Abstract. – OBJECTIVE: To determine the oxidant/antioxidant balance and proinflammatory status in amniotic fluids collected during cesarean section of patients diagnosed with abruptio placentae.

PATIENTS AND METHODS: Twenty-five patients diagnosed with ablatio placentae with intact membranes who went to emergency cesarean section were included in the study. A diagnosis of AP was made in those who had at least one of the following criteria or, in suspicious cases, two findings. (i) Antepartum hemorrhage starting after 20 weeks of gestation, (ii) presence of retroplacental hematoma on ultrasonography, (iii) severe fetal distress or death, (iv) localized or diffuse uterine tenderness or pain. The control group consisted of 25 patients who presented for delivery, who were not diagnosed with AP, and whose membranes were intact. NF-κB, total oxidant capacity (TOC), total antioxidant capacity (TAC), and oxidative stress index (TOC/TAC=OSI) levels were measured in amniotic fluids collected during cesarean section from both groups.

RESULTS: Amniotic fluid TAS values of the AP group were significantly lower than the healthy controls (1.14±0.33 vs. 9.05±3.40, p<0.01). Amniotic fluid TOS values were significantly increased in the AP group (36.1±8.10 vs. 11.4±2.77, p<0.02). OSI values were significantly higher in the AP group (31.6±9.03 vs. 1.26±0.02, p<0.01). Amniotic fluid NF-κB expression of the AP group was approximately 5 times higher than the control group (10.4±2.56 ng/mL vs. 1.86±0.30 ng/mL, p<0.01). High blood pressure and smoking history were significantly higher in the AP group. Gestational age and fetal birth weight of the AP group were lower than the control group.

CONCLUSIONS: Since the increase in amniotic fluid oxidant capacity and proinflammatory cytokine synthesis cannot be neutralized by the antioxidant system, hypoxic cell damage may lead to premature separation of the placenta.

Key Words: Abruptio placentae, Amniotic fluid, TAS, TOS, OSI, NF-κB.

Introduction

Affecting approximately 1 to 2% of all pregnancies, ablatio placentae (AP) remains one of the major causes of feto-maternal morbidity and mortality. AP is defined as the premature separation of the placenta from the implantation site. However, since placental location is a sonographic finding, we do not know clearly whether the placenta has completed its placement in the implantation area by looking at these images alone. Therefore, it is very difficult to answer the question of whether AP occurs in placentas that have completed their placement in the uterus or in placentas with defective implantation. Although the risk of AP increases significantly in the presence of certain risk factors, there is no clear data on the pathophysiology of the disease. In addition to hypertensive diseases such as preeclampsia/eclampsia, multiple pregnancies, smoking and cocaine use, advanced age and high parity are important risk factors for AP.

Chronic poor perfusion at the uteroplacental junction, ischemia-reperfusion injury and subsequent placental infarcts initiate the separation of the placenta. Detection of high alphafetoprotein levels in Down syndrome screening tests is also considered as a separate risk factor for AP. AP can lead to preterm birth or premature rupture of the membrane, which may adversely affect the perinatal outcome. In cases of excessive bleeding or late intervention, maternal morbidity increases significantly. Therefore, urgent delivery may be required in cases of acute abruption. In cases of partial placental separation, the decision to deliver may be urgent, or it may be delayed depending on the gestational week. However, the question of what kind of changes occur at the molecular level, despite all these different risk factors and other accompanying maternal diseases, is the most critical question that needs to be answered. The high incidence of thrombophilia in abruptio placentae cases and its frequent occurrence among members...
of the same family suggests that the disease has a genetic origin. Detection of polymorphisms in oxidative phosphorylation and mitochondrial biogenesis genes suggests that AP is closely related to a defect in oxidative stress pathways. Since mitochondrial damage will interfere with oxidative phosphorylation, it may lead to defective migration of trophoblasts and subsequently placental defects. Trophoblasts, which must have proliferation, differentiation, invasion and migration features in order to acquire hemochorial characteristics, cannot complete their differentiation due to impaired intracellular redox balance and undergo complete or partial separation in the presence of a spontaneous or accompanying disease. Although AP is an ischemic placental disease, the redox balance and proinflammatory content of amniotic fluid has not been investigated as an etiologic culprit to date. Nuclear factor kappa beta (NF-κB) is a cytoplasmic dimer that triggers inflammation and angiogenesis in amnioblasts. This dimer, which is activated in the presence of an exogenous and endogenous stimulus, goes to the nucleus and stimulates genes that activate inflammatory reactions. The relationship between NF-κB and other inflammatory cytokines is necessary for healthy decidualization and implantation. If the redox balance is disturbed in the presence of AP, NF-κB expression may also be disturbed. This study was designed to determine the oxidant/antioxidant balance status in amniotic fluids collected during cesarean section of patients diagnosed with AP. Instead of measuring individual oxidant, antioxidant and proinflammaturar markers and enzymes, the NF-κB, total oxidant capacity (TOC), total antioxidant capacity (TAC), and oxidative stress index (TOC/TAC=OSI) of amniotic fluid were measured and compared with healthy controls.

Patients and Methods

Study participants were selected among the patients who applied to Kayseri City Hospital Gynecology and Obstetrics Service with complaints of onset of labor, antepartum hemorrhage, water discharge or pain. The gestational age was calculated according to the last menstrual period. If the patient had USG performed in the first 14 weeks of pregnancy, it was evaluated together with the date of the last menstrual period and we tried to determine the exact gestational week of pregnancy. Among the applicants twenty five patients diagnosed with ablatio placenta were included in the study. A diagnosis of AP was made in those who had at least one of the following criteria or, in suspicious cases, two findings (i) Antepartum hemorrhage starting after 20 weeks of gestation, (ii) presence of retroplacental hematoma on ultrasonography, (iii) severe fetal distress or death in sonographic and NST evaluation, (iv) localized or diffuse uterine tenderness and pain. Patients who did not have retroplacental hematoma in sonography but who were found to have hematoma in postpartum placental evaluation were also considered as AP. Patients with intact membranes who went to emergency cesarean section with a preliminary diagnosis of AP constituted the study group. Patients with ruptured membranes or discharged amniotic fluid in addition to antepartum bleeding were not included in the study. Patients who started labor at the time of application and decided to have vaginal delivery were also excluded from the study. Patients with placenta previa, acute appendicitis, genitourinary infection, ovarian torsion and fibroid degeneration that may cause pain and bleeding were excluded from the study. In addition to their medical and obstetric histories, participants’ age, parity, smoking or alcohol use, and arterial blood pressure were recorded. Those who had multiple pregnancies and those who used anticoagulants during their pregnancy were not included in the study. Twenty five participants in the control group were selected from the patients who applied to the same maternity ward for delivery but did not have a diagnosis of AP and whose fetal membranes were intact. Since the amniotic fluid sample will be used to evaluate the redox balance, the condition of intact membranes was sought. The patients in the AP and the control groups were tried to be matched in terms of their gestational weeks, but this could not be achieved due to the nature of the AP requiring acute intervention.

All interventional procedures in this study were performed in accordance with both ethical and Helsinki Declaration standards, with the consent of the patients and the approval of the ethics committee. Following the lower segment incision at C/S delivery, before the fetal membranes were cut, amniotic fluid sample was obtained by passing through the membrane with a 10 cc syringe. Fetus and placenta were delivered subsequently. Patients whose amniotic fluid was contaminated with meconium and dense vernix were not included in the evaluation. Patients with heavy bleeding into the amniotic fluid due to the nature of AP
Disrupted redox balance in utero-placental junction may be the main culprit in the occurrence of abruptio placenta

Measurement of TOS, TAS, OSI (TOS/TAS) and NF-κB

All amniotic fluid samples collected during cesarean section were centrifuged at 400 rpm for 5 minutes to remove vernix, debris, and blood. The samples were then stored at -20°C until analysis. NF-κB concentration in amniotic fluid samples was measured by enzyme-linked immunoassorbent assay using human immunoassay kit (Cusabio Biotech Co., Ltd., Wuhan, China). The kit was used to measure the NF-κB in cell culture supernatants and biological fluids. The detection range of the NF-κB kit was 0.3 to 20 ng/mL and the minimum measurable level (sensitivity) was 0.078 ng/mL. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively. TAS and TOS levels were studied in an autoanalyzer by using their own kits (Rel Assay, Mega Medicine Industry, Gaziantep, Turkey). The result of TAS was presented as mmol Trolox Equivalent/L. The results TOS was presented as μmol H$_2$O$_2$ Equivalent/L. By dividing TOS data by TAS the OSI value was calculated OSI (TOS/TAS=OSI). The results of OSI were presented as percentages (%).

Statistical Analysis

Statistical analysis was performed by the use of Statistical Package for the Social Sciences for Windows version 21 (IBM Corp., Armonk, NY, USA). Normality of the distribution was assessed by using Kolmogorov-Smirnov test. Student’s t-test was used for normally distributed variables, Chi-square test for categorical variables and Mann-Whitney U test for abnormal variables. Results were presented as mean ± standard deviation or percentages. A p-value of <0.05 was accepted as significant.

Results

As shown in Table I the age and parity of the patients in the abruptio placenta group were similar to the control group. The gestational week of ablatoio placenta group was significantly lower than the control group (p<0.01). Fetal distress was detected in 6 (24%) of 25 patients in the AP group, while fetal distress was detected in only 1 patient in the control group (4%). The number of patients with arterial blood pressure above 140/90 mmHg was 9 (36%) in the AP group, while it was 3 (12%) in the control group. The blood pressure of the AP group was significantly higher than that of the controls (p<0.02). The number of patients

Table I. Comparison of abruptio placenta and control groups in terms of demographic characteristics and AP risk factors.

<table>
<thead>
<tr>
<th></th>
<th>Abruptio placenta (n=25)</th>
<th>Control (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.6±4.07</td>
<td>26.9±7.04</td>
<td>0.67</td>
</tr>
<tr>
<td>Gestational age (week)</td>
<td>34.7±6.20</td>
<td>37.3±8.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Fetal distress in NST n (%)</td>
<td>6 (24%)</td>
<td>1 (4%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Parity</td>
<td>1.44</td>
<td>1.28</td>
<td>0.46</td>
</tr>
<tr>
<td>Cesarean delivery n (%)</td>
<td>25 (100%)</td>
<td>25 (100%)</td>
<td>0.60</td>
</tr>
<tr>
<td>Blood pressure (&gt;140/90 mm Hg) n (%)</td>
<td>9 (36%)</td>
<td>3 (12%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td>6 (24%)</td>
<td>3 (12)</td>
<td>0.03</td>
</tr>
<tr>
<td>Alcohol use n (%)</td>
<td>2 (8%)</td>
<td>2 (8%)</td>
<td>0.32</td>
</tr>
<tr>
<td>Trauma history n (%)</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Fetal weight (gr)</td>
<td>2306.7±184.9</td>
<td>2970.5±320.6</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table II. Comparison of amniotic fluid TAS, TOS, OSI and NF-κB values of patients in abruptio placenta and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Abruptio placenta (n=25)</th>
<th>Control (n=25)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAS (mmol Trolox Equivalent/L)*</td>
<td>1.14±0.33</td>
<td>9.05±3.40</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TOS (μmol H$_2$O$_2$ Equivalent/L)</td>
<td>36.1±8.10</td>
<td>11.4±2.77</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>OSI (TOS/TAS) (arbitrary unit)</td>
<td>31.6±9.03</td>
<td>1.26±0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NF-κB (ng/mL)</td>
<td>10.4±2.56</td>
<td>1.86±0.30</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The results were given as mean± SD. Mann-Whitney U test was used for comparisons between groups. p<0.05 was accepted as significant.
who smoked in the AP group (24%) was found to be significantly higher than the control group (12%) \((p<0.03)\). There was no difference between the groups in terms of alcohol use. Abdominal trauma history was found in only one person in the AP group. The patients in the control group had no history of trauma. Fetal birth weights of the AP group were significantly lower than the control group \((2,306.7±184.9 \text{ g} \text{ vs. } 2,890.5±320.6 \text{ g}, p<0.04)\).

Table II shows redox balance in amniotic fluid samples. Amniotic fluid TAS values of the AP group were significantly lower than the healthy controls \((1.14±0.33 \text{ vs. } 9.05±3.40, p<0.01)\). Amniotic fluid TOS values were significantly increased in the AP group \((36.1±8.10 \text{ vs. } 11.4±2.77, p<0.02)\). OSI values were significantly higher in the AP group \((31.6±9.03 \text{ vs. } 1.26±0.02, p<0.01)\). Amniotic fluid NF-κB expression of the AP group was approximately 5 times higher than the control group \((10.4±2.56 \text{ ng/mL} \text{ vs. } 1.86±0.30 \text{ ng/mL}, p<0.01)\).

**Discussion**

If mitochondrial biogenesis and oxidative phosphorylation defect are responsible for the formation of AP\(^{12}\), the synthesis and release of these amniotic fluid reactive oxygen derivatives (ROS) will increase and the intracellular redox balance will be disturbed. If the production of ROS in placental site exceeds the neutralization capacity of trophoblasts oxidative stress will occur as the antioxidant defense system will collapse\(^{2,3}\). When the ROS scavenging effect of antioxidant pathways is insufficient, hypoxic cell damage in uteroplacental cells negatively affects trophoblast development and migration\(^{11-15}\). Since the increase in production of ROS and proinflammatory cytokines disrupts the redox balance, invasion of healthy trophoblasts is prevented and the placenta is separated before term, which is known as AP\(^{1}\).

Placental hypoxia activates enzymatic and non-enzymatic antioxidant defense mechanisms and proinflammatory cytokines production in uteroplacental cells. Antioxidant molecules, whose production and secretion increase due to ischemic placental pathology, try to neutralize the increased ROS in the placental bed\(^{2,3}\). Measuring ROS and opposing systems in the presence of AP in a local biological fluid will give clearer information about the etiology of the disease. We decided to perform the measurements in amniotic fluid, as it is an element of the uteroplacental region and is affected by changes in the presence of AP. Since ROS is a short-lived and unstable molecule, its true amount cannot always be determined in measurements\(^{16}\). Therefore, we tried to determine the redox balance in placental cells by measuring amniotic fluid TAS and TOS levels and to reveal its role in AP formation. We found that TOS levels in amniotic fluid samples obtained during cesarean delivery were significantly increased in AP patients. The significant increase in TOS levels is evidence that the amount of ROS responsible for oxidative stress exceeds the neutralization capacity of uteroplacental cells. In ROS neutralization\(^{14,17}\), both amnioblasts and uteroplacental cells use thiol or sulfhydryl (-SH) groups. By measuring TAS levels, we can detect the change of thiol or sulfhydryl (-SH) scavengers in AP. Significantly decreased amniotic fluid TAS levels in the AP group compared to healthy controls suggest that the ROS scavenging ability of uteroplacental cells is reduced. Since thiol and sulfhydryl groups are used in the neutralization of the possible antioxidant defense system, their synthesis and release have decreased.

Oxidative stress index is one of the best indicators of redox balance in amniotic fluid and is calculated as the ratio of TOS to TAS \((\text{TOS/TAS}=\text{OSI})\). We found an approximately 30-fold increase in OSI value in patients in the AP group compared to the control group. This value suggests that the increase in oxidant molecules is far above the neutralization capacity of the antioxidant defense system. However, we do not know clearly whether the increase in OSI is a cause or a result of AP. The main purpose of the developing placenta is to provide easy access to sufficient gas and nutrients for the fetus. For this purpose, trophoblasts invade the spiral arteries to a certain extent\(^{18,19}\). Adequate trophoblast invasion ensures low-resistance and high-volume blood flow between mother and fetus. As in all ischemic placental diseases, defective trophoblast invasion will impair gas and nutrient exchange between fetus and mother in AP, leading to hypoxia first, then insufficient perfusion and then ischemia-reperfusion damage\(^{18,19}\). Ischemic changes in the placental bed cause an increase in free radical production and the release of proinflammatory cytokines\(^{20}\). Increased OSI values in AP cases are an important evidence of ROS overproduction. Increased OSI may damage uteroplacental junctional cells, leading to hypoxic damage and premature separation of trophoblasts. Therefore, it can be said that impaired redox balance in AP cases is a result of insufficient trophoblast invasion.
In ischemic placental diseases with impaired redox balance insufficient trophoblast invasion leads to hypoxia, resulting in an increase in free radical production. Hypoxia stimulates the production of inflammatory cytokines\textsuperscript{10,21}. NF-κB is one of the main proinflammatory dimers secreted in hypoxic tissues. NF-κB is found in the cytoplasm in an inhibited form\textsuperscript{22}. NF-κB works in coordination with prostaglandins and COX enzymes in placenta\textsuperscript{11,23} to control the production of angiogenic factors for feto-maternal gas exchange and fetal access to nutrients. In this study, we detected approximately 5-fold increased NF-κB expression in the AP group compared to healthy controls. This increase in NF-κB may be due to the increase in ROS. An increase in NF-κB above physiological limits may create a pathological inflammatory environment and adversely affect trophoblast development and feto-maternal gas exchange\textsuperscript{24}. The increase in OSI is a sign of ROS production and indicates the onset of inflammation. The increase in TOS, decrease in TAS and increase in NF-κB may cause endothelial damage to be more serious and cause the coagulation system to be activated\textsuperscript{25}. Severe endothelial damage may induce infarction of trophoblasts, inducing premature separation of the placenta. Since uterine transplant patients may be in the risk group for AP, close follow-up of these patients is recommended\textsuperscript{26}.

Conclusions

Our results are of clinical importance as it is the first clinical study investigating amniotic fluid redox balance and proinflammatory cytokine production in AP. Increased ROS production in AP activates the NF-κB pathway, causing ischemic cell damage. Hypoxic cell damage in the endothelium and trophoblasts leads to inadequate placentation, resulting in premature separation of the placenta. ROS overproduction and activation of proinflammatory pathways are important culprits in AP formation. Placental separation can be delayed with ROS scavenging and anti-inflammatory drugs. If our results are supported by comprehensive case-controlled studies, it may be possible to develop new medical agents in the management of AP.

Conflict of Interest

All authors have nothing to disclose.

Informed Consent

Informed consent was obtained from all participants.

Ethics Approval

All interventional procedures in this study were performed in accordance with both ethical and Helsinki Declaration standards, with the consent of the patients and the Ethics Committee of the Kayseri City Training and Research Hospital.

Funding

None.

Authors’ Contributions

All authors contributed to the study conception and design. Material preparation was performed by Mehmet AK and Bertan Demir. Data were collected by Bertan Demir and Mehmet AK. All authors contributed to statistical analysis. The first draft was written by Mehmet AK. All authors approved the final version of the manuscript.

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