Determination of pro-oxidant-antioxidant balance (PAB) assay in mothers with spontaneous abortion

Faezeh Ghasemi, Alireza Kamali, Maryam Shokrpour

**Abstract:** Oxidative stress has been identified to play a vital role in the pathogenesis of spontaneous abortion were characterized as an imbalance between the generation of pro-oxidants, free radicals, and reactive species, and enzymatic and non-enzymatic antioxidant defenses in favor of the former. In the present study, the pro-oxidant-antioxidant balance was assessed in women with spontaneous abortion and compared with healthy age-matched controls. A group of 50 females with spontaneous abortion were considered patients, and a group of age-matched healthy pregnant women were considered controls. The pro-oxidant-antioxidant balance (PAB) assay was carried out on participants’ serum samples. The mean age of the spontaneous abortion group was 30.84 ± 3.82, and controls were 26.53 ± 4.05 years. The obtained PAB values were 236.74 ± 11.37 HK and 148.69 ± 76.50 HK in patients and controls. Our results demonstrate the significant rise of PAB values in subjects with spontaneous abortion compared to healthy controls (p<0.0001). Our study showed that the PAB values might be involved in the termination of spontaneous abortion.

**Key words:** Spontaneous abortion, Oxidative stress, Antioxidants, PAB assay.

**Introduction**

Spontaneous abortion is considered as the fetus’s natural death before 20 gestations. Based on scientific declarations, spontaneous abortion defines by pregnancy termination before complete 20 weeks gestational period while the fetal weight is less than a half kilogram. Massive investigation showed that the rate of spontaneous abortion depends on pregnancy age and miscarriage. According to the Cardone et al. rate of spontaneous abortion increased in elder females compare with younger cases. In North American pregnant females, spontaneous abortion happened in 17%-20% cases younger than 25 years old whereas increased to 30%-35% in those with 35-45 years old. Cramer and Wise published data demonstrated that miscarriage increase spontaneous abortion risk. The incidence of spontaneous abortion in females with no miscarriage is approximately 13 percent and increased to 25%, 30%, and 35% in those who suffered from 1, 2, and 3 miscarriages, respectively. Further investigation illustrated the various spontaneous abortion risk factors. Dickey RP and colleagues represented the higher incidence of spontaneous abortion in one-fourth of females treated with human gonadotropin and clomiphene.

Moreover, fragmentation of sperm DNA, hyperhomocysteineemia, immunological disorders, and insufficient antioxidant defenses have also been observed in females with spontaneous abortion. Oxidative stress, a profound imbalance between pro-oxidants, reactive oxygen species, free radicals, and enzymatic and non-enzymatic antioxidants in favor of the former, plays the leading role in disorders and deficiencies. Despite the essential role of oxygen in metabolism and life persistency, its toxic derivatives can be caused severe damage to cellular compartments.

Reactive species damage DNA, mitochondria, and cell membrane, which affects the cellular compartments pathologically and leads to ultimate cellular demise. In response to oxidative damage, the antioxidant system neutralizes the excess pro-oxidants, maintains physiological balance, and establishes cellular homeostasis. Simsek et al. uncovered the essential role of oxidative damage, higher level of lipid peroxidation, and reactive oxygen species immediately before abortion.

Although the exact causes of spontaneous abortion are not clearly understood, immunological alterations and profound disruption of oxidative stress seem to be involved etiologically. Our study aims to characterize the prooxidant-antioxidant balance (PAB) in patients with spontaneous abortion.

**Materials and methods**

**Subjects**

The presented study was conducted on 80 pregnant females (50 with spontaneous abortion history as a patient group and 30 normal age-matched healthy women without a history of abortion as controls) hospitalized between March 2017 and October 2018 in Taleghani Hospital Arak, Iran. The first day of gestation was determined based on ultrasound obtained data, and obstetricians evaluated the risk of spontaneous abortion in participants. Females who had been exposed to teratogenic drugs were excluded, whereas the participants who had at least 1 terminated abortion before complete 20 weeks gestation were categorized as the patients. Moreover, all participants were signed the written consent to contribute to the survey that the ethics committee approved of Arak Medical School.

**Sample collection**

For all subjects, the age, history of spontaneous abortion, gestational time, deliveries, and abortions were recorded. To carrying out the PAB assay, 2 ml arterial-venous mixed cord

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blood was collected after delivery and abortion. The collected blood samples were centrifuged at 3600 rpm for 10 minutes after clotting to separate sera. To measure the oxidative stress parameters in the rapid, accurate method, the obtained sera were kept at -80°C until analysis. The hemolytic samples were excluded from the PAB assay.

Chemicals

The chemicals were used in our survey include TMB powder (3, 3′, 5, 5′-Tetramethylbenzidine, Fluka), chloramine T trihydrate (AppliChem: A4331, Darmstadt, Germany), peroxidase enzyme (AppliChem: 230 U/mg, A3791), 0005, Darmstadt, Germany), hydrogen peroxide (Merck). Double distilled water was applied for dissolving molecular biology grade reagents that need further preparation.

The pro-oxidant antioxidant balance (PAB) assay

PAB assay was carried out based on our previous studies14,15. Standard solutions were prepared by blending the exact proportion of 250 M H2O2 (0-100%) and uric acid 3 mM and added to the NaOH (10 mM). To prepare TMB cation, 60 mg TMB powder was dissolved in 10 ml dimethyl sulfoxide (DMSO) and was added to 20 ml acetate buffer (0.05 M, pH 4.5). After that, well-mixed 70 µl freshly chloramine T solution (100 mM) was added to the previously prepared solution, shaken immediately, and incubated for 2 hours at room temperature (23°C-27°C) in the dark places.

The horseradish peroxidase working solution was prepared by blending the 25-unit peroxidase enzyme solution into the 20 ml TMB cation and aliquoted into 1 ml microtubes and stored at -20, respectively. TMB working solution was prepared by gently blending 200 µl TMB/DMSO into the 10 ml acetate buffer (0.05 M, pH 5.8).

To determine the PAB values in the subjects’ sample, 200 µl working solution was added to each well of 96-well ELISA. Then, 10 µl participants’ sera, distilled H2O (blank well), and standard solutions were added and mixed gently as well as the plates were incubated in the dark place at 37°C for 15 minutes. The 50 µl of 2 N HCl was recruited as the stop solution, and the PAB was measured at 450 nm wavelength.

The PAB values were demonstrated in arbitrary HK units, the percentage of H2O2 in the standard solution. Meanwhile, unknown samples were then calculated based on the obtained values from the standard drowning curve.

Statistical analysis

The Statistical Package for Social Sciences (SPSS version 20.0, IBM, USA) was used for data analysis while the P-value < 0.05 was considered statistically significant. The Kolmogorov-Smirnov test was carried out to determine the data’s normality, and unpaired sample T-tests were applied for evaluating the statistical analysis.

Results

The mean age of participants with spontaneous abortion and controls were 30.84±3.82 and 26.53±4.05 years, respectively. The higher range and lower range of participants’ PAB value shows 214 – 253.74 HK in females with spontaneous abortion and 2.50 – 264.03 in the standard group. Our obtained data revealed a significantly raised level of PAB values in spontaneous abortion participants compared to the standard group (p-value <0.0001) (figure 1).

No abortion was seen in controls, while in the spontaneous abortion group, 21 cases had had one abortion, and 5 cases had had 3 abortions. Furthermore, 29 cases had delivered at least one normal childbirth (Table 1).

Discussion

Massive investigations demonstrate the role of oxidative stress in the pathogenesis of spontaneous abortion, pre-eclamptic pregnancy, and related pregnancy hypertensive...
The corpus luteum activity is fundamental for embryogenesis and fetal development. The early stages of pregnancy and trophoblast implantation are dependent on corpus luteum activity which relies upon superoxide dehydrogenase-enzyme activity. In the ovary, superoxide dehydrogenase prevents the restrictive reaction of hydrogen peroxide-derived peroxide and superoxide ions on gonadotropin hormones and affects the ovary’s follicular function16-18. Recent investigations demonstrate that the proper fetus implantation seriously depends on low oxygen concentration to complete 10 gestational weeks19. It may protect fetal development and differentiation against reactive species, free radicals, and immunological reactions19. Compelling evidence uncovered the deficient trophoblastic invasion that occurred following the spontaneous abortions23. Therefore, in the same direction as our obtained data, oxidative stress may raise the spontaneous abortions before complete 10 weeks of gestation, which might be the consequence of maternal blood-flow disorders16-18. Higher levels of oxidants such as malondialdehyde increase in females with spontaneous abortion24. Sugino et al. published a report that showed the higher concentration of lipid peroxidation in females with spontaneous abortion suffered from vaginal bleeding25. These observations boost our hypothesis about the role of oxidative stress in spontaneous abortion group and controls.

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Higher lipid peroxidation and oxidative stress biomarkers such as malondialdehyde increase in females with spontaneous abortion24. Sugino et al. published a report that showed the higher concentration of lipid peroxidation in females with spontaneous abortion suffered from vaginal bleeding25. These observations boost our hypothesis about the role of oxidative stress in spontaneous abortion. In other words, higher levels of reactive species, free radicals and consequent oxidative damages lead to placenta oxidation, spontaneous abortion, and fetal termination. A higher level of lipid peroxides such as malondialdehyde may involve in urine evacuation. The corpus luteum activity is fundamental for embryogenesis and fetal development. The early stages of pregnancy and trophoblast implantation are dependent on corpus luteum activity which relies upon superoxide dehydrogenase-enzyme activity. In the ovary, superoxide dehydrogenase prevents the restrictive reaction of hydrogen peroxide-derived peroxide and superoxide ions on gonadotropin hormones and affects the ovary’s follicular function16-18. Recent investigations demonstrate that the proper fetus implantation seriously depends on low oxygen concentration to complete 10 gestational weeks19. It may protect fetal development and differentiation against reactive species, free radicals, and immunological reactions19. Compelling evidence uncovered the deficient trophoblastic invasion that occurred following the spontaneous abortions23. Therefore, in the same direction as our obtained data, oxidative stress may raise the spontaneous abortions before complete 10 weeks of gestation, which might be the consequence of maternal blood-flow disorders16-18. Higher levels of oxidants such as malondialdehyde increase in females with spontaneous abortion24. Sugino et al. published a report that showed the higher concentration of lipid peroxidation in females with spontaneous abortion suffered from vaginal bleeding25. These observations boost our hypothesis about the role of oxidative stress in spontaneous abortion group and controls.

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Conclusions

A significant increase in pro-oxidants and oxidative stress were reported in several investigations. Based on our data, profound disruption of oxidative stress may involve spontaneous abortion even though more surveys are needed.
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