DNA Damage in Perinatal Asphyxia using Micronucleus Assay

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Abstract

This study was conducted to determine the level of DNA damage in perinatal asphyxia us-ing micronucleus(MN) assay and correlate with severity of asphyxia. Eighty term asphyxi-ated and sixty term non-asphyxiated neonates were enrolled in this case /control study. Blood samples were collected within 24 hours of birth for micronucleus assay and estima-tion of serum malondealdehyde (MDA). Micronucleus score was correlated with severity of asphyxia and serum MDA level. There was a significant difference of micronucleus (MN) score and serum MDA level in cases and controls (p value <0.0001). Among babies in vari-ous hypoxic ischemic encephalopathy (HIE) stages the micronucleus score significantly in-creased with severity of asphyxia (p value <0.0001).There was a significant correlation be-tween Apgar score, HIE stages, seizures , micronucleus score and serum MDA level (p value <0.0001). Micronucleus score and serum MDA level correlated well with severity of asphyxia

Key words: DNA damage, Micronucleus, MDA , Perinatal asphyxia
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Introduction

Perinatal asphyxia is a global problem resulting in neona-tal morbidity and mortality [1]. In developing countries the incidence is much higher. Hypoxia leads to specific cellular changes affecting enzymatic activities, mitochon-drial function, cytoskeletal structures, membrane transport and antioxidant defences. The highly reactive oxygen species(ROS) and reactive nitrogen species(RNS) react with several living cell contents eg phospholipids, sugars, aminoacids and nucleic acids leading to lipid peroxidation DNA strand breaks, base methylation etc. The conse-quence could be immediate and long term complications [2]. The DNA damage can be quantified by cytokinesis block micronucleus assay (CBMN). Micronucleus can be originated by fragments or lagging of chromosome dur-ing cell division. It is morphologically similar to main nuclei but smaller in size hence the name. It can be made visible by freezing the cytoplasmic division of dividing cell by addition of Cytochalasin (Cyto-B) [3]. Oxidative stress can be assessed by serum malondealdehyde (MDA) level [4]. We tried to correlate the severity of asphyxia to oxidative stress and DNA damage.

Patients and Methods

The study was conducted in the Cytogenetic unit of De-partment of Anatomy in collaboration with Departments of Pediatrics and Biochemistry from February 2008 to July 2010. The study was approved by the institute re-search council and human ethical committee. Term as-phyxiated appropriate for gestational age babies were taken as cases. Gestational age and weight matched ba-bies without asphyxia were taken as controls. Perinatal asphyxia was diagnosed when more than three of the fol-lowing criteria were present viz; (1)Apgar score less than 6 at 5 mts. (2) meconium stained liquor (3) changes in the fetal heart rate (4) clinical evidence of HIE (5)evidence of multiorgan dysfunction. Preterm or post term babies, large (LGA) or small (SGA) for gestational age babies, those with congenital malformations and delivered of mothers with significant illness were excluded. The CBMNassay was

carried out in conventional RPMI sup-plemented with phytohaemagglutinin (Sigma), on blood lymphocytes using cytochalain-B (Sigma) [5]. Heparini-zed whole blood 0.2 ml of was inoculated in the culture media and incubated at 37ºC for 44 hours. The Cyto-chalasin-B (Sigma) was prepared at a final concentration of 3µl /ml. Binucleated lymphocytes were harvested for 72 hours. The buffy coat was dispersed with hypotonic solution (0.075 M KCl). The cell pallets were fixed with 3:1 methanol acetic acid. The slides were stained with Giemsa stain for three minutes. The micronucleus index for each sample was analysed by counting 1000 binucle-ated cells based on the scoring criteria outlined by HUMN project [6]. The binucleated cells were scored blindly using 400X magnification of the Epiflurescent microscope BX 51 (Olympus). Thiobarbituric acid reac-tive substances(TBRAS) which measure MDA present in the serum was estimated for assessment of oxidative stress.

Statistical Analysis

The statistical analysis has been performed using unpaired student t-test for comparison of parametric test, Mann-whitney for nonparametric test and One way ANOVA (Kruskal Wallis test) for multiple comparison. Pearson correlation coefficient was used for association between the groups. All the data was analysed by Graph Pad (In-Stat, San Diego, USA)

Results

There were 80 cases and 60 control babies. Among the cases there were 50 male babies 30 females while there were 32 male and 28 female babies among controls. Birth weight and gestational age of the asphyxiated babies were not significantly different from the controls. The age, par-ity and mode of delivery were comparable among the groups. The mean and SD of Apgar score [7] in cases were significantly lower than the controls(4.9±1.624 vs 8.633±0.604). Based on Sarnat and Sarnat score [8] 21 babies were in HIE stage -1 , 40 in HIE stage-2, 19 in HIE stage-3. Among the cases 59 (73.75%) developed seizure, 50 were discharged, 2 left against medical advice (AMA) and 28 expired. All the control babies were dis-charged

The serum MDA level in asphyxiated babies was found to be significantly increased (6.709±1.695 vs.3. 683 ± 0.536) The serum MDA level (P value <0.0001) posi-tively correlated with severity of asphyxia.

Table 1. Micronucleus score and serum MDA in As-phyxiated and control babies.

Groups	Micronucleus Score	Serum MDA
Control (n=60)	1.212±0.775	3.683±0.536
Hypoxia (n=80)	3.959±2.816	6. 709±1.69
HIE-1 (n=21)	1.579±0.901	4.667±0.950
HIE-2 (n=40)	2.944±1.013	7.117±0.529
HIE-3 (n=19)	8.263±1.327	8. 99±0.5395
Seizure present(n=59)	4.782±2.787	1.780±0.86
Seizure absent(n=21)	1.579±0.901	4.667±0.950

*P value <0.0001 **P value <0.0001

It has been observed that there was a significant differ-ence between micronucleus score in cases and controls (3.959±2.816vs 1.212±0.775). MN score was signifi-cantly increased with severity of asphyxia (P value <0.0001). In babies with seizure there was a significant increase of MN score compared to non-seizure babies

Table 2. Correlation coefficient® among various parameters

Parameter	Correlated Variables			
	HIE	Seizure	MNi	Serum MDA
Apgar Score (n=80)	-0.6314**	-0.7580**	-0.7570**	-0.8325**
HIE Staging (n=80)		0.9599*	0.8123*	0.9004*
Seizure (n=59)			0.8232*	0.7702*
MNi (n=80)				0.9201*

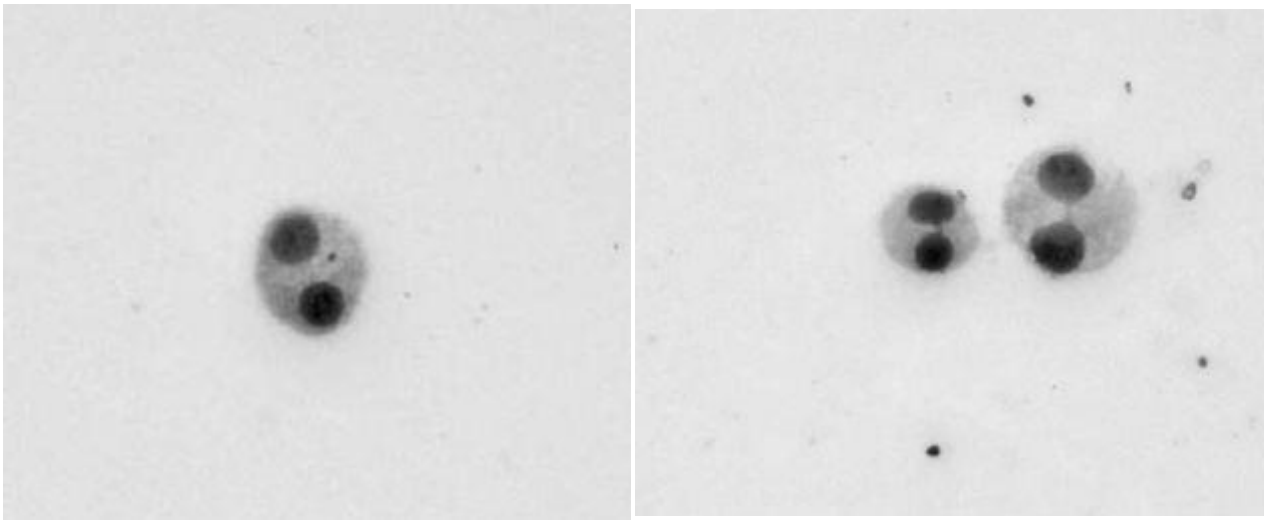

Figure 1. Images of 40 X Geimsa stained A – Binucleated lymphocytes with micronucleus (←) and B- Nucleoplasmic bridge (→) in Perinatal asphyxia. y = 0.3818x + 5.0162R2 = 0.52030123456789100246810Micronucleus ScoreSerum MDA level in micromol/lit.

Figure 2. Correlation Coefficient between micronucleus and serum MDA level r value 0.9201 and p value <0.0001

Discussion

Perinatal asphyxia is one of the major causes of neonatal mortality in developing countries like India. Perinatal asphyxia is characterised by impaired gas exchange which leads to hypoxemia, hypercarbia and metabolic acidosis.

Impaired gas exchange and insufficient perfusion to vital organs leads to severe neurological insult resulting in death and long term disability. The molecular mechanism behind the neurological deficit is not fully evaluated.

Two mechanisms of the DNA damage have been sug-gested. It is envisaged that H2O2 which crosses the bio-logical membrane easily can penetrate the nucleus and react with ions of iron or copper to form hydroxyl radical (OH.) [9]. Other explanation is the ability of oxidative stress to cause DNAdamage by trigerring a series of metabolic events within the cell that lead to the elevation of nuclease enzymes which cleave the DNA backbone. The intracellular free Ca+ interact with Ca+ dependent endonuclease leading to fragmentation of DNA , resem-bling the mechanism that of apoptosis (programmed cell death) [10].

The micronucleus score of cases and controls were sig-nificantly different. Normal and HIE-1 babies have only single strand breaks which could be repaired and the chance of formation of micronuclei is less. There was significant increase in MN score in higher HIE stages. The DNA damage in severe hypoxic ischemic encephalo-pathy is unable to repair. There was deletions, interchro-matid and intrachromatid exchanges. These drastic insta-bility in the genome was visible as micronucleus, nucleo-plasmic bridges and nuclear buds in the next generations of cells. This will arrest further growth of cell and the normal functioning leading to apoptosis and necrosis of the cell. This also leads to alteration in the normal synthe-sis of DNAand gene encoding reflected in the neurologi-cal behaviour of the child [11].

Increased micronucleus score has been observed in women having ovarian cyst and after chemical exposure [12,13]. There was a significant increase in the micronu-clei in cerebral palsy children compared with normal children and in double hemiplegia compared to hemi-paresis patients. The endomutagen cause cerebral palsy in birth asphyxia with excess generation of glutamate. These excess glutamate production leads to the formation of clastogenesis and aneuploidgenesis [14].

Peroxidation of lipids have been reported to damage DNA. It produces wide range of reactive oxygen species includ-ing OH, H2O2, singlet oxygen, peroxyl radicals and alkoxyl radicals. Lipid peroxidation also decomposes to give a huge range of products including carbonyl com-pounds such as malondealdehyde and the unsaturated al-dehyde4-hydroxy-2 transnonenal. If the aldehydes are generated in the vicinity of DNA, they may be able to combine with it to form distinctive products. The associa-tion between oxidative stress and DNA damage have been observed by many authors. MDAreact with adenine, cytosine and guanine , the guanine MDA adduct has been identified in the urine. The above findings is a strong evidence of peroxidation of lipids causing DNA damage [15]. In Perinatal asphyxia we found that the serum MDA level was significantly increased compared to controls. The oxidants and antioxidant level in hypoxic ischemic encephalopathy were found to be significantly higher [16,17]. The MDA level in cord blood of babies with meconium stained liquor with or without birth asphyxia was significantly higher than the controls [18]. The uri-nary creatinine andMDA values were significantly high in asphyxiated babies and their levels correlated with out-come [19]. Markers like serum MDA and protein car-bonyl will be beneficial in predicting the outcome of peri-natal asphyxia [20].

There was a negative correlation between micronucleus score and Apgar score and also MDA level and Apgar score. It has been observed that Apgar score inversely related to DNA damage and oxidative stress. The correla-tion between micronucleus index and HIE stages was positive and also there was a positive correlation between HIE stages and serum MDA values signifying that oxida-tive stress induces DNA damage in perinatal asphyxia. This results in conditions like epilepsy, motor dysfunc-tion, loss of memory, abnormalities in sympathetic and parasympathetic system in later life. Immediate newer therapies like cerebroprotective agents being tried follow-ing asphyxial insult to prevent childhood sequlae.

Reference

1. American Academy of Pediatrics, American College of Obstetrician and Gynecologists. Relationship between perinatal factors and neurological outcome. In: Ploand RL , Truman RK (Eds) Guideline of perinatal care 3rd ed. EK , Illinios. Amarican Academy of Pediatric. 1992; 221-24.
2. Volpe JJ. Hypoxic –ischemic encephalopathy: Clinical Aspects. In Volpe jj , ed. Neurology of the Newborn, Philadelphia, Saunders ; 2000: 332-382
3. Fenech M. The cytokinesis block micronucleus te-chanique and its application to genotoxicity studies in human populations. Environmental health perspective supplements. 1993; 101: 101-107.
4. Satoh K. Serum lipid peroxide in cerebrovascular dis-order determined by a new calorimetric method. ClinChem Acta 1978; 90: 37-43.
5. Rooney DE, Czepulkowski BH, Gosden GM, Davidson C, Robertson M. Human Cytogenetics. Constitutional analysis, A practical approach. Lymphocyte culture, Oxford University Press, Walton street USA1992; 1: 31-37.
6. Bonassi S, Fenech M, Lando C, Lin Y, Ceppi M, Chang WP, Holland N et al. Human micronucleus pro-ject: International database comparison for results with the Cytokinesis-block micronucleus assay in human

lymphocytes1. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei.Environmental and molecular mutagenesis 2001; 37: 31-45.
7. Apgar V. A proposal for new method of evaluation of the New born infant. Current researches in Anesthesia and Analgesia 1953; 32: 260-267
8. Sarnat HB, Sarnat MS. Neonatal encephalopathy fol-lowing fetal distress. A clinical and encephalographic study. Arch Neurol 1976; 33: 696-705.
9. Halliwell B, Okezie I, Arouma. DNA damage by oxy-gen derived species its mechanism and measurement in mammalian systems.Elsevier Science 1991; 281: 9-20
10. Dypbukt JM, Thor H, Nirtlldrn P. Free radicals. ResCommun 1990; 8: 346-354.
11. Kaufmann WK, Richard S, Paules. DNA damage and cell cycle and checkpoints. FASEB journal USA 1996; 283: 238-247
12. Yesileda E, Sahin I, Ozcans H, Yildrm IH, Yologlu S, Taskapan C. Increased micronucleus frequencies in pheripheral blood lymphocytes in women with polcys-tic ovary syndrome. Euro J Endocrinol 2006; 154: 563-568.
13. Santikoviae M, Joksiae G, Marija.Incidence of micro-nuclei in pregnant women and cord samples before and after bombing in Siberia.Arch oncology2004; 12 (4): 200-202.
14. Gainetdinova DD, Zyatidinova GK, Semenova VV, Pakhalina IA, Kolochkova. Clastogenesis and aneugenesis in children with cerebral palsy.Bulletin of ex-perimental biology and medicine. 2005; 139: 596-599.
15. Halliwell B, Gulliridge JMC. Free radicals in biology and medicine, second edition, Clarendon press, Oxford; 1989.
16. Throat VN, Surykumar AN, Sardeshmukh AS, Sarw-ade SS. Oxidants and antioxidants in hypoxic ischemic encephalopathy. Ind J Clin Biochem 2004; 19 (2): 32-35.
17. Singh SK, Dua T , Tandon A Kumari S, Ray G , Batra S. Status of lipid peroxidation and antioxidant enzymes in hypoxic ischemic encephalopathy. Indian Pediatr 1999; 2: 659-668
18. Bhatia BD, Goel A. Study of free radicals in neonates born through meconium stained amniotic fluid deliveries. Indian Pediatr 2005; 42: 956-957.
19. Banupriya C, Ratnakar, Doureradjou, Mondal N, Bhat VB, Koner BC. Can urinary excretion rate of malondealdehyde, uric acid and protein predict the severity and impending death in perinatal asphyxia. Clinical Biochemistry 2008; 41: 968-973.
20. Mondal N, Bhat VB, Banupriya C, Koner BC. Oxida-tive strss in perinatal asphyxia in relation to outcome. Indian J Pediatr 2010; 77: 515-517.

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