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Evaluation of oxidative stress and antioxidative defense systems in Infants and Young Children with Iron-Deficiency Anemia of Sharkia Governorate, Egypt

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Abstract: Iron deficiency anemia (IDA) is one of the major causes of morbidity and mortality worldwide affecting =opeople of all ages in both developed and developing countries. Evidences from epidemiological and clinical studies suggest a possible correlation between oxidant/antioxidant levels and the anemic disease risk. The present work is to investigate oxidative stress and antioxidative defense systems in anemic patients. A number of 86 patients (45 males and 41 females) and 73 healthy (42 males and 31females) infants and young children, aged 0 months to 6 years, were selected for the study from Maternal and Child Care Centers, Sharkia Governorate, Egypt. Based on analysis, we found that the mean hemoglobin (Hgb) level and mean corpuscular volume (MCV) of normal infants were 13.95±1.12 g/dL and 84.43±4.11fL which significantly decreased by the rates of -42.80% ($p<0.01$) and -23.30% ($p<0.05$) in IDA patients, respectively. The opposite direction was observed for the red blood cell distribution width (RDW) which recorded 12.60±1.55% in normal infants and significantly ($p<0.01$) increased by the rate of 35.24% in IDA patients. The oxidative stress parameters (malonaldialdehyde, MDA and nitric oxide, NO_2) of patients with IDA was significantly higher than controls ($P\leq0.05$) while enzymatic (glutathione peroxidase,GSH-P; superoxide dismutase, SOD and catalase, CAT) activities and nonenzymatic antioxidants levels (glutathione, GSH and vitamins A, C and E) were significantly lower ($P\leq0.05$). Statistically analysis indicated positive correlations between Hb and all enzymatic as well as nonenzymatic antioxidants of IDA patients. The opposite directions were recorded for Hb and oxidative stress parameters (MDA and NO_2). In conclusion, the results of our study support the higher oxidative stress hypothesis in IDA. Iron-rich foods in conjunction with antioxidant vitamins supplementation therapy should be advised by health care providers to all infants from 6 months to 6 years.

Key words: Hemoglobin, malonaldialdehyde, nitric oxide, antioxidant enzymes, antioxidant vitamins, glutathione fractions.

Introduction

Iron deficiency anemia (IDA) is anemia due to not enough iron. It is defined as a decrease in the amount of hemoglobin or red blood cells (RBCs) in the blood (Martins *et al.,* 2001). On the basis of the 1999 –2002 US National Health and Nutrition Examination Survey, IDA is generally characterized by a hemoglobin level of less than 110 g/L plus a measure of poor iron status/ Iron-deficiency (ID) for both male and female children aged 12 through 35 months (Martins *et al.,* 2001; CDCP, 2008 and Cusick *et al.,* 2008).

IDA is one of the most common causes of morbidity and mortality worldwide, affecting people of all ages in both developed and developing countries. Around the world, ID causes approximately half of all anemia cases and affects women more often than men. IDA affect affected 1.2 billion people in 2013 (GBDS, 2013). Also, Christofides et al., (2005) stated that IDA affects approximately 750 million children. In 2013 anemia due to iron deficiency resulted in about 183,000 deaths – down from 213,000 deaths in 1990 (MCD, 2013). In Egypt, micronutrient deficiencies, especially anemia, were a public health problem in Egypt, where the prevalence of anemia reached 40% (EDHS, (2005). Anemia by third trimester of pregnancy represents major health problem in this geographical area of Egypt. Its risk factors include personal, dietary and some aspects related to outcome healthcare delivered at this stage (Elashiry *et al.,* 2014). Also, the study of Afaf et al., (2015) conducted in 2010/11, among 4526 households from eleven governorates and focus on four population groups: mothers (20 – 49.9 yr), children <5 yr (preschool children), children 5-<12 yr (schoolchildren), and adolescents (12-18 yr). They reported that iron deficiency anemia (IDA, low Hb and low ferritin) was recognized among 18.5% of whole sample population, with high prevalence for mothers (25.1%). Furthermore, Al Ghwass et al., (2015) reported that the high frequency of IDA is a severe public health problem in Egypt, especially in children from rural areas, those from low social class and those of low maternal educational level.

Oxidative stress was initially defined by Sies (1985) as a shift in balance between oxidant/antioxidant in favor of oxidants, "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage". So, it reflects an imbalance between the systemic

manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA (Toshniwal and Zarling, 1992; Evans *et al.,* 2005 and Rahman *et al.,* 2012). Several studies indicated that oxidative stress contributes to many pathological conditions, including cancer, neurological disorders, atherosclerosis, Alzheimer's disease, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Halliwell, 1991; Vasavidevi *et al.,* 2006; Chaitanya *et al.,* 2010; Rahman *et al.,* 2012 and Elmaadawy, 2016). Also, increased oxidative stress in IDA subjects was reported in several studies, in which the oxidative status was evaluated using measurement of oxidants, individual antioxidants, or both (Kumerova *et al.,* 1998; Kurtoglu *et al.,* 2003 and Mehmet *et al.,* 2011). In these studies, it has been reported that oxidants were increased and antioxidants were decreased, and as a result, the oxidative/anti-oxidative balance shifted toward the oxidative side in patients with IDA. Thus, increased oxidative stress may contribute to the pathogenesis of patients with IDA (Jong-Ha *et al.,* 2009 and Saadet *et al.,* 2013).

On the other hand, the human body is equipped with antioxidant systems that serve to counterbalance the effect of oxidants (Esra *et al.,* 2012).. For all practical purposes, these can be divided into two categories: enzymatic and nonenzymatic. The major enzymatic antioxidants of the lungs are superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GSH-Px). In addition to these major enzymes, other antioxidants, including heme oxygenase-1, and redox proteins, such as thioredoxins, peroxiredoxins, and glutaredoxins, have also been found to play crucial roles in the body antioxidant defenses. Nonenzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), β-carotene and glutathione (GSH) (Esra *et al.,* 2012 and Rahman *et al.,* 2012).

The literature offers limited data on oxidative stress and antioxidant defense in patients with IDA. Thus, in the present study, we investigated whether IDA exposure can be affected as oxidative stress in

infants and young children blood. Also, the potential relationship between oxidative status and antioxidative defense systems in infants and young children with IDA will be in the scope of this investigation.

Materials and methods

Materials

Chemicals: Standard vitamins (A, C, and E) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals, solvents and buffers, except stipulated, were in analytical grade and purchased from Al-Gomhoria Company for trading Drugs, Chemicals and Medical Instruments, Cairo, Egypt.De-ionized water (Milli-Q 18.2 MΩ) was used in the preparation of the mobile phases, reagent solutions and standards.

Equipments: Throughout this study a SP Thermo Separation Products Liquid Chromatograph (Thermo Separation products, San Jose, CA, USA) was used with a Consta Metvic 4100 pump, a Spectra Series AS100, Spectra System UV 1000 UV/Visible Spectrophotometer Detector, Spectra System FL 3000 and a PC 1000 system software. The columns used (Alltech, Deerfield, IL, USA) were a Spherosorb ODC-2 (5 µm, 150 x 4.6 mm I.d.) for glutathione fractions ; a reversed-phase water Adsorbosil C_{18} (5 µM, 100 mm × 4.6 mm I.d.) for vitamin C; and normal Ultrasphere Si (5 µM, 250 mm × 4.6 mm I.d.) for analysis of vitamins A and E. Also, absorbance and fluorescence for different assays were measured using Labo-med. Inc., spectrophotometer, CA and Schematzu fluorescence apparatus, Japan, respectively.

Study protocol

This study was conducted from August 2015 to April 2016, in three Child Care Centers of Zagazig Governorate, Egypt. These centers serve most infants and children from low and middle socioeconomic standards. The study protocol was carried out in
accordance with the Helsinki Declaration as revised in 1989 and approved by the Local Committee for Scientific Research Ethics. A total of 187 children aged 0 to 6 years who were seeking medical advice for mild acute complaints as upper respiratory tract infections, mild gastroenteritis or other complaints, were enrolled in the study. All subjects were informed about the study protocol and written consent was obtained from all participants. Online statistical calculator was used for

sample size determination guided by power test of 80%, confidence level of 95%, and α error of 5%. The sample size was calculated to be 159 infants. Exclusion criteria were iron treatment before or during the study period infants, any history of chronic illness, or recent blood transfusion. A field pretested interviewing questionnaire was used for data collection which covering the following points: child age, sex, residence (urban or rural) and family size. Sociodemographic status of children and their families and socioeconomic score, which contained social variables were calculated and classified such as mentioned by Fahmy and El-Sherbini (1983).

Study Criteria

Description criteria for IDA are microcytic hypochromic erythrocytes; with a mean corpuscular volume (MCV) <70 fL, **Red blood cell distribution width (RDW) ≥15%**, hemoglobin concentration <11 g/dL, serum iron concentration <40 µg/dL, and serum ferritin concentration <10 µg/dL. Exclusion criteria include acute bleeding, history of blood transfusion within 6 months before the study, existence of diabetes mellitus, coronary artery disease, rheumatoid arthritis, malignancy, systemic or local infection, hypertension, liver diseases, renal dysfunction and usage of supplemental vitamins (Ann et al., 2002 and Bermejo and García-López, 2009).

Hematological analysis

Blood samples were withdrawn from the antecubital vein into glass centrifuge tubes containing oxalate solution (1.34%) as anticoagulant. After centrifugation at 1500 *Xg* for 10 minutes, plasma was withdrawn and used for analysis of blood lipid parameters and vitamins. The erythrocyte residue was washed with three successive portions of sodium chloride solution (0.9%) and then hemolyzed with deionized water for 30 minutes. Hemolysate was then centrifuged at 105,000 X*g* for 30 minutes, and the supernatant fractions was transferred to a clean test tube and analyzed for antioxidant enzymes (Stroev EA, Makarova, 1989).

Serum iron (Fe) and selenium (Se) content samples were determined by the adaptation the method mentioned by Singh *et al.*, (1991). One hundred µl of plasma sample were transferred into a digested glass tube and 2 ml of tri-acids mixture (containing nitric acid:

perchloric acid: sulfuric acid in the ratio of 20: 4: 1 v/v respectively) were added to each tube. The tubes content were digested gradually as follow, 30 min at 70 0C; 30 min at 180 ^{0}C and 30 min at 220 ^{0}C. After digestion, the mixture was cooled, dissolved in MilliQ water, and the volume was increased to 10 ml in volumetric beaker. After filtration in ashless filter paper, aliquots were analyzed for Fe and Se content using of atomic absorption spectrophotometer, type Perkin - Elmer, Model 2380.

Blood hemoglobin (Hb) concentration was determined using cyanmethemoglobin method according to Villanova (1994). The plasma ferritin concentrations and hematocrit value were assayed using specific Kits (Al-Gomhoria Company for Drugs, Chemicals and Medical Instruments, Cairo, Egypt) according to the methods mentioned in Tietz, (1999). The complete blood count was done using Coulter 1660 to determine the erythrocyte indices (mean corpuscular volume [MCV], mean corpuscular Hb [MCH], MCH concentration, and red cell diameter width.

Antioxidant enzymes

GSH-Px and CAT activities were measured as described (Splittgerber and Tappel, 1979, and Aebi, 1974, respectively). SOD activity was measured by Ransod kit (Randox laboratories mmited, Germany). GSH-Rd activity was determined according to the method recommended by the International Committee for Standardization in Haematology (ICSH, 1979). Activities of SOD and GSH-Px enzymes were expressed in international unit per milliliter erythrocyte sediment and one unit of SOD was expressed as the enzyme protein amount causing 50% inhibition in 2- (4-iodophenyl)-3 (4-nitrophenol) 5-phenyltetrazolium chloride ($INTH_2$) reduction rate.

Antioxidant vitamins

All vitamins (A, C, and E) were extracted and analyzed by HPLC techniques as follow: Vitamin A was extracted by adaptation the method of Epler *et al*., (1993). A 0.3 ml of serum were saponified by 0.1 ml of sodium hydroxide solution (60%) and 1- 2 ml ethyl alcohol; heated on a water bath at 85 - 90 ^{0}C under reflux for two hrs untill the serum components were completely dissolved; 1 - 2 ml of ethyl alcohol and about 2 to 4 ml of distilled water were added. The unsuponified portion

was extracted three times by ether using 5 ml in the first and second extraction and 2.5 ml in the third one. The ether extraction was washed 3 to 4 times with water until the washed water became neutral, 0.6 – 0.8 grams sodium sulphate were added and the mixture was left for 90 min and filtrated. After removing the ether from the solution, the residual matter was dissolved in one ml chloroform and diluted to 2.5 ml with the same solvent, 0.1 ml of the chloroform extract was transferred to a small screw-captest tube. The chloroform solution was dried under a stream of nitrogen, re-dissolved in exactly 0.1 or 0.2 ml isopropanol - hexane (1 : 99, v/v), and used for HPLC injection.

Vitamin E (α-tocopherol) was extracted by adaptation the method of Hung, *et al*. (1980). Approximately 50 µl of serum were homogenized in 1.5 ml dioxane-isooctane (20 + 80, v/v) for 1 min, using a Polytron homogenizer (Beckman, Toronto). The homogenate was centrifuged at 10000 rpm for 5 min, and 0.5 ml supernate was placed in a 25 ml round-bottom flask. Remainder of the supernate was discarded. The residue was homogenized with another 1.5 ml dioxane-isooctane solution, centrifuged as before, and 0.5 ml supernate was pooled with the previous supernate and dried under vacuum in a rotary evaporator to near dryness. The residue was extracted 3 times with 0.5 ml acetonitrile and the pooled acetonitrile extracts were filtered through glass wool into a 5 ml screw-cap test tube. The filtrate then was extracted with 1.5, 1, and 0.5 ml isooctane. The isooctane extracts were pooled in a 25 ml round-bottom flask and dried under vacuum in a rotary evaporator. The residue was dissolved in 0.2 - 0.4 ml petroleum ether (bp 30 - 60 ^{0}C) and transferred to a small screw-captest tube. The petroleum ether solution was dried under a stream of nitrogen, re-dissolved in exactly 0.1 or 0.2 ml isopropanol -hexane (1 : 99, v/v), and used for HPLC injection.

Vitamin C (ascorbic acid) was extracted according to the method of Moeslinger *et al*., (1994). One hundred µl of plasma were deproteinized by 400 µl ice-cold 8% perchloric acid which was described as stabilizing ascorbic acid in biological samples, centrifuged at 10000 g for 10 min at 4 ^{0}C, and neutralized by the addition of 4 M potassium hydroxide. The residues was dried under a stream of nitrogen, re-dissolved in exactly 0.1 or 0.2 ml methanol and used for HPLC injection.

The chromatographic conditions for vitamins A and E were flow rate, 1.5 l/min; detection, UV absorption at 265 nm, volume of injection, 20 µl; temperature, room temperature; and the mobile phase composition was an isocratic system of isopropanol : hexane (1:99) while in vitamin C were flow rate, 1 ml/min; detection, UV absorption at 254 nm, volume of injection, 20 µl; temperature, room temperature, and mobile phase composition was an isocratic system of 100 % methanol. Retention times and absorbance ratio against those of standards were used to identify the separated vitamins. Quantitative determination of each vitamin was determined from its respective peak area and corresponding response factor. The percent recoveries of vitamins were also studied by adding each vitamin to serum after sample preparation and HPLC determination. Under such chromatographic conditions, the Mean ±SD values of vitamins A, C and E recoveries were 88.98± 3.07, 86.18 ± 4.11, and 92.56 ± 2.85, respectively.

Nitrite determination

Nitrite was determined flourometric such as described by Misko *et al*., (1993). Ten µl of freshly prepared 2,3-diaminonaphthalene (DAN, 0.05 mg/ml in 0.62 M HCl, protected from light) is added to100 µl of sample and mixed immediately. Nitrate standards (> 98% pure, Sigma) are routinely made fresh, dissolved in DI H_2O, and kept on ice prior to use. After 10 min incubation at 20 ^{0}C, the reaction was terminated with 5 µl of 2.8 N NaOH. The intensity of the fluorescent signal produced by the product is maximized by the addition of base. Formation of the 2,3-diaminonaphthtriazole was measured using a Schematzu fluorescence apparatus with excitation at 365 nm and emission read at 450 nm with a gain setting at 100%.

Nitrite/nitrate detection

Plasma is filtered through an ultrafree microcentrifuge filter unit (14000 rpm for 15 min) to remove the hemoglobin resulting from cell lysis. The filtrate should contain mostly nitrate (recovery greater than 90%) due to the reaction of NO with the iron-heme center of the protein. Nitrate is converted to nitrite by the action of nitrate reductase (from *Aspergillus niger*, Sigma Chemical Co., St. Louis, MO, USA) such as follow: the sample is incubated with 40 µM NADPH (to initiate the

reaction) and 14 mU of enzyme in a final volume of 50 µl of 20 mM Tris buffer (pH, 7.6). The reaction is terminated after 5 min at 20 ^{0}C by dilution with 50 µl of water followed by addition of the DNA reagent for determination of nitrite. Nitrite levels in samples are then calculated by first subtracting the value of the enzyme blank (i.e., nitrate reductase plus NADPH) from the experimental and then calculating the value using a standard curve for nitrite to which NADPH has been added.

Malonaldialdehyde (MDA) content

MDA was measured as described by Buege and Aust, (1978). Half milliliter of plasma were added to 1.0 ml of thiobarbituric acid reagent, consisting of 15% TCA, 0.375% thiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene (BHT) in 0.25 N HCl. Twenty-five microliters of 0.1 M FeSO4.7H2O was added and the mixture was heated for 20 min in boiling water. The samples were centrifuged at 1000 xg for 10 min and the absorbance was read at 535 nm using Labo-med. Inc., spectrophotometer against a reagent blank. The absorbance of the samples was compared to a standard curve of known concentrations of malonaldialdehyde.

Statistical analysis

All measurements were done in triplicate and recorded as mean±SD. Statistical analysis was performed with the Student *t*-test and MINITAB-12 computer program (Minitab Inc., State College, PA).

Results and Discussion

Sociodemographic characteristics of the studied group

Among 189 patients enrolled, 30 (15.87%) children were not analyzed because of unavailability of consent for study or inadequate sample. The remaining enrolled 159 children aged 0 months to 6 years with a mean age of 4.01 ± 1.79 years. They were 87 (54.71%) males and 72 (45.29%) females. The sociodemographic data are shown in Table (1). Based on the biochemical iron status, 86 infants (54.09%) were anemic. As shown in the same data, a logistic regression model was used to assess the effects of the significant explanatory variables in order to distinguish predictors of IDA. It was found that children from rural areas (68.97%), those from low social class (50.00%) and those of illiterate and can read and write mothers (36.03) were the significant risk factors

for IDA in these children. In similar study carried out by Amany and Samaa (2012) found that multivariate analysis revealed that, low level of education, decreased birth spacing and history of anemia before pregnancy were associated with increased risk of anemia ($p<0.05$, OR =18.821, 10.582 and 3.362 respectively). Also, Wu *et al*., (2002)

Table 1. Sociodemographic characteristics and results of multivariate analysis of potential risk factors for IDA among studied children

Variables	Studied group				
	Number (159)	Percentage (%)	IDA		Statistical analysis
			Number (86)	Percentage (%)	
Sex:					
– Male	87	54.71	45	52.33	*
– Female	72	45.29	41	47.67	
Age (years):					
– <1	18	11.32	10	11.63	
– 1-2	34	21.38	17	19.77	p<0.05
– 3-4	47	29.56	28	32.56	
– 5-6	60	37.74	31	36.05	
Level of mothers education:					
– Illiterate	17	10.69	20	23.26	
– Can read and write	29	18.24	11	12.79	
– Primary/preparatory	32	20.13	19	22.09	p<0.05
– Secondary school	51	32.08	23	26.74	
– University or higher	30	18.86	13	15.12	
Family size (person):					
– 1	19	11.95	8	9.30	
– 2-3	65	40.88	39	45.35	p<0.01
– 4-5	52	32.70	29	33.72	
– >5	23	14.47	10	11.63	
Residence:					
– Urban	101	63.52	50	53.47	p<0.05
– Rural	58	36.48	36	68.97	
Social class:					
– Low	59	37.11	43	50.00	p<0.01
– Middle	71	44.65	32	37.21	
– High	29	18.24	11	12.79	

* Non significant ($P > 0.05$).

reported that iron deficiency is responsible for lost productivity and premature death in adults and has been implicated as a cause of perinatal complications such as low birth weight and premature delivery in affected mothers (CDC, 2002). In children, the initial manifestations may be subtle and amenable to treatment. Long-term findings attributable to iron deficiency include increased susceptibility to infection and poor growth (Ioli, 2002). Finally, Elhassaneen *et al.*, (2016) found that children from rural areas (68.12%), those from low social class (65.63%) and those of illiterate mothers (82.46) were the significant risk factors for IDA in Egyptian children of Port Said Governorate.

Hematologic and biochemical parameters of the groups

The hematological findings among normal cases (no anemia) and patients with IDA are shown in Table (2). From such data it could be noticed that the mean hemoglobin (Hgb) level and mean corpuscular volume (MCV) of normal infants were 13.95±1.12 g/dL and 84.43±4.11fL which significantly decreased by the rates of -42.80% ($p<0.01$) and-3.30% ($p<0.05$) in IDA patients, respectively. The opposite direction was observed for the red blood cell distribution width (RDW) which recorded 12.60±1.55% in normal infants and significantly ($p<0.01$) increased by the rate of 35.24% in IDA patients. Such data are partially in accordance with that obtained by Elhassaneen *et al.*, (2016) who studied the prevalence of IDA in Infants and Young Children of Port Said Governorate. Such as metioned by Ann *et al.*, (2002) measurement of Hgb, the concentration of oxygen carrying protein, is a more sensitive and direct test for anemia than others tests such measurement of hematocrit (Hct), the percentage of whole blood that is occupied by RBCs. Hgb measurement is inexpensive, readily available test for anemia and is used most commonly to screen for iron deficiency. Anemia generally is defined as Hgb values below the 5th percentile in a healthy reference population: less than 11.0 g/dL for infants 6 months to 2 years of age. However, Ann *et al.*, (2002) stated that Hgb is a late marker of iron deficiency, is not specific for IDA, and is less predictive as the prevalence of IDA decreases. MCV, the average volume of RBCs, is reported in automated analyses, but it also can be calculated as the ratio of Hct to RBC count. MCV is useful for categorizing anemia as

Table 2. Hematologic and biochemical parameters of normal cases (No Anemia) and IDA patients

Parameters		Normal cases (No anemia, n=73)	IDA Patients (n-86)	Statistical analysis
Hematologic parameters				
Hgb (g/dL)	Range	11.99-16.87	6.97-9.61	p<0.01
	Mean ± SD	13.95±1.12	7.98±1.54	
	% of change	------	-42.80	
RDW (%)	Range	10.83-14.52	15.01-19.79	p<0.01
	Mean ± SD	12.60±1.55	17.04±1.10	
	% of change	-----	35.24	
MCV (fL)	Range	79.79-91.93	58.78-69..42	p<0.05
	Mean ± SD	84.43±4.11	64.76±5.09	
	% of change	------	-23.30	
Biochemical parameters				
Serum iron (µg/dL)	Range	99.09-151.54	30.25-39.70	p<0.001
	Mean ± SD	120.76±30.18	34.76±13.22	
	% of change	-----	-71.22	
Serum ferritin (µg/dL))	Range	105.33-151.98	6.88-9.76	p<0.0001
	Mean ± SD	128.83±21.70	7.95±6.06	
	% of change	------	-93.83	

microcytic, normocytic, and macrocytic. Also, RDW measures variations in the size of RBCs and increases with ID. Ann *et al.*, (2002) mentioned that because of its relatively low specificity, RDW is not as useful alone as a screening test, but it is used frequently in conjunction with MCV to differentiate among various causes of anemia. The studies of Booth and Aukett, (1997) and Ann *et al.*, (2002) concluded that RDW is high in IDA, but low in thalassemia minor.

On the other side, data in Table (2) are shown the iron profile among normal cases (no anemia) and patients with IDA. From such data it could be noticed that the mean serum iron and serum ferritin levels of normal infants were 120.76±30.18 µg/dL and 128.83±21.70 µg/dL which significantly decreased by the rates of -71.22% ($p<0.001$) and -93.83% ($p<0.0001$) in IDA patients, respectively. Such data are partially in accordance with that obtained by Elhassaneen *et al.*, (2016) who studied the prevalence of IDA among infants of Port Said Governorate. In

similar study, Fairbanks (1991) reported that serum iron concentration can be measured directly and generally decreases as iron stores are depleted. However, serum iron may not reflect iron stores accurately because it is influenced by several additional factors, including iron absorption from meals, infection, inflammation, and diurnal variation (Oski, 1993 and Ann *et al.,* 2002). Regarding, ferritin is a storage compound for iron, and serum ferritin levels normally correlate with total iron stores. As iron stores are depleted, serum ferritin levels decline and are the earliest marker of ID (Ann *et al.,* 2002). Additionally, Fairbanks, (1991) and Oski, (1993) stated that serum ferritin is an acute-phase reactant that can become elevated in the setting of inflammation, chronic infection, or other diseases.

Biological oxidative parameters in normal and IDA patients

The oxidants levels among normal cases (no anemia) and IDA patients are shown in Table (3). From such data it could be noticed that the mean MDA (TBARS, nmol/mL) level and NO_2 (nmol/mL) of normal infants were 1.95±0.41 and 2.53±0.11 which significantly ($p<0.01$) increased by the rates of 26.15 % and 33.20% in IDA patients, respectively. Such behavior was observed by Mehmet *et al.,* (2011) in adult IDA patient by other parameters including Total peroxide (TP) and oxidative stress index (OSI). It has long been known that exposure to IDA has been associated with a wide variety of adverse health effects including oxidative stress (Mehmet *et al., (*2011). The studies related to this subject are limited. Therefore, in the present study, we investigated whether IDA exposure can be affected as oxidative stress in children blood. Oxidative stress is a general term used to describe a state of damage caused by reactive oxygen species (ROS) (Nohl and Koltover, 1992). Oxidative damage causes a net stress on normal body function and may result in many specific diseases. It also appears to contribute to the general decline in optimum body functions. The oxidative stress attack against several cellular structures. For example, membranes, free radical mediated injury of lipids and proteins seriously impair permeability and integrity of plasma membrane as well as of inner organelle membranes (Sies, 1991). Mitochondria, as mitochondria are the main site of energy production, their malfunction arising from oxidative damage impairs much essential energy dependent cell function eventually leading to cell death (Nohl and Koltover 1992). Proteins and

Table 3. Oxidants levels of normal cases and IDA patients

Parameters		Normal cases (No Anemia, n=85)	IDA Patients (n-126)	Statistical analysis
MDA (TBARS, nmol/mL)	Range	1.71-2.15	1.90-3.71	p<0.01
	Mean ± SD	1.95±0.41	2.46±0.73	
	% of change	------	26.15	
NO_2 (nmol/mL)	Range	2.04-3.02	3.11-4.68	p<0.01
	Mean ± SD	2.53±0.11	3.37±0.23	
	% of change	------	33.20	

enzymes, both intracellular and extracellular protiens are damaged, and enzymes are inactivated by the oxidative stress (Dean and Sympson 1991). Chromosomes, oxidative stress can produce DNA strand breakage, thus influencing nuclear functions which are of paramount importance for cell Integrity (Fraga *et al*., 1990). Oxidant damage to DNA, protein, and other macromolecules appears to be a major contributing factor to aging and the many degenerative processes associated with it, including cancer, heart disease, cataracts, and cognitive dysfunction (Carney *et al*., 1991 and Fraga *et al*., 1990). The results of the current study confirm that IDA and its ability to induce oxidative stress, may have a key role or involved in the occurrence of other diseases, especially those diseases that cause the oxidative stress. Several studies indicated that OS contributes to many pathological conditions, including cancer, neurological disorders, atherosclerosis, Alzheimer's disease, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Halliwell, 1991; Chaitanya *et al.,* 2010; Rahman *et al.,* 2012 and Elmaadawy, 2016).

Biological antioxidative parameters in normal and IDA patients

Antioxidant defense systems are comprised of enzymatic and nonenzymatic components. Important antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd). Numerous low molecular weight antioxidants have been described. Widely distributed water-soluble samples include reduced glutathione (GSH) and ascorbate;

important lipid soluble antioxidants include α-tocopherol and carotenoids such as β-carotene and xanthophylls (Halliwell and Gutteridge, 1985). The changes in all of antioxidant defense systems of diagnosed to have IDA compared with the normal cases (no anemia) were being investigated in the present study. As far as we know, glutathione fractions, antioxidant enzymes in erythrocytes and vitamins in plasma analysis of subject's children diagnosed to have IDA were made for the first time in this study.

Glutathione fractions

The glutathione fractions level among normal cases (no anemia) and IDA patients is shown in Table (4). From such data it could be noticed that the mean GSH and GSSG levels of normal infants were **7.28±1.11**and **0.69±0.10**µmol/L which significantly decreased by the rates of **-19.73**% (**p<0.01**) and **-11.59**% (**p<0.05)** in IDA patients, respectively. Reduced glutathion (GSH) is a tripeptide-thiol (γ-glutamylcysteinyl-glycine) that has received considerable attention in terms of its biosynthesis, regulation, and various intracellular functions (Reed and Beatty, 1980; Larsson *et al*., 1983). Among these function are two constructing roles in detoxifications includes: 1) as a key conjugate of

Table 4. Glutathione fractions and selenium level of normal cases (No Anemia) and IDA patients

Parameters		Normal cases (No anemia, n=73)	IDA Patients (n-86)	Statistical analysis
GSH (µmol/L)	Range	6.70-10.63	4.23-6.88	p<0.01
	Mean ± SD	7.28±1.11	5.87±0.65	
	% of change	------	-19.73	
GSSG (µmol/L)	Range	0.53-0.87	0.49-0.67	p<0.05
	Mean ± SD	0.69±0.10	0.61±0.13	
	% of change	------	-11.59	
GSH/GSSG	Mean ± SD	10.55±1.11	8.54±0.87	p<0.01
	% of change	------	-19.05	
Se (µmol/L)	Range	1.09-1.59	0.86-1.08	p<0.01
	Mean ± SD	1.31±0.23	0.93±0.17	
	% of change	------	-29.01	

electrophilic intermediates, principally via glutathione-*s*-transferase activities in phase II metabolism, 2) as an important antioxidant includes its role in the activities of GSH-Px and GSH-Rd, and 3) can apparently serve as a nonenzymatic scavenger of oxyradicals (Halliwell and Gutteridge, 1985 and Elmaadawy, 2016).

On the other side, a fall in glutathione fractions observed in IDA patients generally accompanied by a concomitant decreased in the ratio of GSH/GSSG. The mean GSH/GSSG value of normal infants was **10.55±1.11** which significantly ($p<0.01$) decreased by the rate of **-19.05**% in IDA patients. Di Giulio (1991) mentioned that a more fundamental effect of oxyradical-generating compounds, however, is their effect on what can be referred to as the redox status (GSH/GSSG) of cells or tissues. Few studies have been addressed directly the issue of effects of pro-oxidants on redox status. Elhassaneen *et al*., (2004) mentioned that increased fluxes of oxyradicals might be decreased in the GSH/GSSG ratio, due either to direct radical scavenging or to increased peroxidase activity. This effect could also occur indirectly due to reduced NADPH availability (necessary for GSH-Rd activity) resulting, for example, from oxidations in the first step of the redox cycle (Champe and Harvey, 1994; and El-Shafie, 1999). Similar findings are recorded for diseases other than IDA. For example, Nour Eldine (2013) reported that significant reducing ($p>0.05$) in glutathione fractions level in full term neonates diagnosed to have idiopathic hyperbilirubinemia as compared with the control group.

The selenium (Se) level among normal cases (no anemia) and patients with IDA is shown in Table (4). From such data it could be noticed that the mean Se level of normal infants was **1.31±0.23** µmol/L which significantly ($p<0.01$) decreased by the rate of **-29.01**% in IDA patients. Selenium (Se) is an essential trace element. Its importance for human and animal metabolism has become apparent more recently, spurred by the discovery of a Se-dependent enzyme, glutathione peroxidase, and suggestive evidence that selenium plays a role in the prevention of certain forms of cancer (reviewed in Linder, 1991 and Packer, 1992). Similar findings are recorded for diseases other than IDA. For example, Nour Eldine (2013) reported that significant reducing ($p>0.05$) in Se level in full term neonates diagnosed to have idiopathic hyperbilirubinemia as compared with the control group. According to

our knowledge, there is no data reviewed the Se level in IDA patients; subsequently it is a difficult to make comparative studies.

Antioxidant enzymatic components

Data in Table (5) shows the antioxidant enzyme activities of normal cases (no Anemia) and IDA patients. The mean glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), catalase (CAT) and Superoxide dismutase (SOD) activities of normal infants were 16.79±2.11, 14.01±1.32, 157.28±19.76 and 3.85±0.69 U/g Hb which significantly decreased by the rates of -22.04 ($p<0.01$), -16.56 ($p<0.05$), -26.34 ($p<0.01$) and -32.21 ($p<0.01$) % in IDA patients, respectively. We think that IDA causes significant depression on the antioxidant defense potential of erythrocytes and thus, cells cannot cope with oxidant stress incurred by IDA injury. Many studies reported that the IDA induced as a complication by injection with carbon tetrachloride correlated with the significant reducing in antioxidant enzymes and treated with the feeding with some plant parts such Henada (*Jasonia Montana*) lemon balm leaves (*Melissa officinalis*), hawthorn leaves (*Crataegus azorolus*), rose of jericho (*Anastatica hierochuntica*) and corn cob silk (*zea mayz*) (Mohamed, 2012). Similar findings are recorded for diseases other than

Table 5. Antioxidant enzyme activities of normal cases (No Anemia) and IDA patients

Parameters		Normal cases (No Anemia, n=85)	IDA Patients (n-126)	Statistical analysis
GSH-Px (U/g Hb)	Range	13.83-22.76	10.07-18.32	p<0.01
	Mean ± SD	16.79±2.11	13.09±3.54	
	% of change	------	-22.04	
GSH-Rd (U/g Hb)	Range	9.99-19.54	8.51-17.84	p<0.05
	Mean ± SD	14.01±1.32	11.69±2.05	
	% of change	------	-16.56	
CAT (U/g Hb)	Range	141.01-180.55	109.53-140.77	p<0.01
	Mean ± SD	157.28±19.76	115.86±21.62	
	% of change	------	-26.34	
SOD (U/g Hb)	Range	2.88-45.96	1.23-3.71	p<0.01
	Mean ± SD	3.85±0.69	2.61± 0.47	
	% of change	------	-32.21	

IDA. For example, Nour Eldine (2013) reported that significant reducing ($p>0.05$) in antioxidant enzymatic activities in full term neonates diagnosed to have idiopathic hyperbilirubinemia as compared with the control group. SOD, CAT and the enzyme of the glutathione redox cycle i.e. GSH-Px and GSH-Rd are the primary intracellular antioxidants and are considered to be preventive or primary, antioxidant as they prevent free radical chain reaction by decreasing the available concentration of free radical to initiate the process (Hefflner and Repine 1989, Giulio, 1991). All of these vital roles performed by the antioxidant enzymes were affected by the IDA which induced significant decreasing in the levels of such antioxidant enzymes in RBC's. According to our knowledge, there is a dearth of information reviewed the antioxidant enzymatic activities in IDA patients; subsequently it is a difficult to make comparative studies.

Antioxidant vitamins

The reducing in antioxidant enzymes defense potential of erythrocytes was contrary with significant decreasing ($p>0.05$) in antioxidant vitamins in children diagnosed to have IDA (Table 6). The mean values itamins A, C and E levels of normal infants were 1.06±0.12, 49.85±9.81 and 21.96±8.11 µmol/L which significantly decreased by the rates of -23.59 ($p<0.05$), -16.35 ($p<0.05$) and -25.18 ($p<0.05$)% in IDA patients, respectively. According to these results, the decreasing in antioxidant vitamins in plasma could be attributed to their consumption in scavenge, quench and/or trap different ROS resulted from IDA injury. Vitamins include A, E and C, the non-enzymatic antioxidants that prevent or retards the oxidation of sensitive molecules found in the body. Vitamin E is considered as primarily intracellular antioxidants associated with cell membranes (Norman and krinskey, 1992). It is a potent peroxyl radical scavenger (Burton *et al*., 1986) and can protect polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes and in plasma lipoproteins (Jialal *et al*., 1995). Furthermore vitamin E reacts with superoxide radical and singlet oxygen (Burton and Inglod 1981). vitamin A precursor, β-carotene and other carotenoids are bleached when exposed to radicals such as those that arise during lipid peroxidation, which indicates that these pigments; must also intercept active oxygen species. They have antioxidant activity through its property as singlet

Table 6. Antioxidant vitamins level of normal cases (No Anemia) and IDA patients

Parameters		Normal cases (No Anemia, n=85)	IDA Patients (n-126)	Statistical analysis
Vit A (µmol/L)	Range	0.88-2.01	0.73-1.01	p<0.05
	Mean ± SD	1.06±0.12	0.81±0.07	
	% of change	------	-23.59	
Vit C (µmol/L)	Range	37.12-60.33	31.22-44.89	p<0.05
	Mean ± SD	49.85±9.81	41.70±11.62	
	% of change	------	-16.35	
Vit E (µmol/L)	Range	21.05-35.76	16.18-26.44	p<0.01
	Mean ± SD	26.81±6.38	20.06±8.11	
	% of change	------	-25.18	

oxygen (1 O_2) quenchers and their ability to trap peroxyl radicals (Truscott, 1990). They are also able to inhibit free radical reactions (Palozza and Krinsky, (1992). Vitamin C (ascorbic acid) is an important antioxidant. It's antioxidant roles can be' summarized in the following: 1) scavenge $O_2^{\cdot -}$ and $OH^{\cdot}$ with the formation of the sernidehydro-ascorbate free radical that is subsequently reduced by GSH to generate dehydroascorbate and GSSG, as most cells contain a GSH-dependent dehydro ascorbate reductase that generates ascorbate and GSSG, 2) scavenges water-soluble peroxyl (RO_2) radicals (Frei, 1991), 3) repairs and so prevents damage by, radicals arising by attack of OH upon uric acid, inhibits lipid peroxidation by hemoglobin or myoglobin H_2O_2 mixtures and prevents heme breakdown to release iron ions by being preferentially oxidized by ferryl protiens (Halliwell and Gutteridge 1990), 4) reduces α- tocopheryl radicals in membranes back to the lipid-soluble chain-breaking antioxidant α-tocopherol (Slater, 1984), 5) reduces nitroxide radicals, e.g. the radicals formed by attack of O_2 or OH upon desferrioxamine (Hoffman and Garewell 1995), and 6) it also protects plasma lipids against peroxidation induced by activated neutrophils (Frei. 1991). All of these vital roles performed by the antioxidant vitamins were affected by the IDA which induced significant decreasing in the levels of such antioxidant vitamins in plasma.

Correlation analyses of the IDA patients

In the intra correlation analyses, important differences were found as a consequence of IDA. Tables (7) indicated positive correlations between Hb and all enzymatic (GSH-Px, CAT and SOD) plus nonenzymatic (vitamins A, C and E) antioxidants of IDA patients. The opposite directions were recorded for Hb and oxidative stress parameters (MDA and NO_2). Such correlations might be important because they indicated that the factor(s) leading to increase antioxidants (GSH) in the cells might also cause increasing in the levels of serum Hb. On the other hands most important negative correlations were recorded between sxdative stress parameters (MDA and NO_2) and all enzymatic (GSH-Px, CAT and SOD) plus nonenzymatic (vitamins A, C and E) antioxidants. Such correlations indicated that IDA injury leads to the formation of ROS and/or some compounds, which act by redox cycling forming a number of ROS. This is confirmed by the results of this study, which showed that IDA injury causes' reduction in the enzymatic and nonenzymatic antioxidants in serum and RBC's. Subsequently, enzymatic and nonenzymatic antioxidants in serum and RBC's could be used successively as important/new biochemical indicators for IDA assessment.

Table 7. Correlation analysis amongst Hb, oxidative stress and antioxidant defense system parameters of IDA patients (n= 86)

Parameters	**r^2**	**Parameters**	**r^2**
Hb/GSH-Px	0.603*	MDA/SOD	- 0.791**
Hb /CAT	0.684*	MDA/ Vit A	- 0.674*
Hb /SOD	0.710**	MDA/Vit C	- 0.570*
Hb / Vit A	0.742*	MDA/Vit E	- 0.649*
Hb /Vit C	0.701*	NO_2/GSH-Px	- 0.797**
Hb /Vit E	0.669*	NO_2/CAT	- 0.783**
Hb/MDA	- 0.773*	NO_2/SOD	- 0.814**
Hb/ NO_2	- 0.742*	NO_2/ Vit A	- 0.665*
MDA/GSH-Px	- 0.806**	NO_2/Vit C	- 0.603*
MDA/CAT	- 0.750**	NO_2/Vit E	- 0.629*

* Significant ($P \leq 0.05$) , ** Significant ($P \leq 0.01$)

Conclusion

The high frequency of IDA is a severe public health problem in developing countries including Egypt, especially in children from rural areas, those from low social class and those of low maternal educational level. The results of our study support the higher oxidative stress hypothesis in IDA. Iron-rich foods in conjunction with antioxidant vitamins supplementation therapy should be advised by health care providers to all infants from 6 months to 6 years.

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تقييم الاجهاد التأكسدى وأنظمة الدفاع المضادة للأكسدة لدى الرضع والأطفال الصغار المصابين بأنيميا نقص الحديد فى محافظة الشرقية ـ مصر

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قسم التغذية وعلوم الأطعمة – كلية الاقتصاد المنزلى- جامعة حلوان – القاهرة- مصر[٢]

الملخص العربى:

تعد أنيميا نقص الحديد أحد الأسباب الرئيسية للاصابة بالمرض والوفيات في جميع أنحاء العالم والتى تؤثر على الناس من جميع الأعمار في البلدان المتقدمة والنامية على حد سواء. وتشير الأدلة من الدراسات الوبائية والسريرية إلى وجود علاقة محتملة بين مستويات أكسدة / مضادات الأكسدة وخطر مرض أنيميا نقص الحدي. لذلك أجريت الدراسة الحالية بهدف التحقيق في دور الإجهاد التأكسدي وأنظمة الدفاع المضادة للأكسدة في المرضى الذين يعانون من مرض أنيميا نقص الحديد. لذلك تم اختيار عدد ٨٦ مريضا (٤٥ ذكور ، ٤١ إناث) ، ٧٣ من الأصحاء (٤٢ ذكور ،٣١ إناث) من الرضع والأطفال صغار السن الذين تتراوح أعمارهم بين يوم الولادة و ٦ سنوات من مراكز رعاية الأمومة والطفولة بمحافظة الشرقية بمصر لاإجراء الدراسة عليهم. وقد اظهرت التحاليل ان متوسط تركيزالهيموجلوبين ، متوسط حجم الصفائح الدموية للأطفال الطبيعيين ١٣.٩٥ ± ١.١٢ جم / ديسيلتر ، ٨٤.٤٣ ± ٤.١١ قدم لتر والتى انخفضت معنويا بمعدلات ٤٢.٨٠٪ (P <٠.٠١) و ٢٣.٣٠٪ (p <٠.٠٥) في المرضى المصابون بأنيميا نقص الحديد على التوالي. أما عن مقاييس الإجهاد التأكسدي (مالونالديهيد، وأكسيد النيتريك) فقد سجلت إرتفاعا معنويا فى المرضى المصابون بأنيميا نقص الحديد (P≤0.05)في حين سجل إنخفاضا معنويا (P≤0.05) فيما يتعلق بمستويات مضادات الأكسدة الأنزيمية (الجلوتاثيون بيروكسيديز، السوبر أكسيد ديسموتاز، والكاتاليز) وغير الإنزيمية (الجلوتاثيون، والفيتامينات أ، جـ، هـ). وأشار التحليل الإحصائي إلى وجود ارتباطات إيجابية بين مستوى الهيموجلوبين وجميع مضادات الأكسدة الأنزيمية وغير الأنزيمية لمرضى أنيميا نقص الحديد . كما سجلت اتجاهات معاكسة لمستوى الهيموجلوبين ومقاييس المؤكسدات (المالونالدهيد وأكسيد النيتروز). وفى النهاية ، تدعم نتائج دراستنا الحالية فرضية دور الإجهاد التأكسدي في الإصابة بأنيميا نقص الحديد .كما يجب أن ينصح مقدمي الرعاية الصحي بتقديم الأطعمة الغنية بالحديد بالتزامن مع المكملات الغذائية (الفيتامينات المضادة للأكسدة) لجميع الرضع من ٦ أشهر إلى ٦ سنوات.

الكلمات المفتاحية: الهيموجلوبين، المالونالدهيد، أكسيد النتريك، الانزيمات المضادة للأكسدة، الفيتامينات المضادة للأكسدة، المعادن ، أجزاء الجلوتاثيون.