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ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT AND LIPID PEROXIDATION STATUS IN IRON DEFICIENT ANEMIC INDIVIDUALS

Nirjala Laxmi Madhikarmi*, Kora Rudraiah Siddalinga Murthy

*Dep. of Biochemistry, Bangalore University, Bangalore-560001
Email: nirjala4@yahoo.com, nirjala4@gmail.com
Mobile: 91-9731195980

ABSTRACT: The objective of this study was to assess the enzymatic and non-enzymatic antioxidant levels in patients with iron-deficiency anemia (IDA). Forty patients with IDA and forty healthy individuals with their informed consent were selected for the study. The enzymatic antioxidants- catalase, glutathione peroxidase and superoxide dismutase; non-enzymatic antioxidants-vitamin C and E and total antioxidant activity (TAA) were also analyzed. The lipid peroxidation parameter thiobarbituric acid reactive substance was analyzed. The enzymatically antioxidants were found to be statistically increased but vitamin C and E and TAA were significantly lowered. Diminished antioxidant status disturbed oxidant-antioxidant balance alleviating oxidative stress state.

Keywords- Antioxidant, catalase, enzymatic, glutathione peroxidase, iron deficiency anemia.

INTRODUCTION

Anemia is the single most common disorder affecting mankind seen in all parts of the world, developed as well as developing countries. In India nearly 70% people are estimated to be iron deficient. The studies of experimental and human evidences support clear roles for increased oxidative stress due to susceptibility of lipids of red cell membrane to peroxidation.[1,2] A low serum iron alone does not diagnose iron deficiency, a combination of serum iron, total iron binding capacity (TIBC) along with hemoglobin (Hb) are conjunctively essential to diagnose. The normal adult erythrocytes can resist oxidative stress by several antioxidant defense systems. Imbalance between oxidative stress (OS) and antioxidant system is present in iron deficient patients. In light of the several epidemiological, clinical and laboratory investigations supporting the role of OS in anemia, we aimed to study the alterations in the levels of oxidants and antioxidants in patients of iron deficiency anemia (IDA).

World Health Organization (WHO) definitions for anemia differs by age, gender and pregnancy status as follows: for children 6months to 5years of age anemia is defined as a hemoglobin (Hb) level < 11gm/dl, children 5-11 years of age Hb<11.5g/dl, adult male Hb level <13g/dl, non-pregnant females Hb <12g/dl. Severe anemia is defined as Hb <7.0g/dl. Iron deficiency anemia was defined as the presence of anemia and serum ferritin <12ng/ml in children younger than 15 years and female all ages, and serum ferritin < 18ng/ml in males age 15 years and older. Over the past decade, anemia has emerged as a risk factor that is associated with a variety of adverse outcomes in humans, including hospitalization, disability, and mortality.[3-5]

MATERIALS AND METHODS

Study design and subject recruitment- The study group included 80 individuals divided into two groups; iron deficient anemic case (40) and healthy controls (40) with their informed consent. Healthy controls were selected on the basis of their non smoking, non alcoholic habit and were nor without any diseases or sickness.

Height and weight was measured to determine respective body mass index and surface area. Both the groups were devoid of family history of iron deficiency anemia. Similarly, both the groups were on regular intake of fruits and vegetables. The study group subjects age ranged from 15 to 45 years. Description criteria for IDA are hemoglobin concentration < 11.5g/dl in women and <13g/dl in men, plasma iron concentration <45g/dl and total iron binding capacity >60µmol/l.

Blood samples and lysates- Blood (5ml) was collected in tubes containing ethylenediamine tetraacetic acid (EDTA), centrifuged at 4000rpm for 10min and the plasma was carefully separated and stored in a clean and dry vial. After the erythrocyte pellet was washed thrice with chilled physiological saline; 0.5ml of cell suspension was diluted with 2ml cold distilled water to lyse the erythrocytes. Glass-wares used for the experiment were acid washed and all the reading were measured by Shimadzu spectrophotometer. The hemoglobin was determined by the Beacon Diagnostics and iron and total iron binding capacity was assayed by Coral Clinical Systems kit method.

Determination of lipid peroxidation- The total amount of lipid peroxidation products in the plasma of healthy volunteers and patients was estimated using thiobarbituric acid reactive substances (TBARS) methods, which measures the MDA reactive products (Buege and Aust method, 1978).[6] In brief, 0.8ml of plasma and 1.2ml of TBA-reagent was mixed properly and heated in a boiling water bath for 10min. after cooling it, the tubes were centrifuged at 3000rpm after addition of 2ml of 0.2N NaOH to obtain a pink colored adduct formed at 535nm. Results were expressed as nmol/ml.

Enzymatic determination of superoxide dismutase (SOD)- CuZn-SOD activity in hemolysed RBC was determined by Kakkar et al method (1984)[7] based on the 50% inhibition of the formation of nicotinamide adenine dinucleotide (NADH)-phenazine methosulfate-nitroblue tetrazolium formazan at 560nm. One unit of CuZn-SOD activity is defined as the amount of the enzyme required to inhibit the rate of NADH autooxidation by 50%. The enzyme activity was expressed in units per gram of hemoglobin.

Enzymatic determination of catalase (CAT) activity- CAT activity in the whole blood and hemolysed RBC (erythrocytes) were assessed in the erythrocyte lysates by the method as described by Sinha (1972). [8] Briefly, hydrogen peroxide (0.2M) was used as a substrate and the decrease in H_2O_2 concentration at 22^0C in phosphate buffer (0.05 M, pH 7.0) was followed spectrophotometrically at 240nm. One unit were presented as units per gram hemoglobin (U/g Hb).

Enzymatic determination of Glutathione peroxidase (GPx) activity- GPx activity also analyzed in hemolysed RBC lysates by the method of Rotruck et al 1973[9] with modifications. 100µl of enzyme preparation was allowed to react with H_2O_2 in the presence of reduced glutathione. After a specified period of enzyme action; the remaining reduced glutathione content was measured by the method of Beutler and Kelley (1963).[10]

Determination of Vitamin C (Ascorbic acid) - Vitamin C is plasma is estimated by Natelson [11] DNPH method where vitamin C is oxidized to diketogluconic acid which reacts with 2, 4-dinitrophenyl hydrazine to form diphenylhydrazone. The hydrazone dissolves in strong acid solution to form orange-red colored complex. The absorbance was read at 520nm.

Determination of Vitamin E- The vitamin E was measured by Baker and Frank method [12] by the reduction of ferric to ferrous ion which then forms a red colored complex with α, α'-bipyridyl. The absorbance was measured at 490 and 520nm.

Determination of total antioxidant activity-The TAA assay was performed according to Benzie & Strain, 1996.[13] In brief; reductants ('antioxidants') in the sample reduce a ferric–tripyridyltriazine complex, present in stoichiometric excess, to the blue colored ferrous form. The change of absorbance at 593 nm over 4 min is proportional to the combined total antioxidant value of the antioxidants in the sample.

Statistical methods- The packaged program SPSS (Statistical package for social sciences) for windows version 13.0 (SPSS, Chicago, Il, USA) was used for statistical analysis. The results are reported as means ± standard deviation (SD) for the patients and the controls group. Differences between the groups were determined by means of a Student's t-test. Pearson's correlation (r) was performed on the paired data obtained by the individual IDA cases. Significance level was set at $p<0.05$.

RESULT AND DISCUSSION

According to our results, we found decreased hemoglobin and iron status but increased TIBC in the IDA patients which were significant statistically. The enzymatic antioxidants such as CAT, SOD and GPx were increased significantly when compared to the healthy volunteers. The non-enzymatic antioxidants vitamin C, vitamin E and total antioxidant activity were highly reduced in the IDA patients. The lipid peroxidation parameter, TBARS was also highly increased both in plasma and erythrocytes in the patients suffering from iron deficiency. (Table I).

Table I: Biochemical parameter of Iron deficient anemic patients and healthy subjects.

Parameters	IDA-Case (n=40)	Control (n=40)
Age (years)	39.30±14.22*	30.85±10.08
BMI (kg/m^2)	20.82±7.86*	23.12±2.27
SA (m^2)	1.55±0.23*	1.68±0.35
Hb (g/dl)	8.72±1.51*	14.99±2.17
Iron (g/dl)	40.17±0.18*	120.21±12.32
TIBC (µmol/l)	92.23±8.87*	55.27±3.65
TBARS-plasma (nmol/ml)	4.38±1.12*	2.51±0.87
TBARS-RBC (nmol/ml)	4.06±1.81*	1.68±0.65
CAT-WB (U/gHb)	1591.18±38.21*	1017.00±21.35
CAT-RBC (U/gHb)	900.26±41.69*	863.77±23.35
SOD (U/mgHb)	26.02±6.03	25.49±7.28
GPx (U/mgHb)	2.80±2.29*	1.57±0.85
VIT C (mg/dl)	0.18±0.11*	0.41±0.37
VIT E (mg/dl)	0.67±0.19*	1.11±0.12
TAA (µmol/l)	509.14±78.96*	838.97±22.75

*$p<0.05$, the level of significance

On splitting our subjects with respect to gender case-control, we found a significant change in the oxidant and antioxidant parameters. The Hb levels in male and female IDA cases were highly decreased than compared to their healthy counterparts. The enzymatic antioxidant- catalase in whole blood was highly increased in either sex which was significant statistically. The increase of catalase in erythrocyte in male and female IDA was not statistically significant when compared to their healthy subjects.

Similarly, the increase of SOD in IDA cases in both sex were not significant statistically. On the other hand, GPx was increased statistically both in male and female IDA cases when compared to the healthy individuals. The non-enzymatic antioxidants vitamin C & E and TAA were highly decreased in both the male and female cases which were significant statistically. The lipid peroxidation parameter, TBARS both in plasma and erythrocyte were found to be increased both in male and female IDA cases which was also significant statistically when compared with their respective healthy counterparts. (Table II).

Table II: Biochemical parameters of IDA case and control with respect to gender.

Parameters	Male		Female	
Case (n=20)	Control (n=20)	Case (n=20)	Control (n=20)	
Age	39.10±14.86*	31.70±0.82	39.50±14.35*	31.51±1.43
Hb	9.14±1.52*	15.84±2.62	8.92±1.74*	13.14±1.21
TBARS-p	2.74±0.99*	4.18±0.96	2.29±0.71*	4.58±1.27
TBARS-e	4.11±2.11*	1.41±1.10	4.01±1.56*	1.95±0.30
CAT-B	1623.21±41.91*	998.22±24.36	1559.15±36.10 *	1035.79±18.99
CAT-e	908.20±34.35	861.39±23.39	892.31±48.61	866.16±24.56
SOD	24.43±6.37	20.41±3.68	27.60±5.53	30.58±6.41
GPx	2.67±2.85*	0.86±0.48	2.93±1.72*	2.28±0.41
VIT C	0.18±0.10*	0.59±0.47	0.19±0.12*	0.24±0.05
VIT E	0.65±0.09*	1.18±0.11	0.69±0.07*	1.04±0.05
TAA	525.40±90.76*	929.36±27.56	492.89±65.87*	748.58±12.30

Note: *p<0.05, the level of significance, CAT-B, catalase in whole blood; CAT-e, catalase in erythrocyte; TBARS-e, TBARS in erythrocyte, TBARS-p, TABRS in plasma.

For the analysis of correlations between various above described parameters, Pearson's correlation coefficient (r) was analyzed. But, we found significant correlation between few parameters only. (Table III).

Table III: Pearson's Correlation of various biochemical parameter.

Parameters	r	*p*-value
TBARS-p Vs Vit C	0.600	0.000
TBARS-p Vs TAA	-0.517	0.007
Vit C Vs GPx	-0.333	0.036
Vit C Vs TAA	-0.398	0.044
Hb Vs CAT-e	-0.708	0.000
Hb Vs GPx	-0.533	0.000

*p<0.05, the level of significance

Decreased GPx activity in patients with iron deficiency anemia, and suggested that iron could be of crucial importance for erythrocyte GPx activity. In the present study, CAT activity was significantly higher in patients with iron deficiency anemia compared to the control group. In addition we observed a direct positive correlation between plasma TBARS and erythrocyte GPx levels in patients with IDA.

This relationship shows the continuous oxidative stress present in IDA patients. Their result showed that after therapy with iron in IDA patients, only the level of plasma ceruloplasmin was normalized. TBARS remained high as well as antioxidant enzymes. They reported that repletion of IDA with iron promotes oxidative stress. The causative factor for the generation of hydroxyl radical is because of excess iron.

Cellular non-enzymatic antioxidants are also known as free radical scavengers that protect a cell against toxic free radicals. Vitamin C and E is the chief constituent of the aqueous and lipid soluble environment. Therefore, decreased vitamin C & E and TAA levels may reflect a depletion of non-enzymatic antioxidant reserves. On the other hand, they play prominent role in the antioxidant defense system, and in the reactions of catalysis, regulation, electron transportation and in preserving the correct structure of proteins. Decreased levels of total GSH have been reported in various pathologies, including anemia and its associated diseases.[4, 14-15.]

Lipid peroxidation is a unique mode of oxidative injury which is triggered and promoted by different radical and non-radical members of the reactive oxygen species (ROS) family or by the catalytic decomposition of preformed lipid hydroperoxides. Antioxidant enzymes such as SOD, CAT and GPx, are important for cellular protection due to their ability to detoxify free radicals, such as ROS.[16] The human body conserves antioxidants in order to neutralize or limit the oxidative damage caused by free radicals.[2,17] Glutathione is a crucial component of the antioxidant defense mechanism and it functions as a direct reactive free radical scavenger. Iron is necessary for maintaining normal structure and function of virtually all mammalian cells and is also involved in the immune and non-immune host defense. Iron deficiency causes deficiencies of many iron-dependent enzymes and heme containing proteins, and is known to produce many systemic abnormalities.

Oxidative stress that occurs in the cells, as a consequence of an inequity between the prooxidant, antioxidant systems, causes injury to biomolecules such as nucleic acids, proteins, structural carbohydrates, and lipids. In addition, a variety of lipid byproducts are produced as a consequence of lipid peroxidation some of which can exert adverse and/ or beneficial biological effects. It can also disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes. Injure to mitochondria induced by lipid peroxidation can direct to further ROS generation.

Iron deficiency (ID) is the most widespread nutritional problem, and affects over two billion people. It is a particularly common disorder among infants, preschool-aged children, young women and older people, but it can occur at all ages and in any region. A high demand for iron during rapid growth, pregnancy and lactation, accompanied by dietary deficiencies and menstrual blood loss, are the most common causes of iron deficiency in children, young women and elderly population. It affects one's development, growth and resistance to infections, and is associated with mortality among children younger than two years old. The results of this study indicate that iron supplementation is required for the target group.[16-20]

CONCLUSION

The finding of our study signifies the increased lipid peroxidation and decreased antioxidant status in iron deficiency anemia both in male and female cases. Our study was limited to few patients, the vitamins and iron supplementation was not performed. Further research is recommended to identify the specific risk factors for IDA. Further studies on lipid peroxidation and antioxidant status after vitamin and iron supplementation is being carried out in the laboratory.

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