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Amniotic fluid NF-kB concentration in pregnant women with a high risk of prenatal screening test results

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ABSTRACT

Objectives: Nuclear factor-kappa beta (NF-kB) can potentially be related to certain fetal chromosomal abnormalities. This study aimed to determine whether the concentration of NF-kB changes in the amniotic fluid (AF) of pregnant women who have a high risk of fetal down syndrome (DS) results in prenatal screening and diagnosis testing.

Material and methods: 108 patients with an abnormal first trimester combined screening test (FTCST) were subjected to amniocentesis and fetal karyotype analysis between 16 and 20 weeks of gestation. Amniocentesis material obtained from 86 patients conformed with our research criteria and only this was included in the study. Among the 86 amniocentesis results, there were 12 patients with confirmed DS. The karyotypes of the remaining patients were normal. Therefore the total study group was divided into two groups: patients with DS fetal karyotype (Group 1, n = 12) and patients with normal fetal karyotype (Group 2, n = 74). We used the ELISA method to assess the concentration of NF-kB and high sensitivity C-reactive protein (hsCRP) in each sample of AF.

Results: We observed significantly lower NF-kB concentrations in the AF of the women in Group 1 compared with the women in Group 2. Patients in Group 1 also had a higher concentration of hsCRP in their AF when compared with patients in Group 2. The FTCST results for patients in Group 1 showed a significantly higher risk than for those of Group 2. There were no statistically significant correlations detected when comparing the amniotic fluid nuclear factor-kappa beta (AF-NF-kB) levels with other clinical and laboratory parameters.

Conclusions: AF-NF-kB may play a role in the pathogenesis of fetal down syndrome.

Key words: down syndrome, nuclear factor-kappa beta, amniotic fluid, prenatal screening test

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INTRODUCTION

Human chromosomal diseases are characterized by chronic low- or high-grade inflammation (para-inflammation). Evaluation of AF may be a potential source of information about the pathological pathways that are critical in the development of chromosomally abnormal fetuses. The results of this type of research can lead to an understanding of the processes of abnormal fetal development. In addition to karyotype analysis, molecular examination of AF can help us better understand the developmental stages of the fetus in patients with a high risk of fetal DS results in prenatal screening and diagnosis testing. The AF of pregnant women with fetal DS shows a high incidence of biochemical variations [1–3]. Although inflammatory mediators have been well documented in pPROM and spontaneous preterm births with intact membranes, the main marker of inflammation, NF-kB, has not been measured in the AF of pregnancies with a high risk of fetal DS results in prenatal screening tests.

Activation of the NF-kB pathway is involved in the pathogenesis of chronic inflammatory diseases [4]. The deficient expression of immune response or inflammatory genes has been reported in fetuses with DS [3]. NF-kB is a stress-inducible transcription factor that regulates the host's immune and inflammatory responses. Nuclear factor-kB also inhibits apoptosis in response to DNA damage [5]. Stimulation of the NF-kB pathway activates the IkB kinases which phos-

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phorylate the inhibitory protein IkB. The degradation of IkB allows the translocation of NF-kB from the cytoplasm to the nucleus where it induces the expression of related genes [6–8]. For this reason, variations in NF-kB expression in the AF of pregnancies with a high risk of fetal DS results in prenatal screening tests were assessed and compared with those in pregnancies with normal karyotypes. The main objective of our study was to test whether NF-kB concentration alters in the AF of the pregnant women who have a high risk of fetal DS results in prenatal screening tests.

MATERIALS AND METHODS

The study was approved by the Ataturk University Medical Faculty Hospital review board committee (date: 30 November 2017). We retrospectively reviewed the data of 108 patients who had undergone amniocentesis, between 16 and 20 weeks of gestation, for fetal karyotype analysis due to abnormal FTCST, in the perinat clinic of the Ataturk University Medical Faculty Hospital (Erzurum, Turkey), between January 2017 and August 2017. Of these 108 patients, only the amniocentesis material obtained from 86 patients who met the research criteria was included in the study. We measured the levels of NF-kB and hsCRP in the AF. We used the ELISA method to assess the concentration of NF-kB and hsCRP in each sample of AF.

Gestational age was determined based on the date of the woman's last menstrual period and the prenatal ultrasound examination at the first 14 weeks of pregnancy. Those excluded from our study included pregnant women with any chronic or acute diseases, women taking any type of hormonal or anti-inflammatory treatment, and those with vaginal, urinary tract or systemic symptoms suggestive of infection.

All 86 women in our study underwent amniocentesis and fetal karyotype analysis between 16 and 20 weeks of gestation. Ultrasound-guided transabdominal amniocentesis was performed and approximately 20 mL of the patient's AF was aspirated for genetic analysis. 2 mL of the AF was centrifuged for 5 min at 3.000 cycles/min. We added 3 mL of BIOAMF-1 basal medium (Biological Industries, Israel) to the supernatants and stored the samples at +4 for future investigation.

Fetal karyotype with DS was detected in 12 out of 86 samples. The karyotypes of the remaining 74 patients were normal. Therefore we divided the whole study population into two Groups: those patients with DS fetal karyotype (Group 1, n = 12) and patients with normal fetal karyotype (Group 2, n = 74). Table 1 shows the clinical and demographic characteristics of each Group of subjects. Indication of amniocentesis was the only abnormality in the screening test in 62 of the 86 patients. In the remaining twenty-four patients, in addition to an abnormal FTCST result, some soft markers of fetal DS were also detected in the ultrasound examination. Table 2 shows the soft markers of fetal DS in detail. Patients with a FTCST result greater than 1/240 was accepted as abnormal and high risk.

Table 1. Clinical and demographic characteristics of the patients with normal and down syndrome fetal karyotypes

	Patients with down syndrome fetal karyotype (Group 1) (n = 12)	Patients with normal fetal karyotype (Group 2) (n=74)	p value*
age [years]	32.17 ± 4.89	33.12 ± 6.16	0.283
BMI [kg/m^2]	23.37 ± 1.62	25.19 ± 3.00	0.047*
gravidity	2.58 ± 1.37	3.42 ± 1.57	0.072
parity	1.50 ± 1.31	2.14 ± 1.26	0.075
gestational age [weeks]	17.92 ± 1.16	17.88 ± 1.02	0.884
estimated risk for trisomy 21 in first trimester combined screening test result [1: rate]	1:41.83 ± 1:22.33	1:107.84 ± 1:74.36	0.001*
NF-kB [ng/mL]	0.34 ± 0.028	0.64 ± 0.072	0.022*
hsCRP [mg/L]	2.69 ± 1.55	1.62 ± 2.34	0.014*
heamoglobin [g/dl-1]	12.17 ± 0.89	12.02 ± 1.37	0.681
WCC [(µl)]	8545.83 ± 2015.33	8282.57 ± 1994.70	0.746

Results are given in mean ± SD. The Mann-whitney u test and t-test were used. A p value of < 0.05 was considered significant*. BMI — body mass index; NF-kB — nuclear factor-kappa beta; hsCRP — high sensitivity C-reactive protein; WCC — white cell count

NF-kB measurement principle

AF nuclear factor kappa B p65 (NF-kB p65) levels were analysed by enzyme-linked immunosorbent assay. The

Table 2. Percentages of fetal down syndrome soft markers detected before amniocentesis

	n = 24	[%]
choroid plexus cysts	5	20.8
cleft lip and palate, gastroschisis	1	4.1
lateral cerebral ventriculomegaly	2	8.3
hydrops fetalis	5	20.8
cystic hygroma	2	8.3
fetal hyperechogenic bowel	4	16.6
increased maternal age	2	8.3
echogenic intracardiac focus	3	12.5

ELISA kit we used uses the Sandwich-ELISA method. The ELISA was validated for AF and samples were assayed in duplicate. We used the Human NF-kB p65 ELISA Kit (Elabscience Biotechnology Inc., China). The sensitivity of the kit was 0.10 ng/ml, the detection range was 0.16 -10 ng/mL, and the coefficient of variation was < 10%. The ELISA is specific for NF-kB p65 detection in biological fluids, and we did not observe any significant cross-reactivity or interference between the Human NF-kB p65 and analogues. The micro ELISA plate provided in the kit we used had been pre-coated with an antibody specific to Human NF-kB p65. Standards, or samples, were added to appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific to Human NF-kB p65 and the Avidin-Horseradish Peroxidase (HRP) conjugate were successively added to each micro plate well and incubated. After incubation, free components were washed away. Then the substrate reagent was added to each well; and only those wells that contained Human NF-kB p65, the biotinylated detection antibody and the Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by adding Stop Solution and appeared yellow in color. The optical density (OD) was measured with spectrophotometry at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of Human NF-kB p65. The concentration of Human NF-kB p65 in the samples was calculated by comparing the OD of the samples with the standard curve. We also performed hsCRP determination: using an immunoturbidimetric assay, we measured hsCRP in the AF which was detected on the BioTek ELx800 Elisa Reader and BioTek ELx50 Elisa Washer.

Statistical analysis

The normal distribution of data was assessed by using the Kolmogrov-Smirnov test and found to be normal. The continuous variables were analyzed by the Mann-Whitney U test. The categorical data were analyzed by the student t test. Results were expressed as mean and standard deviations (SD). For statistical analysis, calculations, and graphic illustrations the IBM SPSS Statistics 22.0 package was used. Statistical significance was accepted if the p value was less than 0.05 ($p < 0.05$).

RESULTS

The clinical and demographic characteristics of the pregnant women with normal and DS fetal karyotypes are presented in Table 1. Table 1 also presents the values of the mean concentrations and standard deviations of AF-NF-kB levels for each study Group. Table 2 shows the percentages of fetal DS soft markers that were detected before amniocentesis. The fetal DS soft markers detected before amniocentesis in Group 1 were not significantly different from those detected in Group 2. Our results show that there were significant decreases in the concentration of AF-NF-kB levels in the AF from women with fetal DS compared with the study patients with healthy fetuses. Patients with fetal DS also had higher AF concentrations of hsCRP when compared with patients with a healthy fetus with normal karyotypes. The FTCST showed a significantly higher risk in patients with fetal DS than in those patients with normal fetal karyotypes. The BMI of patients with fetal DS was lower when compared with patients with a healthy fetus. We did not find any statistically significant differences between the study Group and the contral Group when we compared other clinical parameters. Likewise, no significant correlation between AF-NF-kB levels and clinical and/or demographic parameters was detected.

DISCUSSION

Altered NF-kB expression may be involved in human diseases [4]. Likewise, the NF-kB pathway likely plays a role in the pathogenesis of DS. Because many immune responses and inflammatory genes are expressed in DS abnormalities in the regulation of the amniotic fluid, NF-kB may be involved in the development of fetuses with DS. Therefore, measurement of NF-kB in pregnancies with fetal DS could lead to a better understanding of the influence of DS on pregnancy and fetal outcomes. Here, we showed for the first time that there is a functioning NF-kB pathway in the AF of pregnant women both with and without fetal DS. It has been reported that the expression capacity of the immune response genes of cells with trisomy 21 is lower than that of euploid cells [3]. Concordantly, cytokines such as IL-1b (interleukin-1 beta) and TNF-α (tumor necrosis factor-alpha) induce the NF-kB pathway [6–8] which can amplify the inflammatory response in fetal parts. A recent study by Monika et al. showed significantly decreased AF IL-1b levels in women with fetal DS [9]. They also reported that AF TNF-α levels in women with fetal DS were not different from those in healthy pregnant women

with normal fetal karyotytpes. Moreover, other cytokines, such as interleukin-4, monocyte chemotactic protein-2, matrix metalloproteinase-1 and -9, transforming growth factor alpha and vascular endothelial growth factor-2 are all decreased in the AF of patients with fetal DS [9]. Likewise, Piotr Laudanski et al. showed that there are significant alterations in the chemokine concentrations of AF of women with fetal DS [10]. In accordance with these findings we showed for the first time that AF-NF-kB levels, which are the principal regulatory molecules of inflammation, decrease in women with fetal DS, suggesting that inhibition of the NF-kB pathway may be involved in the pathogenesis of fetal DS. NF-kB also stimulates the expression of inflammatory enzymes such as nitric oxide (NO), and the inducible cyclooxygenase-2 (COX-2) [7]. Therefore, by affecting the amniotic fluid, NO, COX-2 and IL-1b expression and decreased levels of NF-kB in the AF of women with fetal DS may contribute to abnormal fetal development and perinatal outcomes.

NF-kB also controls the degree of cellular apoptosis and proliferation [11]. Many antiapoptotic genes are directly activated by NF-Kb [12]. Hence, decreased AF-NF-kB levels in women with fetal DS can lead to activation of apoptosis in fetal tissues. Concordantly, activation of apopitotic genes in the fetus and fetal parts may cause the loss of fetal growth control. The design and development of drugs that increase NF-kB levels and that induce the expression of antiapoptotic cellular proteins, can thus reduce apoptosis in the fetus and its parts. Failed NF-kB activation of the inflammatory genes may be an important contributor to the pathogenesis of abnormal fetal development in women with fetal DS. It is not possible to compare the results of our study with any other research, because of the lack of publications about AF-NF-kB levels in women with fetal DS. There are some possible explanations for the role of decreased AF-NF-kB levels in the pathophysiology of fetal DS. We know that NF-kB is a multifunctional molecule and that it does not only participate in the immune and inflammatory processes, but that it also affects other processes such as apopitosis and embryogenesis. Decreased AF-NF-kB levels are likely to be due to alterations in regulatory proteins that lead to activation of the NF-kB pathway. Hence, trisomy 21 may cause low expression levels of NF-kB in the nucleus of fetal cells, and consequently, NF-kB may be decreased in amniotic fluid.

NF-kB plays an important role in fetal developmental processes such as immune responses and apopitosis which are largely affected by a fetus with trisomy 21 during pregnancy. In the present study, the AF-NF-kB levels of women with fetal DS were lower than those of women with a healthy fetus. This could indirectly confirm the role of NF-kB in the development of the fetus and its parts, that is disrupted in people with DS. Evaluation of AF-NF-kB levels as potential biomarkers of fetal DS can help us understand the pathophysiology of trisomy 21. However, when reviewing the literature, there still exists no relevant research focused on the role of NF-kBs in the pathogenesis of DS. Therefore, it is difficult to make a definitive conclusion about the variations in the AF levels of NF-kB.

CONCLUSIONS

A better understanding of the role of the AF-NF-kB pathway may provide opportunities for the development of new drugs to prevent DS or its long term complications. The reduced levels of NF-kB in the AF might be dependent on embryonic cells with trisomy 21. In order to clarify the exact role of NF-kB in DS, additional factors that correlate with the clinical and laboratory findings should be measured.

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Conflict of interest

The authors have no competing interests to declare.

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