

The Predictive Value of the Pentraxin 3 Concentration in Cumulus Cell Culture Media for the Embryo Implantation

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ABSTRACT

OBJECTIVE: Many studies on the interrelation of cumulus cells and oocytes, and research on cumulus protein factors continue. This study aims to investigate the relationship of Pentraxin 3 level with embryo implantation in the cumulus culture fluid.

STUDY DESIGN: A total of 31 women with idiopathic infertility who underwent intracytoplasmic sperm injection treatment were prospectively evaluated. Cell suspensions containing 5 million/mL cumulus cells were obtained post-hyase and incubated for 24 hours in a culture medium. A possible association between the culture media Pentraxin 3 concentrations and embryo implantation was analyzed.

RESULTS: The cumulus cell culture media Pentraxin 3 concentrations were significantly higher in the pregnant group compared to the non-pregnant group (98.9 ng/mL vs 53.2 ng/mL, respectively, $p=0.005$). There was a significant positive correlation between the culture media Pentraxin 3 concentrations and embryo implantation ($r=0.500$, $p=0.005$). The culture media Pentraxin 3 concentration was a significant predictor for successful embryo implantation (AUC=0.845, $p=0.006$). A cut-off value of 64.25 ng/mL had an 86% sensitivity and 80% specificity to predict embryo implantation. There was no pregnancy under the cut-off value of 60 ng/mL, whereby seven women had good quality grade 1 oocytes according to the traditional morphological criteria.

CONCLUSION: The cumulus cell culture medium Pentraxin 3 concentration was predictive for successful embryo implantation. Low Pentraxin 3 levels (<60 ng/mL) were associated with failure of conception.

Keywords: Cumulus granulosa cell, In vitro fertilization, Pentraxin 3

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Introduction

One of the most important parameters in assisted reproductive technologies is the selection of the embryo with high implantation capacity. The traditional selection criteria in-

clude morphological criteria, early cleavage, fragmentation rate, and blastocyst structure (1). With the help of advances in technology, new biological markers have been discovered for the selection of the oocyte and embryo (2).

Objective markers with high predictive value are essential for the selection. Moreover, it is important to maintain the viability of the oocyte during the molecular analysis for the selection. For that reason, the follicle fluid and cumulus ooforus complex are very suitable for molecular analyses (3).

Pentraxin 3 (PTX3) is an extracellular matrix protein expressed by somatic and immune cells. PTX3 is also an inflammatory molecule and synthesized in the liver and various blood cells. PTX3 was suggested as a marker for preeclampsia (4). Rovere-Querini et al. (5) have shown that the plasma levels of PTX3 in placenta tissue were significantly higher in preeclamptic compared to non-preeclamptic women. Lekva et al. (6) have shown that serum PTX3 is low in gestational diabetes.

In cumulus cells, PTX3 is secreted in case of inflammatory stimulus (7). Recent studies showed that PTX3 is directly associated with the stability of the extracellular matrix supporting the cumulus cells (8).

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Pentraxin 3 protein has been shown to play an important role in the optimal expansion of the cumulus cells and supporting hyaluronic acid (9). It also plays an important role in *in vivo* fertilization (10). Animal studies have shown that it is instrumental in the maturation of the oocyte and transtubar transport (11).

Considering the previous data, we aimed to evaluate the possible association between PTX3 concentration in cumulus cell culture medium and oocyte maturation and embryo quality as well as fertilization and implantation rates.

Material and Method

This study was conducted between November 2014 and March 2015 in Zeynep Kamil Hospital's *in vitro* fertilization (IVF) unit in Istanbul. Primary human cumulus cells were obtained with informed patient consent after approval from the Zeynep Kamil Women Hospital Ethics Board at October 2014/728 number. All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standard. The study was performed in accordance with approved guidelines. Informed consent was obtained from all individual participants included in the study. A total of 31 couples with unexplained infertility, who were treated in our clinic, were included. Inclusion criteria were female, age <35, and normal sperm count test parameters. The couples with female factors, women <18 and >36 years were excluded from the study.

The controlled ovarian stimulation protocol for *in vitro* fertilization (IVF) patients consisted of follicular phase GnRH antagonist (Ganirelix; Merck, Turkey) down-regulation. Gonadotropin stimulation began on menstrual cycle day 2 with human menopausal gonadotropin (hMG; Menopur, Ferring, Turkey) and recombinant FSH (Puregon, Merck, Turkey). Following ovulation induction, the antral follicles were counted. Oocyte pick-up (OPU) was performed 36 hours after the human chorionic gonadotropin (HCG) (Pregnyl®) injection. The OPU procedure was performed under ultrasound guidance. The oocytes were incubated for 2-4 hours in a human tubal fluid (HTF) fertilization medium (Life Global) followed by a denudation procedure with hyaluronidase (Life Global). After the ICSI procedure, the oocytes were kept in an HTF fertilization medium (Life Global).

In our study, the oocytes with clear, light, and nongranulated cytoplasm, normal thickness of zona pellucida, and normal-sized polar body were scored as oocyte score 1; whereas oocytes with two or more of the unfavorable criteria such as a dark or granulated cytoplasm, thick zona pellucida, and large or small polar body were classified as score 2 oocytes.

The fertilization check was performed 16-18 hours after the ICSI procedure, and the embryos were cultured in the

cleavage medium (Life Global). The evaluation of the embryos was performed according to the European Society of Human Reproduction and Embryology (ESHRE), the American Society of Reproductive Medicine (ASRM), and the Alpha 2011 Consensus (12). According to the cumulative evaluation of the embryos, women who have >70%, 70-20%, and <20% of good quality embryos were scored with 1 (best), 2, and 3 (worst), respectively.

Once the granulosa cells were removed from the oocytes, the remaining cumulus cells were washed twice with the G-1 PLUS medium (Vitro Life). The cumulus cells were counted in a Neubauer chamber and were distributed to the containers, with 5 million cells/mL in each container. Every container was filled up to 1 mL with G-IVF PLUS medium (Vitro Life) and was incubated for 24 hours. The cell culture suspensions were centrifuged at 5000 rpm for 10 minutes, and the supernatants were labeled. All samples were frozen at -80°C until the analysis. A total of 260 oocytes were obtained from 31 patients in our study. 201 of these oocytes were in the metaphase 2 stage. Oocyte denudation was performed separately in oocytes obtained from each selected patient, and the resulting cumulus cells were washed twice in HTF medium (1500 rpm/10 min) and the cell concentration was adjusted to 5 million cells/ml and HTF medium and 10% albumin were added and cultured for 24 hours. Supernatants were collected, numbered for each oocyte, and frozen at -80°C until it was assayed. PTX3 determinations were made by the ELISA method only in the transferred embryo culture supernatant. Embryo transfer was done on the 3rd day with 1 or 2 embryos. In 2 embryo transfer cases, each embryo's PTX3 value was given as the mean value. Enzyme-linked immunosorbent assay, human PTX3 by sandwich assay (Abcam, UK, ab 214570). The sensitivity of the test was 3.4 pg/ml, and the range was 15.6 pg/ml-1000. Kit includes human PTX3 capture antibody, HPTX3 lyophilized recombinant protein for control, cell extraction buffer, tetramethylbenzidine substrate, stop solution and anti-tag coated microplates (96 wells). This kit can use for human tissue culture supernatants, serum, or plasma. Embryo transfer was performed in 1 or 2 embryo transfers. In 2 embryo transfer cases, each embryo's PTX3 value was given as the mean value.

The semen analysis was performed according to the World Health Organization (WHO) 2010 criteria and the good quality sperm were isolated by the density gradient method (13). The oocytes were yielded from the follicles with a diameter of >18 mm. The fertilization was achieved by ICSI and the embryo was followed up.

Preparation of the patients

All patients were stimulated with the antagonist protocol. Controlled ovarian stimulation (COS) was initiated on cycle day 2 or 3 and continued until the day of ovulation induction. A fixed dose of rFSH was used for the first 6 days, 150 IU according to ≤36 years of age. After 6 days of stimulation, the

rFSH doses were adjusted according to ovarian response evaluated using transvaginal ultrasonography. A daily GnRH antagonist (Ganirelix-Orgalutran®; MSD, Ballerup, Denmark) dose of 0.25 mg was used starting on stimulation day 6 and was administered until the day of ovulation induction (14,15). The follicle growth was followed up by regular ultrasonographic examinations. By adequate growth of the follicles (>18mm), recombinant chorionic gonadotropin was used to induce ovulation. The oocytes were picked up 36 hours after the HCG injection. Pregnancy was defined by the demonstration of the heartbeat-positive embryo with ultrasound.

Statistical analysis

Homogeneous parametric variables were expressed as mean \pm standard deviation, minimum, and maximum; non-homogeneous parametric variables were expressed as median, minimum, and maximum. Homogenous parametric variables were evaluated with a t-test and ANOVA. Non-homogenous variables were evaluated with the Mann-Whitney-U-Test. Non-parametric variables were evaluated with a chi-square test. Statistical analyses were performed using SPSS 22 (Statistical Package for Social Science) for Windows 10.0. A $p < 0.05$ value was accepted as significant.

Results

The comparison of the demographic and clinical features of the pregnant ($n=7$) and non-pregnant ($n=24$) groups is presented in table I. The cumulus cell culture media PTX3 concentrations were significantly higher in the pregnant group compared to the non-pregnant group (98.9 ng/mL vs 53.2 ng/mL, respectively, $p=0.005$, Mann Whitney-U). There was no significant difference in patient ages, BMI, Gonadotrophin doses, days of follicle induction, the total number of oocytes, the number of MI and GV oocytes between the two groups.

The rate of oocyte score 2 was significantly higher in pregnant compared to non-pregnant women ($n=17$ vs $n=4$, $p=0.049$).

The multivariate regression analysis showed that PTX3 was associated with pregnancy (β quotient = 0.385, $p=0.009$) (Table II).

The PTX3 concentration had a significant predictive value for pregnancy, as shown by the ROC analysis (AUC=0.845, $p=0.006$) (Figure 1). A level of 64.25 ng/mL had a sensitivity of 86% and a specificity of 80%, respectively (Table III).

Table I: The comparison of the demographic and clinical features of the pregnant and non-pregnant groups

	Non-pregnant (n=24) mean \pm SD	Pregnant (n=7) mean \pm SD	<i>p</i>
Age (year)	30.00 \pm 3.83	32.00 \pm 3.51	0.182
BMI (kg/m ²)	25.98 \pm 3.79	25.60 \pm 4.63	0.488
Antral follicle (n)	12.83 \pm 4.36	12.00 \pm 4.04	0.532
Gonadotropin dose (IU)	2158 \pm 945	2135 \pm 1113	0.800
Duration of follicle induction (days)	9.2 \pm 2.0	8.5 \pm 1.9	0.532
Endometrial thickness (mm)	10.97 \pm 2.42	9.90 \pm 0.99	0.274
Total number of oocytes (n)	8.25 \pm 4.56	8.29 \pm 3.03	0.661
MII oocyte (n)	6.13 \pm 4.26	6.57 \pm 2.44	0.627
Number of MII oocytes/total number of oocytes	0.71 \pm 0.26	0.79 \pm 0.14	0.438
M1 (n)	1.50 \pm 0.97	1.40 \pm 0.54	0.859
GV (n)	3.25 \pm 2.05	2.00 \pm 1.41	0.533
Pentraxin 3 (ng/mL)	53.22 \pm 21.92	98.98 \pm 70.45	0.005

Table II: The multivariate regression analysis

Model	Non-standardized quotient		Standardised quotient β	<i>t</i>	<i>p</i>
	β	Standard error			
Constant	-0.358	0.129		-2.782	0.010
Pentraxin concentration	0.004	0.001	0.385	2.796	0.009

Table III: Sensitivity and specificity values according to various cut off levels

Pentraxin level	Sensitivity	Specificity
60.00 ng/mL	100%	67%
64.25 ng/mL	86%	80%
95 ng/mL	14%	96%

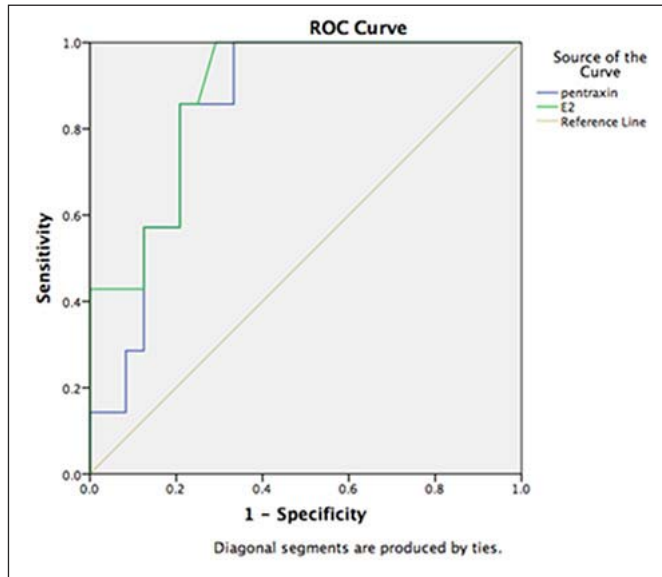


Figure 1: The ROC Analysis for the predictive role of the PTX3 concentration for pregnancy

There was no pregnancy under the cut-off value of 60 ng/mL, whereby seven women had score 2 oocytes according to the traditional morphological criteria (Table IV).

The PTX3 concentration was significantly higher in the grade 1 embryo group compared to the grade 2 and grade 3 embryo groups (Table V).

Table VI shows pregnancies due to the embryo transfer day. There was no statistical difference between the groups.

Discussion

The extracellular matrix surrounding the cumulus cells contains hyaluronic acid. Recent studies have shown that hyaluronic acid is associated with the stability of the cumulus cells, and PTX3 protein regulates matrix synthesis (16,17). During ovulation, the synthesis of the cumulus matrix increases initially and decreases with the increased protease secretion of the follicular wall (18,19,20). Salustri et al. (1996) have shown that the PTX3 had a role in the cumulus expansion and matrix organization as well as normal IVF procedure (16). Varani et al. detected the presence of subfertility in PTX3 knockout mice (7). The studies on knockout mice have shown that the reduction of the PTX3 is associated with failure of cumulus oophorus expansion and in vitro fertilization (21). In 2013, Huang et al. (22) evaluated the association between the cumulus gene expression of 7 different genes, including glutathione peroxidase 3 and PTX3, and the fertilization and embryo development in women with polycystic ovaries. The authors have shown that decreased PTX3 expression was associated with a high multinucleation rate and an abnormal fertilization rate (22). The microarray findings obtained from the cumulus culture cells of the women with PCOS showed that PTX3 is associated with fertilization (22).

Table IV: The oocyte scores and PTX3 levels in the pregnant and nonpregnant groups

	Nonpregnant	Pregnant	Total
PTX <60 ng/mL, OS 2	8	0	8
PTX <60 ng/mL, OS 1	7	0	7
PTX >60 ng/mL, OS 2	4	1	5
PTX >60 ng/mL, OS 1	5	6	11

PTX3, pentraxin 3; OS, oocyte score. OS 2 indicates bad quality oocytes, whereas OS 1 indicates good quality oocytes.

Table V: Comparison of the PTX3 levels according to the embryo grading

	n	Mean \pm Standard Deviation (ng/mL)	Minimum (ng/mL)	Maximum (ng/mL)	<i>p</i>
Grade 1 embryo (the best quality)	20	77.9 \pm 44.4	48.8	257.6	0.028
Grade 2 embryo	7	39.3 \pm 16.5	13.6	55.0	
Grade 3 embryo (the worst quality)	4	33.9 \pm 19.9	4.7	47.8	
Total	31	63.5 \pm 41.7	4.7	257.6	

Table VI: Pregnancies due to the embryo transfer day

n	Non pregnant	pregnant	Total	<i>p</i>
2. day	3	1	4	0.417
3. day	16	6	22	
5. day	5	0	5	
Total	24	7	31	

However, Li et al. (23) could not detect any association between the cumulus PTX3 expression and embryo quality.

Zhang et al. evaluated gene expressions in cumulus cells by microarray analysis and suggested that PTX3 could be used as a potential marker to predict oocyte quality (24). In our study, PTX3 was found to be related to oocyte and embryo quality.

Gebhart et al. (21) evaluated the potential associations between the expression of various genes in human cumulus cells and clinical pregnancy and live birth rates. Considering the gene expressions, they tried to detect the genes related to oocyte development. They assessed the genes such as aldolase, fructose-bisphosphate A, lactate dehydrogenase A, phosphofructokinase protein, and pyruvate kinase muscle 2, which play roles in the metabolic process; genes such as apoptotic cell-induced activity, gremlin 1, prostaglandin-endoperoxide synthase 2, and steroid sulfatase, which play roles in signaling pathways; and hyaluronic acid synthetase 2, PTX3, TNF α induced protein 6, and versican, which are related to the extracellular matrix. They found that PTX3 mRNA expression was significantly higher in the pregnant group (21). This finding is in accordance with our study.

In our study, we detected that the pentraxin secretion in cumulus cell culture was significantly higher in the embryo groups with successful implantation. This finding is in accordance with the gene expression studies (21-23). The use of PTX3 in addition to inadequate morphological criteria may be beneficial to select the oocytes with a higher fertilization capacity in assisted fertilization technologies.

Experimental studies show that PTX3 expression may be affected by gonadotropin stimulation (25-27). However, Paffoni et al. (28) have found that human follicular fluid PTX3 levels did not change with ovarian stimulation and were not related to the oocyte quality. Due to the inconsistent results in previous studies, we preferred to recruit women who underwent the same stimulation protocol.

In our study, the multivariate regression analysis showed that PTX3 was associated with pregnancy. The ROC curve showed that a cut-off value of 64.25 ng/ml had a sensitivity of 86% and specificity of 80% to predict the pregnancy. Moreover, a cut-off value of 60 ng/ml had a 100% sensitivity for the prediction of pregnancy. However, publications are scarce about PTX3 and pregnancy rates in the literature.

Another interpretation of our results is that the PTX3 levels lower than 60 ng/ml may be an indicator that achievement of pregnancy is difficult. In other words, rather than saying high levels of PTX3 may predict pregnancy, it is more accurate to say low PTX3 levels may predict failure of pregnancy. Namely, there was no pregnancy in our study with a PTX3 level under 60 ng/ml in 15 women. However, according to the

morphological criteria, seven of these 15 women had an oocyte score of 1 but did not conceive. In these seven women, the morphological score showed a good prognosis, although PTX3 levels indicated a bad prognosis. This finding shows the inadequacy of the morphological criteria.

The selection of embryos with a good morphology is known to be associated with higher pregnancy rates (29), as is also shown in our study. We also detected that pentraxin secretion was increased in cumulus cells of the good-quality embryos. Seli et al. (30) performed a study on the predictive value of fifth-day embryo morphology on pregnancy and compared metabolomic viability with morphological scoring. They have shown that a combination of metabolomic viability and morphological scoring had a higher predictive value for pregnancy than morphological scoring alone (30). Table IV also shows the oocyte score and pregnancy relationship according to pentraxin level. When the oocyte score is above 60 pg/ml, pregnancy occurs. Again, pregnancy can not be seen in cases with an oocyte score of 1 in the same table and below 60 pg/ml of PTX3. In this case, the PTX3 level in the culture supernatant was found to be superior to the oocyte morphology score in determining pregnancy. In addition, Table V shows that the embryo score and PTX3 value are closely related. The mean ptx value of grade 1 embryos transferred in 20 cases was 77.9 pg/ml. Grade 2 and grade 3 embryo transfer rates were 39.3 and 33.9 pg/ml, respectively. These results indicate that morphological assessment is still valuable in IVF applications.

The ovulation process is governed by many endocrine and paracrine factors. Examination of various factors necessary for egg production in the investigation of unexplained female infertility will provide a better understanding of this issue. Studies have shown that PTX3 has an important role in the GDF signal transduction cascade in the cumulus-oocyte interaction during the periovulatory period (31). It has been found that PTX3 is synthesized in the periovulatory follicle before ovulation by cumulus oophorus and plays a role in the organization of a hyaluronan-rich matrix required for successful fertilization (32). With mouse studies, it has been shown that when PTX3 is not synthesized, there is a normal amount of hyaluronan synthesis, but matrix stabilization does not occur. Studies with mice cumulus culture have shown that exogenous PTX3 administration restores matrix organization (9). We think that this study will make an important contribution to unexplained female infertility and will bring an important perspective in the selection of embryos with implantation ability in embryo development.

Our study group consisted of pairs with unexplained infertility. Sperm and oocyte factors were eliminated by conventional methods. However, it is well known that the implantation process is multifactorial. Endometrial receptivity, as well as sperm DNA integrity, plays an essential role in implantation. In addition to the WHO criteria, sperm DNA and matu-

ration analysis may be used to evaluate sperm quality to exclude the malefactor in such studies. This may be addressed as a weakness of our study. Larger scaled studies are needed to define the sensitivity and specificity rates.

Conclusion

Cumulus cell PTX3 concentration has predictive value on embryo grade and pregnancy. Low PTX3 levels (<60 ng/ml) were associated with failure of conception, which has not been accurately predicted according to the traditional morphological oocyte scoring.

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Consent for publication: We understand that the text and any pictures published in the article will be freely available on the internet and may be seen by the general public. The pictures and text may also appear on other websites or in print, may be translated into other languages, or used for commercial purposes. We have been offered the opportunity to read the manuscript

Ethics: Ethics approval and consent to participate; Primary human cumulus cells were obtained with informed patient consent after approval from the Zeynep Kamil Women Hospital Ethics Board by the number, Oct 2014/214 KD.

Availability of data and material: Study data are recorded in the spss program, can be given at any time

Authors' contributions: Design of study; T.I., E.M., O.G., Experimentation: Y.S., Statistics: O.G.

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