КЛИНИЧКИ ИСТРАЖУВАЊА

ЗГОЛЕМЕНИ ВРЕДНОСТИ НА IL-8 ВО РАН ВТОР ТРИМЕСТАР И НИВНАТА ПОВРЗАНОСТ СО ПРЕДВРЕМЕНО РАЃАЊЕ

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Извадок

Цитокините (IL-1, IL-6, IL-8, TNF- Alfa) се од исклучително значење во бременоста и тие се продуцираат од страна на постелката во амнионската течност и се зголемени доколку постои интраутерина инфламација. Целта на студијата беше да се докаже соодносот на покаченото ниво на IL-8 во амнионската течност во почетокот на раниот втор триместар (16-22 г.н.) и предвременото породување (< 37 г.н.). Материјал и методи: Во оваа проспективна студија беа вклучени 150 гравидни пациентки, каде постоеше медицинска индикација за изведување на амниоцентеза (напредната мајчина возраст, висок ризик на PRISCA I, суспектни аномалии на фетусот, вирусни инфекции, или по желба на мајката). По потпишана согласност за учество во студијата, сите пациентки беа анализирани, односно на сите пациентки им беше направен ехо преглед, вагинална цервикометрија, и беа земени дополнителни 5 мл амнионска течност при изведување на амниоцентезата. Сите пациентки беа следени сè до нивното породување, каде точно беше нотирана гестациската недела на породување, а потоа споредена со нивото на IL-8. Резултати: Сите 150 пациентки беа во периодот од 16-22 гестациска недела. Кај 20 од вкупно 150 пациентки констатиравме предвремено раѓање, додека, пак, 120 пациентки се породија во термин. 139 пациентки имаа зачнато природно, додека 9 со ИВФ и ЕТ, од кои три се породија предвремено. 80% од пациентките кои се породија предвремено имаа зголемени вредности на IL-8. Средната вредност на должина на цервиксот кај оние кои се породија во термин беше 32,1 мм, додека кај оние превремено породени беше 30,7 мм. Заклучок: Оваа студија јапотврди оправданоста за испитување цитокини како метод за откривање на асимптоматски промени кај пациентки кое ќе се породат предвремено.

CLINICAL SCIENCE

INCREASED LEVEL OF IL-8 IN AMNIOTIC FLUID IN EARLY SECOND TRIMESTER LINKED WITH PRETERM PREGNANCIES

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Key words: preterm birth, amniotic fluid, IL-8, amniocentesis, infection

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Abstract

Cytokines (IL-1, IL-6, IL-8, TNF- alfa) are of crucial importance during pregnancy; they are produced by the placenta in the amniotic fluid and they are elevated in case of intrauterine inflammation. The aim of the study was to prove the ratio between the increased IL-8 in the amniotic fluid in the beginning of the second trimester (16-22 g.w.) and premature birth (< 36.6 g.w.). Material and methods: This was a prospective study that included 150 pregnant patients that had clinical indication for amniocentesis (advanced mother's age, abnormal test of PRISCA I, suspicious anomalies of the fetus, virus infection or mother's wish). They all gave a signed consent on being informed about the aims of the study, and following the protocol, they were analyzed and examined i.e. all patients underwent ultrasound examination, vaginal cervicometry. Five ml. of amniotic fluid during the process of amniocentesis was taken for the purpose of the study. All patients were followed until they gave birth, and the exact week of gestation was noted and compered with the IL-8 level. Results: All 150 patients were in the period of 16th-22nd gestational weeks. Twenty of the total of 150 patients had preterm delivery. A total of 139 patients conceived naturally and 9 patients underwent in vitro fertilisation (IVF) and embryo transfer (ET). In those with IVF and ET, 3 had preterm birth. 80% of patients that had preterm birth had increased IL- 8 levels. Median cervical length in those who gave birth at term was 32.1 mm and in those who gave preterm birth was 30.7mm. Conclusion: The study has confirmed the reason for examining cytokines as a method of discovering asymptomatic changes in patients who would give a premature birth.

Introduction

Intraamniotic fluid infections caused by viruses, bacteria or mycoplasmas are frequently followed by increased perinatal morbidity and mortality. Cytokines are key substances regulating a number of biological processes including reproductive and inflammatory processes1. An association between intraamniotic infections, rising concentrations of inflammatory cytokines in amniotic fluid and preterm labor is often present2. Many researches are driven to find reliable markers for intraamniotic fluid infections with a high predictive value that make possible detection of patients with intraamniotic infection3,4.

IL-8 is secreted and is an important mediator of the immune reaction in the immune system response, IL8 or chemokine (C-X-C motif) ligand 8. CXCL8 is a cytokine produced by macrophages and other cell types such as epithelial cells and endothelial cells5. Endothelial cells store IL-8 in their storage vesicles6. In humans, the interleukin-8 protein is encoded by the CXCL8 gene. IL-8 is initially produced as a precursor peptide of 99 amino acids, which then undergoes cleavage to create several active IL-8 isoforms7.

Interleukin-8 is a key mediator associated with inflammation where it plays a key role in neutrophil recruitment and neutrophil degranulation3, 5. As an example, it has been cited as a proinflammatory mediator in infections linked with preterm pregnancies and its presence in amniotic fluid.

The presence of IL-8 in amniotic fluid, as well as IL-6, suggests these cytokines are involved in important immunobiological events relevant to the third trimester of gestation1, 7.

Fetal development in uncomplicated pregnancies occurs in the absence of amniotic fluid infections and offspring microbial colonization starts after uterine contractions and rupture of amniotic membrane, therefore all earlier studies have suggested that both uterine cavity and amniotic fluid stay as “sterile womb”8.

However, the current evidence for a sterile intrauterine environment is inconclusive and the extent to which, if, and how maternal infections influence fetal immunologic development and the state of the infant is not still clear6, 7. Therefore, this study investigated the presence of an IL-8 in amniotic fluid in the second trimester of uncomplicated pregnancies and determined the amniotic fluid IL-8 load.

Midtrimester amniocentesis is the most widely used procedure for prenatal diagnosis of fetal cytogenetic abnormalities. However, the fluid and cells obtained at the time of the procedure are not currently being used to assess the risk of other causes of adverse pregnancy outcomes such as preterm birth, fetal growth restriction and preeclampsia. Preterm delivery occurring after preterm labor is a significant cause of neonatal morbidity and mortality9.

Antenatal upper genital infection is known to be strongly associated with preterm labor and delivery. Because such infection is often subclinical, intractable preterm labor may be the first sign of this pathologic process10.

Most women with preterm labor, who are later demonstrated to have chorioamnionitis, usually have no symptoms such as fever, abdominal pain, peripheral blood leukocytosis and fetal tachycardia11, 12. Therefore, identifying women with intrauterine infections is a major challenge.

Different amniotic fluid markers have been evaluated to determine intrauterine infection in women with symptoms of preterm delivery. Recently, levels of many cytokines such as IL-6, IL-8, C-reactive protein (CRP), matrix metalloproteinases-8, angiogenin were studied in the amniotic fluid at the time of the genetic amniocentesis for prediction of preterm delivery13, 14.

The aim of this study was to prove the ratio between the increased IL-8 in the amniotic fluid in the beginning of the second trimester (16-22 g.w.) and premature birth (< 36, 6g.w.).

Materials and methods

This prospective study was conducted at the University Clinic of Gynecology and Obstetrics, Ss Cyril and Methodius University, Medical Faculty, Skopje, Republic of North Macedonia at the Department of High Risk Pregnancy. The study included 150 pregnant women that had clinical indication for amniocentesis (advanced mother's age, abnormal test of PRISCA I, suspicious anomalies of the fetus, virus infection or mother's wish), during the period fromJ une 2018 to December 2018. Before entering the study, all patients gave their informed consent to participate in the study. The study was previously approved by the Ethics Committee of the Ss. Cyril and Methodius University, Medical Faculty, Skopje, Republic of North Macedonia. The examination was a prospective study. Pregnant women are were selected to enter the study between their 16-22 g.w. and were being followed until the end of the pregnancy. Each woman underwent obstetric ultrasound by which the gestational week was determined and it was confirmed that there were no exclusion criteria for the patient to enter the group of respondents.

The pregnant women were followed on Voluson 730pro for ultrasonography. Ultrasound cervicometry was done and the length of cervix was measured with a vaginal transducer. The results were recorded in the personal document for each patient. Each patient was taken a detailed anamnesis adapted to the needs and information needed for the research.

After the patient was examined, she was hospitalized and prepared for the procedure of amniocentesis.

The amniocentesis itself took place in the ultrasound and diagnostics ward at the Department of High Risk Pregnancy. Each amniocentesis was done in special sterile conditions with highly determined protocol and was controlled by an ultrasound. It was done in the period between 16-22 gestational weeks. Before the intervention, the whole procedure was described to the patients. A 22 gauge spinal needle was inserted into the amniotic cavity transabdominally under sonographic guidance and 5 ml of amniotic fluid was aspirated for the needs of the study. Each sterile syringe was marked with the name and surname of the patient, immediately after the intervention. Patients were discharged from hospital on the same day.

Inclusion criteria: Single pregnancy, patients who need amniocentesis in their early second trimester due to clinical indication (advanced mother's age, abnormal test of PRISCA I, suspicious anomalies of the fetus, virus infection or mother's wish), pregnancy from 16-22 gestational week, patients who had no signs of miscarriage (spontaneous abortion) while the amniocentesis was being made.

Exclusion criteria: Positive test of amniocentesis- abnormal karyotype, multiple pregnancies, patients who would not be able to be contacted and there would be no information on the pregnancy outcome, confirmed fetal anomalies or patients where pregnancy was prematurely terminated due to other reasons such as trauma, preeclampsia, placental abruption etc.

Biological samples and their analysis

Amniotic liquid: After amniocentesis, the amniotic fluid specimens were centrifuged at 1500 g for 10 min immediately after collection, and then frozen and stored at – 20°C for further analysis. The IL-8 concentration in the amniotic liquid was measured by a device - Immulite 2000 HP, Immulite 1000 HP Diagnostic Products Corp, at the Institute of Immunology and Human Genetics. This technique was realized and the analyses of the results were done in accordance with the manufacturer's instructions.

Statistical analysis

A database in the statistical program SPSS for Windows 23.0 was created for the purpose of data statistical analysis obtained during the study. The numerical, i.e. the quantitative parameters were shown with an average, standard deviation, median and inter-quarter rank. Qualitative i.e. attributive parameters are shown by distributing frequencies. For comparing women who gave premature birth with those who gave birth at

term, Mann-Whitney test was used. Statistical significant values were set at the value of $p<0.05$.

Results

This study included 150 patients who underwent amniocentesis during which 5 ml of amniotic fluid was taken for examination of IL-8. Also, ultrasound examination and cervicometry were done. All patients were in the period of 16th-22nd gestational week. Twenty of the total of 150 patients gave premature birth, and 120 gave birth at term. A total of 139 patients conceived naturally and 9 patients underwent IVF and ET. In those with IVF and ET, 3 gave preterm birth. 80% of patients that had preterm birth had increased IL- 8 levels. Median cervical length in those who gave birth at term was 32.1 and in those who gave preterm birth was 30.7.

Nine of all patients had pregnancy with IVF and ET. In the group that gave birth before 37 weeks of gestation, 15% (3) had undergone treatment with IVF and ET. In the group that gave birth after 37 weeks of gestation, 4.7% (6) had undergone treatment with IVF and ET. Differences between patients that had IVF and ET and those that conceived naturally, and the gestational week of delivery were not significant (p=0.19) (Table 1).

Table 1. Distribution of patients according to way of conceiving and gestational week at birth

Way of conceiving	Gestational week at birth			p-level
	n	At term	Preterm	
		N (%)	N (%)	
Normal	139	122 (95.31)	17 (85)	X^2=1.67
IVF et ET	9	6 (4.69)	3 (15)	p=0.19 ns

Yates Chi-square

Cervical length of women who gave preterm birth (< 37 g.w.) was not significantly shorter than that in women who gave birth at term (30.7 ± 2.7 vs.32.12 ± 3.8; p=0.11) (Table 2).

Table 2. Cervical length and gestational week at birth

Term of delivery	Descriptive Statistics (cervical length)			p-level
	n	mean ± SD	min-max	
Term	128	32.12 ± 3.8	22 – 40	t=1.6
Preterm	20	30.7 ± 2.7	27 – 39	p=0.11 ns

T-tests

Cervical length shorter than 30 mm was measured in those women that gave birth before 37 weeks of gestation (preterm) – 60% (12), opposite of those that gave birth at term 40.6% (52). However, there was no significant difference in both groups regarding cervical length. It means that in women with cervical

length longer than 30 mm we found not enough evidence for statistically significant difference (p=0.1).

Patients that gave preterm birth had significantly higher amniotic fluid levels of IL -8 in comparison with those that gave term birth (p<0.0001) (Table 3). Mean amniotic IL-8 level in the preterm group (before 37 w.g) and at term (after 37 w.g.) was 1585.7 ± 834.7 and 360.65 ± 459.3 pg/ml, respectively. The median was 1598 and 198 pg/ml, respectively.

Table 3. IL-8 levels and gestational week of delivery

Term at delivery	Descriptive Statistics (IL-8)			p-level
	n	mean ± SD	median (IQR)	
Term	128	360.65 ± 459.3	198.2(78.4 – 427.2)	Z=5.99
Preterm	20	1585.7 ± 834.7	1598.8(832.5-2251.6)	p=0.00000

Mann-Whitney

Normal range of cytokine IL-8 in amniotic fluid was measured in 20% (4) of preterm births and 88.3% (113) of term births. Higher values, up to 1780 pg/ml, were found in 35% (7) of preterm births and 8.6% (11) of term births. Higher than 1780 pg/ml values had 45% (9) of preterm births, and3.1% (4) of patients that gave term births (Figure 1).

Statistically significant difference was registered in those patients whose IL-8 levels in amniotic fluid were up to 700 pg/ml to 1780pg/ml and higher than 1780 pg/ml. There was a statistical confirmation as significance (p<0.001) of IL-8 levels between groups of patients that gave birth at term and those that gave preterm births. (Table 4).

Table 4. Differential values of IL-8 in term and preterm patients and their significance

IL-8 pg/ml	Term at birth			p-level
	n	In term N (%)	preterm N (%)	
< 700		113 (88.28)	4 (20)	X2=54.6 p=0.0000
701 – 1780		11 (8.59)	7 (35)	
>1780		4 (3.13)	9 (45)	

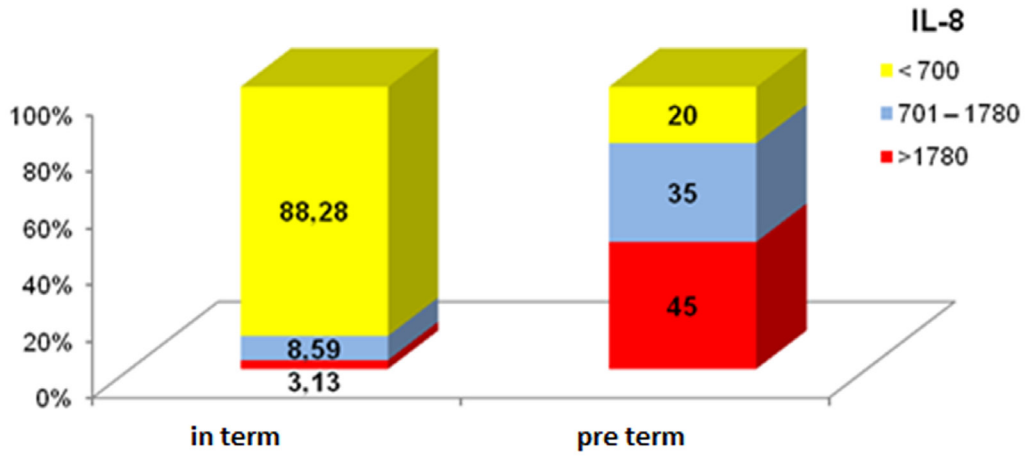

Figure 1. IL-8 in term and preterm births

Discussion

Premature birth is a significant cause of neonatal morbidity and mortality worldwide, and it places an enormous economic burden on healthcare resources. Identifying patients at risk of preterm delivery at early stage of pregnancy is allowing interventions that result in lower preterm birth rates and associated decrease in neonatal morbidity and mortality15. Early diagnosis of intraamniotic infection remains a problem. To permit intervention, a test should detect infection before it is clinically apparent. Ideally the test should be rapid, simple, inexpensive, and capable of being performed in hospital at any time of day. Ultimately a valuable test must be sufficiently accurate to warrant the risks of intervention, particularly at early gestational ages2, 16, 17.

Previous authors found that amniotic fluid IL-8 levels increase from 17 weeks to pregnancy until term18, 19, 20. A peak in mean amniotic fluid IL-8 concentration was found at about 22 weeks of gestation followed by decline, which resulted in a mean concentration of 460 pg/ml at term21. An increased amniotic fluid IL-8 level has been found by others to be as accurate as or more accurate than that of gram stain for the early detection of intraamniotic infection in the symptomatic patients with spontaneous preterm delivery22. With a threshold of 700 pg/ml, Romero et al. found a sensitivity of 87% compared to 65% of the gram stain23, whereas Kirshon and Rosenfeld reported 91% sensitivity at 1800 pg/ml threshold compared to 66% of the gram stain24. While amniotic fluid IL-8 levels seem to serve as an index of intraamniotic infection during the peripartum period in preterm and term pregnancies, their possible value as a diagnostic tool in earlier phases of pregnancy is still uncertain9,15,17,25,26. However, in some recent studies subclinical intrauterine inflammatory cytokine response such as increased CRP, IL-6, matrix metalloproteinases and angiogenin concentration already might be present very early in the gestation27. The relative risk of preterm delivery in the presence of elevated concentrations of inflammation markers (IL-6, CRP, TNF-a, angiogenin) at the time of genetic amniocentesis has been consistently reported to be elevated28.

The results obtained in this study, support the expected hypothesis that the increased IL-8 in the amniotic fluid, although in asymptomatic patients, still affects the outcome of the pregnancy i.e. its increase leads to premature birth. The examination is more valuable since we know that 5-18% in total of the full number of births in our Clinic belongs to this group. The results have confirmed that risk factors for premature birth include shortened cervix (Table 2) and presence of increased values of the inflammatory marker IL- 8 in the amniotic fluid.In the group of patients who gave premature birth, the average value of gestational week was from 32nd to 36th, whereas in the group with normal values, the most common findings showed delivery on time, i.e. in the 37th gestational week (Figure 1). The examinations in which amniotic fluid is used for researches of cytokines, are relatively new and done to a small series of patients29. In the examined group, changes have been observed in other parameters i.e. the cervical length, but not in the number of leukocytes, and the values of glucose in the amniotic liquid which suggests that the increased cytokines i.e. IL-8 as a risk factor affect the outcome and time of giving birth30. However, a large amount of research has been devoted to determining risk factors and predictors of preterm delivery and while advances have been made, no single method has been identified that can consistently predict which patients will delivery prematurely2,17,31.

Conclusion

This study is the first one done in Republic of North Macedonia aimed at examining any kind of changes in the amniotic fluid, regardless of gestational age. The study has so far confirmed the reason for examining cytokines as a method to discover asymptomatic

changes in patients who would give a premature birth. To date, there have been few studies that have addressed the same hypothesis. The increased levels of IL-8 connected with preterm birth in the pathophysiological pathways may provide an understanding as to why various therapeutic interventions succeed in some people and fail in others. They may also explain differences in sensitivity to various stimuli that trigger preterm labor. If these results are true, then the implication is that there are innate and differentially distributed risk factors among populations with respect to immune responses.

The further course of the study will additionally determine the values and frequency of changes in premature birth. The expected results are those shown in patients who do have certain inflammatory agent (increased IL-8), shortened cervix and will have more common complications i.e. it would be expected that there is an increased risk of a premature birth. The benefit of the study lies in detecting asymptomatic cases, so that this complication can be prevented on time. This type of examination would contribute to reduction of premature births, which goes along with a high rate of morbidity and mortality as well as high costs at the Clinic regarding these complications. It would be useful to create an algorithm for multidisciplinary treatment of these patients. These results should provide fertile grounds for further research in this area.

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