Investigation of ADAMTS-1, BAX and IL-10 Expressions in Granulosa Cells Determinanting Fertilization Success in IVF

OBJECTIVES: To investigate the expressions of ADAMTS-1, BAX and IL-10 in granulosa cells in human oocytes during in vitro fertilization (IVF) and to evaluate their role in determining fertilization success.

MATERIALS AND METHODS: The study included 30 patients with primary or secondary infertility and polycystic ovary syndrome (PCOS) who were diagnosed in the IVF center of Memorial Hospital, Diyarbakır, Turkey. Blood samples were taken for measurement of Anti Müllerian Hormone (AMH), and granulosa cell diameter and cell core diameter were measured in 20 cells collected from each patient. Degeneration scoring and BAX, ADAMTS-1, IL-10 expressions in granulosa cells were evaluated. Immunohistochemical techniques were used for the evaluation of these proteins.

RESULTS: There were significant differences in the expression levels of ADAMTS-1, BAX and IL-10 in patients with different fertility statuses. Patients with higher AMH levels had lower expression levels of ADAMTS-1 and BAX, while patients with lower AMH levels had higher expression levels of IL-10. This suggests a potential relationship between the expression of these proteins and the success of fertilization.

CONCLUSIONS: The expression levels of ADAMTS-1, BAX and IL-10 in granulosa cells may be important indicators of fertilization success in IVF.

KEY WORDS: Granulosa Cells; Fertility; ADAMTS-1; IL-10; BAX; immunohistochemistry.

INTRODUCTION

During in vitro fertilization, fertilization occurs when sperm is injected into micro-droplets containing secondary oocytes. Success rate depends on sperm-oocyte interaction, penetration through external cumulus layers and zona pellucida, oocyte binding and fertilization ability with plasma membrane (Varras, 2013). A proper successful ovulation required cumulus oocyte complex (COC) formation and rupture of epithelium lined the ovarian surface. During this step, extracellular matrix (ECM) components including versican and hyaluronic acid are elevated (Richards et al., 2005).

Granulosa cells are somatic cells surrounding each developing oocyte necessary for follicle and oocyte development. The relation of GCs with the oocyte maintains essential nutrients for oocyte development and accumulation of oocyte secreted metabolites (Su et al., 2009). Moreover, GCs are involved in hormonal activity with the production of estradiol during follicular growth and secretion of progesterone after ovulation (Dzafic et al., 2013). GCs shape changes into four different layers; the outermost layer is the membrana granulosa, the inner most layer is the periantral, the intermediate layer is the cumulus oophorus, and the layer aligned next to the oocyte corona radiata in a pre-ovulatory follicle. These layers have different function i.e. they secrete different molecules (Nguyen et al., 2012).

ADAMTS-1 is a metalloproteinase that contain disintegrin domain and thrombosponding type I motifs (Kuno et al., 1997). Disintegrin domain prevents integrins bind their ligands leading to blockage of intracellular signaling events. This suggests they may deteriorate interactions between cells and collagenous matrix or interactions of granulosa cells with each other (Fujiwara et al., 1996). ADAMTS-1 is a critical molecule involved in degradation of versican, ovulation, vasculogenesis and expansion of COC formation. In an experiment on ADAMTS1-null mice, oocyte and ovarian vasculature was degenerated, ovarian network was less organized (Richards, 2005).
Cytokine function in the ovary has been described as promoting processes of follicular growth, steroidogenesis, recruitment and activation of leukocytes necessary for ovulation and tissue remodelling during ovulation, luteinization, and luteolysis (Büscher et al., 1999). Ovarian interleukins (ILs) mediate follicle development, fertilization, embryo development and implantation. IL-10 and vascular endothelial growth factor levels promote angiogenesis and corpus luteum formation (Gómez et al., 2010) IL-10 is a general anti-inflammatory molecule that is present at low levels in circulating blood. It exerts key functions for the maintenance of the pro-inflammatory and anti-inflammatory balance in the body to prevent inflammatory and autoimmune pathologies (Pestka et al., 2004; Saraiva & O’Garra, 2010; Ouyang et al., 2011).

The aim of this study was to investigate certain cellular components involved in the proliferation, differentiation and functional characteristics of granulosa cells in the success of fertilization of human oocytes during in vitro fertilization via immunohistochemical techniques.

MATERIAL AND METHOD

Experimental procedure. This study was performed between September 2018 and April 2019 in the IVF center of Memorial Hospital Obstetrics and Gynecology Clinic including 30 patients in the assisted reproduction program with the complaint of not having children, in addition to diagnosis of primary or secondary infertility, polycystic ovary syndrome (PCOS), male factor and unexplained infertility diagnosis when starting the Ovum-Pick-Up (OPU) procedure, the patient was first sedated. Before the procedure, the bladder was emptied in order to reach the ovaries more easily during transvaginal oocyte collection and to avoid possible bladder injury. After sedation, the patient was placed in the lithotomy position and covered sterile. Patients with a body mass index of between 18 and 35 kg/m² were selected for IVF or intracytoplasmic sperm injection for various indications between 18 and 45 years of age. When the mean diameter of the prominent follicle reached 18 mm, the final matured follicle was induced with 1000 IU of urinary Hcg or 250 mcg of recombinant hcg, and oocyte collection was planned after 34-48 hours. A gel was placed on the ultrasound probe and a sterile plastic sheath is passed before starting the OPU. A biopsy clip was inserted over the sheath, where the needle was passed and guided during aspiration. 16 or 17 gauge needles designed for OPU operation were used. The ideal aspiration pressure for the procedure was 100-150 mmHg.

The follicle fluids and granulosa cells that came with these fluids were examined by histopathological and immunohistochemical methods

Histopathologic procedure. The granular cells obtained from the patients were preserved at +4 °C. Materials were sent to the histology laboratory for routine histological procedures. For each sample, the fluid with granular cells was subjected to high-speed centrifugation, the supernatant was discarded then 10 % neutral formaldehyde was added (pellet:formaldehyde, 1:3). After a fixation of 2 h, the supernatant was discarded again and tube was reversed to filter paper. The pellet was placed on a filter paper using a spatula and Eosin staining solution was added using a Pasteur pipette. After the cell aggregate turned into red, it was wrapped in the filter paper and placed in a cassette. After routine histological follow-up, 4-5 μm sections were cut from the paraffin blocks with a microtome (Leica, Germany). Harris Hematoxylin and Eosin staining was applied to sections and examined under light microscope (Imager A2 Zeiss, Germany).

Immunohistochemical staining. Samples were fixed with 10 % formaldehyde solution, decalcified with 5 % ethylenediamine-tetraacetic acid (EDTA), dehydrated in a graded series of ethanol, and then embedded in paraffin wax. Then, 4–5 μm thick sections were cut with a microtome (Leica, Germany) and placed on coated slides. Sections were brought to distilled water and washed three times for 5 min in phosphate buffered saline (PBS, pH 7.4) (catalogue number # 10010023, Thermo Fisher Scientific, US). To unmask antigen sites, slides were incubated with EDTA solution in microwave for 110 minutes at 3x90 °C. The sections were washed in three times for 5 min in PBS and incubated with hydrogen peroxide (catalogue # TA-015-HP, Thermo Fisher Scientific, US) for 20 min. Ultra V block (TA-125-UB, Thermo Fisher Scientific, US) was applied to the sections for 8 min prior to the addition of the primary antibodies, which were left on overnight ADAMTS-1 monoclonal antibody 1:100, BAX monoclonal antibody 1:100, IL-10 monoclonal antibody 1:100. The sections were washed three times for 5 min in PBS and then were incubated with biotinylated secondary antibody (catalogue # TP-125-BN, Thermo Fisher Scientific, US) for 14 min. After washing with PBS, streptavidin peroxidase (catalogue # TS-125-HR, Thermo Fisher Scientific, US) was applied to the sections for 15 min. The sections were washed three times for 5 min in PBS. Diaminobenzidine (catalogue # TA-012-HDC, Thermo Fisher Scientific, US) was applied to sections for up to 20 min as a chromogen. Control slides were prepared using the same procedure, without primary antibodies. Counterstaining was done using Harris’s haematoxylin for 45 s, dehydrated through
ascending alcohol and cleared in xylene (Product Number: HHS32 Sigma, hematoxylin solution, Harris Modified, Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO, 63103, USA). Slides were mounted with Entellan® (lot: 107961, Sigma-Aldrich, St. Louis, MO, USA) and examined under a light microscope (Olympus, Germany).

**Statistical analysis.** Age information was obtained from the patients in the study were evaluated statistically. AMH amount from blood samples taken from 30 patients (control 15 and experiment 15) and granulosa cell diameter and cell core diameter were measured in 20 cells from each collected cells. In addition, degeneration scoring and BAX, ADAMTS-1, IL-10 expressions were scored in granulosa cells. After the normal distribution test, independent t test was applied. P <0.05 was considered statistically significant.

**RESULTS**

Significant differences were observed in the morphometric parameters of granulosa cells in the experimental group compared to the control group (p <0.01). AMH in blood showed a significant decrease in the experimental group (Table I) (Figs. 1 and 2).

| Parameter                          | Groups          | N  | Mean   | Std. Error Mean | p value |
|------------------------------------|-----------------|----|--------|-----------------|---------|
| Granulosa cell diameter            | Control         | 20 | 10.29  | 0.30            | <0.01   |
|                                   | Experimental    | 20 | 6.15   | 0.27            |         |
| Diameter of granulosa cell nucleus | Control         | 20 | 5.31   | 0.19            | <0.01   |
|                                   | Experimental    | 20 | 2.98   | 0.16            |         |
| Degeneration in granulosa cell     | Control         | 20 | 1.05   | 0.14            | <0.01   |
|                                   | Experimental    | 20 | 3.25   | 0.16            |         |
| Bax expression                     | Control         | 20 | 0.90   | 0.14            | <0.01   |
|                                   | Experimental    | 20 | 3.10   | 0.16            |         |
| AMH                                | Control         | 15 | 5.16   | 0.12            | <0.01   |
|                                   | Experimental    | 15 | 1.40   | 0.10            |         |
| Age                                | Control         | 15 | 29.07  | 0.49            | <0.01   |
|                                   | Experimental    | 15 | 38.33  | 0.73            |         |
| ADAMTS-1 expression               | Control         | 15 | 0.47   | 0.13            | <0.01   |
|                                   | Experimental    | 15 | 1.93   | 0.28            |         |
| IL-10 expression                   | Control         | 15 | 1.80   | 0.17            | <0.05   |
|                                   | Experimental    | 15 | 2.87   | 0.26            |         |
Histopathological observations. In the examination of histological sections taken from normal control group patients; the granulosa cells were found to be eosinophilic, with the chromosome-rich nucleus structures and the stoplasms of the polygonal cells where the nucleus settled out of the rounded cells. Cell size was found to be normal. In the samples taken from PCOS patients, there was a decrease in nucleus dimensions, changes in shape, small losses in stoplasmic areas and degeneration areas in granulosa cells. Deformations were observed with decrease in cell size (Figs. 3a and 3b).

Immunohistochemistry results. BAX expression was negative in stoplasma of granulosa cells and nuclei of the control group. Granulosa samples obtained from idiopathic patients showed a degerative changes in stoplasm of granulosa cells and chromatin particles in nucleus BAX expression (Figs. 4a and 4b). In the control group, ADAMTS-1 expression was observed in stoplasm of granulosa cells while ADAMTS-1 expression was negative in the nucleus. ADAMTS-1 expression levels were increased in the supranuclear region in idiopathic patients (Figs. 5a and 5b). IL-10 expression was moderate in granulosa cells in control group patients, whereas IL-10 expression was significantly increased in atypical flat granulosa cells in idiopathic patients (Figs. 6a and 6b).

Fig. 3. A. In the examination of histological sections taken from normal control group patients; the granulosa cells were found to be eosinophilic, with the chromosome-rich nucleus structures and the stoplasms of the polygonal cells where the nucleus settled out of the rounded cells. Cell size was found to be normal. B. In the samples taken from PCOS patients, there was a decrease in nucleus dimensions, changes in shape, small losses in stoplasmic areas and degeneration areas in granulosa cells. Deformations were observed with decrease in cell size.

Fig. 4. A. BAX expression was negative in stoplasma of granulosa cells and nuclei of the control group. B. Granulosa samples obtained from idiopathic patients showed a degerative changes in stoplasm of granulosa cells and chromatin particles in nucleus BAX expression.
DISCUSSION

Ovulatory process is very similar to that of inflammation since inflammatory cytokines are produced, prostaglandins are induced, immune cells (leukocytes, neutrophils, and macrophages) are recruited (Care et al., 2013). Oocyte develops in follicle fluid (FF) which may contain potential biomarkers for the development of follicle, fertilization of oocyte, development and quality of embryo, proper implantation and successful pregnancy (Revelli et al., 2009). There is an important communication between granulosa cells and an oocyte in a developing follicle, and gene expression in maturing GCs is closely related to the production of competent oocytes (Cillo et al., 2007; Hamel et al., 2008; Jiang et al., 2010). GCs play a critical role in pathologic folliculogenesis not only in normal folliculogenesis but also in both benign disorders such as polycystic ovary syndrome (Yilmaz et al., 2018), early ovarian failure (POF), also called early ovarian failure. Some authors have found that granulosa cell apoptosis is the primary factor leading to follicular atresia and affects oocyte structure and oocyte development potential (Han et al., 2006; Elmore, 2007; Li et al., 2009). The incidence of apoptotic bodies was considered as a predictive marker of assisted reproductive technology (ART) outcome.
It has been suggested that the emergence of apoptosis in the aspirated granulosa cells reflects the adequacy of the oocyte in pregnancy rates (Giampietro et al., 2006). Oocytes with a low rate of apoptotic granulosa cells were observed to have normal morphology whereas high apoptotic cumulus granulosa cells were reported to have abnormal morphology (Assey et al., 1994). Recent studies support the idea that an increase in apoptotic ratio of cumulus granulosa cells, a decrease in intracellular junction and loss of microvilli can cause low maturation and abnormal oocyte morphology and development so affect IVF or ICSI results. Moreover, studies on the issue also exhibited that egg maturation, fertilization, the quality of the corresponding embryos and also egg fertilization ability were closely related to the apoptosis of cumulus granulosa cells (Høst et al., 2000; Raman et al., 2001). Clavero et al. (2003) found that the rate of apoptosis in granulosa cells was not associated with the maturity of the oocyte and the ability for fertilization in ICSI or the quality of follicles during ovulation induction. In patients assisted with IVF, an increase in the number of apoptotic GCs isolated from follicular fluid pool was observed. Evidence from the previous studies suggested that ratio of apoptosis in GCs was increased in elderly IVF patients. This has been associated with the decreased oocyte quality, fertilization, pregnancy and live birth rates (Sifer et al., 2002).

Lobach et al. (2019) reported that to show apoptosis in human preovulatory follicles, caspase-3 expression in aspirated GC cells can be used an indirect marker since it negatively affects follicular growth and oocyte yield. The same group also predicted for future research that to illustrate all mechanism of apoptosis in preovulatory follicles and explain the reason of high cell loss which leads to poor ovarian response. Different biomarkers should be examined and that was the starting point in our study to examine bax proapoptotic gene expression. A significant increase in BAX expression was observed in chromatin and degenerative cytoplasmic structures in granulosa cell nuclei obtained from idiopathic patients. Granulosa cells (GC) apoptosis may cause ovarian atresia and GC dysfunction may play a role in PCOS pathology. Increased increase in apoptotic granulosa cells will have a negative effect on embryo development with low oocyte quality and low oocyte quality.

Reported in studies, cytokines and inflammatory proteins regulate expansion of cumulus cell oocyte complex for successful ovulation. These events begin with changes in LH level and activation of downstream molecules (Richards et al., 2015). Furthermore, infertility studies reveal that GCs increased expression of proinflammatory cytokines (Köks et al., 2010). Adams et al. (2016) stated that there was a significant difference in the proinflammatory gene expression between control and PCOS groups. Besides elevated GCs cytokine expression and increased number of immune cells, these results also showed that GCs interact with each other by cellular and paracrine ways in their in vivo periovulatory environment. Interleukins have been shown to play an important role in the evaluation of ovarian reserve function of intermittent hormone in immune diseases (Knauff et al., 2009). Sun et al. (2018) reported that the higher the IL-6 and IL-21 levels, the lower the AMH level, the worse the ovarian reserve function. In our study, IL-10 expression was found to be very high in idiopathic patients and AMH value was found to be low compared to control (Table I).

An extracellular metalloprotease ADAMTS-1 we investigated here is induced by ovarian hormones in ovarian follicles and is required for fertility. The ADAMTS-1 protease domain mediates directions for cellular degradation and dissolution of the follicle wall, while the thrombospondin and disintegrin regions control their localization and the directions of inflammation, differentiation, and neovascularization, and thus have been reported to provide for the co-ordination of many follicle ruptures and the formation of the corpus luteum (Robker et al., 2000). It has been shown that ADAMTS-1 is primarily secreted by wall granulosa cells, localized to the ECM of expanded cumulus oocyte complex, and induced significantly by LH in ovulating follicles (Russell et al., 2003). It has a critical role in the expansion of ADAMTS-1 versican (a large extracellular matrix proteoglycan), expansion of COC formation, ovulation and angiogenesis. In ADAMTS-1 null mice, oocyte and ovarian vascular degeneration and a decrease in the organization of the ovarian network have been identified (Brown et al., 2006). Sun et al. stated that ADAMTS-1 has reshaped the dynamic structure of versican catabolism required for subsequent degradation of the COC matrix in order to ensure successful fertilization of the ovulation-associated follicle wall and tissues. They also investigated ADAMTS-1 expression in granulosa cells of patients with polycystic ovary syndrome by immunocytochemistry and reverse transcription polymerase chain reaction. In this context, they showed a reduced expression of ADAMTS-1 in patients with PCOS compared to women laying eggs. In our study, ADAMTS-1 expression was moderate in the supranuclear layer of granulosa cells from idiopathic patient groups.

Despite the results, there are a few limitations in clinical contribution. Apoptosis of granulosa cells help ovulation mechanisms by paracrine signaling and endocrine pathways, may disrupt the ovulation process in humans. It was thought that apoptosis induced by human GCs might be an indicator of egg quality. Moderate expression of
ADAMTS-1 was thought to be related to failure of ovulation, deterioration of oocyte quality and decreased fertilization rate. A reduction in AMH levels may be associated with defects in granulosa cells and correlated with increased granulocytes secreting significantly lower AMH than healthy people and increasing IL10 expression levels.

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RESUMEN: Las células de la granulosa (GC) son componentes esenciales de los folicículos y tienen un papel en la regulación del desarrollo de éste. El objetivo del estudio fue investigar ciertos componentes celulares involucrados en la proliferación, diferenciación y características funcionales de las células de la granulosa en el éxito de la fertilización de los ovocitos humanos durante la fertilización in vitro (FIV) a través de técnicas inmunohistoquímicas. En este estudio, se incluyeron 30 pacientes diagnosticados con infertilidad primaria o secundaria, síndrome de ovario poliquístico en el centro de FIV del Departamento de Obstetricia y Ginecología del Hospital Memorial. La cantidad de Hormona Anti Mülleriana (AMH) en la sangre, el diámetro de las células de la granulosa y el diámetro del núcleo celular se midieron en 20 células obtenidas de cada paciente. Además, se evaluó la puntuación de degeneración y las expresiones BAX, ADAMTS-1, IL-10 en células de granulosa (p < 0.01). Se estimó que la apoptosis inducida por los GC humanos podría ser un indicador de la calidad del huevo. Se estimó que la expresión moderada de ADAMTS-1 estaba relacionada con el fracaso de la ovulación, el deterioro de la calidad de los ovocitos y la disminución de la tasa de fertilización. La disminución en los niveles de AMH puede estar asociada con defectos en las células de la granulosa. Por lo tanto, el aumento de las células de granulocitos puede explicar la disminución significativa de la secreción de AMH y el aumento de los niveles de expresión de IL10 en personas sanas.

PALABRAS CLAVE: Células de Granulosa; Fertilidad; ADAMTS-1; IL-10, BAX; Inmunohistoquímica.

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Corresponding author: Yunus Çavuş,
Department of Obstetrics and Gynecology at Memorial Hospital
Diyarbakır
TURKEY

Email: ycavus1212@gmail.com

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