International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

*Original Research Article*

# Evaluation of Oxidative Stress and Antioxidant Status in Iron Deficiency Anemia

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*Received: 12/08//2014* *Revised: 13/10/2014* *Accepted: 13/10/2014*

## ABSTRACT

Anemia is a pathological condition in which there is a decrease in red blood cell mass or decrease amount of hemoglobin. Iron deficiency anemia (IDA) is the most common cause for it among all the others. IDA occurs when iron loss occurs and/or the dietary intake or absorption of iron is insufficient. Oxidative stress is an imbalance between free radicals and antioxidant molecules; an increase in oxidants and/or a decrease in antioxidant capacity which is one of the potential biochemical mechanisms involved in the pathogenesis of iron deficiency anemia. The aim of this study is to investigate oxidative stress and antioxidant status in patients with IDA. A total of 50 subjects (20 subjects with IDA and 30 healthy controls) were included in our study with the mean age of 32.38±13.74 years. Serum total antioxidant capacity, serum total peroxide level, oxidative stress index and iron indices {Hemoglobin, Mean Cell Volume (MCV), Mean Cell Hemoglobin Concentration (MCHC) were compared between the control and IDA subjects including serum total protein, albumin, uric acid and bilirubin. There were no significant differences between IDA subjects and controls with respect to age, gender and body mass index (BMI) Protein, Albumin, Uric acid and bilirubin in IDA subjects and controls were significantly higher in controls subjects than IDA.TP and OSI were significantly higher in IDA subjects than controls, while TAC was significantly lower. In conclusion, our study clearly revealed a positive association between total peroxide level and total antioxidant status in IDA and healthy subjects. IDA subjects have significantly higher level of total peroxide and OSI index and significantly lower level of the total antioxidant status than that of the healthy control subjects. With regard of the other individual antioxidant such as total protein, albumin, uric acid and total bilirubin were significantly decreased in IDA subjects than that of the healthy control subjects. Hence, increase in the total peroxide and OSI represents oxidative stress. These pre-disposal factors may in part, contribute to the greater risk of severe anemia to fatality.

***Keywords:*** Iron deficiency anemia, oxidative stress, antioxidant status, total antioxidant capacity, total peroxide, iron indices

## INTODUCTION

Anemia is a pathologic condition; decrease in erythrocytes (red blood cell) count or a decrease in the hemoglobin concentration. Iron deficiency anemia is the most prevalent nutritional disorder. It is characterized by microcytic hypochromic anemia with reduced blood hemoglobin

levels (<10g/dl), resulting from blood loss, low dietary intake or decrease absorption of iron. [(1)] It is one of the most prevalent deformity affecting approximate 500-600 million peoples and most common in woman than in man, [(2)] Anemia can be classified based on MCV, MCH, MCHC and red cell morphology in the blood smear.

Table 1: Classification of anemia based on MCV, MCH, MCHC and red cell morphology in the blood smear.

| S.N. | Types of anemia | MCV | MCH | MCHC | RBC count | Example |
|---|---|---|---|---|---|---|
| 1 | Normochromic normocytic | Normal | Normal | Normal | Reduced | Acute blood loss, aplastic anemia |
| 2 | Hypochromic microcytic | Reduced | Reduced | Reduced | Reduced | Iron deficiency anemia, thalassemia |
| 3 | Normochromic macrocytic | Elevated | Elevated | Normal | Reduced | Pernicious anemia, megaloblastic anemia |
| 4 | Normochromic microcytic | Reduced | Reduced | Normal | Reduced | Chronic infections |

Iron is an essential element in all living cells and plays an important metabolic role, particularly in electron transfer reactions. The adult human body contains about 4 grams of iron. Approximately 75% of total body iron is associated with hemoglobin, which is responsible for oxygen transport. [(3)] Free radicals known as reactive oxygen species (ROS) are potentially able to induce cellular injury e.g. hydroxyl radicals, superoxide radicals, hydrogen peroxide and singlet oxygen. The antioxidants are capable of scavenging free radicals directly, which consists of endogenous antioxidative agents; superoxide-dismutase, catalase, glutathione peroxidases-reductase, glutathione, uric acid, NADPH, albumin, bilirubin and exogenous antioxidative agents; tocopherols, ascorbate, carotenoids, thiols, polyphenols, and other micronutrients. [(4)] In some circumstances, the oxidative or antioxidative balance swings towards the oxidative status as a result of an increase in ROS and/or impairment in the antioxidant mechanism. Thus, oxidative stress develops. [(4)] Typically, Red cell membrane lipids are susceptible to the lipid peroxidation by the reactive oxygen species i.e. The oxidation of lipids which produces free radicals that steals electrons from the lipids in cell membrane(free radical chain reaction), resulting cell damage. [(5)] Reduction in serum iron concentration in the body causes inadequate synthesis of hemoglobin with the subsequent reduction of the erythrocyte proliferation. Since, cellular energy metabolism is $O_2$ dependent; anemia has wide range of clinical consequences. [(6)]

- Decrease in the life span of the red blood cell in circulation that further intensifies the anemic condition.
- An increase in membrane stiffness and a decrease in deformability, which decreases the ability of the RBCs to pass through the spleen without being removed.
- Iron deficiency accelerates the suicidal death of RBCs (eryptosis), which is initiated by an increase in red cell cytosolic calcium, which stimulates phospholipids scrambling and increased exposure of phosphatidylserine to the outer surface of the membrane.
- Phosphatidylserine on the outer surface of the membrane is recognized by macrophages and results in the removal of erythrocytes from circulation.

The oxidative status was evaluated using measurement of oxidants, individual antioxidants, or both. [(7)] In our studies, it has been hypothesized that oxidants will

increase and antioxidants will decrease, and as a result, the oxidative/anti-oxidative balance will shift toward the oxidative side in patients with IDA. Thus, increased oxidative stress may contribute to the pathogenesis of patients with IDA. In this study, we evaluated the oxidative status in IDA patients and normal individual using measurements of Total Antioxidant Capacity (TAC), Total Peroxide (TP) level and Oxidative Stress Index (OSI).

## MATERIALS AND METHODS

The study population included native residents of Pokhara Valley and surrounding areas and those who have migrated from other parts of Nepal. A total of 50 individuals participated in the present study conducted between October and November 2011. The number of male and female participants was 20 and 30respectively. The participants were divided into two groups according to the disease condition i.e. IDA and healthy controls relying in the iron indices. One group was individuals with Hb<10gm/dl and the other group was control individuals with Hb>12gm/dl. There were 20 IDA subjects (5 Males and 15 Females) and 30 control subjects (15 Males and 15 Females). The age range of participants was 15 to 59 years. The clinical examination consisted of a personal interview. Recruitment of participants for the investigation was performed by principal investigators. Those individuals, who were iron deficient without any other anemia, pregnancy, menstruation, chronic diseases, were selected while recent blood donors and person on iron supplements therapy were excluded for the study. The participants were asked to fill the questionnaire, 5 ml of the venous blood was collected in EDTA vacationer and examined for the iron indices then samples were centrifuged at 1500 g for 10 min .The serum was preserved in deep freeze (-44°C) for the measurement of total peroxide, total antioxidant capacity, oxidative stress index, total protein, albumin, uric acid and bilirubin. Total peroxide, total antioxidant capacity, total protein, albumin, uric acid and bilirubin were determined by semi-automated analyzer.

**Measurement of Total Peroxide concentration:** FOX2 method is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples to produce a colored ferric-xylenol orange complex, the absorbance of which can be measured by semi-automated analyzer at 560nm. The total peroxide content of plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of $H_2O_2$ as a standard. FOX2Reagent is prepared by adding 9.8 mg ammonium ferrous sulphate in 100 ml of FOX2 reagent blank, 100 ml of FOX2 reagent blank is prepared by mixing 250mM $H_2SO_4$ and 90 ml of solution A and Solution A is prepared by mixing 79.2 mg BHTto 90 ml methanol then adding 7.6 mg xylenol orange). 200 µl serum is added to 1800 µl of FOX2, mixed and incubated 30 minutes at room temperature. Then, centrifuged at 4000 RPM for 30 minutes. Absorbance of supernatant was taken at 560 nm spectrophotometrically. Total peroxide content was determined by plotting the standard curve of $H_2O_2$.

**Measurement of Total antioxidant capacity:** Erel method is based on the concentration of hydroxyl radical produced during reaction. In the reaction, ferrous ion solution (Reagent 1) is allowed to reacts hydrogen peroxide (Reagent 2) to produce radicals such as brown-colored dianisidinyl radical cation and hydroxyl radical, potent radicals are then cleared by the reaction by antioxidative effect of the sample against. The change (decrease) in color produced after addition of sample is directly

proportional to antioxidant capacity of body. Reagent 1( Clark and Lubs solution ,75mM, pH 1.8) is prepared by mixing 800ml KCl solution (5.591 g of KCl dissolved in 1000 ml of deionized water , 75mM) and 200ml HCL solution (Reagent grade HCl ;36.5%, 6.41ml diluted in 1000ml with deionized water, pH of 1.8),3.17 gm. of Ortho-dianisidine dihydrochloride and 0.01764 gm. of ammonium ferrous sulphate was added and dissolved making the final concentration making 45 µM. while Reagent 2 (Hydrogen peroxide solution 7.5mM) is prepared by mixing 0.641 ml of $H_2O_2$ solution (30%Merck) was diluted to 1000ml of reagent 1. During measurement, 200 µl of Reagent 1, 5 µl of Sample volume (serum or other fluids, pure or complex antioxidant solutions) and 10 µl of Reagent 2 is mixed and incubated. The first absorbance is measured before the mixing of R1 and R2 (as sample blank) and the last absorbance is taken (about 3– 4 min after the mixing) at Wavelength 444 nm. The results are expressed as mmol Ascorbic acid Equivalent/L Ortho-dianisidine dihydro-chloride is carcinogenic and toxic and while preparing it gloves and mask should be strictly used.

**Total serum protein:** Biuret colorimetric test based on principle where cupric ions react with protein in alkaline solution to form a purple complex. The absorbance of this complex is proportional to the concentration of protein in the test.1000ul of working reagent and 20ul of serum was mixed and incubated for 10 min at 20-25 ºC. Absorbance of the sample and standard were measured against the reagent blank within 30min at 546nm (520-580nm) using colorimeter.

Serum albumin: BCG colorimetric test based on principle where bromocresol green forms a colored complex with albumin in citrate buffer. The absorbance of this complex is proportional to the concentration of albumin in the test. 1000ul of working reagent and 10ul of serum was mixed and incubated for 5 min at 20-25 ºC. Absorbance of the sample and standard were measured against the reagent blank immediately at 578nm using colorimeter.

**Serum uric acid:** Uricase-POD(enzymatic, end point) colorimetric test based on principle where Uricase converts uric acid to allantoin and hydrogen peroxide, the hydrogen peroxide formed further reacts with a phenolic compound and 4aminoantipyrine by the catalytic action of peroxidase to form a red colored quinolonemine dye complex. The absorbance of this complex is proportional to the concentration of uric acid in the test. 1000ul of working reagent and 10ul of serum was mixed and incubated for 5 min at 37 ºC. Absorbance of the sample and standard were measured against the reagent blank within 30 min at 520nm using colorimeter.

**Total bilirubin:** Jendrassic and Groff colorimetric test based on principle where bilirubin reacts with diazotized sulphanilic acid (DSA) to form a red azodye. The absorbance of this dye at 546 nm is directly proportional to the bilirubin concentration in the sample. Water soluble bilirubin glucoronites react directly with DSA whereas the albumin conjugated indirect bilirubin will only react with DSA in the presence of accelerator. 1000ul of working reagent and 10ul of serum was mixed and incubated for 5 min at 37 ºC. Absorbance of the sample and standard were measured against the reagent blank within 30 min at 520nm using colorimeter.

Patients'data were collected in prescribed forms containing history, clinical findings, available laboratory data, and socio-economic background. The data was analyzed by the statistical software SPSS version 16.0. Data were presented mean ± standard error mean (SEM). Correlations

analysis was done using Pearson coefficient. Comparison of parameters between IDA and healthy controls was performed with Mann-Whitney test. Two tailed probability values were calculated throughout, and $p<0.05$ was considered statistically significant.

## RESULTS

There were no significant differences between IDA subjects and controls with respect to age, gender and body mass index (BMI)(all p>0.05).

Table 2: Demographicand hematological parameterin controls and IDA patients

| Parameters | IDA(N=20) | Controls (n=30) | P |
|---|---|---|---|
| Age | 33.05±3.59 | 31.93±2.23 | ns |
| BMI | 24.8±1.8 | 24.6±2.1 | ns |
| Hb (g/dl) | 8.39±0.42 | 14.49 ±0.24 | P<0.001 |
| PCV | 26.77 ±1.63 | 39.07±1.39 | P<0.001 |
| MCV(fl) | 76.41 ±1.65 | 89.78±1.28 | P<0.001 |
| MCHC | 33.17 ±0.76 | 35.39±0.18 | P<0.001 |
| Ferritin | 5.62±0.16 | 3.74± 1.26 | P<0.001 |

BMI: Body mass index; Hb: Hemoglobin; PCV: Pack cell volume; MCV: Mean corpuscularvolume; IDA: Iron deficiency anemia.Values are mean ± SD; ns: non significant

Protein, Albumin, Uric acid and bilirubin in IDA subjects and controls were significantly higher in controls subjects than IDA.

Table 3: Biochemicalparameterin controls and IDA patients

| Parameters | IDA(N=20) | Controls | P |
|---|---|---|---|
| Protein (g/dl) | 5.09±0.31 | 6.82±0.21 | P<0.001 |
| Albumin(g/dl) | 4.99±0.29 | 7.27±0.25 | P<0.001 |
| Uric Acid (mg/dl) | 1.61±0.38 | 2.67±0.12 | P<0.001 |
| Total Bilirubin (mg/dl) | 0.47 ±0.06 | 0.70±0.08 | P<0.001 |

TP and OSI were significantlyhigher in IDA subjects than controls (bothp<0.05), while TAC was significantly lower (p<0.05).

Table 4: Oxidative and antioxidative parameters in controls and IDA patients

| Parameters | IDA(N=20) | Controls (n=30) | P |
|---|---|---|---|
| TP(m molTrolox Eq./L) | 7.4 ±0.6 | 5.2±0.38 | P<0.001 |
| TAC(µmol H2O2 Equiv./L) | 1.62±0.18 | 2.30 ±0.90 | P<0.001 |
| OSI (Arbitrary Unit) | 5.80±7.60 | 5.20±4.09 | P<0.001 |

## DISCUSSION

The present study has shown that the levels of PCV, MCV, MCHC, red blood cell counts found decreased in all iron deficient anemic groups. [8] It was shown that in iron deficiency anemia, red cells have increased membrane rigidity, decreased deformability, and increased susceptibility to hemolysis and may result in immune recognition and eventual removal of red blood cells from the circulation. [9] The term "oxidative stress" is used to describe a number of chemical reactions involved in production of free radicals known as ROS e.g. hydroxyl radicals , superoxide radicals, hydrogen peroxide and singlet oxygen that are potentially able to induce cellular injury. A number of major cellular defense mechanisms exist to neutralize and combat the damaging effects of these reactive substances. The organism has enzymatic, such as superoxide dismutase (SOD), CAT, glutathione peroxidase (GSH-Px), and non-enzymatic (e.g. vitamin C, vitamin E) antioxidant mechanisms that work as scavengers against ROS. [10] Other major individual antioxidant components of plasma are albumin, uric acid and bilirubin. Free sulfhydryl groups of proteins are mainly responsible for antioxidant response of them. Uric acid serves as a potent antioxidant by radical scavenging and reducing activities. [11] Oxidant levels and antioxidant enzyme activities such as SOD, GSH-Px and CAT have been evaluated in several studies. [12-14] The antioxidant system is a combine effort of all the antioxidants in serum which protects body against oxidant attack. Thus, measurement of individual antioxidants has minimum reflection on true antioxidant In this regard; measurement of TAC along with individual measurement should be essential in evaluating the true antioxidant status and has bencharity in several studies. [12-14] Colorimetric methods are widely used for measurement of oxidative status and different antioxidants while other methods are fluorescence or chemiluminescence. The fluorescence and chemiluminescence methods necessitate classy techniques which might unavailable in many routine clinical biochemistry

laboratories. Even when these technologies are available, their routine usage is limited .In the present study, we observed that the patients with IDA are exposed to increased oxidative stress. There was significant increase in the total peroxide ($p$<0.01) and oxidative stress index ($p$<0.01) in the IDA subjects than in the healthy control subjects. There was significant decrease in the total antioxidant status in IDA subjects than in the healthy controls.

## CONCLUSION

The study was a prospective cohort cross sectional study carried out in the community population of Pokhara Valley and its surrounding. Since, there have been no studies in the oxidative stress and total antioxidant status in our study area before; we found it likely that our population in this respect represents that of Nepal. Our studies constituted of 50 voluntarily participated subjects and were divided into 20 IDA and 30 healthy controls. Our study clearly revealed a positive association between total peroxide level and total antioxidant status in IDA and healthy subjects. This is supported by the result: IDA subjects have significantly higher level of total peroxide and OSI index and significantly lower level of the total antioxidant status than that of the healthy control subjects. With regard of the other individual antioxidant such as total protein, albumin, uric acid and total bilirubin except uric acid other were significantly decreased in IDA subjects than that of the healthy control subjects. Hence, increase in the total peroxide and OSI represents oxidative stress.

## ACKNOWLEDGEMENT

We wish to acknowledge all the writers, editors and publishers of reviewed literature, We express our gratefulness to Professor Dr. Duk Bahadur Chhetri, pathologist, Western Regional Hospital, Ramghat, Pokhara, Nepal and Associate Professor Dr. Nirmala Jamarkattel, Director, School of Health and Allied Sciences, Pokhara University for their consistent support to conduct this research project, We also extend our special thanks to Mr. Niranjan Shrestha, Lecturer in biostatistics, School of Health and Allied Sciences and Mr. Jagat Khadka, Laboratory Incharge, Padma Nursing Hospital, I appreciate the help from faculty and staff of Department of medical lab technology, School of Health and Allied Sciences, Pokhara University and I am also grateful to all the participants who are involved directly and indirectly in this research work.

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How to cite this article: Chaudhary N, Parajuli A, Gurung B et. al. Evaluation of oxidative stress and antioxidant status in iron deficiency anemia. Int J Health Sci Res. 2014;4(11):216-222.

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