Evaluation of Oxidant/Antioxidant System, IL-6 and IL-10 Parameters and SOD-Enzyme Activity in Pregnancy with Down Syndrome in Amnion Fluid Analysis

Amniyon Sıvı İncelenmesinde Down Sendromu Tespit Edilmiş Gebelerde Oksidan/Antioksidan Sistem, IL-6 ve IL-10 Parametrelerinin SOD ENzim Aktivitesi ile birlikte Değerlendirilmesi

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ABSTRACT

Background: Down Syndrome (DS) can be diagnosed with prenatal invasive diagnostic tests to the risky population. In this study, it was aimed to propound the status of the oxidant/antioxidant system and interleukin-6 (IL-6)/interleukin-10 (IL-10) levels in the amniotic fluids of pregnant women with DS diagnosed child by amniocentesis method.
Methods: According to the results of the genetic examination of the amniotic fluid obtained from the pregnant women who have been admitted to Zekai Tahir Burak Women's Health Training and Research Hospital, Genetic Center, and have undergone amniocentesis, amniotic fluid of 18 pregnant women who are carrying babies with DS and amniotic fluid of 36 pregnant women with healthy babies were included in the study under two groups. Study group (Group 1) consists of the amniotic fluid of the pregnant women carrying babies with DS while control group (Group 2) consists of the amniotic fluid of the pregnant women carrying healthy babies. In the samples taken, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and xanthine oxidase (XO), catalase (CAT), adenosine deaminase (ADA), nitric oxide (NO), nitric oxide synthase (NOS) have been determined by spectrophotometric methods, as the measurements of IL-6 and IL-10 tests were carried out by ELISA method. In statistical evaluation, Student's t test and spearman correlation analysis were used. Results for P <0.05 were considered statistically significant.
Results: According to the findings of the study, SOD level in Group 1 increased statistically significantly when compared to Group 2 (p <0.05). However, CAT and IL-6 in Group 1 were found to be statistically significantly reduced compared to Group 2 (p <0.05). There was no significant difference between the two groups in terms of MDA, GSH-Px, XO, NO, NOS, ADA and IL-10 levels (p> 0.05).
Conclusion: Consequently, SOD enzyme activity was found to be increased in the group with DS. We think that SOD gene localized on the 21st chromosome caused this result. In addition, compared to the control group, the absence of CAT and GSH-Px in the amniotic fluids of pregnant women carrying babies with DS, that can eliminate the excessive amount of H_2O_2 caused by the activity of the SOD enzyme causes a serious oxidative stress. In parallel with these data, using the SOD enzyme as a marker in prenatal diagnosis can be a useful approach. For this purpose, further studies should be carried out with higher numbers.

Keywords: Down syndrome, interleukin-6/interleukin-10, oxidant/antioxidant system, prenatal diagnosis, superoxide dismutase.

ÖZET

Amaç: Down Sendromu açısından riskli popülasyona invaziv tanı testleri uygulanarak prenatal tanı konabilmektedir. Bu araştırma amniyosentez ile DS tanısı konmuş bebek sahibi gebelerin amniyon sıvılarında oksidan/antioksidan sistem ve interlökin-6 (IL-6)/interlökin-10(IL-10) düzeylerinin durumunun ortaya konması amaçlanmıştır.
Yöntemler: Zekai Tahir Burak Kadın Sağlığı Eğitim ve Araştırma Hastanesi Genetik Merkezi'ne başvurup amniyosentez yapılan gebelerin amniyon sıvılarının genetik inceleme sonuclarına gore DS'lu bebeğe sahip olan 18 gebenin amniyon sıvısı ve sağlıklı bebeğe sahip 36 gebenin amniyon sıvısı calışma kapsamına alınarak iki grup oluşturuldu. Çalışma grubunu (Grup 1), DS'lu bebeğe sahip gebelerin amniyon sıvısı kontrol grubunu (Grup2) ise sağlıklı bebeğe sahip gebelerin amniyon sıvısı oluşturmaktadır. Alınan örneklerde malondialdehit (MDA), superoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px) ve ksantin oksidaz (XO), katalaz (CAT), adenozin deaminaz (ADA), nitrik oksit (NO), nitrik oksit sentaz (NOS) spektofotometrik yontemlerle IL-6 ve IL-10 testlerinin ölcümleri ise ELISA yöntemiyle belirlendi. İstatiksel değerlendirmede Student t testi ve spearman korelasyon analizi kullanıldı. P<0,05 icin sonuclar istatistiksel olarak anlamlı kabul edildi.
Bulgular: Araştırmanın bulgularına göre Grup 1'de SOD düzeyi, kontrol grubuyla karşılaştırıldığında istatiksel olarak anlamlı derecede artmış bulundu(p<0,05). Bununla birlikte Grup 1'de CAT ve IL-6, Grup 2 ile karşılaştırıldığında istatiksel olarak anlamlı derecede azalmış olduğu tespit edildi (p<0,05). Her iki grup arasında MDA, GSH-Px, XO, NO, NOS, ADA ve IL-10 düzeyleri bakımından anlamlı bir farklılık gözlenmedi (p>0,05).
Sonuç: Sonuc olarak DS'lu grupta SOD enzim aktivitesi artmış olarak bulunmuştur. Bu sonuca 21. kromozom üzerinde yer almakta olan SOD geninin neden olduğunu düşünmekteyiz. Ayrıca kontrol grubuna kıyasla DS'lu bebeğe sahip gebelerin amniyon sıvılarında SOD enziminin aktivitesi sonucunda oluşan aşırı miktardaki H2O2'i temizleyecek duzeyde CAT ve GSH-Px'ın bulunmaması ciddi bir oksidatif strese yol acmaktadır. Bu veriler doğrultusunda SOD enzimini prenatal tanıda belirteç olarak kullanmak yararlı bir yaklaşım olabilir. Bu amacla daha yuksek sayıda yapılmış calışmaların gercekleştirilmesi gerekmektedir.

Anahtar Sözcükler: Down Sendromu, İnterlökin-6/İnterlökin-10, oksidan/antioksidan sistem, prenatal tanı, süperoksit dismutaz

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INTRODUCTION

Down Syndrome (DS), also known as Trisomy 21, occurs as a result of triploidy, mosaicism or translocation of all or part of the 21st chromosome in humans and is seen in approximately 1 out of 700 liveborns (1). Down Syndrome is the most common cause of genetic mental retardation. Various health problems such as premature aging, cardiovascular system disorders and Alzheimer's-like dementia are observed in this disease (2). Various screening protocols are applied to high-risk pregnant women determined based on maternal age, ultrasonography findings and biochemical markers (3-6). In order to make a definitive diagnosis in pregnant women who are at risk for fetal chromosomal anomaly, invasive prenatal diagnosis methods such as chorionvillus sampling and early amniocentesis in the first trimester (11th-14th weeks), amniocentesis in the second trimester and cordocentesis in later weeks can be implemented. Amniocentesis is typically performed between weeks 15 and 17 of pregnancy under ultrasonography control (7). There are many molecules in the amniotic fluid such as proteins (albumin, globulin), hormones (estrogen, progesterone, testosterone), prostaglandins, catecholamines (dopamine, adrenaline, noradrenaline, serotonin), enzymes, electrolytes, glucose, creatinine and bilirubin (8). Various studies have shown that oxidative stress markers of individuals affected by Down syndrome are increased (9,10). It has also been reported that this oxidative stress increase observed in DS started in the prenatal period (11). It was observed that the activity of the superoxide dismutase (SOD) enzyme increased in the amniotic fluid of pregnant women who had been diagnosed with DS as a result of amniocentesis (12). Nitric oxide (NO) levels in amniotic fluid of pregnant women with Down syndrome were examined and it was observed that they were high (13). Cytokines are small glycoproteins produced by a number of cells, that regulate the inflammatory system, immune system, and hematopoiesis. IL-1, TNF-α, IFN-g, IL-6 proinflammatory; IL-4, IL-10, IL-12 and IL-13 are anti-inflammatory cytokines (14). Studies in humans have reported that abortus is accompanied by an increase in IL-2 and INF-γ concentrations and a decrease in IL-10 concentration. In infants with small gestational age (SGA), IL-10 levels in mothers' amniotic fluids were examined and found to be increased (15). In addition, studies performed have shown that when there is inflammation in the amniotic cavity, the concentration of IL-6 in the amniotic fluid increases, and the presence of high concentration of IL-6 in the amniotic fluid plays a role in the occurrence of neonatal complications (16). Recent evidence suggests that placental trophoblast cells produce IL-6 and IL-6 is an immune regulator in fetal-maternal interaction (17). These studies show that cytokines and humoral factors are influential factors in the placentation and vascularization phase and that they are probably effective on the prognosis of pregnancy.

In this study, we aimed to put forth the oxidant/antioxidant status and interleukin-6 (IL-6)/interleukin-10 (IL-10) levels in the amniotic fluid of pregnant women who are diagnosed with DS as a result of amniocentesis performed at risk pregnant women.

MATERIAL and METHODS

Patient selection

For this research, required permission was obtained from the Ankara University Faculty of Medicine Clinical Research Ethics Committee. Informed consents of all volunteers participating in the study were obtained. The materials used in this study were created by taking the amniotic fluid from the pregnant women who were at risk for DS in the double and triple tests performed between 16 and 20 weeks of gestation with ultrasonography, among the pregnant women who are admitted to Zekai Tahir Burak Women's Health Training and Research Hospital Genetic Center since September 15th, 2013, and determining that they had a fetus with Trisomy 21 as a result of karyotype analysis. In our study, two groups were formed by collecting amniotic fluid of pregnant women with DS and normal karyotype babies. In the study group (Group 1), the amniotic fluid of the pregnant women carrying babies with DS, and in the control group (Group 2) the amniotic fluid of the pregnant women carrying healthy baby were collected, thus two groups have been formed. In order for the success and reliability of our study to be high, the number of samples was determined by doing Two-Sample T-Test Power Analysis. Accordingly, when the number of cases with DS was 18 and our control group was 36, our power analysis was found to be 82 % with 0.05 error.

Collected amniotic fluids were stored at -80° C until the day of study. After study and control groups were created, we studied MDA, SOD, GSH-Px, XO, CAT, ADA, NO, NOS.

The principle of MDA level measurement is based on the measurement of the absorbance of the pink colored complex formed by MDA and TBA at a wavelength of 532 nm (18).

The SOD level measurement principle is based on the principle that the superoxide radical formed by the xanthine-xanthine oxidase system reduces the nitroblue tetrazolium (NBT) compound in the environment when it cannot be eliminated by the SOD enzyme (19).

On the other hand, GSH-Px reacts with reduced glutathione (GSH) and H_2O_2, and produces oxidized glutathione (GS-SG) and H_2O. Following this reaction, the GSH reductase enzyme reduces GS-SG again. Meanwhile, it oxidizes the NADPH in the environment and converts it to NADP. As GS-SG is produced with GSH-Px activity, the amount of NADPH decreases, the decrease in NADPH absorbance at 340 nm wavelength is calculated in direct proportion to the GSH-Px enzyme activity (20).

The XO enzyme degrades the xanthine to form uric acid. The principle of the experiment is based on measuring the optical density of uric acid released by XO at a wavelength of 293 nm. XO activity is calculated over the amount of uric acid formed per unit time (21).

Hydrogen peroxide is the substrate for catalase. The principle of the catalase measurement method is based on the fact that the absorbance value given by the hydrogen peroxide at 240 nm wavelength decreases during the reaction catalyzed by the CAT enzyme and that this decrease is spectrophotometrically monitored (22).

The principle of the method used in determining ADA enzyme activity is based on the fact that the ammonia released during the formation of inosine from the adenosine by the ADA enzyme, reacts with alkaline hypochlorite and phenol nitroprusside and on the absorbance of the blue color produced is measured spectrophotometrically at 628 nm wave length (23).

The measurement of the NO level is based on the principle of NO diazotization of sulfanilic acid in acidic medium followed by reaction with naphthylenedienediamine. NO is formed through the nitric oxide synthase (NOS) enzyme during citrulline formation from L-arginine. The principle of the experiment is based on the reaction of NO formed of L-arginine with sulfanilic acid diazotization in acidic medium and radical naphthylethylenediamine (24,25).

IL-6 and IL-10 tests were studied with the ELISA method using Platinum ELISA kits. For the study, ELISA reader and washer (ChemWell® 2910 Automated EIA and Chemistry Analyzer), Platinum ELISA kits were used. All chemicals used in measuring oxidant and antioxidant markers were provided from Sigma–Aldrich and Merck Chemistry companies.

Statistical Analysis

The analysis of the data was done using SPSS for Windows 15 package software. Since parametric assumptions are provided, the data are expressed as mean ± standard deviation. In the comparison of these values, the difference between the two averages was investigated by the Student's t-test. Results for $P < 0.05$ were considered statistically significant.

RESULTS

Amniocentesis was performed in patients with the risk of Down Syndrome during routine controls conducted among pregnant women, and two study groups were created by taking amniotic fluid from 18 pregnant women carrying babies diagnosed with DS and 36 pregnant women carrying healthy babies. In the study, the results of SOD, GSH-Px, XO, CAT, ADA and NOS enzyme activities and MDA and NO levels are given in Table 1 as mean ± standard deviation values (mean ± sd). Accordingly, when Group 1 and Group 2 were compared, a statistically significant difference was found between the SOD level in Group 1 and the SOD level in Group 2 ($p < 0.05$). SOD level in Group 1 was found higher than Group 2. In the study, a statistically significant difference was found between the catalase levels between the groups ($p < 0.05$). Catalase level in Group 1 was found lower than Group 2 (Table.1).

Table 1: Oxidant - Antioxidant Parameters of Amniotic Fluids in Group 1 and Group 2

Oxidant-Antioxidant Parameters	Group1 (n=18) (mean ± sd)	Group 2 (n=36) (mean ± sd)	P value
Catalase (IU/ml)	1,71±1,35	3,62±3,16	0,034*
MDA (nmol/ml)	0,86±0,31	0,92±0,31	0,323
SOD (U/ml)	6,28±1,35	5,33±1,61	0,012*
GSH-Px (IU/ml)	0,094±0,02	0,098±0,02	0,566
ADA (mIU/ml)	2,70±0,97	3,10±3,80	0,149
XO (mIU/ml)	42,33±13,46	47,74±20,78	0,114
NO (mM)	46,82±12,30	45,78±10,67	0,749
NOS (IU/ml)	10,19±2,41	10,78±2,63	0,434

* p <0.05 was considered statistically significant. Group 1 = Study (DS) group. Group 2 = Control

Mean ± standard deviation values (mean ± sd) of IL-6 and IL-10 parameters belonging to amniotic fluids by groups are shown in table 2. Accordingly, a statistically significant difference was found between IL-6 level in Group 1 and IL-6 level in Group 2. IL-6 level in Group 1 was found to be lower than IL-6 level in Group 2.

Table 2: IL-6 and IL-10 Parameters of Amniotic Fluids in Group 1 and Group 2

Parameters	Group1 (n=18) (mean ± sd)	Group 2 (n=36) (mean ± sd)	P value
IL-6 (pg/ml)	60,39±46,73	88,40±47,72	0,017*
IL-10 (pg/ml)	31,32±6,29	35,93±34,24	0,425

DISCUSSION

DS is the most common cause of genetic mental retardation. In addition to mental retardation, many health problems such as early aging, cardiovascular system disorders, Alzheimer's-like dementia, various dysmorphic features, congenital malformations, speech and memory problems accompany this disease (1,2,26). It has been proved that oxidative stress plays a major role in the pathogenesis of DS in very early stages and in the emergence of clinical findings. On the other hand, some oxidative stress markers have been observed to be risen in amniotic fluids of pregnant women carrying babies diagnosed with DS (27,28). Increased oxidative stress causes increased expression of some genes encoded by chromosome 21 (Figure 1).

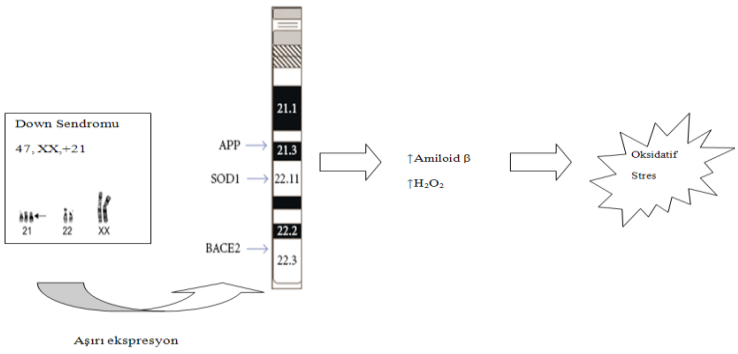

Figure 1: Oxidative stress and Down Syndrome. Amyloid precursor protein (APP), beta secretase (BACE2) - Taken from Perluigi et al. (27).

SOD, BACE2, APP, CBC (cystathion beta synthetase) and ligands and receptors of the interferon family are genes encoded by the 21st chromosome. Since Cu, Zn-SOD (SOD1), which is a subtype of SOD enzyme, is encoded in chromosome 21, its expression and activity increases in DS. SOD constitutes the first step in antioxidant defenses by dismutating the superoxide radical and converting it to H_2O_2 and molecular oxygen. The released H_2O_2 is converted into water with CAT and GSH-Px. As a result of the triploidy of the 21st chromosome, an imbalance occurs between SOD1, CAT and GSH-Px ratios. As a result, H_2O_2, which cannot be removed from the medium, accumulates in large quantities and severe oxidative stress occurs (29). In our study, we found that, in line with the literature, SOD enzyme activity increased significantly in the DS group compared to the control group. Despite this, when we compare CAT enzyme activity that tries to remove H_2O_2 accumulated in the medium with the control group, we found that it decreased significantly in the DS group. When we look at the GSH-Px levels, we found a slight decrease in Group 1, although there was no statistically significant difference between the two groups. Although these data we obtained are compatible with previous studies, they can be explained by the increase of SOD activity encoded on the extra 21st chromosome observed in DS. H_2O_2, which accumulates in the medium with increased SOD activity, creates oxidative stress. The levels of antioxidant enzymes (CAT, GSH-Px) that remove H_2O_2 in the medium do not increase, but on the contrary, their activity decreases, causing oxidative damage to increase. Netto et al. thought that the neurodegenerative symptoms observed in DS in 2004 may be related to SOD, an antioxidant enzyme and S100B encoded by the 21st chromosome, and the increase in their activity may be a valuable marker in regard to the prenatal screening. The researchers conducted a study by collecting amniotic fluids of pregnant women with healthy babies and pregnant women carrying babies diagnosed with DS. For the study, they collected amniotic fluid from 26 pregnant women carrying babies with normal karyotypes and amniotic fluid from 71 pregnant women carrying babies with DS and measured SOD enzyme activity and S100B levels. As a result, they found that SOD enzyme activity and S100B level were significantly higher in amniotic fluid in DS group compared to healthy group (12). Baeteman et al., in their study, reported that SOD activity in amniotic fluid in pregnant women with DS was higher than normal ones (30). Tranquilli et al. collected amniotic fluids from 15 pregnant women with DS babies and 15 pregnant women with healthy babies and examined NO and vascular endothelial growth factor (VEGF) levels. They found that the NO level was significantly higher in the DS group compared to the normal group, while the VEGF level was lower. Increased NO synthesis in pregnant women carrying babies with Down Syndrome may increase with high level of hCG or as a response to low levels of VEGF by immature placenta (13). In our study, when NO levels were analyzed, although there was no statistically significant difference between the two groups, we found that there was a slight increase in the DS group compared to the control group. In our study, no statistically significant difference was found in the NOS levels between the two groups. However, we found that there was a decrease in DS group compared to Group 2. Brooksbank et al. showed in their study that there was a significant increase in in vitro lipoperoxidation in the cerebral cortex of fetuses with DS, estimated by MDA (31). No significant difference was found between the groups in terms of MDA and Xanthine oxidase levels. Uslu et al. measured in their study serum ADA activities in second trimester pregnant women at high age and at risk of Down Syndrome. Compared to the control group, ADA activities were found to be low in those with low risk of Down Syndrome, in all pregnant women, and in pregnant women at high age risk, and in women with high risk of Down Syndrome (32). When ADA enzyme activities were examined in our study, no statistically significant difference was found between the two groups.

Despite all the evidence and hypotheses it is still unclear how all pro-inflammatory cytokines play a role in Alzheimer's disease and dementia seen in DS. Heyser et al. reported in their study that IL-6 production was high in astrocytes of transgenic mice with learning disabilities (33,34). Carta et al. showed that the levels of chemokines and cytokines increased in patients with DS. They also found that there was a positive correlation between IL-6 level and the degree of mental retardation in the DS group. Based on these data, they suggested that the degenerative processes observed in Alzheimer's disease in DS are similar and that inflammatory chemokines may play a role in these processes (35). In their study, Kameda et al. showed that placental trophoblast cells produce IL-6 and IL-6 plays a role as an immunoregulator in fetal-maternal interaction (36).

Complement C9 is part of the α-1B-glycoprotein acute phase response and is a protein that has protective role in fetal development. The presence of these proteins in the amniotic fluid prevents intraamniotic infection and subsequent complications. Their oxidation causes them to lose their biological functions and fail to fulfill their functions to protect the fetus. These results explain why pathological conditions such as acquired immune deficiency and autoimmune diseases occurring in DS are observed more frequently (34,35).

Despite all the evidence and hypotheses how all pro-inflammatory cytokines play a role in Alzheimer's disease and dementia seen in DS is still unclear. Heyser et al. reported in their study that IL-6 production was high in astrocytes of transgenic mice with learning disabilities (36,37). Carta et al. demonstrated that the levels of chemokines and cytokines increased in patients with DS. In their study, the researchers aimed to investigate whether affective disorders and mental attenuation observed in DS are related to proinflammatory cytokines. They examined 19 patients with DS and 87 patients with perinatal ischemic injury, and found that cytokines and chemokines were risen in patients with DS, but there was only a significant difference in the level of MIF-1α. They also found that there was a positive correlation between IL-6 level and the degree of mental retardation in the DS group. Based on these data, they suggested that the degenerative processes observed in Alzheimer's disease and DS are similar and that inflammatory chemokines may play a role in these processes (38). In another study, Park et al. did not find any changes in IL-6 plasma levels in adult DS patients. Again, other researchers found that IL-8, TNF-α levels increased, while others found that cytokines and chemokines levels did not display any change (39).

Kameda et al. showed in their study that placental trophoblast cells produce IL-6 and IL-6 plays a role as an immunoregulator in fetal-maternal interaction (36). When we look at IL-6 levels in our study, we found a statistically significant decrease in DS group compared to the control group. Although there was no statistically significant difference between the groups in terms of interleukin 10 levels, we detected a slight decrease in DS group compared to control group.

Despite the increased interest in immune system changes observed in DS, the role of cytokines is still not fully explained. Most of the studies have been performed in adults with DS. For a better understanding of the role of cytokines in DS, Śmigielska-Kuzia et al. put emphasis on the levels of plasma proinflammatory cytokines of children and adults with DS [interleukin-1α (IL-1α), IL-2, IL-6, TNF-α and soluble tumor necrosis factor receptor 1 (sTNFR1)]. Researchers suggested that IL-1α, IL-2, IL-6, TNF-α concentrations were increased in the serum of children and adults with DS compared to the control group (39).

In our study, SOD in Group 1 increased statistically significantly compared to the control group ($p < 0.05$). However, CAT and IL-6 decreased significantly in Group 1 compared to the control group ($p < 0.05$). There was no significant difference between the two groups in terms of MDA, GSH-Px, XO, NO, NOS, ADA and IL-10 levels ($p > 0.05$).

In conclusion, SOD enzyme activity was found to be increased in the group with DS. We think that SOD gene localized on the 21st chromosome caused this result. In addition, compared to the control group, the absence of CAT and GSH-Px in the amniotic fluids of pregnant women carrying babies with DS, that can eliminate the excessive amount of H_2O_2 caused by the activity of the SOD enzyme causes a serious oxidative stress. In parallel with these data, using the SOD enzyme as a marker in prenatal diagnosis can be a useful approach. For this purpose, further studies should be carried out with higher numbers.

Conflict of interest

No conflict of interest was declared by the authors.

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