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Determination of maternal serum pro-inflammatory cytokine changes in intrauterine growth restriction

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Abstract. – OBJECTIVE: To evaluate maternal serum inflammatory marker changes in intrauterine growth restriction (IUGR) pregnancies.

PATIENTS AND METHODS: 50 healthy pregnant women and 50 patients diagnosed with IUGR were enrolled. Maternal serum high sensitivity C-reactive protein (hsCRP), erythrocyte sedimentation rate (ESR), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-10 levels were measured before delivery and neonatal outcomes were evaluated.

RESULTS: Birth weight, Apgar scores, and cord blood gas pH were lower in the IUGR group ($p<0.001$, $p<0.001$, $p<0.001$ and $p=0.006$, respectively). While the levels of ESR, hsCRP, IL-6, and TNF-α were higher, the IL-10 level was found to be lower in the IUGR group ($p<0.001$, $p=0.033$, $p<0.001$, $p=0.004$ and $p<0.001$, respectively). As ESR, hsCRP, and IL-6 levels increased, birth weight, Apgar scores, and cord blood gas pH decreased ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, $p=0.02$, $p=0.002$, $p=0.001$, $p=0.03$, $p<0.001$, $p<0.001$, $p<0.001$ and $p=0.02$, respectively). As TNF-α level increased, only birth weight and Apgar score at the 1st minute decreased ($p=0.006$ and $p=0.048$, respectively). As IL-10 level decreased, birth weight, Apgar scores, and cord blood gas pH decreased ($p<0.001$ for all). IL-6 (>3.2 pg/ml) had a sensitivity of 100%, specificity of 100%, PPV of 100% and NPV of 100%.

CONCLUSIONS: While birth weight, Apgar score and cord blood pH decreased in IUGR cases, ESR, hsCRP, IL-6 and TNF-α levels increased. Combined measurement of these markers can be used for the diagnosis of IUGR.

Key Words:
Intrauterine growth restriction, IL-6, IL-10, TNF-alpha.

Introduction

Intrauterine growth restriction (IUGR) describes a decrease in growth rate that can occur in fetuses. Although the exact causes of IUGR are unknown it affects approximately 30 million newborns each year[1]. It is thought[2,3] that genetic factors play a role in approximately one-third of cases, and maternal, fetal, or placental factors account for the remaining two-thirds. IUGR fetuses are more vulnerable to perinatal morbidity, such as sepsis, asphyxia and perinatal death[3,4]. In addition, IUGR is a significant risk factor for the development of diabetes mellitus and cardiovascular diseases in childhood and adulthood[5]. For these reasons, it is important to understand the pathogenesis of IUGR and take the necessary precautions.

Immunological balance is of great importance during pregnancy. It is known[6] that some biomarkers and cytokines are responsible for endothelial damage and placental dysfunction in pregnancy. Furthermore, the alteration of the immune balance mediates perinatal complications, such as preterm delivery or abortion[7,8]. There are few studies[9] in the literature investigating the relationship between inflammation and IUGR. In a study comparing C-reactive protein (CRP) levels in maternal serum and umbilical cord blood during delivery, CRP levels were higher in the IUGR group. In addition, there are studies[10] showing that high CRP levels detected in the early weeks of pregnancy can predict which infants will be small for their gestational age at birth. Recent studies[11,12] have been conducted on the effect of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, IL-12, IL-18, and IL-23, and anti-inflam-

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matory cytokines such as IL-4, IL-10, and IL-13 in the pathogenesis of IUGR. However, these studies[13] could not identify which of the pro- or anti-inflammatory cytokines are significant in the development IUGR, probably due to the complicated and heterogeneous nature of the disease.

This study was planned to determine the pro-inflammatory and anti-inflammatory response in IUGR by examining inflammation-related biomarkers, such as CRP and erythrocyte sedimentation rate (ESR), and cytokines, such as IL-6, IL-10, and TNF-α.

Patients and Methods

This prospective study included pregnant women who applied to an outpatient clinic or emergency service between February 1, 2021, and August 1, 2021 for delivery in our hospital. Local Ethics Committee approval was obtained (Approval No.: 2021/01-33). Informed consent also was obtained from all patients. The inclusion criteria for this study were: (1) delivery carried out in our hospital, (2) diagnosis of IUGR *via* ultrasonographic examination, and (3) volunteering to participate in the study. IUGR was diagnosed if the estimated fetal weight calculated *via* the Hadlock[14] formula was below the 10th percentile. The presence of maternal systemic diseases or medication use were criteria for exclusion from the study. A control group was formed of healthy volunteer pregnant women who applied to our hospital for elective cesarean delivery.

Demographic characteristics such as age, body mass index, and obstetric history of all patients were recorded. A venous blood sample was taken from the mother before delivery. High sensitivity CRP (hsCRP), ESR, IL-6, IL-10, and TNF-α levels were analyzed from maternal serum. Approximately 2 mL of blood was taken from the mother and placed in gel vacuum tubes. Afterwards, the plasmas were separated at 4°C and 14,000 rpm in a refrigerated centrifuge (HetticZentrifugen Universal 320, Tuttlingen, Germany) and stored under suitable conditions. hsCRP measurement was made with the immunoturbidimetric method using original Roche diagnostic kits on Cobas C501 device (Basel, Switzerland). Human CRP agglutinate was used with latex particles coated with monoclonal anti-CRP antibodies. Aggregates were analyzed turbid metrically in mg/L. Westergren's method was used for ESR measurement. ESR was expressed in mm/hr. TNF-α, IL-6 and IL-10 levels in serum were studied using ELISA kit (Bioassay Technology Lab., Shanghai, China) in accordance with the manufacturer's instructions. 40 µl of plasma sample was placed in micro wells coated with specific antibodies, followed by 10 µl of specific antibody. Then, after adding 50 µl of streptavidin-HRP, it was incubated at 37°C for 60 minutes. After the wells were emptied, they were washed 1x with wash buffer. In the next steps, substrate A and B solution were added. The plates were read at 450 nm after the stop solution was added. ELISA procedures were performed on DS2 automated ELISA instruments (Dynex Technologies Inc, Chantilly, VA, USA). After absorbance values were obtained, total levels were determined with controls according to the instructions. Neonatal outcomes such as birth weight and Apgar scores at the 1st and 5th minute were recorded. After each delivery, blood gas was collected from the umbilical cord and pH values were evaluated.

Statistical Analysis

All statistical analyses were performed using the SPSS v. 17 (SPSS Inc., Chicago, IL, USA) program and $p<0.05$ was considered significant. Distributions of numerical parameters were evaluated with the Kolmogorov-Smirnov test. While IL-6 and IL-10 were parameters showing normal distribution, other parameters did not show normal distribution. Normally distributed data were expressed as mean ± standard deviation. Data that did not show normal distribution were expressed as median (25th-75th percentile). The Student's *t*-test was used to compare IL-6 and IL-10 levels between groups, and the Mann-Whitney U test was used to compare other parameters. Correlations between inflammatory markers and obstetric outcome parameters were analyzed with the Spearman's correlation test. Receiver operating characteristic (ROC) analysis was performed for the significant parameters in terms of prediction of IUGR. Area under the curve (AUC) was determined by ROC analysis. The optimal cut-off values were selected using Youden's index[15] and specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) were estimated.

Results

Demographic characteristics, obstetric results, and inflammatory markers of the IUGR (n=50) and control groups (n=50) are given in Table I. While

Table I. Demographic and obstetrics characteristics and inflammatory markers of IUGR and control groups.

	IUGR (n=50)	Control (n=50)	p
Age (years)	28 (25-31)	29 (25-32)	0.69
BMI (kg/m^2)	25.2 (24.7-25.9)	25.3 (24.8-26.6)	0.38
Gravida	2 (1-3)	2 (1-3)	0.13
Parity	1 (0-2)	1 (0-1)	0.14
Abortus	0 (0-0)	0 (0-0)	0.28
Birth weight (g)	1,470 (980-2,230)	3,300 (3,180-3,400)	**<0.001**
Apgar score (1st min)	7 (6.8-8)	8 (8-9)	**<0.001**
Apgar score (5th min)	8 (8-9)	9 (9-9)	**<0.001**
pH (cord blood gas)	7.3 (7.3-7.4)	7.4 (7.3-7.4)	**0.006**
ESR (mm/hr)	6 (3-9)	3 (2-3)	**<0.001**
hsCRP (mg/L)	0.4 (0.3-3.0)	0.4 (0.3-0.4)	**0.033**
IL-6 (pg/mL)	8.5 ± 1.5	1.6 ± 0.4	**<0.001**
IL-10 (pg/mL)	6.2 ± 2.6	11.7 ± 3.3	**<0.001**
TNF-α (pg/mL)	1.5 (0.8-2.5)	0.9 (0.5-1.3)	**0.004**

BMI: Body mass index; ESR: Erythrocyte sedimentation rate; hsCRP: High sensitivity C-reactive protein; IL: Interleukin; IUGR: Intra-uterine growth restriction; TNF: Tumor Necrosis Factor.

both groups were homogeneous in terms of demographic characteristics, it was observed that the neonatal outcome results – birth weight, Apgar scores, and cord blood gas pH – were lower in the IUGR group than in the control group (p<0.001, p<0.001, p<0.001, and p=0.006, respectively). While the levels of the inflammatory markers ESR, hsCRP, IL-6, and TNF-α were higher in the IUGR group, the IL-10 level was found to be lower in the IUGR group (p<0.001, p=0.033, p<0.001, p=0.004, and p<0.001, respectively).

Correlations between outcome variables and inflammatory markers are shown in Table II. When the correlations between the parameters were examined, it was observed that as ESR, hsCRP, and IL-6 levels increased, birth weight, Apgar scores, and cord blood gas pH decreased *(*p<0.001, p<0.001, p<0.001, p<0.001, p=0.02, p=0.002, p=0.001, p=0.03, p<0.001, p<0.001, p<0.001, and p=0.02, respectively*)*. As TNF-α level increased, only birth weight and Apgar score at the 1st minute decreased (p=0.006 and p=0.048, respectively). When IL-10 level decreased birth weight, Apgar scores, and cord blood gas pH decreased (p<0.001 for all).

Diagnostic performances of the optimal cut-off values of biochemical parameters for the diagnosis of IUGR are shown in Table III. Levels of ESR, hsCRP, IL-6, IL-10 and TNF-α had AUC of 0.82, 0.62, 1.0, 0.93 and 0.67, respectively for the diagnosis of IUGR (p<0.001, p=0.04, p<0.001, p<0.001 and p=0.004, respectively). ESR levels higher than 3.5 mm/hr had a sensitivity of 72%, specificity of 96%, PPV of 94.7% and NPV of 77.4%. hsCRP levels higher than 0.7 mg/L had a sensitivity of 42%, specificity of 100%, PPV of 100% and NPV of 63.3%. IL-6 levels higher than 3.2 pg/ml had a sensitivity of 100%, specificity

Table II. Correlations between outcome variables and inflammatory markers (r).

	Birth weight	Apgar score at 1st min	Apgar score at 5th min	pH (cord blood gas)
ESR (mm/hr)	-0.52**	-0.59**	-0.61**	-0.39**
hsCRP (mg/L)	-0.24*	-0.31**	-0.33**	-0.22*
IL-6 (pg/mL)	-0.73**	-0.66**	-0.58**	-0.23**
IL-10 (pg/mL)	0.62**	0.60**	0.55**	0.37**
TNF-α (pg/mL)	-0.27**	-0.20*	-0.15	-0.03

*p<0.05, **p<0.01
ESR: Erythrocyte sedimentation rate; hsCRP: High sensitivity C-reactive protein; IL: Interleukin; TNF: Tumor Necrosis Factor.

Table III. Diagnostic performances of the optimal cut-off values of biochemical parameters for the diagnosis of IUGR.

	AUC	Sensitivity%	Specificity%	PPV%	NPV%
ESR >3.5 mm/hr	0.82	72	96	94.7	77.4
hsCRP >0.7 mg/L	0.62	42	100	100	63.3
IL-6 >3.2 pg/mL	1.0	100	100	100	100
IL-10 <8.7 pg/mL	0.93	80	86	85.1	81.1
TNF-α >1.0 pg/mL	0.67	66	60	62.3	63.8

AUC: Area under the curve; ESR: Erythrocyte sedimentation rate; hsCRP: High sensitivity C-reactive protein; IL: Interleukin;NPV: Negative predictive value; PPV: Positive predictive value; TNF: Tumor Necrosis Factor.

of 100%, PPV of 100% and NPV of 100%. IL-10 levels lower than 8.7 pg/ml had a sensitivity of 80%, specificity of 86%, PPV of 85.1% and NPV of 81.1%. TNF-α levels higher than 1.0 pg/ml had a sensitivity of 66%, specificity of 60%, PPV of 62.3% and NPV of 63.8%.

Discussion

This study determined that neonatal outcomes including birth weight, Apgar score, and cord blood gas pH were lower in the IUGR group. ESR, hsCRP, IL-6, and TNF-α were higher in the IUGR group, but IL-10 levels were lower in that group. In addition, IL-6 was identified as a promising diagnostic marker for the diagnosis of IUGR (AUC=1; sensitivity 100%, specificity 100%, PPV of 100%, NPV 100% with a cut-off value of 3.2 pg/ml)

Optimal adaptation of the maternal immune system is essential for a healthy pregnancy. In this adaptation process, changes occur in the innate and adaptive immune systems through acute-phase reactants and cytokines[16]. In the literature, there are inconclusive studies of changes in acute-phase reactants in pregnant women whose fetuses experience IUGR. In a study conducted by Karli et al[9], maternal serum CRP levels were found to be higher in the IUGR group than in healthy controls. However, in a study by Kara et al[17], no significant difference in hsCRP values was shown in IUGR patients compared to normal pregnant patients. Similarly, in another study[18], CRP levels were also found not to differ in patients diagnosed with IUGR. Interestingly, in a prospective study[10], CRP levels measured before the 20th week of gestation were found to be a potential predictor of small for gestational age births. Similarly, Gandevani et al[19] reported another prospective study showing that CRP levels measured between 14-20 weeks of gestation were associated with fetal birth weight. There is also a meta-analysis[20] showing that using aspirin, an anti-inflammatory drug, before 16 weeks of gestation in high-risk patients reduced the development of IUGR. Our findings determined that hsCRP and ESR levels were higher in the IUGR group. Given the results from the literature and our findings, we argue that it is likely that there is systemic inflammation in patients with IUGR.

There are conflicting data regarding the alteration of pro-inflammatory and anti-inflammatory cytokines in IUGR. Like with hsCRP, Kara et al[17] reported that IL-6 values in IUGR were not significantly different from normal pregnant women. Likewise, in another study[21], maternal pro-inflammatory TNF-α and IL-6 levels in serum showed no increase in the IUGR group. However, in the study conducted by Bartha et al[11], TNF-α values in maternal serum were found to be higher in IUGR patients with placental insufficiency; this difference was not seen in IUGR patients without placental insufficiency. Also, maternal serum IL-6 levels were found to be similar in IUGR and healthy control groups. In another study[22,23] examining placental cytokine mRNAs, decreased IL-10 mRNAs were found in the IUGR group. In a study conducted by Al-Azemi et al[12], IL-4 was found to be higher in the IUGR group. In addition, IL-6 and TNF-α levels were found to be higher in patients with IUGR and placental insufficiency, whereas anti-inflammatory IL-10 levels were found to be lower. This finding was interpreted as the pro-inflammatory process dominant in IUGR, and this process was aggravated in the presence of placental insufficiency. Our results showed that IL-6 and TNF-α levels were higher in the IUGR group, although IL-10 levels were lower. Also, IL-6 was found to be a potential biomarker for the diagnosis of IUGR (AUC=1; sensitivity 100%, specificity 100%, PPV of 100%, NPV 100% with a cut-off value of 3.2 pg/ml). Thus, as with hsCRP and ESR above, it can be said that our findings indicate that a pro-inflammatory process is involved in cases of IUGR.

Limitations

There are some limitations in our study. There was no study investigating ESR values in IUGR. To our knowledge, we have evaluated the association between ESR and IUGR for the first time. In addition, pro-inflammatory and anti-inflammatory cytokines were evaluated simultaneously in our study. Furthermore, our study results cannot be generalized due to the small sample size. Sensitivity, specificity, PPV and NPV values determined for IL-6 in the diagnostic performance analysis were affected by the small sample size. Therefore, the findings that can be obtained from larger samples are of great importance in proving the accuracy of our results.

Conclusions

Pro-inflammatory cytokines, ESR, hsCRP, IL-6, and TNF-α levels were higher while anti-inflammatory IL-10 levels were lower in IUGR patients. Thus, it is clear that there is a pro-inflammatory state contributing to the pathogenesis of IUGR. With further studies, it will be possible to identify the specific causes of IUGR and eliminate this inflammation, thereby hopefully preventing the development of IUGR.

Conflict of Interest

The authors declare that there is no conflict of interest.

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None.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation was performed by Elif Seren Tanrıverdi. Data were collected by Pınar Kırıcı, Fatma tanılır Çağıran. Statistical analysis was performed by Pınar Kırıcı and Zercan Kalı. The first draft was written by Pınar Kırıcı. The article was edited and finalized by Pınar Kırıcı. All authors approved the final version of the manuscript.

Ethics Approval

Ethics Committee approval was received from the Clinical Research Ethics Board of the Adıyaman University (Date: 19.01.2021, Approval No.: 2021/01-33).

Informed Consent

The informed consent was obtained from all patients before the study.

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