

Catalytic ferrous iron in amniotic fluid as a predictive marker of human maternal-fetal disorders

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Amniotic fluid contains numerous biomolecules derived from fetus and mother, thus providing precious information on pregnancy. Here, we evaluated oxidative stress of human amniotic fluid and measured the concentration of catalytic Fe(II). Amniotic fluid samples were collected with consent from a total of 89 subjects in Nagoya University Hospital, under necessary medical interventions: normal pregnancy at term, normal pregnancy at the 2nd trimester, preterm delivery with maternal disorders but without fetal disorders, congenital diaphragmatic hernia, fetal growth restriction, pregnancy-induced hypertension, gestational diabetes mellitus, Down syndrome and trisomy 18. Catalytic Fe(II) and oxidative stress markers (8-hydroxy-2'-deoxyguanosine, 8-OHdG; dityrosine) were determined with RhoNox-1 and specific antibodies, respectively, using plate assays. Levels of 8-OHdG and dityrosine were higher in the 3rd trimester compared with the 2nd trimester in normal subjects, and the abnormal groups generally showed lower levels than the controls, thus suggesting that they represent fetal metabolic activities. In contrast, catalytic Fe(II) was higher in the 2nd trimester than the 3rd trimester in the normal subjects, and overall the abnormal groups showed higher levels than the controls, suggesting that high catalytic Fe(II) at late gestation reflects fetal pathologic alterations. Notably, products of H₂O₂ and catalytic Fe(II) remained almost constant in amniotic fluid.

Key Words: amniotic fluid, catalytic ferrous iron, oxidative stress, pregnancy

Perinatal diagnostic procedures utilizing amniotic fluid as the matrix, in search of chromosomal abnormalities, anomalies, and infectious diseases, confer precious clinical information in humans. Therefore, they are important because they allow not only obstetricians and pediatricians to be ready for any needed treatments of the baby but also allow the parents to understand the baby's condition and be prepared to accept the baby. During the 2nd trimester of human pregnancy, fetal renal function develops and urinary excretion gradually increases. By the 3rd trimester, approximately 80% of amniotic fluid consists of fetal urine, and the other 20% of amniotic fluid is derived from oral, nasal, tracheal and pulmonary cells of fetus and the amnion. Thus, amniotic fluid is in direct contact with the fetal oropharynx, lungs, gastrointestinal tract, skin, and urinary system.⁽¹⁾ Amniotic fluid also contains nutrients and growth factors, provides mechanical cushioning and antimicrobial effectors that protect the fetus and allow an assessment of fetal maturity as well.⁽²⁾

Oxidative stress is associated not only with various human diseases but also with physiological conditions. Previous studies

have shown that maternal oxidative stress increases in serum according with the progression of pregnancy^(3,4) and that pregnancy complications are associated with both maternal and fetal oxidative damage. For example, using F₂-isoprostane as a marker molecule, subjects with preeclampsia,⁽⁵⁾ fetal growth restriction⁽⁶⁾ and Down syndrome⁽⁷⁾ demonstrate oxidative stress in amniotic fluid at 15 to 18 weeks of gestation. In subjects with gestational diabetes mellitus (DM; type 1 or insulin-treated), the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in amniotic fluid tested at term were also increased and associated with fetal hypoxia demonstrated by increased erythropoietin in amniotic fluid.⁽⁸⁾ The cell-free mRNA levels in amniotic fluid suggest that oxidative stress is increased in Down syndrome.⁽¹⁾

Excess iron is a major cause of oxidative stress because catalytic Fe(II) can generate hydroxyl radicals in the presence of H₂O₂ through the Fenton reaction, resulting in oxidative damage to biomolecules. Indeed, iron overload, which is associated with the presence of catalytic iron, leads to a variety of diseases including DM,⁽⁹⁾ liver cirrhosis,⁽¹⁰⁾ and cancer.⁽¹¹⁾ Overdose of iron supplementation in pregnant women may result in oxidative stress.^(12,13) Previous studies showed that non-protein bound iron (NPBI) in the plasma of the umbilical vein is higher in neurologically abnormal neonates than in normal neonates.⁽¹⁴⁾ There have been only two studies on NPBI in the amniotic fluid of normal pregnancies. Determinations were made with high-performance liquid chromatography (HPLC) using nitrilotriacetic acid to capture iron bound to low-molecular-weight proteins and/or nonspecifically bound to the proteins, followed by two filtration steps and by using 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridone as the high-affinity iron ligand which forms a colored complex with ferric iron with the absorbance at 450 nm.^(15,16) Thus far, there have been no studies performed on the levels of catalytic Fe(II) in amniotic fluid during abnormal pregnancies.

In this study, we aimed (i) to determine the levels of oxidative stress and catalytic Fe(II) concentration with H₂O₂ in amniotic fluid during pregnancy and (ii) to compare those in normal pregnancies with those in abnormal pregnancies. This study measured two markers: 8-hydroxy-2'-deoxyguanosine (8-OHdG) and dityrosine. 8-OHdG is a major product of oxidative DNA damage, and it is excreted almost entirely to urine.⁽¹⁷⁾ Dityrosine is a dimer of tyrosine and is produced by the oxidation of tyrosine residues in protein via peroxynitrite, metal-catalyzed oxidation or UV-irradiation.⁽¹⁸⁾ Recently, dityrosine was identified in the atherosclerotic lesion of apo-E-deficient mice⁽¹⁹⁾ and in the liver of

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rats injured by acetaminophen.⁽²⁰⁾ In the present study, we reveal, for the first time, the biologic significance of catalytic Fe(II) and oxidative stress markers in human amniotic fluid.

Materials and Methods

Human amniotic fluid samples. This study was reviewed and approved for the protection of human rights by the Institutional Review Board of Nagoya University Graduate School of Medicine. Prior to amniocentesis or caesarean section, written informed consent was obtained from all the participants. During the period from January 1, 2009 to August 31, 2013, we collected amniotic fluid from pregnant women who underwent amniocentesis for chromosomal analysis or caesarean section at Nagoya University Hospital.

Amniocenteses were performed between the 16th and 18th gestational weeks (early gestation group). Using a transabdominal approach, approximately 23 ml of amniotic fluid was obtained from each subject. Twenty ml of the amniotic fluid were used for karyotype examination and the remaining 3 ml were used for the present study. Except for early gestation group, the amniotic fluid samples were collected at caesarean section. At caesarean section, amniotic fluid samples were aspirated after uterine incision and prior to the rupture of the amniotic membrane. Then, amniotic fluid samples were stored at 4°C until centrifuge processing on the same day. Amniotic fluid samples were centrifuged for 10 min at $3,000 \times g$ to remove cellular and particulate matters, and the supernatant was stored at -80°C until assay. Each amniotic fluid specimen was subjected to three freeze-thaw cycles at the maximum.

Subject groups. (i) Control group (late gestation group) ($n = 13$): women at term gestation (>37 weeks' gestation) with uncomplicated pregnancies and underwent caesarean section for breech position or previous history of uterine surgery; (ii) middle gestation group ($n = 6$): women delivered by cesarean section at 26 to 34 weeks of gestational age because of maternal disease such as cervical cancer and pituitary tumor but without obstetrical complications; (iii) early gestation group ($n = 12$): women who underwent amniocentesis for screening of karyotype at 16–18 weeks due to one or more of the following reasons (advanced maternal age, abnormal levels of maternal serum markers for Down syndrome such as α -fetoprotein, human chorionic gonadotropin and unconjugated estriol, fetal ultrasound anomalies, previous history of chromosomal anomalies, or maternal request), and all newborns of the samples used in the present study were delivered at term without any complications; (iv) pregnancy-induced hypertension group ($n = 10$) was defined by gestational hypertension with or without proteinuria that resolved after delivery. Gestational hypertension is defined as systolic blood pressure of >140 mmHg or diastolic blood pressure of >90 mmHg, starting after the 20th weeks of gestation in previously normotensive women. Proteinuria is defined as >300 mg protein in a 24-h urine collection or continuously more than 2+ proteins on a voided urine. This diagnostic criterion is determined by JSOG (Japan Society of Obstetrics and Gynecology);⁽²¹⁾ (v) gestational DM group ($n = 8$): according to protocols in our hospital, according to the guideline of JSOG, all pregnant women receive a screening test for gestational DM at the first and second trimester. If the casual blood sugar level exceeds 100 mg/dl or more, a 75 g oral glucose tolerance test is performed. If the test is positive, the woman is diagnosed as gestational DM and treated only with diet therapy or concomitant with insulin administration by diabetes specialists of our hospital; (vi) fetal growth restriction group ($n = 12$) was defined as infant birth weight less than -1.5 standard deviation (SD) based on a reference for the Japanese population; (vii) congenital diaphragmatic hernia group ($n = 12$): fetuses were diagnosed with ultrasound and magnetic resonance imaging prenatally, which was confirmed after birth; (viii) Down syndrome

group ($n = 7$): prenatal diagnosis was done with G-banding chromosomal analysis using amniotic fluid based on ultrasound findings, followed by a definitive diagnosis with chromosomal analysis after birth; (ix) trisomy 18 group ($n = 9$): prenatal diagnosis was done G-banding chromosomal analysis using amniotic fluid, followed by a definitive diagnosis after birth. Almost all the cases were hydramnion.

Determination of 8-OHdG. Amniotic fluid samples were thawed and used for the determination of 8-OHdG levels with a commercial enzyme-linked immunosorbent assay (ELISA) kit (New 8-OHdG Check; Nikken Zeil, Fukuroi, Shizuoka, Japan) according to the manufacturer's instructions. The amount of 8-OHdG in each subject was calculated by comparison with a standard curve; a typical standard curve ranged from 0.5–200 ng/ml. The measurements were done in duplicate.

Determination of dityrosine. Amniotic fluid samples were thawed and used for the determination of dityrosine with a commercial ELISA kit (Dityrosine analysis kit; Nikken Zeil) according to the manufacturer's instructions. The amount of dityrosine in each subject was calculated by comparison with a standard curve; a typical standard curve ranged from 0.05–12 μM . The measurements were done in duplicate.

Determination of catalytic Fe(II). Recently, a highly specific probe, named RhoNox-1, for labile Fe(II) was developed. RhoNox-1 was a kind gift from Tasuku Hirayama and Hideko Nagasawa.⁽²²⁾ Previously we reported RhoNox-1 dose-dependently reacted only with Fe(II) but not with Fe(III) and it showed positive linear relationship ($r = 0.977$, $p < 0.0001$).⁽²³⁾ To make Fe(II) standard solution, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Wako, Osaka, Japan) was dissolved in deionized water (MilliQ). RhoNox-1 was diluted with dimethylsulfoxide to make 1 mM solution, which was further diluted with 10 mM phosphate-buffered saline, pH = 7.2, to make a 1 μM solution. Amniotic fluid samples were thawed and used without dilution. To 100 μl of Fe(II) standard solution (0, 0.156, 0.31, 0.625, 1.25, 2.5, 5, 10 μM) or 100 μl of amniotic fluid samples, 100 μl of RhoNox-1 (1 μM) were added within the 96 well black-microplate well (#MS-8096K, Sumitomo Bakelite Co., Osaka, Japan), which was followed by an incubation at room temperature for one hour under cover with aluminum foil. Thereafter, fluorescence intensity was measured with a microplate reader (Power Scan4, DS Pharma Biomedical, Osaka, Japan) with excitation wavelength at 530 nm and with emission wavelength at 575 nm. The concentration of catalytic Fe(II) in each sample was calculated in comparison to a standard curve. The measurements were done in duplicate.

Determination of hydrogen peroxide (H_2O_2) concentration. Amniotic fluid samples were thawed and used for H_2O_2 determination with a commercial kit (Hydrogen peroxide fluorometric detection kit; Enzo Life Science, Farmingdale, NY) according to the manufacturer's instructions. The H_2O_2 concentration in each subject was calculated by comparison with a standard curve. The measurements were done in duplicate.

Statistical analysis. Statistical analysis of the data was performed with statistical package for the social sciences (SPSS) for Windows (ver. 20.0; SPSS, Chicago, IL) and Excel for Windows 2010 (Microsoft, Redmond, WA). Differences between the means were assessed using the Mann-Whitney test, Student's t test or Bonferroni test. A p value of <0.05 was considered statistically significant.

Results

Patients. Demographic data and clinical characteristics of each group are shown in Table 1 and 2. In Table 1, the birth weight in preterm delivery group was significantly lower than the controls. However, the birth weights of fetuses in the preterm delivery group were appropriate for the length of gestation. In Table 2, no significant differences were observed in maternal age among the

Table 1. Demographic data of normal pregnancy groups

	Early gestation (n = 12)	Middle gestation (n = 6)	Late gestation (n = 13)
Age (years)	38.3 ± 4.1 [†]	32.5 ± 3.3	32.8 ± 5.6
Gestational age at amniocenteses (w)	16.4 (16.0–17.9) [†]	29.6 (26.1–34.0) [†]	37.5 (37.3–38.9)
SBP at amniocenteses (mmHg)	115.6 ± 15.9	110.4 ± 10.9	112.5 ± 13.3
DBP at amniocenteses (mmHg)	71.1 ± 10.9	63.8 ± 14.8	73.5 ± 10.4
Maternal BMI at amniocenteses	22.3 ± 4.5*	25.6 ± 3.7	26.2 ± 3.4
Birth weight (g)	NA	1,401 (1,024–1,950)**	2,830 (2,450–3,450)

Values are shown as the mean ± SD or median (minimum–maximum). * $p < 0.05$, ** $p < 0.01$ vs Normal 37–38 w (control) by Student's *t* test; [†] $p < 0.01$ vs Normal 37–38 w (control) by Mann-Whitney test. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; NA, not applied.

Table 2. Clinical characteristics of abnormal pregnancy groups

	Control (n = 13)	PIH (n = 10)	GDM (n = 8)	FGR (n = 12)	CDH (n = 12)	Down syndrome (n = 7)	Trisomy 18 (n = 9)
Age (years)	32.8 ± 5.6	34.8 ± 5.0	33.8 ± 3.6	31.8 ± 5.0	29.5 ± 5.0	37.6 ± 4.1	36.3 ± 5.0
Gestational age at caesarian section (w)	37.5 (37.3–38.9)	37.1 ^{††} (28.0–37.7)	38.3 (37.0–40.6)	37.4 (33.3–38.1)	38.1 (37.0–39.9)	34.4 ^{††} (29.0–37.7)	32.1 ^{††} (28.3–37.9)
SBP at delivery (mmHg)	112.5 ± 13.3	151.0 ± 13.3**	119.1 ± 8.3	115.8 ± 13.3	109.4 ± 12.4	113.1 ± 7.0	114.4 ± 14.0
DBP at delivery (mmHg)	73.5 ± 10.4	94.0 ± 9.9**	73.8 ± 8.7	71.2 ± 8.0	68.6 ± 7.1	70.0 ± 3.6	72.9 ± 13.1
Maternal BMI at amniocenteses	26.2 ± 3.4	24.4 ± 3.7	29.4 ± 4.8	23.4 ± 2.4 [†]	24.6 ± 1.8	23.5 ± 1.4 [†]	24.1 ± 2.7
Birth weight (g)	2,830 (2,450–3,450)	2,504 (620–3,160)	3,017 (2,498–3,336)	1,912** (1,190–2,402)	2,638 (2,250–3,050)	2,262* (1,046–3,160)	1,492** (955–2,060)

Values are shown as the mean ± SD or median (minimum–maximum). * $p < 0.05$, ** $p < 0.01$ vs Normal 37–38 w (control) by Student's *t* test; [†] $p < 0.05$, ^{††} $p < 0.01$ vs Normal 37–38 w (control) by Mann-Whitney test. PIH, pregnancy induced hypertension; GDM, gestational diabetes mellitus; FGR, fetal growth restriction; CDH, congenital diaphragmatic hernia.

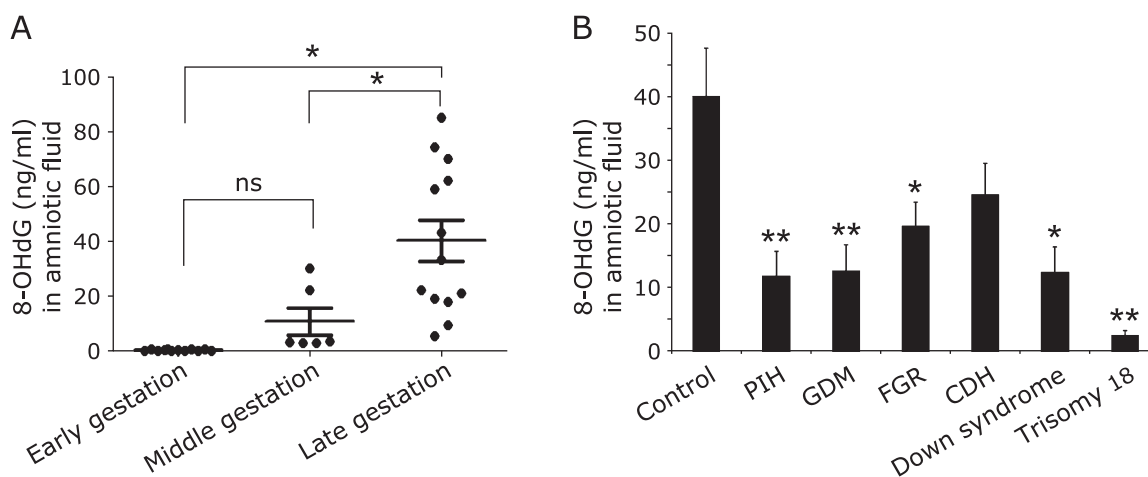


Fig. 1. 8-OHdG levels in amniotic fluid. (A) 8-OHdG levels in amniotic fluid were increased according with the progression of gestational period (mean ± SEM; * $p < 0.01$ vs control with Bonferroni test). (B) Comparison of control with abnormal pregnancies. 8-OHdG levels in abnormal pregnancies were significantly lower than the controls (mean ± SEM; * $p < 0.05$ by Student's *t* test; ** $p < 0.01$ by Mann-Whitney test). PIH, pregnancy induced hypertension; GDM, gestational diabetes mellitus; FGR, fetal growth restriction; CDH, congenital diaphragmatic hernia.

groups. Women with pregnancy-induced hypertension revealed significantly higher blood pressure than the control group and the other groups. Fetal growth restriction and Down syndrome groups showed significantly lower maternal body mass index (BMI) than the control group ($p < 0.05$). Groups of pregnancy-induced hypertension, Down syndrome and trisomy 18 delivered earlier than the controls ($p < 0.05$). The fetal growth restriction group showed significantly lower birth weight than the controls. Pregnancy-induced hypertension and Down syndrome groups

also showed significantly lower birth weight, but it was attributable to their shorter gestational ages at delivery. The trisomy 18 group presented significantly lower birth weight due to the disease.

Oxidative stress markers. The results from measuring the levels of oxidative stress markers, 8-OHdG and dityrosine, are presented in Fig. 1 and 2. In the normal pregnancy, both 8-OHdG and dityrosine levels increased as gestation progressed. Then, the marker levels of the controls were compared with those of abnormal pregnancies (Fig. 1B and 2B). In abnormal pregnancies,

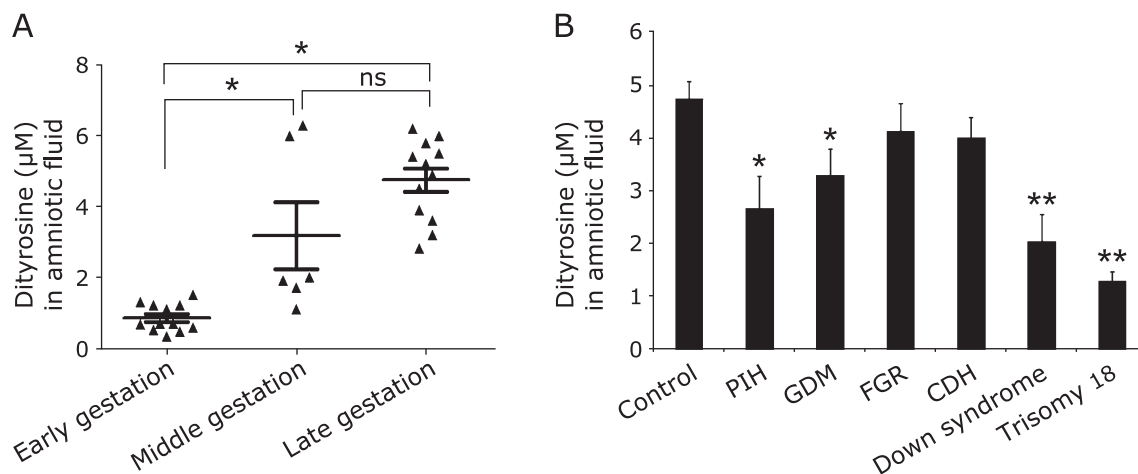


Fig. 2. Dityrosine levels in amniotic fluid. (A) Dityrosine levels in amniotic fluid were increased as gestation progressed (mean \pm SEM; * p <0.01 with Bonferroni test). (B) Comparison of control with abnormal pregnancies. Dityrosine levels abnormal pregnancies were significantly lower than the controls (mean \pm SEM; * p <0.05; ** p <0.01 with Student's *t* test). PIH, pregnancy induced hypertension; GDM, gestational diabetes mellitus; FGR, fetal growth restriction; CDH, congenital diaphragmatic hernia.

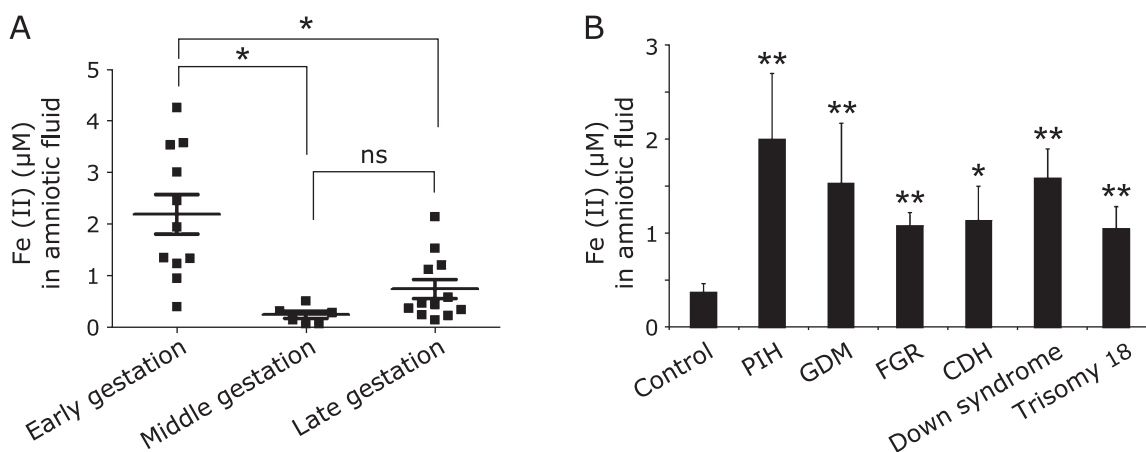


Fig. 3. Levels of catalytic Fe(II) in amniotic fluid. (A) Catalytic Fe(II) levels in amniotic fluid of normal pregnancies were decreased according with the progression of gestational periods (mean \pm SEM; * p <0.01 with Bonferroni test). (B) Comparison of control with abnormal pregnancies. Catalytic Fe(II) levels in amniotic fluid of abnormal pregnancies were all significantly higher than the controls (mean \pm SEM; * p <0.05; ** p <0.01 by Mann-Whitney test). PIH, pregnancy induced hypertension; GDM, gestational diabetes mellitus; FGR, fetal growth restriction; CDH, congenital diaphragmatic hernia.

8-OHdG levels were lower than the controls. Especially, the pregnancy-induced hypertension, gestational DM, fetal growth restriction, Down syndrome and trisomy 18 groups revealed significantly lower 8-OHdG levels. The dityrosine levels of the control group were higher than those of abnormal pregnancy groups. Thus, dityrosine and 8-OHdG levels presented a similar pattern. Especially, the dityrosine levels of pregnancy-induced hypertension, gestational DM, Down syndrome and trisomy 18 groups were significantly lower than the controls.

Catalytic Fe(II). The catalytic Fe(II) levels are summarized in Fig. 3. The catalytic Fe(II) concentration in amniotic fluid was significantly higher in the 2nd trimester than the 3rd trimester. All the abnormal pregnancy groups revealed significantly higher catalytic Fe(II) levels than the controls.

H₂O₂ concentration in amniotic fluid. H₂O₂ concentration in amniotic fluid is shown in Fig. 4. In the normal pregnancy, H₂O₂ levels showed no significant differences (Fig. 4A). H₂O₂ levels in the control group were higher than abnormal groups.

Especially, the levels in gestational DM, fetal growth restriction, Down syndrome and trisomy 18 groups were significantly lower than the controls (Fig. 4B).

To assess the balance of hydrogen peroxide and catalytic Fe(II), both levels were multiplied. The results are shown in Fig. 5. In the normal pregnancy, the products of each groups showed no significant differences (Fig. 5A). Further, the products of abnormal pregnancies also showed no significant differences compared to controls and also among them (Fig. 5B).

Discussion

The present study, for the first time, showed that oxidative stress in amniotic fluid, as determined by the 8-OHdG and dityrosine levels, increased as gestation progressed. This result is distinct but agrees with a previous study showing the increase in maternal oxidative stress with progression of gestation in normal pregnancies.⁽⁴⁾ The levels of the two oxidative stress markers in

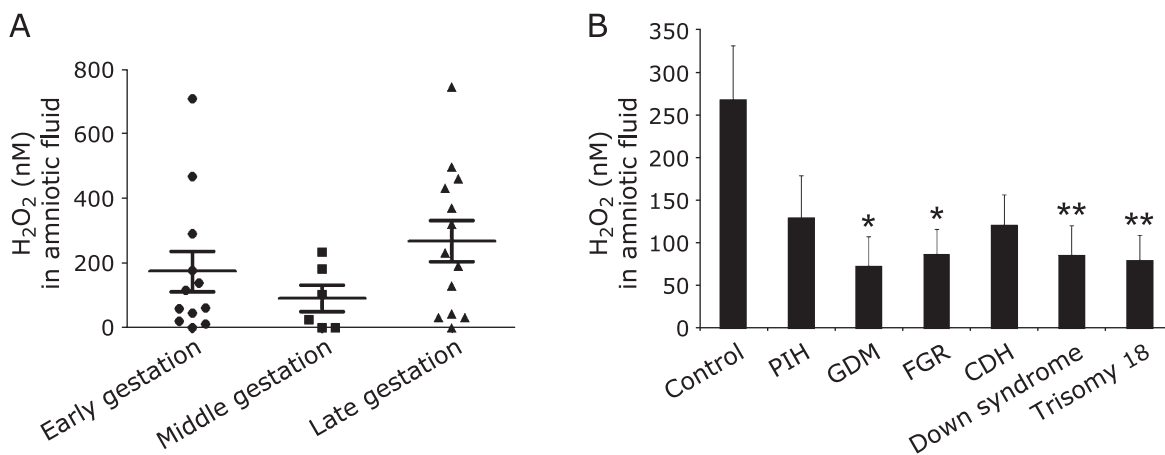


Fig. 4. Concentration of hydrogen peroxide (H₂O₂). (A) The levels of H₂O₂ in amniotic fluid of normal pregnancies showed no significant differences among each pregnant stages (mean \pm SEM; Bonferroni test). (B) Comparison of control and abnormal pregnancies. The levels of control were higher than abnormal pregnancies (mean \pm SEM; * p <0.05 by Mann-Whitney test, ** p <0.05 by Student's t test).

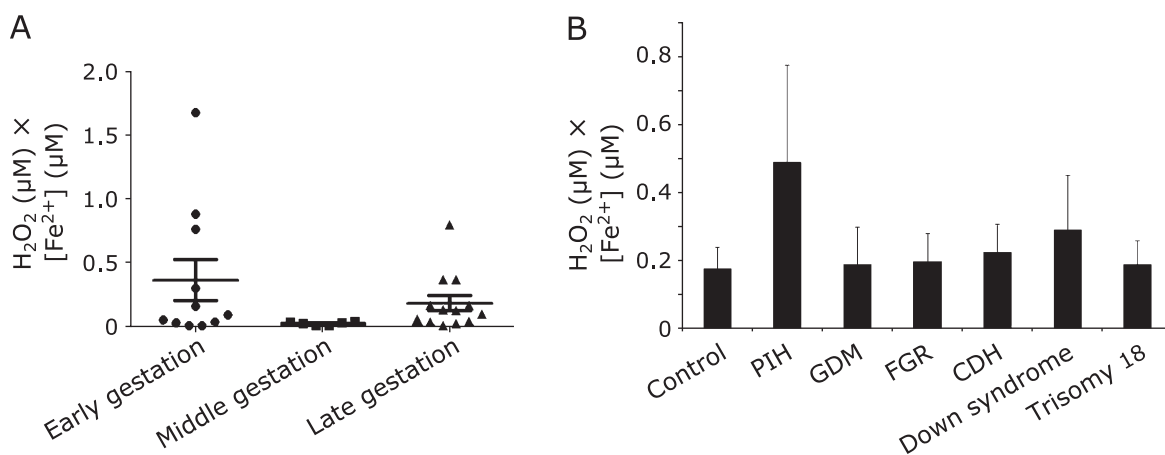


Fig. 5. Product of H₂O₂ and catalytic Fe(II). (A) Products of normal pregnancy showed no significant differences (mean \pm SEM; Bonferroni test). (B) Comparison of control group with abnormal pregnancies. Products also showed no significant differences (mean \pm SEM; Mann-Whitney test).

amniotic fluid of abnormal pregnancies were lower than that of the normal pregnancy. These results are in contrast to the previous reports.^(1,5-8) We speculate that the inconsistencies are caused by the different gestational ages when the amniotic fluid was sampled. Previous reports used amniotic fluid of the 2nd trimester as controls.^(1,5-7) However, we used the amniotic fluid of the 3rd trimester, specifically near-term or term pregnancy, as controls. Because amniotic fluid of the early 2nd trimester is derived primarily from maternal serum, amnion and placenta, whereas it is derived primarily from fetal urine in the 3rd trimester,⁽²⁾ the biological significance of samples taken in the two different trimesters is likely to differ. Here, we propose that the levels of marker molecules in the later pregnancy stage parallel the fetal activities, including oxygen consumption, energy production and metabolism of toxic substances, making the time-dependent increases plausible. We hypothesize here that 8-OHdG in amniotic fluid may be a limiting factor of the duration of intrauterine life because 8-OHdG itself is mutagenic.⁽²⁴⁾ Indeed, the activity levels of fetuses affected by oxidative stress are much lower than those of normal fetuses.

There are two previous studies on 8-OHdG levels in amniotic fluid.^(8,25) Escobar *et al.*⁽⁸⁾ used amniotic fluid from patient with

diabetic pregnancies at term, reporting that the levels of 8-OHdG correlate with fetal chronic hypoxia concomitant with erythropoietin increase in amniotic fluid. They showed the levels of 8-OHdG were 0.22 to 11.01 nM. Lam *et al.*⁽²⁵⁾ used amniotic fluid of normal pregnancy at term, which reported the levels of 8-OHdG as 0.27 to 0.85 nM. These results are single or double-digits less than the levels of 8-OHdG of this study. Whereas 8-OHdG was measured by HPLC in both previous reports, we used ELISA. Both methods have merits and weaknesses. ELISA can measure numerous samples simultaneously, saving time in comparison to HPLC and also detects DNA fragments with 8-OHdG. Notwithstanding, the measurements of 8-OHdG by HPLC highly correlates with those by ELISA.⁽²⁶⁾ We speculate that we measured not only 8-OHdG but also DNA fragments including dimers and polymers containing 8-OHdG. Our study is the first to measure that 8-OHdG in amniotic fluid of normal pregnancy at the 2nd trimester and that of various abnormal pregnancies at the 3rd trimester.

In the present study, we have measured catalytic Fe(II) in amniotic fluid for the first time. RhoNox-1 is a recently developed, highly selective luminescent probe for free Fe(II).⁽²²⁾ Our results revealed that catalytic Fe(II) decreased as gestation progressed. Previously, Gazzolo *et al.*⁽¹⁵⁾ measured non-protein bound iron in

amniotic fluid in the 2nd trimester with HPLC, showing the increase with progression of gestation from the 15th to the 18th weeks. Our present data correspond to similar levels as theirs in the 2nd trimester. Notably, we showed that catalytic Fe(II) levels decrease in the 3rd trimester. However, we also have to consider the difference in the iron included for measurements because RhoNox-1 is specific to Fe(II) and not to Fe(III).

The levels of catalytic Fe(II) in abnormal pregnancies, such as those with pregnancy-induced hypertension, gestational DM, fetal growth restriction, congenital diaphragmatic hernia, Down syndrome and trisomy 18, were significantly higher than in normal pregnancies. Previous studies suggested that abnormal pregnancies are associated with oxidative stress, suggesting that the presence of catalytic iron induces oxidative stress by the Fenton reaction. However, our study showed low oxidative stress by 8-OHdG and dityrosine in the amniotic fluid of abnormal pregnancies. Therefore, we conclude that the source of catalytic Fe(II) is damaged fetuses, among whom cellular death is presumably abundant. In this situation, removal of H₂O₂ with enzymes, such as peroxidases, would be important to prevent further deleterious reactions.

In this study, we also measured H₂O₂ levels in amniotic fluid. H₂O₂ levels were very low (~nM levels), and the levels in abnormal pregnancies were lower than the controls. We suspect that Fenton reaction will not easily occur in amniotic fluid due to the minimal H₂O₂ levels. However, we believe that catalytic Fe(II) will be taken up by the fetus by swallowing, which may accelerate Fenton reaction within the abnormal fetus. Notably, the product of H₂O₂ and catalytic Fe(II) showed no significant differences among each group. We believe that this may represent a balance between H₂O₂ and catalytic Fe(II) levels. An antioxidant capacity may be different in each fetus, depending on gestational ages and conditions. There are many reports on the antioxidant levels in amniotic fluid, such as superoxide dismutase, glutathione peroxidase,⁽²⁷⁾ and vitamins C and E⁽²⁸⁾ but with a wide variations.

There are several limitations in our study, including the relatively small size of samples. Due to ethical concerns, it is impossible to obtain amniotic fluid of every pregnant woman. Amniocentesis is not suitable for screening because it is an invasive examination. Differences in the total volume of amniotic

fluid in each pregnancy is also the problem. This pertains particularly to Down syndrome and trisomy 18, which were often complicated with hydramnion, preterm delivery, or intrauterine fetal death. In the current study, the measurements were not corrected with any standard molecules. The levels of 8-OHdG in urine are usually corrected by urinary creatinine. However, amniotic fluid is derived not only from fetal urine but also from different sources of fetuses and mothers. Conversely, amniotic fluid volume is affected by fetal renal function and swallowing ability. It would be necessary in further research to establish which molecule is most effective to correct the 8-OHdG levels in amniotic fluid in treatment settings.

In conclusion, our results revealed that catalytic Fe(II) in the amniotic fluid of abnormal pregnancies is significantly higher than in normal pregnancies and thus can be one of the markers of abnormal pregnancy. Accordingly, high catalytic Fe(II) may result in additional oxidative stress in abnormal pregnancies. Removing excess catalytic iron in amniotic fluid may help to decrease the oxidative stress in abnormal pregnancies and to improve fetal prognosis.

Acknowledgments

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Abbreviations

DM	diabetes mellitus
ELISA	enzyme-linked immunosorbent assay
HPLC	high-performance liquid chromatography
NPBI	non-protein bound iron
8-OHdG	8-hydroxyl-2'-deoxyguanosine

Conflict of Interest

No potential conflicts of interest were disclosed.

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