6
bLH (mIU/mL)	1-18	7
bE_2 (pg/mL)	13.4-736	47
Gonadotropin treatment duration (day)	4-14	9.4±1.7
Total gonadotropin dose (IU)	750-4125	2171±754
hCG application day	9-22	13±2.3
Endometrium thickness at hCG application day	5-13	10±1.9
E_2 at hCG application day (pg/mL)	202-9400	3135

BMI: body mass index; bFSH: basal follicle stimulating hormone level in third day of the cycle; bLH: basal luteinizing hormone level in third day of the cycle; bE2: basal estradiol level in third day of the cycle; hCG: human chorionic gonadotropin; SD: standard deviation

Total number of oocytes retrieved from the patients ranged between 4 and 34 (median: 14). An average of 12 (minimum:2 – maximum:31) of these oocytes were mature (M II), others were immature oocytes.

PAPP-A values ranged between 55 and 1833 mIU/L (median: 517) in the follicles marked as 'first follicle', while in the follicles marked as 'second follicle' PAPP-A values ranged between 145 and 1909 mIU/L (median: 556). Serum PAPP-A (sPAPP-A) values ranged between 0 and 13 mIU/L with a median of 2.

Relation between quality of embryos developed from the oocytes retrieved from first follicles and PAPP-A values of first follicles (PAPP-A I), and relation between quality of embryos developed from the oocytes retrieved from second follicles and PAPP-A values of second follicles (PAPP-A II) are compared with Spearman Correlation Quotient and no statistically significant correlation was found (p>0.05).

Embryos developed from oocytes retrieved from first and second follicles are classified seperately according to their quality (A, B, C, D quality and unusable), and PAPP-A of these follicles are compared with Kruskal-Wallis test. Owing to low number of embryos in C and D groups, these two groups were merged. In both groups (first follicles and second follicles) PAPP-A values did not differ significantly between follicular fluid of A, B, C+D and unusable quality embryos (Table 2).

Table 2. Comparison of PAPP-A values of follicular fluids in first and second follicles according to embryo quality

	Embryo 1-PAPP-A (mIU/L) (developed from first follicles)		Embryo 2–PAPP-A (mIU/L) (developed from second follicles)	
Quality	**n_1**	**Median (mínimum-maximum)**	**n_2**	**Median (mínimum-maximum)**
A	22	437 (55-1833)	21	619 (145-1909)
B	7	812 (225-1067)	9	911 (153-1094)
C+D	4	392 (143-992)	7	520 (177-1097)
Unusable	22	700.5 (139-1432)	18	442 (167-1112)
p*		0.298		0.576

*Kruskal Wallis Variance Analysis, n1: number of embryos in different quality developed from first follicles, n2: number of embryos in different quality developed from second follicles

PAPP-A of follicular fluids of which embryos at two different qualities are obtained in the same patient are compared by Wilcoxon test. Significant difference was not found between PAPP-A of follicular fluids of A and B quality embryos (9 patients, p=0.314), PAPP-A of follicular fluids of C/D and A quality embryos (6 patients, p=0.075), PAPP-A of follicular fluids of unusable embryos and A quality embryos (8 patients, p=0.4), and PAPP-A of follicular fluids of unusable embryos and B quality embryos (6 patients, p=0.917).

Relationship between PAPP-A I, PAPP-A II and sPAPP-A values of patients were compared with Spearman Correlation Quotient, PAPP-A I and PAPP-A II values were found to be similar (p<0.001), while significant relationship was not found between other parameters. Correlation between PAPP-A I and PAPP-A II were evaluated by interclass correlation quotient (ICC), which showed a significant relation (ICC:0.656; p<0.001).

Relationship between pregnancy and PAPP-A values were assessed by Mann-Whitney U test. Serum PAPP-A levels during oocyte retrieval were significantly lower in patients who achieved pregnancy when compared to patients who could not achieve pregnancy (p<0.05) (Table 3). For this relation, ROC curve was drawn, and area under curve was calculated (Figure 1). Youden's index demonstrated optimum intersection point as 1.5, so that, achieving pregnancy was more likely when sPAPP-A was ≤1.5 (sensitivity:43.48%; spesivity:87.5%).

Other parameters were compared between patients at whom pregnancy was achieved and at whom pregnancy was not achieved. Statistically significant difference was not found between two groups, except infertility period was shorter in patients who achieved pregnancy (Table 4).

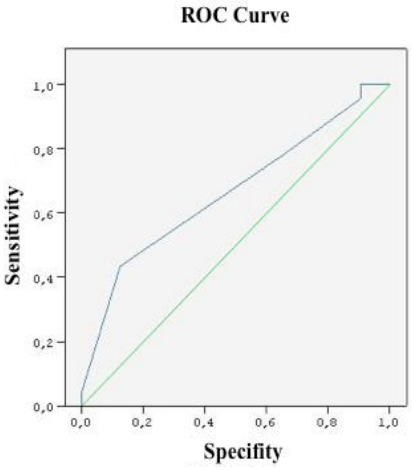

Figure 1. Relation between serum PAPP-A values and achieving pregnancy

Table 3. Relation between serum PAPP-A values and pregnancy

	PAPP-A serum Median (minimum-maximum)
Pregnancy not achieved	2 (1-13)
Pregnancy achieved	2 (0-7)
p	0.029

*Mann-Whitney U test

Discussion

Proteolysis of IGFBP-4 through PAPP-A, is an important mechanism regulating steroidogenesis in human granulosa cells and ovarian follicular development. Earliest biochemical change that could be demonstrated in the follicle recruited to be dominant follicle is the increase of PAPP-A level which leads to decrease in IGFBP 4 and 5 and increase in free IGF levels. These changes are denoted to be working in synergy with FSH to increase production of E2 (1,5,11,12).

In recent studies, significant relationship was found between intrafollicular PAPP-A concentrations and some hormone levels such as AMH, E2, and androgens. PAPP-A, seems to play a more important role than thought before. Shift of PAPP-A expression from theca cells to granulosa cells as the follicle develops to dominant follicle was demonstrated. PAPP-A was expressed along with aromatase in the granulosa cells of antral and preovulatory follicles. Also, intrafollicular PAPP-A concentration was found to be directly proportional to E2 and progesterone, and inversely proportional to testosterone and androstenedione. PAPP-A was thought to regulate local IGF-II activity and play the key role in ovarian steroidogenesis and follicular growth (14-16).

However, in late follicular maturation, while ovarian IGFBP-4 mRNA levels increased substantially after HCG, IGF-II and PAPP-A mRNA did not change significantly (17). In another study, it is reported that PAPP-A activity diminished dramatically after HCG application although follicular PAPP-A concentration remained in high levels (16). This suggested that, IGFBP-4 and PAPP-A relation did not play an important role in oocyte maturation and embryo development, unlike its role in early follicular growth (1).

Table 4. Comparison of other parameters in patients who could achieve pregnancy and who could not achieve pregnancy

	Pregnancy not achieved (n=32) **Mean±SD / Median (Minimum-Maximum)**	**Pregnancy achieved (n=23)** **Mean ±SD / Median (Minimum-Maximum)**	**p**
Age*	28.2±4.1	28.2±4.4	0.958
BMI (kg/m^2)**	24.9 (21-39)	23.9 (16.5-37)	0.062
Infertility period (year)**	6.5 (2-17)	3.7 (1-12)	**0.017**
Basal FSH (mIU/mL)**	6.1 (2.6-12)	6.1 (4.3-10.1)	0.823
Basal LH (mIU/mL)**	5.3 (1.5-18)	5 (1-15.5)	0.880
Basal E_2 (pg/mL)*	47.2±18.6	45.5±21	0.753
Gonadotropin administered day **	9 (4-14)	9 (7-13)	0.871
Total gonadotropin dose (IU)**	2025 (750-4125)	1837.5 (1425-3375)	0.441
HCG cycle day**	12 (9-17)	12 (10-22)	0.691
ET at HCG day (mm)*	10.3±2	9.7±2	0.305
E_2 at HCG day (pg/mL)**	2693 (416-8930)	2370.5 (202-9400)	0.658
Number of oocytes retrieved**	13 (4-34)	15 (5-28)	0.203
Number of M2 oocytes**	12.5 (2-31)	14 (4-24)	0.301
Fertilization rate (%)*	56.9±12.8	59.3±11.5	0.698
Number of transferred embryos**	3 (0-5)	3 (1-3)	0.593

*Student's t test (mean±SD is denoted), **Mann Whitney U test (median is denoted), BMI: Body mass index, SD: standard deviation, FSH: follicle stimulating hormone, LH: luteinizing hormone, E2:estradiol, HCG: human chorionic gonadotropin, ET: endometrium thickness

It was previously suggested that embryo development was being regulated by embryonic and maternal IGFs in preimplantation period (18). However, effect of IGF system in follicular fluid on embryo quality and development is not clear.

Wang et al. suggested that high levels of IGF-II, IGFBP-3, IGFBP-4 and low PAPP-A in follicular fluid at the time of oocyte retrieval was related to better oocyte maturation and early embryo development (in 48 hours after oocyte retrieval),

while high IGFBP-1, IGFBP-4 and low IGF-I were more favorable for late embryonic development (in 48-72 hours after oocyte retrieval) (1). Firouzabadi et al., however, reported that PAPP-A in follicular fluid could not be a determiner of fertilization and good embryo quality. It was also stated in that study that follicular fluid PAPP-A levels did not differ between GnRH long protocol and GnRH antagonist protocol cycles (19). Stanger et al. denoted that PAPP-A levels could demonstrate follicle maturity, but could not predict fertility potential of the ovum (20).

Our study also did not demonstrate a significant relationship between follicular fluid PAPP-A levels and embryo quality. Only long protocol cycles were included in the current study in order to minimize possible contribution of different treatment protocols on the results, but the situation might be different for other ovulation induction protocols.

All of the embryos obtained after stimulation and included in our study were not good quality, therefore, some of them were not transferred. However, PAPP-A values of follicular fluid from these embryos were also included in the analysis and compared with follicular fluid of good quality embryos. For this purpose, not only relationship between embryos and their follicular PAPP-A values were evaluated, but also, subgroups of embryos were formed according to their quality and PAPP-A values of their follicular fluid were compared. Furthermore, in order to avoid intervention of other possible factors, different quality embryos of the same patient were classified into subgroups, and PAPP-A of their follicular fluid were also compared and significant difference between PAPP-A values was not found. This outcome is not compatible with the results of Wang (1), however it seems to support the reports of Firouzabadi ve Stanger (19,20). Different sample sizes and embryo numbers included in the studies might be a possible explanation fort his conflict.

As all embryos included in the study were not transferred, relationship between follicular fluid PAPP-A levels and pregnancy results was hard to determine. However, as PAPP-A levels between two follicles of the patient were not statistically different, PAPP-A value of one follicle might be considered to represent a pool of all follicular fluid obtained from the patient. Furthermore, similar PAPP-A of follicular fluids from which different quality of embryos are obtained demonstrate that follicular PAPP-A values were not related to embryo development itself rather than achieving pregnancy.

Serum PAPP-A levels were very low when compared to follicular fluid. However, it was demonstrated in previous studies that, despite substantial amount of PAPP-A in ovarian follicles of women undergoing IVF, intrafollicular PAPP-A did not increase serum PAPP-A concentrations significantly (21).

Results of our study also did not demonstrate a relationship between serum and follicular fluid PAPP-A levels. Pregnancy rates were not related to follicular fluid PAPP-A, but sPAPP-A values were significantly lower in patients achieving pregnancy.

Main limitation of our study is small sample size. Avoiding transfer of poor quality embryos obtained from the studied follicles is another limitation, because we could not observe their progress in terms of pregnancy. However, as significant difference was not found between follicular PAPP-A levels, we consider that our results concerning pregnancy are not highly affected by this condition.

In conclusion, this study did not demonstrate a statistically significant relationship between follicular fluid PAPP-A levels and embryo quality and pregnancy rates. Serum PAPP-A at the time of oocyte retrieval was significantly lower in patients achieving pregnancy. However, further studies with larger study populations are needed.

Ethics Committee Approval: Ankara University Local Ethics Committee permission was obtained with the letter dated 08.06.2009 and Decision number 153-4844.

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