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# Assessment of Redox Imbalance in Idiopathic Fetal Growth Restricted Pregnancies

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## Abstract

**Background:** Fetal growth restriction (FGR) is one of the most significant causes of perinatal morbidity and mortality. The known causes are poor maternal nutrition, hypertensive disorders complicating pregnancy, maternal medical disorders (multiple pregnancy, congenital fetal malformations), and lifestyle influences. However, in vast majority of cases, the cause still remains unknown. Recent studies have suggested the role of oxidative stress in the pathophysiology of FGR. The aim of this study was to evaluate non-enzymatic oxidative stress biomarkers in maternal and cord blood of idiopathic FGR cases.

**Methods:** A total of 100 women subjects aged 18 to 35 years, who fulfilled the recruitment criteria, were enrolled in the study after taking an informed written consent. Non-enzymatic oxidative stress was measured by the quantification of 8-hydroxy-2-deoxy-guanosine (8-OHdG), malondialdehyde (MDA), protein carbonyl, reduced glutathione (GSH) and ferric reducing ability of plasma (FRAP) in maternal and cord blood samples of FGR pregnancies of idiopathic origin and compared with those of normal healthy mother-infant pairs.

**Results:** The levels of 8-OHdG, MDA and protein carbonyl were significantly higher in the 'idiopathic' FGR group as compared to the controls, where as the GSH and FRAP were significantly lower.

**Conclusion:** Increased oxidative stress has been found significantly associated with the increased risk of developing idiopathic FGR.

**Keywords:** Fetal growth restriction; Redox imbalance; Maternal and cord blood

## Introduction

Fetal growth restriction (FGR) is the second leading contributor to perinatal mortality after prematurity [1]. The incidence is between 3-10% in developed countries, which increases to approximately 20% in developing countries [2]. FGR neonates have an increased perinatal and infant mortality and are more prone to cardiovascular disease, obesity and diabetes in adulthood [3]. There are multiple maternal, fetal and placental disorders that affect fetal growth. The known causes are poor maternal nutrition, hypertensive disorders complicating pregnancy, maternal medical disorders (multiple pregnancy, congenital fetal malformations), and lifestyle influences [4]. However, in vast majority of cases, the cause still remains unknown. Recent literature suggests the role of increased redox imbalance as an ultimate step in etiology of these cases of idiopathic origin [5,6].

Free radicals are generated as by-products of aerobic respiration and metabolism. Mammalian cells have evolved a variety of enzymatic mechanisms to control free radicals production, one of the central elements in signal transduction pathways involved in cell proliferation, differentiation and apoptosis. Imbalances between free radicals production and antioxidant systems induce oxidative stress or redox imbalance that negatively impacts reproductive processes. High levels of free radicals during embryonic, fetal and placental development are a feature of pregnancy. Consequently, redox imbalance has emerged as a likely promoter of several pregnancy-related disorders, such as fetal growth restriction, low birth weight, preeclampsia, preterm labor, spontaneous abortions, embryopathies etc. [7].

Oxidative stress or redox imbalance plays a key role in the pathophysiology of placenta-related disorders, most notably FGR. The decrease in the free radical defense system makes lipids, proteins and DNA vulnerable for oxidative damage [5]. Increased oxidative stress causes breaks on the DNA strands, increases lipid peroxides and platelet aggregation which results in abnormal placental circulation and oxygenation of umbilical blood thus ending in cellular damage and fetal growth restriction [6]. A growing body of literature indicates that oxidative stress is closely associated with FGR. FGR is associated with a late life increased prevalence of metabolic syndrome, a condition associating obesity with hypertension, type-2 diabetes mellitus and cardiovascular disease [8]. The growth hormone (GH)/insulin-like growth factor (IGF) axis is significantly affected by IUGR (Intrauterine Growth Restriction) and some of these alterations may lead to permanent pathological programming of the IGF axis which may play a role in the future occurrence of insulin resistance and hypertension [9]. Utero-placental insufficiency, a decline in oxygen and nutrient supply to the fetus and oxidative stress-induced trophoblast cell death are among the identified disorders also associated with FGR [10].

In this study, we have carried out analysis of maternal and umbilical cord blood in term fetal growth restricted pregnancies of idiopathic origin and compared them with normal healthy pregnancies in order to observe differences in the DNA damage, lipid peroxidation, protein oxidation and antioxidant potential.

## Materials and Methods

This is a prospective case-control study conducted in the

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Department of Obstetrics and Gynaecology and the Department of Biochemistry at the University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India. A total of 100 subjects, aged 18 to 35 years, who fulfilled the recruitment criteria, were enrolled into the study after taking an informed written consent. Ethical clearance was obtained from Hospital Ethical Clearance Committee.

### Participant recruitment

After an exhaustive work-up to exclude any known etiology, fifty women with term 'idiopathic' growth restricted pregnancies who delivered small for gestational age (SGA) neonates (birth weight was less than 10$^{th}$ percentile) constituted the study group. For each case, a matched control was recruited. Thus, fifty women with term low risk pregnancies between 37 to 40 weeks and delivering normal weight neonates were taken as control group. Considering type 1 error at 0.05 and power at 80% to detect the difference of 5 nmoles/ml with standard deviation of around 20, the sample size worked out to be a minimum of 30 in each group.

A detailed history, clinical examination and relevant investigations were carried out in order to exclude the known causes of FGR i.e. maternal medical disorders like hypertensive disorders, severe anaemia, diabetes, renal, liver, heart diseases, substance abuse, obstetrical complications like premature rupture of membranes, antepartum hemorrhage, multiple pregnancy. Pregnancies where the neonates had fetal distress, congenital malformations, difficult delivery and birth injury were also excluded from the study as these conditions might alter the oxidative stress parameters.

The mode of delivery, information on placental morphology and weight were recorded after delivery. The neonate's date and time of birth, sex, weight, Apgar score and anthropometric features were also recorded at the time of birth.

### Sample collection

The maternal blood samples were collected from the recruited subjects at the time of delivery and the cord blood was withdrawn from the umbilical vein before the birth of placenta. Five millilitre (ml) of each maternal and cord blood was collected in plain and EDTA tube. The collected serum from plain vial was kept at -20°C until analysis of MDA, FRAP and protein carbonyl and of EDTA vial for analysis of 8-OHdG and GSH levels. All the tests were performed within 12 hours of sample collection.

### Estimation of DNA damage and biochemical markers

**Measurement of DNA damage:** In order to detect oxidative DNA damage, DNA from the whole blood was purified using HI media DNA extraction kit. After the digestion with alkaline phosphatase and nuclease, the concentrations of 8-OHdG (marker of oxidative DNA damage) were analyzed by ELISA using commercially available kit purchased from Caymen Chemicals, US as per protocol. The kit can measure 8-OHdG values ranging from 33 to 3000 pg/ml. Briefly, plates were marked as blank, non-specific binding (NSB), maximum binding (B0), total activity (TA), standard and samples. 100 µl of EIA buffer was added to NSB and 50 µl of EIA buffer was added to B0 wells respectively. 50 µl of standards and samples in duplicates were added to their respective well. 50 µl of 8-OHdG AChE tracer was added to each well except TA and blank wells. 8-OHdG monoclonal antibody (50 µl) was added to each well except TA, NSB and blank wells. Plate then incubated for 18 h at 4°C. Following incubation, the plate was washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate for AChE) was added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. Since the concentration of the 8-OHdG tracer is held constant while concentration of 8-OHdG varies, the amount of 8-OHdG tracer that is able to bind to the 8-OHdG monoclonal antibody, is inversely proportional to the concentration of 8-OHdG.

**Measurement of lipid peroxidation:** The lipid peroxide levels in plasma of maternal blood were measured using a thiobarbituric acid (TBA) reactive substances assay, which monitors MDA production based on the method of Satoh [11]. The MDA-TBA adduct formation was measured spectrophotometrically at 532 nm. The concentration of MDA was expressed as nmol/ml.

**Estimation of plasma protein carbonyl (PC) levels:** Plasma PC levels were measured according to the method on spectrophotometer detection of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl to form protein hydrazones [12]. The results were expressed as nanomoles of carbonyl groups per milligram of protein.

**Measurement of ferric reducing ability of plasma:** Ferric-reducing ability of plasma (FRAP) was determined by measuring the ability of plasma to reduce $Fe^{3+}$ to $Fe^{2+}$ by the method of Benzie and Strain [13]. The complex between $Fe^{2+}$ and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) gives a blue color with absorbance at 593 nm. The concentration of FRAP was expressed in µmol/L.

**Measurement of reduced glutathione:** The total reduced glutathione (GSH) content in blood was measured by the method of Tietze using 5,5' dithiobis-2 nitrobenzoic acid (DTNB) [14]. In this method, GSH was oxidized by DTNB and then reduced by GSH reductase, with NADPH as hydrogen donor. The oxidation of GSH by DTNB was detected photometrically by a change of absorption at 412 nm. The concentration of blood GSH was expressed as µmol/dL.

### Statistical analysis

Statistical analysis of the data was performed with SPSS-15 using student's t-test. A p-value of <0.05 was considered as statistically significant. The values were reported as mean ± SD.

## Results

### Demographic parameters

Table 1 depicts the demographic parameters of the population

| Parameters | | FGR (n=50) | Controls (n=50) |
|---|---|---|---|
| **Age in years (mean ± SD)** | | 23.82 ± 2.8 | 23.16 ± 3.8 |
| **Period of gestation (weeks)** | | 38.3 ± 0.9 | 38.3 ± 0.9 |
| **Occupation** | Housewife (%) | 92 | 100 |
| | Working (%) | 8 | 0 |
| **Religion** | Hindu (%) | 90 | 84 |
| | Muslim (%) | 10 | 16 |
| **Residence** | Urban (%) | 100 | 100 |
| | Rural (%) | 0 | 0 |
| **Living style** | Colony (%) | 90 | 96 |
| | Slum (%) | 10 | 4 |
| **Water Supply** | Govt. directly (%) | 60 | 76 |
| | Ground directly (%) | 30 | 20 |
| | Govt. after filtration or boiling (%) | 10 | 4 |
| **Dietary habits** | Vegetarian (%) | 64 | 54 |
| | Non-vegetarian (%) | 36 | 46 |
| **Obstetrical profile** | Primigravida (%) | 64 | 60 |
| | Multigravida (%) | 36 | 40 |

**Table 1:** Maternal characteristics of FGR and control subjects.

| Parameters | | FGR (n=50) (mean ± SD) | Controls (n=50) (mean ± SD) | *p*-value |
|---|---|---|---|---|
| General physical examination | Height (cm) | 153.8 ± 4.8 | 154.1 ± 4.4 | 0.732 |
| | Weight (kg) | 52.3 ± 4.6 | 55.7 ± 7.6 | 0.009* |
| | BMI (kg/m²) | 22.2 ± 2.3 | 23.4 ± 2.3 | 0.013* |
| Placental weight (grams) | | 396.0 ± 56.1 | 548.5 ± 76.8 | <0.001* |
| Neonatal outcome | Birth weight (kg) | 2.1 ± 0.1 | 3.0 ± 0.2 | <0.001* |
| | Length (cm) | 47.9 ± 1.2 | 50.1 ± 1.1 | <0.001* |
| | Head circumference (cm) | 33.4 ± 0.7 | 34.0 ± 0.4 | <0.001* |
| | Chest circumference (cm) | 29.9 ± 0.8 | 32.5 ± 0.8 | <0.001* |
| | Mid-arm circumference (cm) | 8.9 ± 0.5 | 10.5 ± 0.7 | <0.001* |

* Significant

**Table 2:** Examination and neonatal outcomes of FGR and control subjects.

| Parameters | FGR (n=50) (mean ± SD) | Controls (n=50) (mean ± SD) | P value |
|---|---|---|---|
| DNA damage (pg/ml) | 71.16 ± 10.48 | 56.96 ± 6.64 | <0.001* |
| MDA (nmol/ml) | 3.19 ± 0.78 | 2.20 ± 0.30 | <0.001* |
| Protein carbonyl (nmol/mg protein) | 0.24 ± 0.14 | 0.13 ± 0.03 | <0.001* |
| FRAP (mmol/ml) | 484.58 ± 145.84 | 607.86 ± 100.27 | <0.001* |
| GSH (µmol/dL) | 181.24 ± 57.91 | 242.28 ± 31.77 | <0.001* |

**Table 3:** DNA damage and biochemical markers in maternal blood of FGR and control subjects.

| Parameters | FGR (n=50) (mean ± SD) | Controls (n=50) (mean ± SD) | P value |
|---|---|---|---|
| DNA damage (pg/ml) | 66.31 ± 10.26 | 56.81 ± 5.99 | <0.001* |
| MDA (nmol/ml) | 3.03 ± 1.08 | 2.35 ± 0.41 | <0.001* |
| Protein carbonyl (nmol/mg protein) | 0.24 ± 0.16 | 0.12 ± 0.02 | <0.001* |
| FRAP (mmol/ml) | 474.52 ± 91.60 | 534.82 ± 147.21 | 0.010* |
| GSH (µmol/dL) | 183.20 ± 52.97 | 241.58 ± 38.48 | <0.001* |

* Significant, n=number of subjects

**Table 4:** DNA damage and biochemical markers in cord blood of FGR and control subjects.

recruited into the study. All the women who participated in the study were of relatively homogenous group and the majority was young housewives, residing in urban colonies, belonging to lower-middle and upper-lower socio-economic class. They consumed water supplied by the government without filtration or boiling. The dietary habits were not different in two groups. Almost 60-64% of women were primigravidas. None of them were smokers or alcohol abusers (data not shown). As expected, the mean weight, weight gain during pregnancy and the body mass index was significantly lower in the FGR cases as compared to the normal controls. All the subjects had normal vaginal delivery without any maternal or fetal complications. The morphology of the placenta was normal but the placental weight was significantly less in the FGR group compared to the controls. As a consequence of the recruitment criteria the anthropometric parameters of the FGR group neonates were significantly lower than the normal term neonates ($p<0.05$) (Table 2).

## DNA damage and biochemical markers

The markers of DNA damage (8-OHdG), lipid peroxidation (MDA) and protein oxidation (protein carbonyl) were studied in the maternal and cord blood (Tables 3 and 4). The maternal and cord blood levels of 8-OHdG, MDA and protein carbonyl are shown in figures 1 and 2. The mean maternal blood levels of 8-OHdG in FGR were 71.16 ± 10.48 pg/mL as compared to 56.96 ± 6.64 pg/mL in the control group. The corresponding levels in the cord blood were 66.31 ± 10.26 pg/mL in FGR and 56.81 ± 5.99 pg/mL in controls. The mean MDA level of the maternal blood from the FGR cases was 3.19 ± 0.78 nmol/ml as compared to 2.20 ± 0.30 nmol/ml in the controls and the corresponding values from the cord blood were 3.03 ± 1.08 nmol/ml versus 2.35 ± 0.41 nmol/ml. The mean maternal blood levels of protein carbonyl in FGR were 0.24 ± 0.14 nmol/mg as compared to 0.13 ± 0.03 nmol/mg in the control group. The corresponding levels in the cord blood were 0.24 ± 0.16 nmol/mg in FGR and 0.12 ± 0.02 nmol/mg in controls. The levels of 8-OHdG, MDA and protein carbonyl were found to be significantly increased ($p<0.001$) in both maternal and cord blood samples of FGR cases as compared to full term normal controls.

The anti-oxidants protect the tissues against oxidative stress. The levels of FRAP and GSH measures the antioxidant status in the body. The maternal and cord blood levels of FRAP and GSH are shown in tables 3 and 4 (Figure 3). The mean level of FRAP from the maternal blood was 484.58 ± 145.84 mmol/mL in FGR as compared to 607.86 ± 100.27 mmol/mL in controls, and the corresponding values in cord blood was 474.52 ± 91.60 mmol/mL in FGR versus 534.82 ± 147.21 mmol/ml in controls. The mean maternal levels for GSH were 181.24 ± 57.91 µmol/dL in FGR as compared to 242.28 ± 31.77 µmol/dL in controls. The corresponding values in the cord blood were 183.20 ± 52.97 µmol/dL in FGR compared to 241.58 ± 38.48 µmol/dL in controls. The maternal and cord blood levels of FRAP and GSH were found to be significantly lower in FGR cases in comparison to full term normal controls ($p<0.001$).

## Discussion

Pregnancy, itself is a state of increased oxidative stress which is

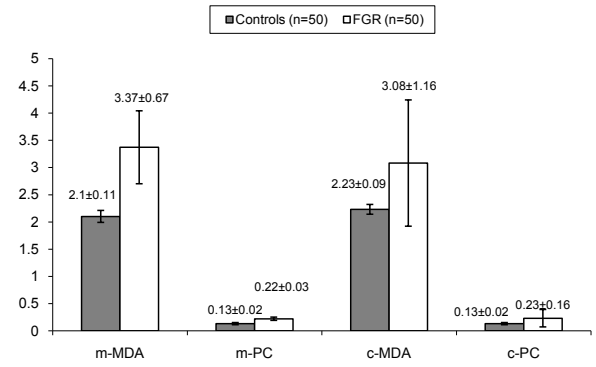


**Figure 1**: Maternal and cord blood levels of MDA and Protein carbonyl in FGR and Controls.

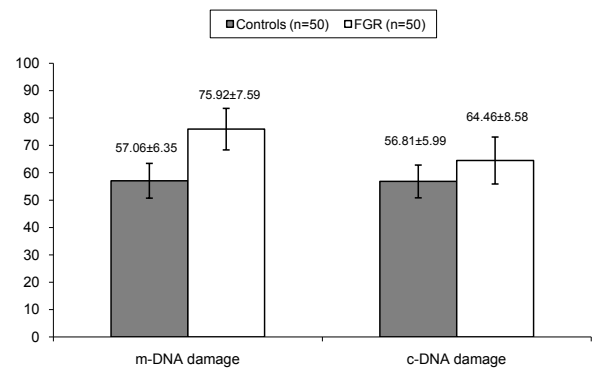


**Figure 2:** Maternal and cord blood levels of DNA damage in FGR and Controls.

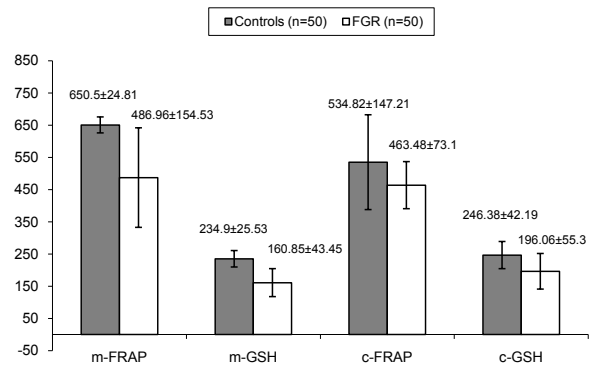


**Figure 3:** Maternal and cord blood levels of antioxidants in FGR and Controls.

aggravated in conditions like FGR [15]. On the other hand oxidative damage of biological substances such as lipids, proteins and DNA has been implicated as a cause of certain diseases in adults as well as neonates and has also been implicated in adverse pregnancy outcome like fetal growth restriction, preeclampsia and preterm births [16].

Oxidative damage to lipids is called lipid peroxidation, wherein polyunsaturated fatty acids and their residues of membrane phospholipids are oxygenated in the presence of free radicals, mainly leading to the production of MDA [17]. The MDA levels have been found to be increased in the pregnancies complicated by intrauterine hypoxia [18]. The anti-oxidant protection, as conferred by the levels of FRAP and GSH and the enzyme activities are inadequate to defend the body against oxidative stress and thus lead to increased lipid peroxidation damage.

Protein carbonyls are formed by the production of carbonyl groups on protein side-chains and via oxidative cleavage of proteins [19]. As they are chemically stable, they serve as a marker for detection and storage. A high concentration of carbonyl groups indicates a high risk of protein destruction. Our study shows a significant increase in the protein carbonyl levels in FGR pregnancies indicating increased protein destruction and thus increased DNA damage. It also shows a significant increase in oxidative DNA damage in growth restricted pregnancies of idiopathic origin resulting in birth of SGA neonates [20].

In the present study, thus we have observed that lipid peroxidation, protein destruction and DNA damage was significantly increased in neonates born to mothers having FGR pregnancies of unknown etiology. The mothers of this group expressed elevated MDA, endogenous protein and DNA damage and proteolytic activity during pregnancy indicating increased oxidative stress and decreased anti-oxidant activity (shown by decreased levels of FRAP and GSH) as compared to normal pregnancies. The study by Hracsko et al. [5] evaluated oxidative stress markers and DNA damage in neonates with FGR. The results of their study were similar to ours but the evaluation was done only in the cord blood and not maternal samples, moreover the sample size was limited only to 29 cases. Biri et al. [6] studied the role of oxidative stress in maternal, cord blood and placentas of 25 subjects, out of which only 13 were FGR which were also preterm (33-34 weeks) and 12 were controls (>37 weeks period of gestation). The results of their study were similar to present study but limited by small sample size. Also, they did not evaluate DNA damage in their study. Study of Kamath et al. [21] was restricted to the estimation of the lipid and protein oxidation markers in maternal and cord blood of 13 FGR cases of variable etiologies and 15 controls between 33-39 weeks, the results of which were similar to this study. Previous studies from our lab have also reported significant association between increased oxidative stress and adverse pregnancy outcomes [22,23]. These studies have shown that increased oxidative stress may be associated with environmental chemicals and genetic polymorphism.

The present study scores over other existing scientific literature as it has a larger sample size including only 'idiopathic' cases of FGR pregnancies and estimated DNA damage in addition to other biochemical markers in both maternal as well as cord blood. However, this study has certain limitations such as analysis of mitochondrial and anti-antioxidant enzyme levels.

## Conclusion

To conclude, the present study found a significant association of increased redox imbalance as evident from DNA damage and biochemical biomarkers with growth restricted pregnancies of idiopathic etiology. Further studies with a larger number of subjects are required to prove the cause-effect relationship and to study the effect of therapeutic or prophylactic role of anti-oxidants in preventing the damage induced by oxidative stress. Moreover, the achievement of a successful pregnancy represents one of the fundamental functions of existence. Thus, studies showing association between increased redox imbalance and adverse pregnancy outcomes needs to be complemented by currently available genome-wide approaches, evaluation of interaction between genes, genes and environment and the contribution of paternal and embryonic genotypes. Further prospective studies will be necessary to assess the predictive potential of markers identified through these and other strategies.

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