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# ROLE OF OXIDATIVE STRESS IN OUTCOME OF PERINATAL ASPHYXIA IN TERM NEONATES

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**ABSTRACT** **BACKGROUND** Perinatal asphyxia (PA) may result in significant functional and biochemical changes due to hypoxia/ischemia of various organs. This study was done to evaluate the presence of oxidative stress in perinatal asphyxia and correlate it with outcome

**METHOD:** This case-control study was conducted on 76 term neonates [38 cases with asphyxia and 38 controls]. Cord blood sample was collected from cases and controls. Another venous sample was collected after 48-72 hours from cases only. Serum Malondialdehyde (MDA) and serum Protein carbonyl (PC) levels were estimated in all samples.

**RESULTS:** The median MDA levels of cases at birth were significantly higher in cases than in controls. The levels of MDA nearly doubled in cases at 48-72 hours ($p<0.001$).The median PC levels at birth were significantly higher in cases [as compared to controls ($p<0.01$). Increase in PC levels at 48-72 hours was not significant

**CONCLUSION:** Oxidative stress may be responsible for multi-organ dysfunction in perinatal asphyxia which may be associated with adverse outcome.

**KEYWORDS :**

## INTRODUCTION

Perinatal asphyxia (PA) resulting from hypoxic ischemic events during fetal to neonatal transition is characterized by prolonged anaerobic metabolism and exhaustion of ATP. This may result in significant functional and biochemical changes due to hypoxia/ischemia of various organs. Upon –reoxygenation, there is a burst of Reactive Oxygen Species (ROS) which may exaggerate the damage to the brain tissue resulting in hypoxic-ischemic encephalopathy. Many cases of perinatal asphyxia develop hypoxic ischemic encephalopathy (HIE). Oxidative stress during PA may play a role in pathogenesis of HIE which may be less severe with limitation of oxygen supplementation [1,2].

ROS readily react with nearly all cellular biomolecules resulting in structural damage and inhibition of their normal function. Oxidative stress may be assessed through measurement of biomarkers associated with oxidative damage to lipids, proteins and DNA in different body fluids and tissues [3].

Lipid oxidation generates hydroperoxides, which subsequently undergo fragmentation to produce a broad range of reactive intermediates, such as malondialdehyde (MDA) and other lipid peroxidation products. [4] Most commonly, lipids and lipoproteins in biological membranes are major peroxidation targets, hence assays for lipid peroxidation are commonly used for estimation of the oxidative status. Malondialdehyde (MDA) is one of the most commonly studied low-molecular- weight end-product of perocxidation of polyunsaturated fatty acids which is highly cytotoxic because of its ability to bind proteins or nucleic acids very quickly [5].

Oxidative cleavage of the peptide backbone by formation of protein-protein cross-linkages, α-amidation pathway, cleavage of glutamyl residues, along with lipid peroxidation in cell membranes give rise to reactive aldehydes and ketones known as protein carbonyls (PC) [6-8]. Protein carbonyls represent an irreversible form of protein modification and have been demonstrated to be relatively stable with respect to the degradation/clearance in hours/days) in contrast to lipid peroxidation products that are removed within minutes [9,10]. PC is the most commonly used, and also the most general indicator of oxidative protein damage [11,12].

This study was planned to evaluate the presence of oxidative stress in term neonates with perinatal asphyxia and correlate it with outcome of the disease.

## MATERIALS AND METHODS:

This case control study was conducted in a tertiary care hospital in New Delhi. The study was approved by the Institute Ethics Committee. Thirty eight term neonates with evidence of perinatal asphyxia (APGAR score <6 at 5 minutes, need for assisted ventilation at birth for >3 minutes or clinical evidence of HIE) were enrolled as cases. For the control group, thirty-eight healthy term neonates (APGAR score >6 at 5 minutes, no assisted ventilation at birth, clinically well and neurologically normal) born within 24 hours of the index case were selected. The cases and controls were enrolled in the study after obtaining informed consent. Neonates with congenital malformations, intrauterine growth retardation, and maternal chorio-amnionitis were excluded from the study.

Sample of umbilical cord blood (3-4 ml) was obtained in plain tube from all cases and controls at the time of delivery, immediately after cord clamping. A second venous sample (3-4 ml) was collected from cases only at 48-72 hrs of life . There were ethical concerns for collecting samples at 48-72 hours from healthy controls and hence it was not taken. Serum was separated by centrifugation and stored at –80°C till further analysis.

The clinical parameters like sex, gestational age, birth weight, mode of delivery, need of resuscitation, fetal heart rate status before delivery, presence or absence of meconium-stained liquor, APGAR score, presence of gestational diabetes, pregnancy-induced hypertension, were noted at the time of birth.

Serum Malondialdehyde (MDA) by Thiobarbituric acid reactive substances (TBARS) assay and serum Protein carbonylation (PC) levels [ELISA] were estimated in all samples. Statistical evaluation was carried out on spss 17.0 and P-value<0.05 was considered significant.

Classification of HIE was done using Sarnat and Sarnat scoring and HIE was classified as mild (Grade I), Moderate (Grade II) and severe (Grade III). The scoring was based on the consciousness level, tone of muscles, seizures, pupil size , duration/ respiration in neonates [13].

All the subjects (cases and controls) were followed till admission in the hospital (discharge in healthy state/ discharge with neurological complications /death). Worst HIE during the stay was recorded.

**STATISTICAL ANALYSIS:**

The following statistical tests were used in this study. SPSS PC 17 was used for analysis of data.

- Kolmogorov- Smirnov test was used to detect parametric nature of data.
- Comparison of data was done by Mann- Whitney 'U' test for non-parametric data.
- Paired t-test was used to compare follow-up results in patients of asphyxia.
- Spearman's rank correlation coefficient was used for correlation analysis of non-parametric data .

**RESULTS:**

**Maternal Demo Graphic And Clinical Profile**

The cases and control neonates were comparable with respect to maternal age (25.66 ± 4.9 vs 25.4 ± 4.5 years ) , gestational age (38.5 ± 1.1 vs 38.7 ± 1.3 weeks) . There was a significant difference in the mode of delivery (Cesarean versus vaginal), presence of maternal medical illnesses (diabetes, hypertension, severe anemia, heart disease and infection) and obstetric problems (Gestational diabetes mellitus, Pregnancy-induced hypertension and oligohydramnios) between the two groups (p=0.01).

**Demographic And Clinical Profile Of Cases And Controls**

No significant difference was observed between Mean birth weight (2.79 ± 5.6 kg vs 2.81 ± 4.5 kg), head circumference (33.5 ±1.78 cm vs 34.73 ± 4.06 cm) and sex distribution (22(M)/16(F) vs 20(M)/18(F) of cases and controls.

Overall 84.2 % (32 out of 38) cases had evidence(s) of fetal distress. The mean APGAR score of the cases at 1 minute was 2.6 ±0.99 (Range 1-5) which was significantly lower than healthy controls (9.0 ± 0.1). Even at 5 and 10 minutes, the APGAR score continued to be significantly lower in cases [median value 4 (1-6) and 6 (3-9)] respectively. In cases with perinatal asphyxia, twenty-eight subjects developed HIE [fourteen patients presented in HIE stage 3 (36.8%) followed by seven patients in stage 1 (26.3%) and three patients in stage 2 (10.5%)].

Among the cases, 26.3% (stage I) did not show any features of encephalopathy. 63.1 % cases with perinatal asphyxia (Stage I and Stage II) were discharged from the hospital, out of which 52.6% had normal neurological function and 10.5 % had neurological abnormalities at the time of discharge. All patients with stage 3 HIE had an adverse outcome (death).

The median MDA levels of cases at birth were significantly higher [6.9 nmol/L (range: 0.46-38.4 nmol/L)] than controls [1.8 nmol/L (range: 0.07-20.6 nmol/L)] ($p<0.05$). The levels of MDA nearly doubled in cases at 48-72 hours [13 nmol/L (range: 0.9-61.5 nmol/L)] ($p<0.001$). [table 1] The levels of MDA at 48-72 hours were significantly higher in subjects with grade 2/grade 3 HIE (n=18) than those with grade1 HIE or no encepaholpathy (n=20). MDA levels at 48-72 hours showed a significant positive correlation with HIE staging (r=0.8, P<0.001, Spearman's Rank Correlation).

The median PC levels at birth were significantly higher in cases [0.009 ng/mg of protein (range: 0.002-0.04ng/mg of protein)] as compared to controls [ 0.006 ng/mg of protein (range: 0.003-0.1 ng/mg)] ($p<0.01$). [Table 1]. Increase in PC levels at 48-72 hours was not significant and was mainly seen in HIE grade 3 patients.

**Table 1: Levels Of Serum Malondialdehyde (mda) And Serum Protein Carbonyls (pc) In Controls And Perinatal Asphyxia Cases.**

| | Controls (n=38) | Cases (n=38) |
|---|---|---|
| **MDA (nmol/ ml of serum): (median, range)** | 1.8 (0.07-20.6) | 6.9 (0.46-38.4)* |
| **a. At birth (cord blood)** | ----- | 13.0 (0.9-61.5)*§ |
| **b.48-72 hrs after birth (venous blood)** | | |
| **PC (ng/mg of protein): (median, range)** | 0.006 (0.003-0.1) | 0.009 (0.002-0.04)* |
| **a. At birth (cord blood)** | ----- | 0.01 (0.004-0.17)* |
| **b.48-72 hrs after birth (venous blood)** | | |

*p < 0.01 by Mann Whitney U test in comparison to controls

§p<0.001 by Paired t-test

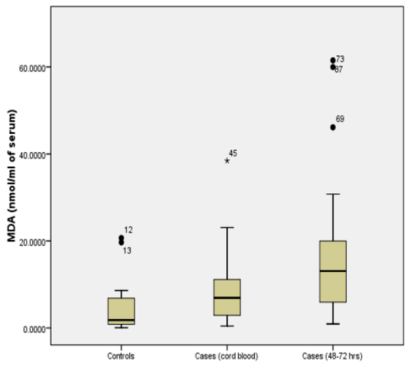


**Figure 1 : Box plot showing MDA levels in cases and controls**

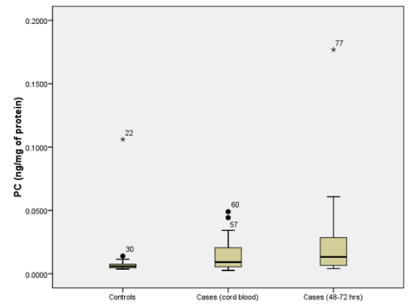


**Figure 2: Box plot showing Protein Carbonyl levels in cases and controls**

**DISCUSSION**

Serum MDA levels and protein carbonyl levels were significantly higher (4-times and nearly 1.5 times respectively) in cases in cord blood and in the 48-72 hour samples in comparison to controls. Similar results were seen in earlier studies carried out by Mondal et al, Thorat, Bhatia et al and Zitnanova [14-17]. Kumar etal [18] have also demonstrated an increase in plasma and Cerebrospinal Fluid MDA levels in newborns with perinatal asphyxia .The higher level of serum MDA and protein carbonyls in perinatal asphyxia group indicates that oxidative stress prevails in this condition. The increase in serum MDA and PC may be explained by the fact that free radical-induced peroxidative damage to poly-unsaturated fatty acids in membranes leads to critical cell injury. If not interrupted, it may lead to irreversible damage to the cells [19] as seen in cases of HIE stage III in present study.

Mean serum MDA level in cases increased significantly (nearly doubled ) during next 48-72 hours as compared to cord blood levels. Kumar etal, have also demonstrated a progressive increase in plasma MDA levels with greater severity of hypoxic ischemic encephalopathy. These changes in MDA levels were noticed despite up-regulation of anti-oxidant enzymes in that study [18]. But the rise in serum protein carbonyl level in cases was not statistically significant. The difference in rise in these two oxidative stress parameters might due to the differences in their (a) sensitivity, (b) kinetics or (c) both. Serum MDA is a sensitive but less specific parameter to assess oxidative stress while protein carbonyl is less sensitive but more specific. The difference in the kinetics of MDA and protein carbonyl may also contribute to differential rise of these parameters in serum after 48-72hr of asphyxia induced tissue damages. MDA molecule being water soluble and very small in size comes out rapidly from the oxidatively damaged tissues creating a buildup of MDA in serum subsequent to tissue damage due to an increased influx into blood. The proteins being very large molecules, even after being carbonylated cannot come out of the oxidatively damaged tissues so readily. So the measure of serum protein carbonyl is predominantly a measure of carbonylation of serum proteins by free radicals and it is less contributed by tissue protein carbonylation. Hence, subsequent rise in serum protein carbonyl after 48-72 hours is less because of elimination of source of free radicals in blood following birth as perinatal asphyxia hardly persists beyond few minutes after birth.

The side chains of all amino acid residues of proteins, particularly lysine and arginine are susceptible to oxidation by ROS action and are involved in "carbonyl" stress [19]. In animal models also, an increase on protein carbonyls has been demonstrated around 6 hours after advent of hypoxic –ischemic events and is associated with poor neuro-development outcomes [20, 21]. The altered protein molecules act as trap for free radicals, which may initiate chain reactions that may worsen the brain damage.

There was a significant correlation between HIE staging and serum MDA level at 48-72 hours of birth. The probable reason for significant rise in plasma MDA levels at 48-72 hours after birth in stage III hypoxic ischemic encephalopathy may be because of excessive multi-organ damage in this stage. In stage III HIE, probably there is excessive lipid peroxidation in different organs of the body and release of MDA in plasma [18].

The increase in susceptibility of neonatal brain to oxidative stress may be due to (i) increased concentration of fatty acids in neonatal brain which are vulnerable to lipid peroxidation by free radicals (ii) higher concentration and availability of unbound Iron ($Fe^{2+}$), which can contribute to development of free radicals and (iii) due to cellular structure of immature brain cells which are particularly sensitive to oxidative damage and preferential death mediated by free radicals [22].

**CONCLUSION:**

The changes observed in MDA and PC levels in perinatal asphyxia indicate that oxidative stress may be responsible for tissue damage and multi-organ dysfunction and is associated with adverse outcomes in neonates with perinatal asphyxia.

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