BIOCHEMICAL STUDIES ON PHENYLHYDRAZINE INDUCED EXPERIMENTAL ANEMIC ALBINO RATS

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ABSTRACT

INTRODUCTION: The present study evaluated the modulatory effects of diphenylhydrazine induced experimental wistar albino rats and also to assess various biochemical parameters in whole blood and red blood cell lysate.

MATERIALAND METHODS: Twenty male albino rats weighing 180-200 gm were selected for the study and divided in two groups; ten phenylhydrazine dihydrochloride (PHZ) induced anemia and ten healthy control. Thiobarbituric acid reactive substances and lipid hydroperoxide were measured as lipid peroxidation parameter. The antioxidant vitamins A, C and E and enzymatic antioxidants; catalase, glutathione peroxidase and superoxide dismutase were also assessed.

RESULTS: Phenylhydrazine induced anemic rats showed a significant increase in the lipid peroxidation and decrease in the antioxidants as compared to healthy rats.

CONCLUSION: The study concludes that phenylhydrazine induced experimental anemic albino rats showed increased oxidative stress than compared with healthy albino rats.

KEYWORDS: Albino rats; Anemia; Antioxidant; Lipid peroxidation; Oxidative stress; Phenylhydrazine

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INTRODUCTION

Anemia is one of the most widespread disorders of blood which affect the populations of all ages throughout the world. It is a public health problem that affects populations both rich and poor countries. It is a pathologic condition, in which there is a decrease in red blood cell mass or a decrease in the amount of Hb. Further, iron deficiency affects the production of other proteins containing Fe^{2+}, such as cytochromes, myoglobin, catalase and peroxidases. Worldwide at any given moment, more individuals have iron deficiency anemia than any other health problem. Non-communicable diseases kill more than 36 million people each year. Mostly a bane of middle and low income countries, they share four risk factors: tobacco use, physical inactivity, the harmful use of alcohol and unhealthy diets.[1,2]

However, the incidence of this disorder is higher in the developing countries than in the developed countries[1] due to poverty and lack of hygiene. The situation is aggravated by factors such as nutritional deficiencies and high prevalence of parasitic gastrointestinal infections which cause loss of blood. Number of other conditions, such as malaria and haemoglobinopathies are also responsible, often in combination[2,3]. Wistar albino rats (Rattus norvegicus) is one of the most commonly used animals in biomedical research. Over the years, rats have been used in many experimental studies, which have added to our understanding of genetics, diseases, the effects of drugs, and other topics in health and medicine. Animal research holds the key for solution to AIDS, cancer, heart disease, aging, congenital defects and also healthier, longer and better life with much less pain and suffering for humans.[4]

Lipid peroxidation has been identified as a basic deteriorative reaction in the cellular mechanism of the disease and is initiated by the free radicals which oxidize the PUFAs leading to the formation of conjugate dienes ultimately resulting in the production of hydroperoxides, cyclic peroxides and malondialdehyde. Acting as the first line of defense against the production of such hydroperoxides is a naturally occurring antioxidant glutathione, which is a major source of free thiol in most living cells. GSH also participates in diverse biological processes such as detoxification of xenobiotics and modulation of enzyme activity by disulphide interchange. Alterations in the normal GSH status influence the normal redox status of the cell and hence may lead to lipid peroxidation[5-7].

MATERIAL AND METHODS

Adult male Albino rats were selected for animal experiment. The animals were housed in a stainless steel cage (2 animals per cage) and acclimatized with free access to tap water and standard diet at controlled temperature (22-24° C) and humidity (50 60%), on a 12 hr dark / light cycle for at least one week before the experiments. The acclimatized rats weighing 150 - 200 g were purchased from Shri Raghavendra Animal House, Vijaya Nagar, Bangalore, India. All experiments on rats were carried out in absolute compliance with the ethical guidelines for care and use of laboratory animals. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee, and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. All the chemicals and reagents used during experimentation were of analytical grade.

The vitamin mixture composition according to Williams and Mills (1970)[8] (%) : vitamin A acetate, 0.18; vitamin D_2, 0.0125; DL α tocopherol acetate, 2.2; ascorbic acid, 4.5; inositol, 0.5; choline chloride, 7.5; menadione, 0.225; p-aminobenzoic acid, 0.5; niacin, 0.425; riboflavin, 0.1; thiamine hydrochloride, 0.1; calcium pantothenate, 0.3; biotin, 0.002; folic acid, 0.009; vitamin B_{12}, 0.000135.

The salt mixture composition according to Hubbel *et al* (1937)[9] (%) : Calcium carbonate, 6.86; calcium citrate, 30.83; calcium phosphate monobasic, 11.28; manganese carbonate, 3.52; magnesium sulfate, 3.83; potassium chloride, 12.47; dipotassium phosphate, 21.88; sodium chloride, 7.71; copper sulfate, 0.00777; manganese sulfate, 0.02008; potassium aluminum sulfate, 0.00923; potassium iodide, 0.00405; sodium fluoride, 0.05070.

The normal diet contained; casein 210g, starch 640g, ground nut oil 80ml, salt mixture 40g, vitamin mixture 30g and 390mg $FeSO_4$. $7H_2O$. Iron deficient diet was prepared by excluding $FeSO_4.7H_2O$ and the amount was replaced by starch. Control wistar albino rats fed on normal standard laboratory diet (10 animals). Anemia was induced in experimental animals by intraperitoneal (IP) injection of phenylhydrazine dihydrochloride (PHZ, 10mg/kg weight) and was fed on iron deficient diet for 15 days. The animals were sacrificed on the 16th day by cervical dislocation.

Blood (3ml) was collected from posterior venacava in tubes containing ethylenediamine tetraacetic acid (EDTA) for analysis. Plasma was collected by centrifuging blood

containing anticoagulant at 4000 rpm for 10min, carefully separated and was stored in a sterile and dry vial for analysis. After plasma separation, erythrocyte pellet was washed thrice with chilled physiological saline (0.01M phosphate buffer saline, PBS; pH 7.45 containing 0.85% sodium chloride); 0.5ml of cell suspension was diluted with 2ml cold distilled water to lyse the erythrocytes. The lysed RBC was again centrifuged at 4000 rpm for 10min to get clear supernatant (RBC hemolysate) and the pellet was discarded.

The hemoglobin level in whole blood (EDTA) was determined by the method of Cyanmethemoglobin using Drabkin's solution. Serum iron and total iron binding capacity (TIBC) was determined by Ferrozine method. The lipid peroxidation product, malondialdehyde (MDA) was determined by the method of Buege and Aust (1978)[10]. The malondialdehyde concentration in RBC lysate was determined by the method of Donnam (1950) [11]. The lipid hydroperoxide (LPHO) was determined according to Jiang (1992)[12] using FOX reagent. The nitrite and nitrate concentration was determined using reduced cadmium by the method of Cortas and Wakid (1990)[13].The nitrate was reduced to nitrite by activated cadmium. Nitrite was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine according to Cortas and Wakid (1990).

Enzymatic Antioxidant Assays: Superoxide dismutase (Cu/Zn-SOD) activity in hemolysed RBC and various tissues homogenate were determined by the method of Kakkar *et al* (1984)[14]. Catalase (CAT) activity in the whole blood and hemolysed RBC (erythrocytes) were assayed in the erythrocyte lysates by the method of Sinha (1972)[15]. Glutathione peroxidase (GPx) activity was analyzed in hemolysed RBC lysates and various tissues homogenate by the method of Rotruck (1973)[16].Determination of NonEnzymatic Antioxidant: Vitamin A was estimated by the method of Bessey (1946)[17]. Vitamin C (Ascorbic acid) was estimated by the method of Natelson (1971)[18]. Vitamin E was estimated by the method of Baker and Frank (1968)[19]. Total antioxidant activity (TAA) was performed according to Benzie and Strain (1996)[20]. Glutathione reduced (GSH) was determined according to the method of Beutler and Kelley (1963)[21]. The total protein was estimated by the method of Lowry (1951)[22] using Folin-Ciocalteau Reagent. Erythrocyte fragility test was carried out by the method of Dacie and Lewis (1968)[23]. The packaged program SPSS (Statistical package for social sciences) for windows version 13.0 (SPSS, Chicago, Il, USA) was used for statistical analysis.

RESULTS

Free radicals are substances that easily react with biomolecules and also responsible for the pathogenesis of several diseases including anemia. Oxygen derivatives, which constitute a large portion of FRs, give rise to reversible or irreversible damages to nucleic acids, proteins, amino acids, lipids, carbohydrates and connective tissue macromolecules. A direct correlation between anemia and oxidative stress is shown before by many researchers. In this study, we aimed to investigate correlation between free radicals and antioxidants in healthy and anemic rats. For this purpose SOD, CAT, GPX, LPHO, TBARS, VIT A, C and E and TAA levels were estimated in healthy and induced anemic rats. On analysis, after injection of phenylhydrazine dihydrochloride (10 mg/kg body weight) haemoglobin levels in experimental rats were highly decreased as shown in Table 1: ($p<0.005$, the level of significance) when compared to healthy animals.

Table 1: Lipid peroxidation and enzymatic antioxidants parameters of albino rats

Groups / Variables	Whole blood		Red Blood Cell lysate	
	Control	Anemia	Control	Anemia
Hb (g/dl)	14.21±1.32	5.6±0.89*	-	-
Lipid peroxidation parameters :				
TBARS (nmol/ml)	3.03±1.50	6.04±0.14	2.19±0.35	3.18±0.29
LPHO (nmol/ml)	1214.45±30.18	1591.87±41.60	478.35±20.85	4958.23±26.88*
NO_2 (μmol/l)	380.48±2.09	1076.48±52.31*	50.26±4.05	201.37±15.56**
NO_3 (μmol/l)	6523.38±29.8	7869.44±10.03	1477.8±21.43	2166.80±22.41
Non-enzymatic Antioxidants parameters :				
GSH (mg/dl)	60.47±12.60	33.66±2.84*	-	-
VIT A (μg/dl)	2682.21±15.0	3571.44±16.48**	438.11±16.49	452.43±11.61**
VIT C (mg/dl)	17.23±0.12	9.50±0.74*	9.18±1.52	2.31±0.47
VIT E (mg/dl)	88.56±0.45	33.6±0.23**	43.23±0.26	153.58±0.41**
TAA (μmol/l)	51.94±0.58	30.29±5.062**	4.08±0.75	9.84±5.17**
TP (mg/dl)	87.61±5.83	40.63±9.24**	48.84±1.63	56.30±3.50
Enzymatic Antioxidants parameters :				
CAT (U/mgHb)	424.71±13.46	873.39±25.46**	150.16±5.29	289.40±14.39
SOD (U/gHb)	-	-	21.66±1.84	93.67±3.64*
GPx (U/gHb)	-	-	2.71±1.03	7.91±0.05**

Note : Hb-Hemoglobin, TBARS-thiobarbituric acid reactive substances, LPHO-lipid hydroperoxides, NO_2-nitrite, NO_3-nitrate, GSH-glutathione, VIT A-vitamin A, VIT C-vitamin C, VIT E-vitamin E, TAA-total antioxidant activity, TP-total protein, CAT-catalase, SOD-superoxide dismutase, GPx-glutathione peroxidase, (Level of significance- $*p<0.005$, $**p<0.05$)

The TBARS levels were increased in experimental anemic rats both in whole blood and RBC lysates but the increase was not statistically significant whereas, increase in LPHO levels were increased both in whole blood and RBC lysates but it was

significant only in RBC lysates. Nitrite level was increased both in whole blood and RBC lysates but the increase in nitrate was not significant. Non-enzymatic antioxidants were statistically significant in whole blood sample in anemic rats whereas, only few parameters showed significance in RBC lysates. The enzymatic antioxidant, catalase was significant only in whole blood at $p< 0.05$. Superoxide dismutase and glutathione peroxidase was statistically significant in RBC lysates. On osmotic fragility test carried out in control and PHZ induced anemic rats (Fig I :) showed increasd hemolysis in PHZ treated albino rats as compared to healthy controls.

DISCUSSION

Testing drugs in animals before doing so in human helps researchers find potential toxic side effects, as well as understand the metabolism of drug compounds and consequent effects seen throughout the body. It is well known since long time that carbohydrates, proteins and lipids are biologically active macromolecules involved in various metabolic and energy yielding processes of cellular systems. Metabolic disturbances of any of these molecules are known to contribute to several chronic pathological states. Amino acids, free as well as in the form of constituents of proteins, play an important role in maintaining the structural integrity and folding nature of proteins and any imbalance or inadequacy of these components influence protein synthesis, thereby affecting the protein turnover. Free radical mediated lipid peroxidation is involved in many pathological processes and biological systems possess self-defensive mechanism against these peroxides mediated through enzymatic and non-enzymatic systems. The use of animals in research is prevalent because they share at least 200 common illnesses and diseases with humans.[4,24]

The RBC are intrinsically prone to oxidative stress because they are exposed to high oxygen tension, and have a characteristic structural composition with polyunsaturated fatty acid in the membrane, besides the presence of haemoglobin bound iron. However, membrane and cytoplasmic compartments of RBC have an efficient antioxidant mechanism that maintains their integrity. A detoxifying system consists of reduced glutathione (GSH), SOD, CAT. GPx and Vit E prevent oxidative damage. In addition, there is also a system consisting of NADPH-dependent methemoglobin reductase, ascorbic acid, glutathione reductase (GR), whose main role is the repairing of damage that follows oxidative stress.[25] In normal physiological state, oxidants generated during metabolism play a significant role in maintaining oxidant-antioxidant ratio. In pathological state, an increase in the reactive free radicals creates an imbalance in this ratio thereby making macromolecules vulnerable to oxidative damage. As a result, proteins undergo rapid oxidation leading to the alterations in their structural integrity and in assessing oxidative damage lipid peroxidation and protein oxidation are generally used as biomarkers.[26]

Lipid peroxidation has been identified as a basic deteriorative reaction in the cellular mechanism of the anemia and is initiated by the free radicals which oxidize PUFAs leading to the formation of conjugate dienes ultimately resulting in the production of hydroperoxides, cyclic peroxides and malondialdehyde. Acting as the first line of defence against the production of such hydroperoxides is a naturally occurring antioxidant glutathione (GSH), a major source of free thiol in most living cells. GSH in addition also participates in diverse biological processes such as the detoxification of xenobiotics and modulation of enzyme activity by disulfide interchange.[27] However, red cells have a potent antioxidant protection that modifies GSH, GPx, Vitamins A, C and E. Synergistic and co-operative interactions of these antioxidants rely on the sequential degradation of peroxides and free radicals as well as on mutual protections of enzymes. Oxidative stress arises when there is an imbalance between radical generating and radical scavenging activity; it may therefore increase formation of oxidation products. Oxidation of erythrocytes induces membrane injury, methemoglobin formation and eventually destruction of the cell. Lipids especially PUFAs are sensitive to oxidation, leading to the term lipid peroxidation, of which, MDA is the most abundant. The accumulation of MDA in tissues or biological fluids is indicative of the extent of free radical generation, OS and tissue damage.[28]

All cells contain iron which is of fundamental biological importance, being a micronutrient that is active in oxidative metabolism, cell growth and reproduction, as well as in oxygen transportation (hemoglobin) and storage (myoglobin). Nevertheless, levels of iron are not always sufficient, because they are influenced by physiological and dietary factors. Oral administration of 10 mg/kg phenylhydrazine for 8 days reduced hematological indices by 50%. PHZ altered the function of RBC by hemolysis characterized by 76.06% decrease in RBC, 46.27% decrease in Hb concentration, 65.24% decrease in WBC and 42.68% decrease in PCV.[29-30]

Markovic *et al.*[31] reported decreased GSH concentration

followed by the accumulation of superoxide anion and hydrogen peroxide in the plasma in phenylhydrazine treated anemic rats. On the other hand there was no change in lipid peroxidation in either the plasma, or the RBC of PHZ treated rats. The high levels of ROS cause the elevation of Heinz body formation in the RBC of PHZ treated rats, indicating that proteins could be the main site of PHZ-induced damage of RBC. GSSG / 2GSH ratio is significantly higher in the plasma of PHZ-treated rats, which indicates an inefficient metabolism of the glutathione system and could be one of the causes of ROS accumulation. Plasma of PHZ-treated rats is the main site of oxidative stress mainly because the glutathione antioxidative mechanisms are less efficient in the scavenging of ROS, but there is good protection of this mechanism against lipid peroxidation.

Feeding iron deficient diet (35mg iron/kg) during gestation and or lactation results in significant decrease in non heme iron as early as in 7 days old pups showing a stage of early iron deficient which later entered into the stage of IDA as shown by reduced PCV and Hb levels.[32] This iron deficiency did not show any effect on body and brain weights of developing rats. However, there was a significant reduction in the activities of brain glutamate dehydrogenase, glutamate adenine deaminase and GABA-transferases enzymes, associated with GABA metabolism, in gestationally and lactationally iron deficient pups. However, earlier reports have reported the involvement of iron in synthesis and packaging of neurotransmitters, their uptake and degradation into other iron containing proteins which may directly or indirectly alter brain function. Iron deficiency during developmental stage of brain may cause irreversible disturbances and damage to GABA neurotransmitters system.[28-33]

Feces of rat fed with iron fortified diet were darker than rats without iron fortified diet. Iron and calcium are known to enhance the qualities of blood, bones, teeth and blood formation and also of cardiac function.[33] They also play a predominant role in oxygen and electron transport. Fortified diet leads to the development of good immune system in rats and in humans which contributes extensively to freedom from frequent sickness and illness, resulting in good health as a result of consumption of high quality food.

Increased TBARS in iron deficient group was reported by Hamed *et al*. (2010)[7] than normal healthy controls. Supplementation of iron in the form of ferrous ammonium sulfate form freeze dried juice mixtures to iron deficient rats produced significant reduction of TBARS. Iron may induce OS only if it is given to non iron deficient subjects or if it was in ferric form. Rats fed on both freeze dried fruit juice and ferrous ammonium sulfate showed decreased TBARS level as compared to those fed only ferrous ammonium sulfate diet. The presence of antioxidant component; ascorbic acid, -carotene, α-tocopherol, polyphenols and condensed tannins in fruit juices has a beneficial effect and results in decreased TBARS level.

Mittal (2002)[34] reported eight weeks of iron deficiency in rat did not significantly change the gross weights of brain and liver. There was no effect on hemoglobin and hematocrit. The non-heme iron in liver and brain decreased significantly. The activities of glutamate dehydrogenase, glutamic acid decarboxylase and GABA-transferase in brain were decreased. The effects of iron deficiency on the levels of iron, copper, zinc and magnesium in the brain, liver, kidney, heart and lungs of albino rats was investigated by Oladiji (2003).[35]

The effect of the iron deficient diet on iron absorption is short-lived. Exposure to the ordinary amounts of dietary iron in the standard diet after a 2-week period of iron deprivation reduces iron absorption to normal levels in 3 days. Even when dietary iron deficiency is extended over a period of 3-10 months, iron absorption returns to control levels after 5 days feeding of an iron-replete diet. The lack of dietary iron appears to be of little importance in the control of iron absorption of humans, in contrast to the rats .[27,36] Rats are continuously growing and are presumably in positive iron balance; thus, their need for iron is comparatively greater than man's. Further, rats excrete iron much more rapidly than do humans. Rats lose 0.23 % of their body iron per day, while humans lose 0.10 % per day. Thus it takes man 23 days to excrete the same proportion of body iron the rat loses in 1 day. Extrapolation would indicate that man would thus have to be deprived of iron for 115 days to acquire a net iron deficit comparable to that which that rat acquires in 5 days of iron-deficient feeding. Iron absorption in rats is increased by dietary iron deprivation, erythropoiesis in the rat is unaffected by dietary iron deprivation that increases iron absorption by more than a factor of four, iron absorption is not increased in bled rats of an amount of iron equivalent to that lost in 5 days of iron derivation, these findings are compatible with the concept that iron-absorption is controlled by depletion of iron from a specific pool, separate from the hepatic and erythrocytic pools, iron absorption in human subjects was unaffected by dietary iron deprivation for 13 days .[36-39]

CONCLUSION

Phenylhydrazine induction causes destruction of red blood cells by oxidation stress and many joint changes at cellular level resulting in anemia. The study suggests that damage caused by induction of phenylhydrazine is more in whole blood than RBC. The induction of PHZ decreases antioxidant defense mechanism resulting in decreased antioxidant levels. Nevertheless, there is need for thorough understanding of the phenylhydrazine on various other tissues (brain, heart, kidney, liver, lungs, spleen, etc) and are yet to be studied.

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