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ROLE OF CYTOKINES AND THEIR PRESENCE IN THE AMNIOTIC LIQUID AS A SIGN OF EARLY DETECTION OF PREMATURE BIRTH IN PREGNANT WOMEN

УЛОГА НА ЦИТОКИНИТЕ И НИВНО ПРИСУСТВО ВО АМНИОНСКА ТЕЧНОСТ КАКО ЗНАК ЗА РАНА ДЕТЕКЦИЈА НА ПРЕДВРЕМЕНОТО ПОРОДУВАЊЕ КАЈ ТРУДНИЦИ

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Abstract

Introduction. Cytokines play a significant role in the pregnancy. They are very powerful and important mediators of the cell growth as well as regulators of the immune and inflammatory reactions. Several cytokines (IL-1, IL-6, IL-8, TNF- alfa) are of crucial importance during the pregnancy since they are produced by the placentain the amniotic fluid, in case there is intrauterine inflammation. Inpatients with premature birth, the intrauterine inflammation and infection is often present and leads to inflammatory syndrome of the human fetus. The intrauterine infection of the choriodecidual space and the amniotic fluid are the most common reasons for this obstetric complication, hence the most commonly examined etiologic factor.

Aim. The study was conducted in order to prove the ratiobetween the increased level ofIL-6in the amniotic liquid at the beginning of the second trimester (16-22 g.w.) and the premature birth (< 36 g.w.).

Methods. This is a case control study that has included 36 patientsso far.The pregnant women wererecruited fromthe Clinic of Gynaecology and Obstetrics. They all gave a signed consent on being informed about the aims of the study, and following the protocol, they were analyzed and examinedi. e. all patients under went ultrasound examination, vaginal cervicometry; cervical and vaginal swabs were taken and 5 ml. amniotic fluid during the process of amniocentesis.

The study was performed at the Clinic ofGynaecology and Obstetrics, the Institute of Microbiology and Parasitologyas well as the Institute ofImmunology and Human Genetics.

Results. The results obtained inthisstudy have confirmed the role of the cytokines i.e. they have shown an increase when there is inflammation in the intrauterine cavity which could lead in future to premature birth. There was an association between the risk of premature birth and positive cervical and vaginal swabs, length of cervix, and not a single case showed positive amnio-culture.

Keywords: premature birth, amniotic fluid, cytokines, IL-6, amniocentesis,pregnancy

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Апстракт

Вовед. Цитокините играат многу важна улога во бременоста. Тие се многу силни и важни медијатори на клеточен раст и регулатори на имунолошки и инфламаторни реакции. Неколку цитокини (IL-1, IL-6, IL-8, TNF-alfa) се од исклучително значење во бременоста и истите се продуцираат од страна на постелката во амнионската течност, доколку постои интраутерина инфламација. Кај пациентки кај кои настанува предвремено породување, интраутерина инфламација и инфекција е многу често присутна и води до инфламационен синдром и на плодот. Интраутерина инфекција на хориодецидуалниот простор и амнионската течност е најчеста причина за настанување на оваа обстетричка компликација, а со тоа и најчесто испитуван етиолошки фактор.

Цел. Оваа студија беше спроведена за да се докаже соодносот на покаченото ниво на IL-6 во амнионската течност во почетокот на вториот триместар (16-22 г.н.)ипредвременото породување (<36 г.н.).

Методи. Во рамките на студијата досега се обработени 36 пациентки. Станува збор за case control студија. Трудниците се регрутирани на Клиниката за Гинекологија и Акушерство каде по потпишаната информирана согласност по протокол истите се обработени односно на сите пациентки им е направен ехо преглед, вагинална цервикометрија, земени се цервикални и вагинални брисеви и 5 мл амнионска течност при изведување на самата амниоцентеза. Студијата се изведува на Клиниката за гинекологија и акушерство, Институтот за микробиологијаи

паразитологија и Институтот за имунологија и хумана генетика.
Резултати. Во рамки на студијата добиените резултати ја потврдуваат улогата на цитокините односно нивно зголемување при постоење на инфламација во интраутеринатa празнина која во иднина би довела до предвремено породување. Испитуваната група на овие пациенти покажа поврзаност при зголемување на цитокинот IL-6 и предвремено породување. Исто така покажа поврзаност на ризикот од предвременото породување со позитивните цервикални и вагинални брисеви, должина на цервискот, а во ниеден случај не се доби позитивна амниокултура.

Клучни зборови: предвремено породување, амнионска течност, цитокини, IL-6, амниоцентеза, бременост

Introduction

Cytokines play an important role during the pregnancy [1-3]. They are very powerful mediators for the cell growth as well as regulators of immune and inflammatory reactions. They are either polypeptides or glycopeptides which act through specific receptors in the cell it self and the cell membrane [4]. They can be positive or negative regulator of the immune response. They are messengers which together with hormones and neurotransmitters belong to the group of most important communication materials between cells [5]. Cytokines transfer the information to the target cell which coincides with its receptor. That is how activation and change in the target cells occur [7,8].
Cytokines act as powerful molecules which are released from the cells and then transported to other parts of the organism and act to the function of other cells, which leads to numerous biological reactions. Each live cell with a core in the human organism creates cytokines whose type and quantity of secretion depends on the type and extent to which cells differentiate i.e. degree of their activation stage. The creation of cytokines is encouraged by antigen specific activation of lymphocyte T4 [4].
Cytokines include a group of interleukines, tumor factors of growth and interferons. This division is made depending on the biological and structural differences, but onthe similarity of these mediators, too. Interleukins were named after their function in the mutual communication with the leukocytes. Today we know 29 types of interleukins which are marked with numbers from IL-1 untilIL-29 [8].
Interleukin 6(IL-6) belongs to the inflammatory cytokines and is secreted during inflamed conditions. It is created by many immunogenic cells but also by many non-immunogenic cells and organs which help the control of the inflamed reactions [9].
The gestational tissue including placenta, extravillous trophoblast, amnion, and mother's deciduas are produced by cytokines themselves [7]. These cytokines are considered to affect the outcome of the pregnancy [8-10].
It is thought that the increased level of IL-6, IL-1, IL-2, IL-8, TNF- alphain the amniotic fluidleads to a bad outcome of the pregnancy, but depending on the cause of the increase [2,11-13].
The amniotic fluidis a sterile environment in a normal pregnancy [14,15]. It is a complex body liquid which has an important role in every pregnancy. Its functions are nutritive, protective but also diagnostic for the fetus [16]. Its content changes during the progression of the pregnancy. It contains exclusively important and complex substances which are essential for the normal fetal development [17,18].
The amniotic fluidhas been used for a long time for diagnosing the intra-amniotic inflammation which is closely related to the occurrence of premature birth. The indicators that suggest presence of inflammation are: increased level of matrix metalloproteinases (MMPs) (e.g.MMP-9) [21-23], increased interleukins (e.g. IL-6, IL-1), TNF- α, Granulocyte-colony stimulating factor (G-CSF), increasedLe, low glucose level, etc [24]. The main cytokines for identification of the intra-amniotic inflammation, most closely related to premature birth are the (IL-6) interleukins [9].
Premature birth is present in 5 to 18% of the pregnancies and is the main reason for the neonatal morbidity and mortality [25]. It is in fact every birth which occurs after a possible viability of the fetus i.e. the 24^{th} gestational week, but before the full 37^{th}. The spontaneous occurrence of the contractions or premature bursting of the placenta is considered a reason in around two thirds of these deliveries. Each delivery before the 24^{th} gestational week is considered a miscarriage. Currently, the 23^{rd} gestational week is considered to be a grey zone [3,27].
According to the time of occurrence of premature births, there are three gestational periods. Late premature birth from 32^{nd} -37^{th} g.w., early premature week from 28^{th} to 32^{nd} g.w., and extremely early premature birth under the 28^{th} g.w., i.e. from 24^{th} to 28^{th} g.w. [28].
The etiology of occurrence of premature birth varies depending on the gestational age [29].
Inpatients who have premature birth, the intrauterine inflammation and infection are present and can lead to inflammatory syndrome to the fetus. The subclinical intrauterine infection of the choriodecidual space and amniotic fluidis the most common reason for occurrence of this obstetric complication, and it is the most common examined etiologic factor [1,25]. The uterine cavityis normally sterile but the vagina contains normal bacteria flora. Depending on the concentration of bacteria and vagina resistance, bacteria can ascend from the vagina to the cervix and get to fetal membrane. They might activate the decidua in order to produce inflammation, hence to activate inflammatory mediators

that would later increase the prostaglandins; and they directly affect the myometrium and provoke contractions. The placenta around the fetus might weaken and burst. The neonatal sepsis, mother postpartum endometrial histological chorioamnionitis are diagnoses which are significantly more common in premature birth, especially in those occurring before the 32nd g.w [26].
Apart from the infection, there are other reasons for occurrence of premature birth such as: overstretching of the uterine wall, surgical procedures of the genital organs, abnormal uterine cavity, cervical weakness and idiopathic [27].
If the asymptomatic change in the amniotic fluid, i.e. the increased level of the cytokines is discovered on time, it will contribute toearly therapeutic intervention. Until now, there are no official data in Macedonia from the examinations of the amniotic fluidin pregnancy, especially when patients have not had any symptoms and changes [27].
The aimof the study wasto prove the ratio between the increased IL-6 in the amniotic fluidatthe beginning of the second trimester (16-22 g.w.) and premature birth (< 36 g.w.).

Material and methods

The study included 36 patients of the planned 150, during the period from 01.06.2018 to 01.08.2018.All patients wererecruited fromthe Clinic ofGynecology and Obstetrics. Prior to inclusion in the study, the pregnant women gave their written consent to participate in the study. The study was previously approved by the Ethics Committee at the Faculty of Medicine in Skopje. The examination wasa case control study. Pregnant women wereselected to enter the study between their 16-22 g.w. and werebeing followed until they gavebirth. Each pregnant woman underwent an obstetric ultrasound bywhich the gestational week wasdetermined and confirmed that there wereno exclusion criteria for the patient toenter the examined group.
The pregnant women were followed onVoluson 730pro for ultrasonography. The patients presentedmedical results from vaginal and cervical swabs and in the casewhen such examinationhad not been done, they were sent to the Institute of Microbiology and Parasitology – Skopje. Ultrasound cervicometry was done and the length of cervix was measured with a vaginal transducer and the results were recorded on the personal document for the patient. Each patient was taken a detailed anamnesis and information adapted to the needs for the research.
After examining the patients, they were hospitalized at the Clinic ofGynecology and Obstetrics, and thepreparation for the procedure of amniocentesis followed.
The amniocentesis itself took place in the ultrasound and diagnosis ward, within the Department of pathological 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.Prior tothe intervention, the whole procedure was described to the patients. The amniocentesis was then performed andadditional 5ml amniotic fluidwastaken for further examination.
Each sterile syringe was marked with the name and surname of the patient, immediately after the intervention. Patients were discharged from the hospital the same day.

Inclusion criteria:

1. Single pregnancy
2. 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)
3. Pregnancy from 16-22 gestational weeks
4. Patients who have no signs of miscarriage (spontaneous abortion) while the amniocentesis is being made.

Exclusion criteria:

1. Positive test of amniocentesis- abnormal karyotype.
2. Multiple pregnancies.
3. Patients who will not contacted and there will be no information on the pregnancy outcome.
4. Confirmed fetal anomalies or patients where pregnancy is prematurely terminated due to other reasons not related to the inflammatory processes such as trauma etc.

Biological samples and their analysis:

Amniotic fluid:

In a separated sample of the amniotic fluid, the number of leukocytes and glucose level were measured. These examinations were done in the biochemistry laboratory of the University Clinic of Gynaecology and Obstetrics-Skopje.
The IL-6 concentration in the amniotic fluid was measured by a device-Immulite 2000 HP, Immulite 1000 HP Diagnostic Products Corp, at the Institute for Immunology and Human Genetics.
The realization of this technique and analysis of the results obtained were done in accordance with the instructions from the manufacturer.
An aliquot of 2ml of the sample was sent to the Institute of Microbiology and Parasitology-Faculty of Medi-

cine-Skopje, where the process of coloring a gram and amnio-culture was done, by using standard bacteriological techniques [28].

Statistical analysis

A database in the statistical program SPSSfor Windows 23.0 was createdfor the purpose of analyzing the results obtained in the research.
The numerical, i.e. the quantitative parametersare shown with an average, standard deviation, median and interquarter rank.
Qualitative i.e. attributive parametersare shown by distributing frequencies.
Mann-Whitney test was used for comparing women who gave premature birth and those who gave term birth.
Statistical significant differenceswere set at $p<0.05$.

Results

This study included36 patients who underwent amniocentesis during which 5ml of amniotic fluid was taken for examination of IL-6, amnio-culture, leukocytes and glucose. Also, vaginal and cervical swabs were taken as well as ultrasound examination and cervicometry. All patients were in the period of 16^{th}-22^{nd} gestational week.
Five (13.9%)of the total 36patients gave premature birth (Table 1).
All 5 patients had increased IL- 6 level (Table 1).
Three of the patients had positive primary vaginal and cervical swabs (*Ureaplasmaurealyticum, Gardnerella vaginalis, Candida albicans*). Three patients had shortened cervix, i.e. it was smaller than 30mm and none of them had a positive amnio-culture. Values of leukocytes and glucose were not increased (Table 1).

Table 1. Values of analyzed parameters in women who gave premature birth

Length of cervix	Glucose concentration in amniotic fluidmmol/l	Conc. Of Le in amniotic fluid	Cervical and vaginal swabs	IL-6 concentration in amniotic fluid Pg/ml	Gestational week during giving birth	Amnio-culture
22	1	4	*Ureaplasma urealyticum*	2234	32	Neg.
29	0	2	/	800	35	Neg.
31	1	0	/	867	36	Neg.
30	3	1	*Ureoplasma urealyticum Gardnerella vaginalis*	1322	34	Neg.
28	2	1	*Candida albicans*	922	36	Neg.

The results of this study showed that cervix length was significantly different in women who gave premature birth compared to those who gave birth on time ($p= 0.049$). Significantly shorter cervix was measured in the group of women who gave premature birth.
The average cervix length in this group was 28.0±3.5, median 29, whereas in the other group the average cervix length was 31.03 ± 2.5, and median 31.

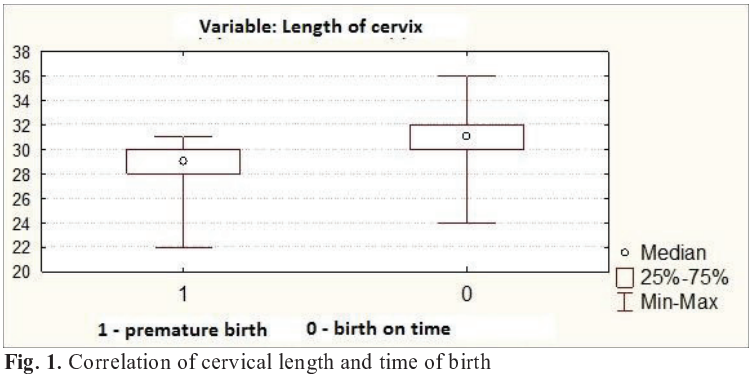

Fig. 1. Correlation of cervical length and time of birth

CytokineIl-6 showed significantly different values in women who gave premature birth and those who gave birth on time ($p=0.00039$). Significantly higher concentration of this inflammatory marker was measured in the group of those with premature birth.
The average value ofIl-6in the group with premature birth was 1229.0 ± 597.5, median 922; average and median value ofIl-6in the group of those who gave birth within their term was 374.52±155.2 and 326 consequently.

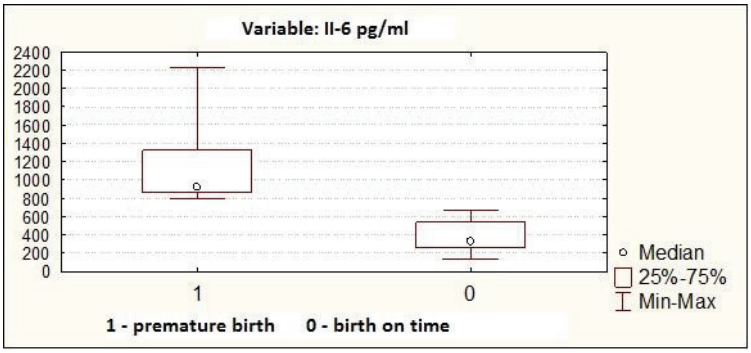

Fig. 2. Correlation of value of IL-6 and time of birth

Discussion

The results obtained in this study, which is still ongoing, support the expected hypothesis that the increased IL-6 in the amniotic fluid, although in asymptomatic patients, still affects the outcome of the pregnancy i.e. its increase leads to premature birth (Figure 1). 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 vaginal and cervical infection [29,30], shortened cervix (Figure 1) and presence of increased values of the inflammatory marker IL-6 in the amniotic fluid. In the group of patients who gave premature birth, the average value of gestational week was from32nd 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 2). The examinations in which amniotic fluid is used for researches of cytokines, are relatively new and done to a small series of patients [31-33]. In our case, some of these results have been partially analyzed. In the examined group, changes have been observed in other parameters i.e. in vaginal and cervical swabs, in the cervix 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-6 as a risk factor affect the outcome and time of giving birth. However, only a small number of the examined subjects planned for the whole study has been analyzed, i.e. 36, which means that we should be careful with the interpretation of the results obtained. In the further course of the study, more detailed results will be presented and they will be more representative due to the larger number of included subjects.

Conclusion

This study is the first one done in 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. 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-6), shortened cervix, presence of microorganisms, and will have more common complications i.e. it would be expected that there is an increased risk of a premature termination of the pregnancy. 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.

Conflict of interest statement. None declared.

References

1. Romero R, Miranda J, Chaemsaithong P, *et al*. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes, *The Journal of Maternal-Fetal & Neonatal Medicine* 2015; 28(12): 1394-1409. DOI: 10.3109/14767058.2014.958463.
2. Romero R, Miranda J, Chaiworapongsa T, *et al*. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. *Am J Reprod Immunol* 2014; 71: 330-358.
3. Kunze M, Klar M, Morfeld CA, *et al*. Cytokines in noninvasively obtained amniotic fluid as predictors of fetal inflammatory response syndrome. *Am J Obstet Gynecol* 2016; 215: 96: e1-e8.
4. Dinarello CA, Gelfand JA, Wolff SM. Anticytokine Strategies in the Treatment of the Systemic Inflammatory Response Syndrome. *JAMA* 1993; 269: 1829-1835.
5. Mantovani A, Bussolino F, Introna M. Cytokine regulation of endothelial cell function: from molecular level to the bedside. *Immunol Today* 1997; 18: 231-240.
6. Papanicolaou DA, Wilder RL, Manolagas SC, Chrousos GP. The pathophysiologic Roles of Interleukin-6 in Human Disease. *Ann Intern Med* 1998; 128: 127-137.
7. Kim CJ, Romero R, Chaemsaithong P, *et al*. Acute Chorioamnionitis and Funisitis: Definition, Pathologic Features, and Clinical Significance. *American journal of obstetrics and gynecology* 2015; 213(40): S29-S52. doi:10.1016/j.ajog.2015.08.040.

8. Kim SM, Romero R, Lee JH, *et al*. About one-half of early spontaneous preterm deliveries can be identified by a rapid matrix metalloproteinase-8 (MMP-8) bedside test at the time of mid-trimester genetic amniocentesis. *J Matern Fetal Neonatal Med* 2016; 29(15): 2414-2422. Published online 2015 Dec 7. doi: 10.3109/14767058.2015.1094049.
9. Chaemsaithong P, Romero R, Korzeniewski SJ, *et al*. A point of care test for interleukin-6 in amniotic fluid in preterm prelabor rupture of membranes: a step toward the early treatment of acute intra-amniotic inflammation/infection, *The Journal of Maternal-Fetal & Neonatal Medicine* 2016; 29(3): 360-367. DOI: 10.3109/14767058.2015.1006621.
10. Chaemsaithong P, Romero R, Korzeniewski SJ, *et al*. A point of care test for the determination of amniotic fluid interleukin-6 and the chemokine CXCL-10/IP-10, *The Journal of Maternal-Fetal & Neonatal Medicine* 2015; 28(13): 1510-1519. DOI: 10.3109/14767058.2014.961417.
11. Chaemsaithong P, Romero R, Korzeniewski SJ, *et al*. A point of care test for interleukin-6 in amniotic fluid in preterm prelabor rupture of membranes: a step toward the early treatment of acute intra-amniotic inflammation/infection, *The Journal of Maternal-Fetal & Neonatal Medicine* 2016; 29(3): 360-367. DOI: 10.3109/14767058.2015.1006621.
12. Ghezzi F, Franchi M, Raio L, *et al*. Elevated amniotic fluid C-reactive protein at the time of genetic amniocentesis is a marker for preterm delivery *American Journal of Obstetrics and Gynecology* 2002; 186(2): 268-273.
13. Harirah H, Donia SE, Hsu CD. Amniotic fluid matrix metalloproteinase-9 and interleukin-6 in predicting intra-amniotic infection. *Obstet Gynecol* 2002; 99(1): 80-84.
14. Figueroa R, Garry D, Elimian A, *et al*. Evaluation of amniotic fluid cytokines in preterm labor and intact membranes. *J Matern Fetal Neonatal Med* 2005; 18(4): 241-247.
15. El-Bastawissi AY, Williams MA, Riley DE, *et al*. Amniotic fluid interleukin-6 and preterm delivery. *Obstetrics & Gynecology*, Volume 95, Issue 6, Pages 1056-1064.
16. Underwood MA, Gilbert WM, Sherman MP, Amniotic F. Not Just Fetal Urine Anymore. *Journal of Perinatology* 25: 341-348. doi:10.1038/sj.jp.7211290.
17. Gervasi MT, Romero R, Bracalente G, *et al*. Midtrimester amniotic fluid concentrations of interleukin-6 and interferon-gamma-inducible protein-10: evidence for heterogeneity of intra-amniotic inflammation and associations with spontaneous early (<32 weeks) and late (>32 weeks) preterm delivery. *Journal of perinatal medicine* 2012; 40(4): 329-343. doi:10.1515/jpm-2012-0034.
18. Martinez-Varea A, Roberto R, Xu Y, *et al*. Clinical chorioamnionitis at term VII: the amniotic fluid cellular immune response DOI 10.1515/jpm-2016-0225 Received July 7, 2016. Accepted August 10, 2016.
19. Kacerovsky M, Vrbacky F, Kutova R, *et al*. *PLoS One*. 2015; 10(5): e0126884. Published online 2015 May 20. doi: 10.1371/ journal. pone.0126884.
20. Philip N. Baker, Louise Kenny Obstetrics by Ten Teachers,19th Edition, March 25, 2011.
21. Gervasi MT, Romero R, Bracalente G, *et al*. Viral invasion of the amniotic cavity (VIAC) in the midtrimester of pregnancy. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies. *The International Society of Perinatal Obstetricians* 2012; 25(10): 2002-2013. doi:10.3109/14767058.2012.683899.
22. DiGiulio DB, Romero R, Amogan HP, *et al*. Microbial prevalence,diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008.
23. Park CW, Lee SM, Park JS, *et al*. The antenatal identification offunisitis with a rapid MMP-8 bedside test. *J Perinat Med* 2008; 36: 497-502.
24. Vousden N, Chandiramani M, Seed P, Shennan A. Interleukin-6bedside testing in women at high risk of preterm birth. *J MaternFetal Neonatal Med* 2011; 24: 1301-1304.
25. Conde-Agudelo A, Papageorghiou AT, Kennedy SH, Villar J. Novel biomarkers for the prediction of the spontaneous pretermbirth phenotype: a systematic review and meta-analysis. *BJOG* 2011; 118: 1042-1054.
26. Skoll MA, Moretti ML, Sibai BM. The incidence of positive amniotic fluid cultures in patients preterm labor with intact membranes**.** *Am J Obstet Gynecol* 1989; 161(3): 813-816.
27. Cobo T, Palacio M, Martinez-Terron M, *et al*. Clinical and inflammatory markers in amniotic fluid as predictors of adverse outcomes in preterm premature rupture of membranes. *Am J Obstet Gynecol* 2011; 205(126): e1-e8.
28. Romero R, Miranda J, Chaiworapongsa T, *et al*. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol* 2014; 72: 458-474.
29. Mendz GL, Kaakoush NO, Quinlivan JA. Bacterial aetiological agents of intra-amniotic infections and preterm birth in pregnant women. *Front Cell Infect Microbiol* 2013; 3: 58. doi: 10.3389/fcimb.2013.00058. eCollection 2013.
30. Arntzen KJ, Kjollesdal AM, Halgunset J, *et al*. TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. *J Perinat Med* 1998; 26: 17-26.
31. Romero R, Yoon BH, Mazor M, *et al*. A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. *Am J Obstet Gynecol* 1993; 169: 839-851.
32. Bobitt JR, Hayslip CC, Damato JD. Amniotic fluid infection as determined by transabdominal amniocentesis in patients with intact membranes in premature labor. *Am J Obstet Gynecol* 1981; 140: 947-952.
33. Romero R, Sirtori M, Oyarzun E, *et al*. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *Am J Obstet Gynecol* 1989; 161: 817-824.