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The association between follicular fluid levels of cathepsin B, relaxin or AMH with clinical pregnancy rates in infertile patients

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ABSTRACT

Objective: The aim of this study was to investigate the relationship of cathepsin B, relaxin and anti-Mullerian hormone (AMH) in follicular fluid (FF) with pregnancy rates in infertility patients.
Study design: Seventy-nine infertile couples who underwent ICSI were included in the study. The FF levels of cathepsin B, relaxin and AMH were measured using ELISA kits.
Results: The FF cathepsin B levels were statistically higher in pregnant patients compared with non-pregnant patients (0.20 ± 0.13 versus 0.13 ± 0.03; $P < 0.001$). There were statistically significant differences in the total number of oocytes (10.00 ± 6.85 versus 5.96 ± 3.94); the mean number of M2 oocytes (8.65 ± 5.63 versus 4.58 ± 3.36) between the pregnant and non-pregnant patients ($P < 0.05$). There were no significant correlations between pregnancy rates and relaxin and AMH ($P > 0.05$). The area under the curve of cathepsin B for prediction of pregnancy was 0.662 ($p = 0.024$, 95% Confidence Interval 0.528–0.797).
Conclusions: This study demonstrated that increased level of cathepsin B in FF significantly correlates with a better chance of clinical pregnancy. Further studies are needed to clarify the role of cathepsin B in the reproductive process of humans.

Introduction

Since the introduction of in vitro fertilization (IVF) in 1978, use of assisted reproductive technologies has spread worldwide. Human reproduction is still not an efficient process as many oocytes fail to produce a live birth [1]. There are several factors that may potentially affect human reproduction, yet oocyte development is mainly influenced by the environment that surrounds the ovarian follicle [2].

Blood plasma elements that pass through the blood follicular barrier, along with the secretory activity of granulosa and thecal cells make up the composition of the follicular fluid (FF) [3] that surrounds the oocyte. Hence, FF has a vital role in determining oocyte quality and an oocyte's potential to achieve fertilization and embryo development [4]. Subsequently, the concentration of some mediators such as cathepsin B, relaxin and anti-Müllerian hormone (AMH) in FF may provide an insight into the likelihood of the success or failure of pregnancy.

The cathepsin family of lysosomal cysteine proteinases is involved in various biological functions. Bettegowda et al. stated that the treatment of cumulus-oocyte complexes during in vitro oocyte maturation with a cell-permeable cysteine proteinase inhibitor (cathepsin) confirms the functional significance of cumulus cell cathepsin expression to oocyte competence [5]. Moreover, cathepsin B was inversely correlated with the quality of oocytes and embryos in a bovine animal study by Balboula et al. [6].

Relaxin is a small peptide (≈6 kDa) and a part of the insulin/relaxin superfamily of hormones [7]. Pig studies showed that the theca interna produces relaxin, and the levels of relaxin in FF increase with follicular size [8]. Data suggest that relaxin may promote follicular growth through intra ovarian autocrine and/or paracrine mechanisms [8,9]. Studies in rats demonstrated that relaxin might also play a role in ovulation, endometrial growth and implantation [10–12]. A study by Feugang et al. demonstrated that relaxin might have a role specifically in early embryo development and oocyte maturation [13].

AMH is in the superfamily of transforming growth factor-β. It is secreted by the granulosa cells (GCs) of growing follicles [14]. Data

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from the literature vary concerning the relation of AMH FF levels with the pregnancy rates after infertility treatments.

The aim of the present study was to investigate the relationship of cathepsin B, relaxin and AMH in FF with clinical pregnancy rates in infertile patients due to the correlations between the above-mentioned markers and various reproductive factors in animal models.

Materials and methods

Study population

Seventy-nine infertile couples who underwent intracytoplasmic sperm injection (ICSI) between March 2012 and December 2012, at the Infertility Clinic of Istanbul University School of Medicine (Istanbul, Turkey) were included in this study.

Patient history, gynecologic exam and conventional infertility evaluation (spermiogram, hormonal profile on the 3rd day of menstruation, hysterosalpingography, etc.) were utilized to evaluate the causes of infertility.

Only the following two etiologies were included in the study: [1] tubal factor and [2] unexplained infertility. The exclusion criteria were: [1] women who had a history of poor response to treatment, [2] male factor, [3] women with endometriosis, hydrosalpinx, ovulatory disorders, polycystic ovary syndrome, or a history of ovarian surgery, [4] women with an antral follicle count (AFC) <5, and [5] women aged >39 years.

Approval from the Ethics Committee of Istanbul University School of Medicine was obtained before data collection commenced. Informed consent was obtained from each patient prior to the procedure.

Controlled ovarian hyperstimulation (COH)

Vaginal ultrasound was performed on all patients on the third day of IVF cycle to evaluate follicular activity and antral follicle count (AFC). COH was started in patients if their ultrasound findings did not reveal follicular cysts over 20 mm. Oral contraceptive pills were not utilized. All patients received GnRH antagonist protocol. Either Puregon (Schering-Plough, NJ, USA) or Gonal-f (EMD Serono, MA, USA) was used in COH. The initial dosage was determined primarily relative to antral follicle count (AFC), and also relative to the patient's age, body mass index (BMI) and response to prior stimulation regime (if applicable). It was then adjusted according to the response of ovarian follicles, which were followed-up via vaginal ultrasound.

GnRH antagonist protocol

The COH was started on the third day of menstrual bleeding with 225–300 IU of recombinant FSH (Gonal F 75 IU ampules; Serono, Geneva, Switzerland) or Puregon (Schering-Plough, NJ, USA) in all patients. Regular pelvic ultrasound scan was performed after starting FSH injections to evaluate ovarian follicle development. Multiple doses of the antagonist protocol Cetrorelix (Cetrotide; Serono, Geneva, Switzerland; 0.25 mg, SC) were injected daily to inhibit a premature LH surge, when the leading follicle was 14 mm in diameter with serum levels of E2 >600 pg/mL and continued until the hCG injection day.

Ovarian follicular development and oocyte retrieval

Ovarian follicular development was observed via vaginal ultrasound at a one- to three-day frequency. hCG was injected to achieve follicular maturation when at least three follicles were found that were $\geq$17 mm in size.

Oocyte retrieval (OCT) took place 35- to 36-h after the hCG injection. All follicles that were $\geq$14 mm in size were retrieved. The number of retrieved oocytes was recorded. For OCT, a 17-guage needle was used while the patients were under general anasthesia. After denudation and a 2-h incubation of the oocyte–corona complexes, ICSI was performed.

Embryo transfer and luteal phase support

During the study, one embryo was transferred to patients aged 35 years in their first two IVF attempts; two embryos were transferred only after two or more failed IVF attempts. In patients who were aged $\geq$35 years, regardless of previous IVF attempts, two embryos were transferred in accordance with the Turkish legislation of elective single embryo transfer (SET). In the present study, grade-1/2 embryos were transferred. The transfer took place either on day 2 or day 3 [15]. The number of grade-3 embryos was recorded to be evaluated.

All patients received vaginal progesterone for luteal phase support on the first morning after oocyte retrieval. If pregnancy occurred, vaginal progesterone support continued until the 12th week of gestation.

Follicular fluid aspiration

Individual aspiration was used to collect oocytes, and each follicle was recovered in a different tube. To avoid contamination from blood, flush medium, or mixed FF during oocyte retrieval, only the FF from the first retrieved leading follicle that contained a single oocyte-cumulus complex was collected. Thus, one FF sample per patient was used for analysis. Samples of FF were centrifuged at 2000*g* for 10 min and the supernatants were stored at $-80\,^{\circ}$C for further analysis.

Assessment of fertilization, embryo quality, and pregnancy

Fertilization results were assessed 18 h after ICSI for the appearance of two distinct pronuclei and two polar bodies. Cleavage was evaluated 24 h after fertilization. Embryo quality was assessed on the second day of fertilization and graded as follows: grade I: symmetric blastomeres and no fragmentation; grade II: blastomeres different in size and shape with $<25\%$ fragmentation; and grade III: blastomeres different in size and shape with $>25\%$ fragmentation.

On the 14th day after embryo transfer, blood levels of β-hCG were measured and recorded. If the β-hCG level was >5 mIU/ml, it was considered as *positive* β-hCG and patients with such levels were regarded as biochemically pregnant. Clinical pregnancy was confirmed by the presence of a fetal heartbeat using vaginal ultrasound at 6 weeks of amenorrhoea.

Determination of cathepsin B, relaxin and AMH expression levels in FF

Expression levels of cathepsin B, relaxin and AMH levels were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Eastbiopharm Co., Ltd., Hangzhou, China) according to the manufacturer's instructions. For Cathepsin B, relaxin and AMH the lower and upper detection limits were estimated as 0.2–60 ng/ml; 10–3000 ng/ml and 0.1–40 ng/ml, respectively.

Determination of protein content in FF by Bradford assay

To avoid potential bias due to volume variability, the protein content of FF was measured by the Bradford method [16]. Firstly, FF sample was diluted into phosphate buffered saline (PBS). The

Bradford method was utilized by mixing 50 μl of the diluted FF sample with the 950 μl of Bradford reagent that contained phosphoric acid, ethanol and Coomassie brilliant blue. The mixture was left at room temperature for 10 min and then the A_{595} was measured. FF Cathepsin B and relaxin levels were expressed as μg/g of protein; FF AMH level was expressed as ng/g of protein.

Statistical analyses

The Statistical Package for Social Sciences 21.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. Comparisons between the two groups were performed using the independent *t* test for parametric conditions and the Mann–Whitney *U* test for nonparametric conditions. Comparisons of more than two groups for nonparametric conditions were performed using the Kruskal–Wallis test. The Fisher exact and Chi-square tests were used for cross-tab analysis. Correlations were measured by Pearson's correlation coefficient (r) and Spearman's Rho (ρ). Receiver operator curves (ROC) were utilized. A p value of <0.05 was accepted as statistically significant.

Results

The demographic, clinical and laboratory features of pregnant and non-pregnant patients are presented in Table 1. None of the parameters were significantly different among the groups in terms of age, BMI, period of infertility, day-3 FSH and estradiol levels (Table 1).

A total number of 79 FF samples without contamination of blood were collected. Twenty-three of the patients who provided FF samples were clinically pregnant, while 56 of the patients were non-pregnant

When the groups were further analyzed according to their pregnancy status (pregnant versus non-pregnant), the findings revealed that FF cathepsin B levels were statistically higher in pregnant patients compared with non-pregnant patients (0.20 ± 0.13 versus 0.13 ± 0.03; $P < 0.001$). The FF AMH and relaxin levels revealed no statistically significant difference between the pregnant and non-pregnant patients (Table 2).

There were statistically significant differences in the total number of oocytes (10.00 ± 6.85 versus 5.96 ± 3.94); mean number of M2 oocytes (8.65 ± 5.63 versus 4.58 ± 3.36); and MII oocyte rate (88.98 ± 10.19 versus 78.14 ± 23.30) ($p < 0.05$), but there was no statistically significant difference in the number of transferred grade-1/2 embryos (1.56 ± 1.15 versus 1.46 ± 1.71) and fertilization rate (81 ± 18.39 versus 76.25 ± 21.79) between pregnant and non-pregnant patients ($p > 0.05$) (Table 3).

Correlations are presented in Table 4. Cathepsin B had a strong positive correlation with the number of retrieved oocytes, number of MII oocytes, MII oocyte rate and fertilization rate ($p < 0.05$), while AMH and relaxin had weak correlations that were not significant ($p > 0.05$).

We further evaluated the area under the curve (AUC) of cathepsin B for prediction of pregnancy, which was 0.662 ($p = 0.024$, 95% CI 0.528–0.797). A threshold of 0.12 μg/g of protein of cathepsin B had a sensitivity of 60.9%, specificity of 53.6%. Cathepsin B-ROC curve is presented in Fig. 1.

Table 1
Characteristics of pregnant and non-pregnant patients.

	Pregnant ($n = 23$)	Non-pregnant ($n = 56$)	*P* value
Age, years	31.55 ± 4.76	33.12 ± 4.67	0.211
BMI, kg/m2	25.1 ± 3.2	25.6 ± 2.3	0.766
Period of infertility, months	87.90 ± 47.69	85.18 ± 60.58	0.859
Day-3 serum FSH, mIU/mL	7.64 ± 4.55	9.11 ± 4.87	0.272
Day-3 serum E2, pg/mL	46.84 ± 20.91	68.67 ± 89.14	0.287
Gonadotropin doses, IU	2175 ± 192.3	2250 ± 268.32	0.862

Table 2
Comparison of FF cathepsin B, relaxin, AMH levels in pregnant and non-pregnant patients.

	Pregnant ($n = 23$)	Non-pregnant ($n = 56$)	*P* value
Cathepsin B, μg/g of protein	0.20 ± 0.13	0.13 ± 0.03	<0.001
Relaxin, μg/g of protein	1.72 ± 1,13	1.88 ± 1.12	0.562
AMH, ng/g of protein	45.90 ± 17.28	50.76 ± 20.16	0.331

Note: FF cathepsin B, relaxin and AMH concentrations were adjusted for protein content.

Comments

In this study we presented a relationship between FF cathepsin B levels and clinical pregnancy in women who underwent ICSI. High FF cathepsin B level related to a better chance of clinical pregnancy.

Cathepsin B, which is a lysosomal cysteine protease that plays a vital role in the degradation of intracellular proteins in lysosomes [17], is present in an extensive range of cells in mammals [5,18] and a well-known decidualization marker in humans [19]. At the last stage of the reproductive process, the lysosomal enzymes are responsible in oocyte re-absorption [20].

In the literature, there are contradicting data about cathepsin B levels and its role on reproductive processes in various animal models. A study by Ireland et al. revealed that gene transcripts for several cathepsins, including cathepsin B, were highly expressed in the cumulus cells surrounding oocytes from prepubertal (a model of poor oocyte quality) versus adult cattle, which implied that these enzymes could affect the quality of oocytes [21]. Waters et al. observed that during the last phase of oocytes maturation in oviparous-vertebrates, the high levels of estradiol commonly found in the plasma of these vertebrates could induce the transcription of cathepsin B gene, like in mammals [22]. It has been demonstrated that cathepsin B is inversely correlated with the quality and developmental competence of cumulus oocytes complexes (COCs) in the bovine animal model in contrast to the present study [4,18]. These studies showed that lysosomal enzymes have an important role in oocyte maturation and increased level of cathepsin B may lead to adverse effects on reproduction. However, this argument was not supported in the present study. FF Cathepsin B levels were higher in women who

Table 3
Comparison of oocyte in pregnant and non-pregnant patients.

	Pregnant ($n = 23$)	Non-pregnant ($n = 56$)	*P* value
N of oocytes	10.00 ± 6.85	5.96 ± 3.94	0.002
N of MII oocytes	8.65 ± 5.63	4.58 ± 3.36	0.001
MII oocyte rate (%)	88.98 ± 10.19	78.14 ± 23.30	0.04
N of transferred embryos (Grade-1/2)	1.56 ± 1.15	1.46 ± 1.71	>0.05
Fertilization rate (%)	81 ± 18.39	76.25 ± 21.79	>0.05

Table 4
Correlations between cathepsin B, relaxin and AMH and retrieved oocyte, MII oocytes and fertilization rate.

Spearman's rho correlation coefficients			
Reproductive factors	Cathepsin B	Relaxin	AMH
N of oocytes	0.033	0.519	0.304
N of MII oocytes	0.037	0.361	0.306
MII oocyte rate	0.032	0.504	0.294
Fertilization rate	0.042	0.258	0.488

had a higher number of retrieved oocytes and MII oocytes in the present study ($P < 0.05$). In addition, FF cathepsin B levels in women who conceived were significantly higher than FF cathepsin B level of women who did not conceive (0.20 ± 0.13 versus 0.13 ± 0.03; $P < 0.001$). This contradicting result may be due to the hormonal milieu of the human ovary, which is hyper-stimulated by huge amounts of gonadotropins compared with normal ovulation cycle during IVF treatment. Patients with an increased number of oocytes may have a better chance to have more embryos and potentially have the opportunity to cryopreserve and transfer these embryos later. Therefore, they may cumulatively have a better chance of clinical pregnancy. Further studies are needed to clarify whether cathepsin B and its particular relationship with E2 contents of FF may play a role in the reproductive process of humans.

Ryan et al. demonstrated the expression and localization of relaxin in the ovaries of mares and that the titres of relaxin increase as the size of the follicle increases [23]. Ohleth et al. investigated whether ovarian follicles from immature pigs produced relaxin and were able to detect relaxin in FF of all sizes [9]. They found a positive correlation between increasing follicle size and relaxin concentration. Consequently, the localization of relaxin in follicular structures led to the hypothesis that this hormone might have a role in the regulation of follicular function. Kim et al. later investigated the effects of relaxin on the maturation of porcine oocytes after IVF [24]. In the present study, there was no significant correlation between FF relaxin level and the number of retrieved or MII oocytes. Furthermore, we found no statistically significant difference in FF relaxin levels between pregnant and non-pregnant women.

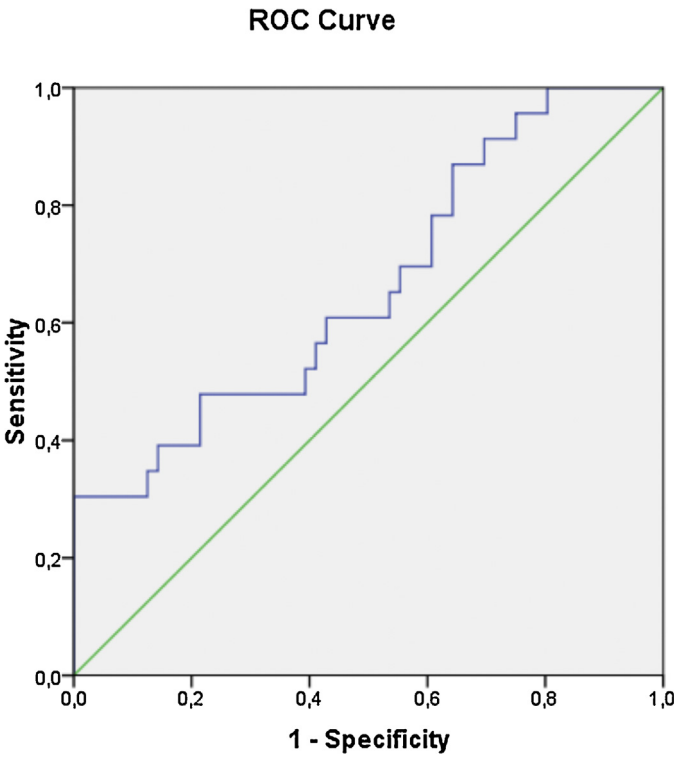

Fig. 1. ROC curve of cathepsin B for prediction of pregnancy.

AMH concentrations convey an inverse relationship with follicular size [25]. It is normally expressed at low levels in primary follicles and increases to maximal concentrations in large preantral and small antral follicles and then declines as the follicular size increases [25]. In the literature, there is no consensus on the relationship between FF AMH levels and positive pregnancy outcome in humans. In the present study, there was no significant difference in FF AMH levels of pregnant and non-pregnant women. Some studies suggested that increased FF AMH level could be a useful marker for predicting clinical pregnancy rates [26,27], embryo implantation rates [26–28], and fertilization rates [26–28]. The study of Pabuccu et al. showed that clinical pregnancy rates, embryo implantation rates and fertilization rates were markedly different among the low, moderate, and high FF AMH groups [26]. In contrast, Fanchin et al. presented no correlation between fertilization rates and FF AMH levels [27]. Similarly, Celik et al. reported no significant relationship between FF AMH levels and clinical pregnancy rates [29]. Meanwhile, Kedem-Dickman et al. showed that AMH is highly expressed in and secreted from cumulus granulosa cells of preovulatory follicles containing premature and atretic oocytes [30]. In addition, FF AMH concentrations of atretic oocytes were significantly higher than FF AMH of MII oocytes in this study. Our data did not reveal a correlation between FF AMH levels and MII oocytes.

In future studies, it may useful to measure gonadotropins in follicular fluid and analyze their correlation to studied marker(s) in order to rule-out any direct effect.

In conclusion, this study demonstrated that increased level of cathepsin B in follicular fluid significantly correlates with a better chance of clinical pregnancy. Further studies are needed to clarify the role of cathepsin B in the reproductive process of humans.

Condensation

This study demonstrated that increased level of cathepsin B in follicular fluid significantly correlates with a better chance of clinical pregnancy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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