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Effect of Fetuin-A and oxidative stress on the occurrence of unexplained infertility

Açıklanamayan infertilite oluşumunda Fetuin-A ve oksidatif stresin etkisi

Tuba Taşkan[1], Taylan Turan[2], Zehra Candan İltemir Duvan[3], Aymelek Gönenç[2]

[1]*Afyonkarahisar Health Sciences University Faculty of Pharmacy, Department of Biochemistry, Afyonkarahisar, Turkey*

[2]*Gazi University Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey*

[3]*Yüksek İhtisas University Faculty of Medicine, Department of Gynecology and Obstetrics, Ankara, Turkey*

Abstract

Objective: Unexplained infertility refers to a diagnosis in which all standard examinations are usually normal and is statistically seen in approximately 30-40% of infertile couples and endometriosis encountered in 25-50% of patients with unexplained infertility. Unexplained infertility is thought to be closely associated with endometriosis and serum and peritoneal fluid levels of Fetuin-A is increased in patients with endometriosis. Fetuin-A is proposed as a diagnostic marker for endometriosis and has anti-inflammatory effects on several diseases. Oxidative stress also is central to the etiopathogenesis of infertility in women. The aim of this study was to evaluate serum Fetuin-A and oxidative stress parameter concentrations impact on unexplained infertility.

Materials and Methods: In the study, serum Fetuin-A, IL-1β, CA I, TAS, TOS levels, and PON and ARE enzyme activities were measured using the Enzyme-Linked ImmunoSorbent Assay in the sera of diagnosed unexplained infertility females (n=44) and controls (n=41).

Results: There was no statistically significant difference between unexplained infertile patients and control groups in terms of serum IL-1β, CA I, OSI, and PON levels ($p>0.05$). Serum Fetuin-A and ARE levels were significantly higher in unexplained infertility compared with the control, whereas serum TAS and TOS levels were lower ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.05$, respectively).

Conclusion: It is thought that increased Fetuin-A levels may be a response to the inflammatory process and increased ARE activity may be a sign of the impaired oxidant-antioxidant balance in unexplained infertility. This may contribute to the pathogenesis of infertility, and the data obtained will make a significant contribution to new works to be done in this sense.

Keywords: Unexplained infertility, Fetuin-A, oxidative stress, carbonic anhydrase I, ARE

Öz

Amaç: Açıklanamayan infertilite, tüm standart muayenelerin genellikle normal olduğu ve istatistiksel olarak kısır çiftlerin yaklaşık %30-40'ında görülen bir tanıyı ifade eder. Açıklanamayan infertilite hastalarının %25-50'sinde endometriozise rastlanmaktadır. Açıklanamayan infertilitenin endometriozis ile yakından ilişkili olduğu düşünülmektedir. Endometriozisli hastalarda serum ve peritoneal sıvıda Fetuin-A düzeyleri artmaktadır. Fetuin-A, endometriozis için tanısal bir belirteç olarak önerilmiştir ve çeşitli hastalıklar üzerinde anti-enflamatuvar etkilere sahip olduğu bilinmektedir. Oksidatif stres de kadınlarda infertilite etiyopatogenezinde önemli bir rol oynamaktadır. Bu çalışmanın amacı, serum Fetuin-A ve oksidatif stres parametreleri konsantrasyonlarının açıklanamayan infertilite üzerindeki etkisini değerlendirmektir.

Gereç ve Yöntemler: Bu çalışmada, açıklanamayan infertilite tanısı konmuş kadınlar (n=44) ve kontrollerin (n=41) serumlarında Fetuin-A, IL-1β, CA I, TAS, TOS seviyeleri ve PON ile ARE enzim aktiviteleri Enzyme-Linked ImmunoSorbent Assay kullanılarak ölçüldü.

Bulgular: Açıklanamayan infertil hasta ve kontrol grubu arasında serum IL-1β, CA I, OSI ve PON düzeyleri açısından istatistiksel olarak anlamlı fark yoktu ($p>0,05$). Açıklanamayan infertilitede kontrole göre serum Fetuin-A ve ARE düzeyleri anlamlı olarak yüksek, serum TAS ve TOS düzeyleri ise daha düşüktü (sırasıyla $p<0,05$, $p<0,01$, $p<0,05$, $p<0,05$).

PRECIS: We have investigated in women with unexplained infertility serum Fetuin-A, IL-1β, CA I, TAS, TOS levels, and PON, ARE enzymes activities' possible effects and relationships among these parameters on disease.

Address for Correspondence/Yazışma Adresi: Aymelek Gönenç PhD,
Gazi University Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey
Phone: +90 312 202 31 52 **E-mail:** aymelek@gazi.edu.tr **ORCID ID:** orcid.org/0000-0001-9661-8291

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Sonuç: Açıklanamayan infertilitede artmış Fetuin-A düzeylerinin inflamatuvar sürece bir yanıt olabileceği ve artmış ARE aktivitesinin bozulmuş oksidan-antioksidan dengenin bir işareti olabileceği düşünülmektedir. Elde edilen bu veriler açıklanamayan infertilitenin patogenezine katkı sağlayacak ve bu anlamda yapılacak yeni çalışmalara ışık tutacaktır.

Anahtar Kelimeler: Açıklanamayan infetilite, Fetuin-A, oksidatif stres, karbonik anhidraz I, ARE

Introduction

Unexplained infertility (UI), which is a major problem of the reproductive system, refers to a failure in achieving a successful pregnancy after at least one year of regular unprotected sexual intercourse despite ovulation, tubal patency, and semen parameters are normal[1]. Many studies have accepted that infertility and endometriosis are associated clinically[2]. Fetuin-A is a multifunctional glycoprotein that is secreted exclusively by the liver parenchymal cells and it is proposed as a diagnostic marker for endometriosis[3]. Fetuin-A is the acute phase protein response to various inflammatory situations and has a protective effect on systemic inflammation[4]. IL-1β is localized in endometrial macrophages and endothelial cells as an inflammatory cytokine. Serum and peritoneal fluid levels of IL-1β have increased in endometriosis[5]. Carbonic anhydrases (CAs, EC 4.2.1.1) are a family of zinc enzymes with 16 isoenzymes that catalyze the reversible hydration reaction of carbon dioxide to bicarbonate. It has critical physiological roles or pathological processes, including the reproductive system. CA has reported that ovarian autoantibodies are associated with UI and premature ovarian failure[6]. Antibodies against autoantibodies including CAs have been shown in endometriosis, in which autoimmune mechanisms may be involved[7].

The resulting oxidative stress is central to the etiopathogenesis of infertility in women and causes health problems in the female reproductivity system, such as UI, endometriosis, PCOS, and recurrent pregnancy loss[8]. Excessive production of ROS affects the body's natural antioxidant defense system and disrupts the environment in which normal physiological reactions occur[9]. Total antioxidant status (TAS), total oxidant status (TOS), and OSI are critical parameters that can be used to assess redox and the degree of oxidative stress status[10]. Paraoxonase-1 (PON1) is synthesized in the liver as a high-density lipoprotein-associated enzyme having both paraoxonase (PON) and arylesterase (ARE) activities. PON and ARE have an antioxidant effect against lipid peroxidation and play a significant role in the anti-inflammatory process. Although there are several reports evaluating the possible link between serum PON activity and oxidative stress in PCOS in literature,[11,12] no study has been found associated with the cases of UI. In this study, it was aimed to evaluate serum Fetuin-A, IL-1β, and CA I levels, antioxidant molecules, and enzyme activities in UI.

Materials and Methods

The study group consisted of 44 female patients diagnosed with UI and 41 controls (women whose cause of infertility is male factor). Patients and controls were chosen from people without systemic diseases. The study protocol has been performed in accordance with the ethical standards described in an appropriate version of the 1975 Declaration of Helsinki, as revised in 2000, and it was approved by the Gazi University Clinical Research Ethics Committee with decision number 512 (Date: 26.02.2018). All participants gave written informed consent.

Peripheral venous blood samples were collected from the study group. The blood samples were dissociated to their serum by centrifugation at 1000x g for 15 min. at 4 °C and immediately placed into sterile Eppendorf tubes. Following this procedure, all serum samples were frozen at -80 °C. The human ELISA kits were used for serum Fetuin-A, IL-1β, and CA I according to the manufacturer's instructions. Serum TAS and TOS values, PON and ARE enzyme activities were measured by spectrophotometer by manual methods.

Serum Fetuin-A, IL-1β, and CA I (Elabscience, USA) were measured according to the ELISA kit procedure. TAS and TOS measurements were applied in serum (Rel Assay Diagnostics, Turkey). TAS reduces the antioxidants in the sample the dark blue-green ABTS radical to the colorless reduced ABTS form and changes in absorbance at 660 nm is related to the total antioxidant level of the sample. The TOS kit principle is oxidant present in the sample oxidizing the ferrous ion-o-dianisidine complex to the ferric ion and the detected color intensity is related to the total amount of oxidant molecules. The oxidative Stress Index (OSI) is a proportional index obtained by dividing total peroxides by total antioxidants with the formulas below.

OSİ (Arbitrary Unit) = TOS (μmol H_2O_2 Equiv./L) / TAS (mmol Trolox Equiv. /L)

Serum PON levels were determined using paraoxon (o,o-diethyl-o-p-nitrophenyl phosphate) as the substrate, which is the active catabolic metabolite of parathion from organophosphate compounds. PON serum activity was measured spectrophotometrically according to the definition of Gan et al.[13]. Based on this method, to prepare a PON reactive mixture solution, freshly prepared 2 mM of paraoxon substrate solution was dissolved in the presence of 2 mM $CaCl_2$ in a total volume of 90 mL 100 mM Tris-HCl buffer (pH 8.0). The assay and blank tubes contained 700 μL of PON reactive mixture solution, and just assay tubes included 20-μL serum, while blank tubes had 20 μL distilled water instead of serum. After incubation for 5 min at 37 °C in a water bath, the changes in absorbance at 3 min were continuously monitored at 412 nm. The amount of p-nitrophenol was calculated from the molar extinction coefficient (18.000 $M^{-1}cm^{-1}$), and the results were expressed as U/mL. Validation was performed by measuring the

same sample at least three times for consistency. Serum ARE levels were measured using phenyl acetate as the substrate, one of the aromatic carboxylic acid esters. ARE serum activity was measured spectrophotometrically according to the definition of Naderi et al.[14]. Briefly, 20 µL of a 1:50 dilution of serum and 20 µL distilled water were added to 700 µL of ARE reactive mixture solution containing freshly prepared 2 mM phenylacetate in 100 mM Tris-HCl pH 8.0 and 2 mM $CaCl_2$ for assay and blank tubes, respectively. After incubation for 5 min at 25 °C in a water bath, the changes in absorbance at 3 min were continuously recorded at 270 nm. The amount of phenol was calculated from the molar extinction coefficient (1310 $M^{-1}cm^{-1}$), and the results were expressed as U/mL. Validation was implemented by evaluating the same sample at least three times for consistency.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS version 22.0 for Windows (SPSS Inc, Chicago, IL, USA). Descriptive statistics are given with mean ± standard deviation. The distribution of the data was examined using the Kolmogrov-Smirnov normality test. "Independent Samples t-test" was applied in the analysis between the two independent groups. The confidence interval was accepted as 95%, and the p-value less than 0.05 was considered statistically significant. The pearson correlation coefficient was used for the relationship among the measured parameters.

Results

The clinical characteristics and biochemical parameters of the study group are given in Table 1. No statistically significant difference was found between the UI patient group and the control group in terms of age, BMI, FSH, TSH, LH, progesterone, estradiol (E_2), number of oocytes, LH/FSH ratio, ALT, and AST levels (p>0.05).

Table 1. Clinical features and biochemical parameters in the study groups

	Control group (n=41)	UI group (n=44)
Age (X ± SH)	30.41±0.75	32.14±0.69
BMI (kg/m^2)	24.85±0.52	25.58±1.56
FSH (mIU/mL)	6.32±0.31	7.20±0.44
TSH (uIU/mL)	2.07±0.14	2.23±0.17
LH (mIU/mL)	3.28±0.46	4.78±0.64
Progesterone (ng/mL)	0.64±0.05	0.60±0.04
E_2 (pg/mL)	34.97±2.40	41.23±2.39
Number of Oocyte	11.32±1.02	12.73±0.89
LH/FSH Ratio	0.55±0.07	0.68±0.08
ALT (U/L)	16.41±1.08	16.20±1.84
AST (U/L)	16.07±0.48	18.41±1.56

Serum levels of Fetuin-A and IL-1β of both groups and statistical comparison between the two groups are given in Figure 1. Serum levels of Fetuin-A were higher in the UI group compared to controls (21.24±0.64 µg/mL, 19.65±0.46 µg/mL, p<0.05). Additionally, IL-1β (2.18±0.46 pg/mL, 1.78±0.40 pg/mL, p>0.05) and CA I (784.86±56.61 pg/mL, 752.13±13.10 pg/mL, p>0.05) concentrations were not found statistically significant between the study groups (Figure 1).

Serum TAS (1.72±0.02 mmol/L, 1.77±0.02 mmol/L, p<0.05) and TOS (6.00±0.16 µmol/L, 6.70±0.31 µmol/L, p<0.05) levels were found to be significantly lower in the UI patient group compared to the control group. No significant difference was found between UI patients and controls in terms of serum OSI levels (3.51±0.12 arbitrary unit, 3.79±0.17 arbitrary unit, p>0.05) (Figure 2).

PON serum concentrations were not statistically different among the study groups (46.76±4.45 U/mL, 41.02±3.37 U/mL, p>0.05). However, ARE levels are higher in UI patients than in controls (98.77±9.26 U/mL, 39.40±3.61 U/mL, p<0.01) (Figure 3).

In the UI group, using bivariate correlation analysis, positive correlations were found between LH/FSH ratio and PON (r=0.325, p<0.05), TOS and OSI, TOS, and CA I (r=0.856, p<0.01; r=1.000, p<0.01 respectively), and OSI and CA I (r=0.856, p<0.01); however, negative correlations between

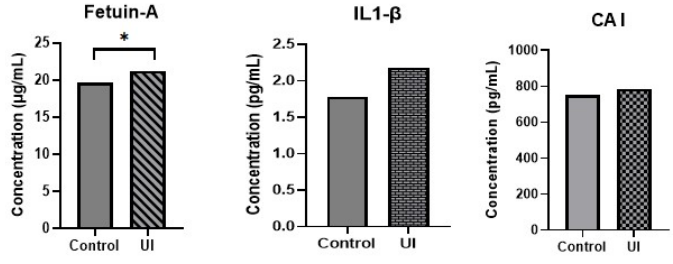

Figure 1. Serum Fetuin-A, IL-1β, and CA I levels in the study groups

** Significantly increased in unexplained infertility patients compared to controls (p<0.05)*

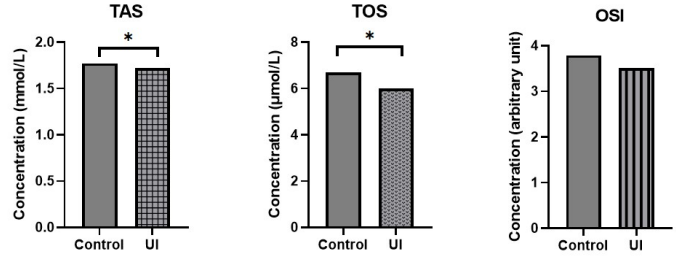

Figure 2. Serum TAS, TOS, and OSI levels in the study groups

** Significantly decreased in unexplained infertility patients compared to controls (p<0.05)*

serum TOS and ARE, TAS and OSI, OSI and ARE, CA I and ARE were found (r=-0.379, p<0.05; r=-0.492, p<0.01; r=-0.339, p<0.05; r=-0.379, p<0.05, respectively) (Table 2).

In the control group, positive correlations were found between age and mean levels of serum TOS, age and OSI, age and CA I (r=0.413, p<0.01; r=0.468, p<0.01; r=0.413, p<0.01, respectively), BMI and TOS, BMI and OSI, BMI, and CA I (r=0.391, p<0.05; r=0.355, p<0.05; r=0.391, p<0.05, respectively), LH and TAS, LH and Fetuin-A (r=0.353, p<0.05; r=0.327, p<0.05, respectively), LH/FSH ratio and TAS (r=0.316, p<0.05), TOS and OSI, TOS and CA I, OSI and CA I (r=0.978, p<0.01; r=1.000, p<0.01; r=0.978, p<0.01, respectively). On the other hand, there were negative correlations between PON and LH/FSH ratio, ARE and TSH (r=-0.361, p<0.05; r=-0.319, p<0.05, respectively) (Table 3).

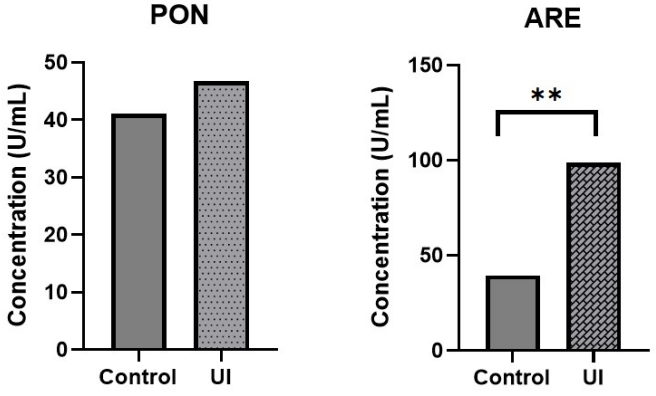

Figure 3. Serum PON and ARE levels in the study groups

*** Significantly increased in unexplained infertility patients compared to controls (p<0.01)*

Table 2. Correlations between the parameters measured in the patient group

UI group (n=44)	CA I	TAS	TOS	OSI	ARE
CA I	-	-	1.000**	0.856**	-0.379*
TAS	-	-	-	-0.492**	-
TOS	1.000**	-	-	0.856**	-0.379*
OSI	0.856**	-0.492**	0.856**	-	-0.339*
ARE	-0.379*	-	-0.379*	-0.339*	-

*Difference according to the control group p<0.05,
**Difference according to the control group p<0.01

Table 3. Correlations between the parameters measured in the control group

Control group (n=41)	CA I	TOS	OSI
CA I	-	1.000**	0.978**
TOS	1.000**	-	0.978**
OSI	0.978**	0.978**	-

*Difference according to the control group p<0.05,
**Difference according to the control group p<0.01

Discussion

In cases of UI, fertility does not occur, although all standards-regarding infertility are normal. Potential reasons for this include endocrinological, immunological, genetic factors, and reproductive physiology diseases[15]. Although it is known that UI is linked to metabolic diseases, it is clear that new biomarkers representing this condition are strongly needed in clinical practice. Various molecules related to the inflammatory pathways in UI include adipokines, inflammatory cytokines, oxidative stress molecules, and transcription factors, which are candidates for new molecular targets for the prognosis and treatment of UI. There is no study in the literature evaluating UI in terms of Fetuin-A, IL-1β, CA I, TAS, TOS, OSI levels, and PON and ARE activities. We mainly investigated these parameters in this study.

Fetuin-A is an important adipokine that is expressed and secreted by the adipose tissue. It has an anti-inflammatory effect in the acute phase response[4]. Although there are some controversial data on the role of Fetuin-A in infertility, it has not been evaluated in UI. In one of these studies at issue, higher circulating Fetuin-A levels were observed in patients with PCOS compared with healthy women by Liu et al.[16] Moreover, they suggested that serum Fetuin-A was associated with the occurrence of PCOS according to binary logistic regression analysis and concluded the results as Fetuin-A may be a useful biomarker for screening PCOS. Obviously, higher levels of serum Fetuin-A in patients with PCOS has been asserted to be connected with PCOS[17]. Similarly, a significant increase in both women undergoing IVF treatment and women with recurrent implantation failure was denoted compared with healthy controls in terms of serum Fetuin-A levels[18,19]. On the other hand, Gurbuz et al.[20] did not find any statistical differences between PCOS and the control group. In our study, serum Fetuin-A levels were found to be statistically higher in UI patients compared with the control group. This result obtained in our study is consistent with the studies conducted by Liu et al.[16], and Sak et al.[17]. We think that increased levels of Fetuin-A may contribute to the etiopathogenesis of UI.

Interleukin-1 beta (IL-1β) exerts various biological functions as a proinflammatory cytokine. To investigate the possible effect of inflammation on the risk of in vitro fertilization and embryo transfer (IVF-ET) failure, serum samples of infertile and pregnant women were collected on the second day of menstruation, and the inflammatory cytokine IL-1β, IL-6, and IL-8 levels were evaluated by Xie et al.[21]. Among these inflammatory cytokines, serum IL-8 levels were found to be higher compared with controls who became pregnant after treatment, while no significant difference was found between the groups between IL-1β and IL-6 levels. In another study by Sequeira et al.[22], IL-1β was measured in the serum and embryo

culture medium of infertile female patients who received IVF treatment, and there was no significant difference between the groups of pregnant and non-pregnant women. Our results showed that serum IL-1β levels were similar in both the patient and control groups. It is seen that the results obtained from our study are compatible with the studies of Xie et al.[21] and Sequeira et al.[22].

Carbonic anhydrase enzymes are common in many tissues and organs in the human body. The ovary is the target of autoimmune attacks in various pathologies that progress toward dysfunction. The presence of Anti CA I antibodies has been shown in recurrent pregnancy losses, endometriosis, and PCOS, and it is thought to be a reliable diagnostic indicator[7,23,24]. In a study by Menteşe et al.[24] with patients with PCOS, it was reported that serum Anti-CA I autoantibodies were higher levels compared with healthy controls. Anti-CA I has not been reported so far in sera obtained from infertility patients with unexplained presence. In our study, although serum CA I levels were higher in the UI patient compared with the healthy control group, no significant difference was found. There is no study in the literature that evaluated the levels of CA I enzyme in UI.

Although it is known that numerous factors that cause oxidative stress in infertility play a role in its etiopathogenesis, the uncertainty regarding its effect on UI continues[25]. It has been reported that oxidative stress plays a role in various etiological factors that may contribute to infertility, such as impaired endometrial receptivity, impaired oocyte quality, premature ovarian failure, endometriosis, tubal disease, pelvic adhesions, and immunological and endocrinological abnormalities. Isbilen et al.[26] found higher levels of TOS and lower levels of OSI in patients with UI, although it was not significant compared with healthy women. Additionally, significantly lower TAS values were reported in healthy fertile women compared with the patient group. Şentürk et al.[27] found significantly higher TOS and OSI levels in women with UI than in fertile women but did not find any difference in TAS values. In our study, decreased TOS and TAS levels were found in patients with UI patients. The data we obtained contradicted the previous results in the literature. Additionally, it was observed that there was no difference between the groups in OSI values, similar to Isbilen et al.[26] and these results contradict the results of Şentürk et al.[27] The significant decrease in our study serum TAS, TOS, and OSI values in UI and the positive correlations between TOS and OSI, TOS and CA I, and OSI and CA I in both the control and UI groups show us the impaired oxidant-antioxidant balance in UI.

In this study, we hypothesized a possible relationship between serum PON/ARE levels and UI development. There are controversial results in the literature. Carlioglu et al.[28] showed lower PON and ARE levels in untreated patients with PCOS than the controls, while these enzyme levels were significantly increased after treatment. Accordingly, it has been reported that PON enzyme activity was decreased in women with endometriosis[29]. Verit et al.[30] suggested that serum PON enzyme level was significantly reduced in women with moderate/severe endometriosis than in women with minimal/mild endometriosis and control groups, and in women with minimal/mild endometriosis compared with controls, correlatively. The authors also found a significant negative correlation between serum PON levels and stage of endometriosis and conclude that serum PON enzyme levels can be used as a diagnostic marker for endometriosis. However, this is inconsistent with the study by San Millan et al.[31], who did not show a significant difference between the PCOS and controls in the serum PON activities. These results were supported by Bragatto et al.[32] for women with endometriosis in minimal/mild and moderate/severe stages for both PON and ARE enzymes, even when the patients were analyzed separately according to the endometriosis stage. In the same way, Younis et al.[33] also demonstrated no differences between the endometriosis, PCOS, and UI groups in baseline and peak serum levels of PON. In our study, serum ARE levels were found to be significantly higher in the UI patient group than in the control group. Similarly, although serum PON levels were increased in patients compared with controls, this increase was not statistically significant. These conflicting results suggest that much is still unknown related to the causes and mechanisms involved in the endogenous production of PON/ARE enzyme. It is thought that increased ARE activity in the body may contribute to the pathogenesis of infertility.

Study Limitations

The findings of this study must be seen considering some limitations. The first is that our study has a small sample size. More precise results can be obtained with a study in which more women with IU participate. The second limitation, in UI, there can be many factors that can also affect oxidative stress, such as lifestyle, medications, malnutrition, obesity, and environmental factors. The third limitation it can be stated that measuring and comparing the parameters measured in the serum also in the follicular fluid may be more helpful in the interpretation.

Conclusion

Considering these data obtained from our study, it is thought that increased Fetuin-A levels may be a response to the inflammatory process and increased ARE activity may be a sign of the impaired oxidant-antioxidant balance in UI and may contribute to the pathogenesis of infertility. It is predicted that Fetuin-A and ARE levels can be considered biomarkers in evaluating the UI status. We believe that more comprehensive studies should be conducted by increasing the number of samples so that the results can be more reliable.

Ethics

Ethics Committee Approval: The study protocol has been performed in accordance with the ethical standards described

in an appropriate version of the 1975 Declaration of Helsinki, as revised in 2000, and it was approved by the Gazi University Clinical Research Ethics Committee with decision number 512 (Date: 26.02.2018).

Informed Consent: All participants gave written informed consent.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: T.T., T.T., Z.C.İ.D., A.G., Concept: T.T., T.T., Z.C.İ.D., A.G., Data Collection or Processing: T.T., T.T., Z.C.İ.D., A.G., Analysis or Interpretation: T.T., T.T., Z.C.İ.D., A.G., Literature Search: T.T., T.T., Z.C.İ.D., A.G., Writing: T.T., T.T., Z.C.İ.D., A.G.

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