

AN IDEAL ESTIMATION TO VALIDATE LIPID PEROXIDATION INDUCING  
OXIDATIVE DNA DAMAGEA. Manoj<sup>\*1</sup>, B. Vishnu Bhat<sup>2</sup>, C. Venkatesh<sup>2</sup> and Z. Bobby<sup>3</sup>Department of Anatomy<sup>1</sup>, Paediatrics<sup>2</sup> and Biochemistry<sup>3</sup>

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**ABSTRACT**

Lipid peroxidation is the oxidative degradation of lipids. It is the process in which free radicals steal electrons from the lipids in cell membranes leading to cell damage. The end products of lipid peroxidation are reactive aldehydes, such as Malondialdehyde (MDA). Malondialdehyde (MDA) is the organic compound colourless liquid which is a highly reactive compound that occurs as the enol. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress. Malondialdehyde reacts with bases adenosine and guanosine in DNA, forming DNA adducts hence it well correlates with DNA damage and chromosomal aberrations. Malondialdehyde and other thiobarbituric reactive substances (TBARS) condense with two equivalents of thiobarbituric acid to give a fluorescent red derivative that can be assayed spectrophotometrically. The methodology for estimation of MDA was standardised for determining the oxidative stress induced DNA Damage in Perinatal Asphyxia. Peripheral veins samples were collected in heparinised tubes. Plasma were separated and stored in deep freezer and estimated the amount of MDA in oxidative stress DNA damages of cases and controls. MDA estimation is recommended for evaluation of oxidative stress induced DNA damage. There was significant elevation of serum MDA level in Perinatal asphyxia.

**KEYWORDS:** Lipid peroxidation, Malondialdehyde (MDA), Deoxynucleic acid (DNA), Thiobarbituric reactive substance (TBRAS).

**INTRODUCTION**

The lipids of mammals are classified into two apolar and polar. The apolar /Triglycerides found in adipose tissue / fat in man which provides energy storage whereas Polar lipid are structural component of cell membrane which control the biophysical state such as polarity, permeability and signalling molecules.<sup>[1]</sup> Lipid damage by Reactive Oxygen Species (ROS) / Free radical (FR) is due to the imbalance between pro-oxidants and antioxidant. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes through a variety of mechanisms including enzymatic reactions and /or auto-oxidation of several compounds, such as catecholamines and hydroquinone. Different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide/insecticides, are sources of ROS production.<sup>[2]</sup>

The two most prevalent ROS that can affect profoundly the lipids are mainly Hydroxyl radical (HO·) and Hydroperoxyl (HO·2). The hydroxyl radical (HO·) is a small, highly mobile, water-soluble, and chemically most

reactive species of activated oxygen. Hydroxyl radicals cause oxidative damage to cells because they unspecifically attack biomolecules. It is generally assumed that HO· in biological systems is formed through redox cycling by Fenton reaction, where free iron (Fe<sup>2+</sup>) reacts with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the Haber-Weiss reaction results in the production of Fe<sup>2+</sup> when superoxide reacts with ferric iron (Fe<sup>3+</sup>). The hydroperoxyl radical (HO·2) plays an important role in the chemistry of lipid peroxidation. This protonated form of superoxide yields H<sub>2</sub>O<sub>2</sub> which can react with redox active metals including iron or copper to further generate HO· through Fenton or Haber-Weiss reactions. The HO·2 is a much stronger oxidant than superoxide anion-radical and could initiate the chain oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function.<sup>[3]</sup>

The process of lipid peroxidation consists of three steps Initiation, Propagation, and Termination. During Initiation step, pro-oxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centered lipid radical (L·). In the Propagation phase, lipid radical (L·) rapidly reacts with oxygen to form a lipid peroxy radical

( $\text{LOO}\cdot$ ) which abstracts a hydrogen from another lipid molecule generating a new  $\text{L}\cdot$  and Lipid Hydroperoxide (LOOH). In the Termination reaction, antioxidants like vitamin E donate a hydrogen atom to the  $\text{LOO}\cdot$  species and form a corresponding vitamin E radical that reacts with another  $\text{LOO}\cdot$  forming non-radical products. Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced.<sup>[4]</sup>

The Lipid Peroxidation Products are due to reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are Lipid Hydroperoxides (LOOH). The secondary products during lipid peroxidation are reactive aldehyde such as Malondialdehyde (MDA), Propanal, Hexanal, and 4-hydroxynonenal. Malondialdehyde (MDA) is the organic compound with the nominal formula  $\text{CH}_2(\text{CHO})_2$ . It is a colourless liquid, malondialdehyde is a highly reactive compound that occurs as the enol with molar mass is  $72.063 \text{ g}\cdot\text{mol}^{-1}$ , density  $0.991 \text{ g/mL}$ , melting point  $72^\circ\text{C}$  ( $162^\circ\text{F}$ ;  $345 \text{ K}$ ) and boiling point  $108^\circ\text{C}$  ( $226^\circ\text{F}$ ;  $381 \text{ K}$ ). MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with Thiobarbituric acid (TBA)<sup>[5]</sup> Oxidative stress causes lipid peroxidation of cell membrane yielding oxidation products that constitute thiobarbituric acid-reacting substances (TBARS). The most abundant of the TBARS is malondealdehyde (MDA), aldehydic lipid peroxidation product formed by the action of ROS on membranes polyunsaturated fatty acid lipid. The TBRAS is frequently measured to assess the extent of oxidative stress.<sup>[6]</sup>

Oxidative damage to DNA is a result of interaction of DNA with hydroxyl radical. Superoxide and Hydrogen peroxide radicals do not directly react with DNA unless transition metal ions are present to allow  $\cdot\text{OH}$  formation. The  $\text{OH}$  radical is so reactive that it can attack all components of DNA. Thus  $\text{OH}$  abstract hydrogen atom from deoxyribose giving sugar radical that fragment in various ways. Reactions of deoxyribose derived radicals can lead to the release of purine and pyrimidine bases from the DNA producing abasic site and strand break. Some altered sugars that remain attached to the DNA can be split to give strand breakage by incubation with alkaline solution called Alkali Labile Sites (ALS). The  $\text{OH}$  can add on to guanine residues at C4, C5 and C8 positions. Addition of  $\text{OH}$  to C8 of guanine produces a radical adduct that has several possible fates. It can be reduced to 8-hydroxyl guanine or undergo ring opening followed by one electron reduction and protonation of to give 2,6 diamino-4hydroxy-5-formidopyrimidine. It can also modify RNA, proteins and other biomolecules.<sup>[7]</sup>

## MATERIALS AND METHODS

The current study was conducted at department Anatomy in collaboration with department of Paediatrics and Department of Biochemistry for the doctoral research of the Principal author during 2008-2011. Blood samples were taken from peripheral vein of asphyxiated and non asphyxiated babies within 24 hours of birth into heparinized tubes (1ml) and processed within one hour.<sup>[8]</sup> The aliquots of plasma were preserved in centrifuge tubes containing PBS stored in deep freezer of refrigerator.

**Table 1: Showing Materials required for MDA Estimation.**

Sl. No	Materials	Specification	Company/ Establishment
1.	Trichloro Acetic Acid (TCA)		MERC
2.	Thiobarbituric Acid		MERC
3.	Sulphuric Acid		MERC
4.	Manganese Sulphate		MERC
5.	Butanol		MERC
6.	Sphectrophotometer		Systronics- India
7.	Cuvitte		
8.	Micropipette	100 micro lit.	
9.	Voltex Mixer	230V/50 HZ	KEMI
10.	Centrifuge	High Speed Refrigerator tube	REMI
11.	Incubator	12A 230 H VAC, 50/60 HZ	HICOOL
12.	Centrifuge tubes	1.5 ml, 10ml	
13.	Microtips		
14.	Analytical Balance	Digital 10 mg-220 gm	Shimadzu

**Table 2: Showing preparation of stock and working solution for estimation of MDA.**

Sl. No.	Reagent	Stock Solution		Double distilled water	Working Solution
		Name of Chemical	Requirement		
1.	Trichloroacetic acid	20% TCA	20 gm	100 ml-	2.5ml
2.	Sulphuric acid	0.05m $\text{H}_2\text{SO}_4$	450 $\mu\text{l}$	100 ml	1ml
3.	Sodium sulphate	2M $\text{NaSO}_4$	28.4g	100 ml	2ml
4.	Thiobarbituric acid	0.22% TBA	0.22 ml	100 ml $\text{Na}_2\text{SO}_4$	1.5ml

### 1. Estimation of Serum MDA Level (Non-enzymatic Oxidative stress parameter).

The methodology was standardised based on the guidelines of Satohk's method 1978<sup>6</sup>. The methods / protocol comprises of sample collection, Inoculation with TCA, Centrifugation, Washing with H<sub>2</sub>SO<sub>4</sub>, Incubation with TBA, Removal of contaminants, Reading with Spectroscope and Calculations.

#### Protocol

##### Sample Collection

The blood samples were collected from peripheral vein into heparinized tubes (1-2ml) and processed within one hour<sup>8</sup>. The heparinised samples has to be centrifuged at 1000 rpm for 30 mts at room temperature. The aliquots of plasma were preserved in centrifuge tubes containing PBS stored in deep freezer of refrigerator.

##### Inoculation with TCA

- To 250 µl plasma, 2.5 ml 20% TCA has to be added in a test tube and allowed to stand for 10 min in order to precipitate protein (Table:2).

##### Centrifugation

- Precipitated proteins have to be separated by centrifugation at 3500 rpm for 10mins (Table:1(10)).

##### Washing with H<sub>2</sub>SO<sub>4</sub>

- Thus obtained protein precipitate has to be washed thrice with 1 ml of 0.05M H<sub>2</sub>SO<sub>4</sub> (Table:2).

##### Incubation with TBA

- After washes, protein precipitate has to be incubated with 1 ml 0.05M H<sub>2</sub>SO<sub>4</sub>, 2ml of 2M NaSO<sub>4</sub> and 1.5 ml of freshly prepared TBA reagent in a boiling water bath for 30 mins (Table:2).
- Subsequently the tubes have to be cooled under tap water to arrest the reaction.

##### Removal of contaminants

- 2 ml Butanol was added to the above and mixed vigorously using vortex mixer (Table:1).
- Further the tubes were centrifuged at 3500 rpm for 10 mins to obtain a clear supernatant (Table:1).

##### Reading with Spectroscope

Absorbance of the supernatant was read in Spectrophotometer at 540 nm with Butanol as the blank (Table:1(6)).

##### Calculation

Plasma MDA in µmol/L = 102.56 \* A

Where, A is the absorbance at 540 nm

Molar extinction coefficient of MDA = 1.56 \* 10<sup>5</sup> mol<sup>-1</sup>/L/cm<sup>-1</sup>



Figure 1: Showing the Captures of Steps of Estimation Serum Malondealdehyde (MDA).

## DISCUSSION

The measurement of MDA values can be made in a variety of biological samples. The use of thiobarbituric acid reactive substances (TBARS) assay has been carried out in plasma, serum, tissues, and occasionally in urine. In the current study we used plasma samples for estimation of MDA in order to determine the Oxidative DNA damage in Perinatal Asphyxia. This paper has been focussing the methodology of MDA estimation in Oxidative stress induced DNA damage rather than analysis with other data's either Indian or foreign which was discussed in other papers of the same author.<sup>[10]</sup> Therefore the succeeding paragraph dealt with actions and effects of the reagents used for the estimation of MDA in oxidative stress.

Thiobarbituric acid reactive substance (TBARS) measures concentration of malondialdehyde produced due to degradation of unstable lipid peroxides. It is a reagent in assaying malondialdehyde.

Trichloroacetic acid (TCA) has two functions first dehydrating the hydration cells around the protein by which the anionic TCA triggers partial protein unfolding through disruption of the electrostatic interactions by determining the native tertiary structure of the protein. TCA is less effective for disordered proteins.

N-butanol is used as a solvent intermediate to create other chemical and also to avoid interference formation by removing contaminants or remaining of haemoglobin from incubation mixture.

Once MDA formed which can be enzymatically metabolized or can react on cellular and tissue proteins or DNA to form adducts resulting in biomolecular damages. MDA adducts are biologically important because they can participate in secondary deleterious reactions of crosslinking by promoting intramolecular or intermolecular protein/DNA crosslinking that may induce profound alteration in the biochemical properties of biomolecules.

## CONCLUSION

To best of my knowledge the evidence of the data suggest that the present method can be used to measure the lipid peroxidation in human peripheral blood lymphocytes which is specific for MDA estimation in which lipid peroxidation causes oxidative stress induced DNA damages in Perinatal Asphyxia.

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## REFERANCES

1. G. Fruhbeck, J. Gomez-Ambrosi, F. J. Muruzabal, and M. A. Burrell, The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation, *The American Journal of Physiology: Endocrinology and Metabolism*, 2001; 280(6): E827–E847.
2. L. Moldovan and N. I. Moldovan, Oxygen free radicals and redox biology of organelles, *Histochemistry and Cell Biology*, 2004; 122(4): 395–412.
3. B. Halliwell and J. M. C. Gutteridge, Oxygen toxicity, oxygen radicals, transition metals and disease, *Biochemical Journal*, 1984; 219(1): 1–14.
4. H. Yin, L. Xu, and N. A. Porter, Free radical lipid peroxidation: mechanisms and analysis, *Chemical Reviews*, 2011; 111(10): 5944–5972.
5. W. A. Pryor, On the detection of lipid hydroperoxides in biological samples, *Free Radical Biology and Medicine*, 1989; 7(2): 177–178.
6. Satoh K. Serum lipid peroxide in Cerebrovascular Disorder determined by a New Calorimetric method. *Clin Chem Acta*, 1978; 90: 37–43.
7. Halliwell B, Okezie I, Arouma . DNA damage by oxygen derived species its mechanism and measurement in mammalian systems. *Elsevier Science*, 1991; 281: 9-19.
8. R. O. Sinnhuber, T. C. Yu, and T. C. Yu, Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity, *Journal of Food Science*, 1958; 23(6): 626–634.
9. S. Younes-Mhenni, M. Frih-Ayed, A. Kerkeni, M. Bost, and G. Chazot, "Peripheral blood markers of oxidative stress in Parkinson's disease," *European Neurology*, 2000; 58(2): 78– 83.
10. Manoj A, Rao RK, Bhat VB, Venkatesh C, Bobby Z. Oxidative stress induced DNA damage in Perinatal asphyxia. *Curr Ped Res.*, 2011; 15(1): 19-23.