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Biochemical Studies of Iron Fortified Nigerian Rice Fed to Phenylhydrazine Induced Anaemic Rats

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ABSTRACT

The blood chemistry, hematological and oxidative stress parameters of anaemic rats fed iron-fortified Nigerian Ofada rice with Ferrazone® (NaFeEDTA) were investigated. Forty male rats were divided equally into 4 groups. Groups 1 and 2 were induced with anaemia using phenylhydrazine and were fed with and without iron fortified diet respectively, while groups 3 and 4 which served as control were non-anaemic and were fed with and without iron fortified diet respectively, for 30 days. The blood chemistry, electrolyte level, hematology, anti-oxidant enzyme activities and tissue histology were determined. Packed cell volume , hemoglobin level and red blood cell count were significantly ($p<0.01$) increased in rats fed with/without iron fortified diet as compared to the control. The activities of aspartate amino transferase, alanine amino transferase, direct bilirubin, creatinine and triacylglycerol levels were significantly increased in anaemic rats fed with iron fortified diet as compared with the control. There was a significant decrease in superoxide dismutase (SOD) activity and malonyldialdehyde (MDA) level of anaemic rats fed with fortified diet as compared with the control group, while there was a significant increase in SOD and catalase activities and a decrease in MDA level of anaemic rats fed with unfortified diet. There was a significant decrease in sodium and potassium levels and increased in the brain iron levels of anaemic rats fed with iron fortified diet as compared with the control. Data of the study show that iron fortification using Ferrazone® affected the hematology, lipid peroxidation, electrolytes and blood chemistry of rats.

Key words: Ferrazone® (NaFeEDTA), Iron fortified rice, anaemic rats, Nigerian Ofada rice, enzymes, blood chemistry, hematology, tissue histology

INTRODUCTION

Iron deficiency and anaemia are major problems affecting billions of people throughout the world. These are more prevalent in the under-developed countries, during rapid growth periods and when requirements and/or losses are higher (UNICEF/UNU/MI, 1999). For these reasons, vulnerable groups to develop iron deficiency and anaemia are infants, young children, adolescents, women of reproductive age and pregnant women (Haas and Brownlie, 2001). Therefore, strategies for combating iron deficiency include control of parasitic infections, improvement of sanitation, iron supplementation and iron fortification (INACG, 2005; Andango *et al*., 2007).

Of these strategies, iron fortification of basic foods is the most economical, more convenient approach and has the advantage that does not require food habit modifications. The major issues

for a successful fortification program are the food vehicle and the iron compound. From the wealth of literature, several iron fortificants have been developed and used in food fortification which include ferrous sulphate, ferrous fumarate, ferrous gluconate, ferrous succinate, elemental iron and Ferrazone® (NaFeEDTA), etc. (Hurrell *et al.,* 1989, 2000; Haas *et al.*, 2005; Ebuehi and Oduwole, 2010).

The selection of the iron compound for fortification is important in order to avoid interactions of iron with the food vehicle, because a minor change in organoleptic characteristics of the food will result in consumer's rejection. Solubility, chemical reactivity, bioavailability and cost are other important issues when selecting a fortificant (Garcia-Casal *et al.*, 1998).

Iron is an essential mineral and functions primarily as a carrier of oxygen in the body, both as a carrier of hemoglobin in the blood and of myoglobin in the muscles. It also aids in immune function, cognitive development, temperature regulation, energy metabolism and work performance (Ebuehi and Oduwole, 2010; Haas *et al.*, 2005). Iron deficiency is the most prevalent nutritional deficiency. Iron deficiency anaemia is characterized by a defect in hemoglobin synthesis resulting in abnormal red blood cells (microcytic) and reduced hemoglobin level (hypochromic) (Provan, 1999).

Rice is the most popular cereal worldwide, serving as a staple food for 39 countries and nearly half of the world's population. Globally, rice accounts for 22% of total energy intake (Juliano, 1993; Ebuehi and Oduwole, 2010). There are two species of rice, namely *Oryza sativa* and *Oryza glabberima*, which are the commonly cultivated varieties in Nigeria (Ebuehi and Oyewole, 2007). Several indigenous cultivars exist in Nigeria, such as Ofada and Abakaliki, etc and are widely consumed by the Nigerians as a major staple. In view of the high prevalence of iron deficiency anaemia in Nigeria, coupled with the increasing population challenges, it has been imperative to introduce iron fortification of rice using Ferrazone® (NaFeEDTA), so as to alleviate the menace of iron deficiency.

The existing literature is replete of information *viz a viz* rice fortification and nutrition, especially in Nigeria and sub-saharan Africa. Therefore, the information generated from the present study may be worthwhile. The aim of the present study is to conduct biochemical studies of iron fortified Nigerian rice fed to phenylhydrazine-induced anaemic Sprague-Dawley rats. The specific objectives include to induce anaemia in rats using phenylhydrazine and iron fortification of a Nigerian indigenous rice using Ferrazone® (NaFeEDTA), blood chemistry of anaemic and non-anaemic rats, hematological, electrolytes and histopathological determination of brain and heart of rats. The iron fortificant used for the study was Ferrazone® (NaFeEDTA). Ferrazone® (NaFeEDTA is a staple , water soluble iron compound that meets JECFA specification for food fortification. It is generally recognized as safe (GRAS) with the US FDA . Ferrazone® (NaFeEDTA) manufacturing is certified to be in accordance with the HACCP requirements.

MATERIALS AND METHODS

Nigerian Ofada Rice and Ferrazone® (NaFeEDTA): Freshly harvest Ofada rice in December 2009, was purchased from a local market in Mushin local Govt. Area, Lagos state, Nigeria. The Ferrazone® (NaFeEDTA), an iron fortificant, used for the study was manufactured by Akzo Nobel Chemicals, The Netherlands. A commercial rat chow containing 21% protein and water were given to the rats and *ad libitum* throughout the 2 weeks of acclimatization period.

Iron fortification of rice: Iron fortification of the rice was carried out using the method of Chitpan *et al.* (2005). One kilogram of rice was milled using a grinding machine and was soaked in one litre of Ferrazone®(NaFeEDTA) solution containing 0.057 g and was uniformly mixed. It was oven dried at 70°C for 2 h and later sun-dried for 48 h.

Feed formulation: The rat feed was formulated using the iron fortified or unfortified rice, full fat soya , soya bean meal, palm kernel meal, bone meal, salt and mineral and vita premixes. Two different diets were formulated, namely the iron fortified rice diet and unfortified rice diet. The proximate composition of the respective diets were as follows; protein 21.0%, carbohydrate, 68.5%, fat, 3.7%, fibre, 5.7%, mineral mix, 0.4% and vitamin mix, 0.7% (Ebuehi and Akinwande, 1992).

Induction of Anaemia: Anaemia was induced in rats by intraperioneal administration of phenylhydrazine (60 mg/100 g b.wt.) daily for two days. The phenylhydrazine hydrochloride solution in 0.01 M potassium phosphate buffer, pH 7.4 was prepared *in situ*. The solution was sterilized by filtration prior to use and intraperitoneally administered daily for 2 days. A confirmatory test using 3 rats was carried in a trial experiment, after which the plasma haemoglobin level was determined to verify that the rats were anaemic.

Animal feeding and treatment: Forty virgin male Sprague Dawley rats (146.37±8.12 g) were used for this study. They were randomly divided into four groups with each group containing 10 rats. Group A consists of anaemic rats fed fortified rice diet with water. Group B contains anaemic rats fed unfortified rice and water and Group C consists of non-anaemic rats fed fortified rice and water *ad libitum,* while Group D comprises non-anaemic rats fed unfortified rice and water *ad libitum* for 30 days.

Collection of blood and tissues: After 30 days of feeding the rats the respective diets, they were allowed to starve overnight and then decapitated. Blood samples were taken by cardiac puncture in Na-EDTA tubes for haematology analysis while some blood samples were collected in fluoride oxalate and lithium heparinised bottles for other analysis. Blood was centrifuged at 3000 g for 15 min to obtain the plasma. The liver, brain and heart were used for enzyme and histological analyses.

Histological assay: Histological examination of the brain and heart of the anaemic and non-anaemic rats fed fortified and non-fortified rice was carried out by fixing the tissues in a large fixative (10% formal saline) and tissues were embedded in paraffin, sectioned and stained with hematoxillin and eosin using standard methods. The slides were read and the photomicrograph was taken at a resolution power HEx400.

Biochemical assay: Biochemical analyses of plasma were assayed for the activities of alanine amino transferase, aspartate amino transferase, alkaline phosphatase, levels of total and direct bilirubin, creatinine and triglycerides were determined using Synchron CX5 autoanalyzer. The total protein concentration was determined by the method of Lowry *et al.* (1951). The mineral composition of Na^+, K^+ and Fe^+ in the brain and plasma of the anaemic and non-anaemic rats fed iron fortified rice were determined using Atomic Absorption Spectrophotometer (AAS).

The antioxidant enzyme, superoxide dismutase was assayed utilizing the technique of Kuratko (1998) and catalase was assayed colorimetrically at 620 nm. Glutathione peroxidase activity and malonyldialdehyde level were determined by the method of Ellman *et al.* (1961).

Haematological assay: Haematological profile, such as haemoglobin level, haematocrit (packed cell volume), red blood cell count, white blood cell count were determined using Synchron CX5 autoanalyzer.

RESULTS

The blood chemistry of anaemic and non-anaemic rats fed with iron fortified rice are presented in Table 1. The activities of aspartate aminotransferase were significantly ($p<0.01$) different in anaemic rats than in the non-anaemic rats, while there were no significant differences in total bilirubin, cholesterol, total protein and glucose levels.

The blood chemistry of anaemic and non-anaemic rats fed without iron fortified rice for 30 days are shown in Table 2. The levels of glucose, total and direct bilirubin were significantly ($p<0.01$) higher in anaemic rat than in non-anaemic rats. However, the AST activity, cholesterol and TAG levels were lower in anaemic rat than the non-anaemic rat, while there were no significant ($p>0.01$) differences in the activities of ALT, ALP, the levels of creatinine and total protein.

Table 1: Blood chemistry of anaemic and non-anaemic rats fed with iron fortified diet for 30 days

Parameters	Anaemic rat	Non-anaemic rat
Aspartate amino transferase (U L^{-1})	63.06±4.78	35.27±2.94
Alanine amino transferase (U L^{-1})	27.14±2.61	20.74±2.76
Alkaline phosphatase (U L^{-1})	27.35±1.58	22.61±1.37
Total bilirubin (mg dL^{-1})	20.91±1.25	16.58±2.51
Direct bilirubin (mg dL^{-1})	12.65±1.73	5.05±0.48
Creatinine (mg dL^{-1})	88.07±4.28	94.76±5.31
Cholesterol (mg dL^{-1})	64.10±2.89	52.08±4.67
Triglyceride (mg dL^{-1})	75.06±2.84	94.11±6.31
Total protein (mg dL^{-1})	69.52±4.87	63.82±5.73
Glucose (mg dL^{-1})	40.16±2.89	35.46±2.89

[1]Values are expressed as Mean±SD of three determinations; [2] Values carrying different superscripts horizontally are significant different ($p<0.01$)

Table 2: Blood chemistry of anaemic and non-anaemic rats fed without iron fortified rice for 30 days

Parameters	Anaemic rat	Non-anaemic rat
Aspartate amino transferase (U L^{-1})	54.47±2.31	69.10±2.60
Alanine amino transferase (U L^{-1})	23.16±1.74	28.43±1.53
Alkaline phosphatase (U L^{-1})	15.80±2.96	23.67±1.83
Total bilirubin (mg dL^{-1})	17.10±1.43	5.82±1.15
Direct bilirubin (mg dL^{-1})	8.24±1.15	2.67±0.38
Creatinine (mg dL^{-1})	69.38±2.56	84.09±7.11
Cholesterol (mg dL^{-1})	53.70±1.64	46.58±3.94
Triglyceride (mg dL^{-1})	50.81±2.30	99.46±6.87
Total protein (mg dL^{-1})	56.49±5.31	60.75±2.96
Glucose (mg dL^{-1})	54.06±3.20	41.72±2.15

[1]Values are expressed as Mean±SD of three determinations; [2]Values carrying different superscripts horizontally are significant different ($p<0.01$)

Table 3: Electrolytes level in brain and plasma of anaemic and non-anaemic rats fed with or without iron fortified rice

	Brain electrolytes (mg kg^{-1})						Plasma electrolytes (mg L^{-1})	
	Anaemic			Non-anaemic			Anaemic	Non-anaemic
Rice	Na^+	K^+	Fe^{2+}	Na^+	K^+	Fe^{2+}	Fe^{2+}	Fe^{2+}
Iron fortified rice	115.7±6.30	75.4±6.10	12.95±0.66	167.2±12.80	80.05±7.16	11.75±0.82	43.6±1.80	36.07±1.27
Without iron fortified diet	125.04±4.36	64.27±2.93	4.61±0.30	117.08±3.15	64.28±2.58	6.71±0.52	19.14±1.16	25.37±1.89

Table 4: Haematological indices of anaemic rats fed with or without iron fortified 'ofada' rice for 30 days

	Iron fortified rice		Without iron fortified	
Indices	Anaemic	Non-anaemic	Anaemic	Non-anaemic
PCV (%)				
Before	42.1±2.34	35.67±1.50	38.64±1.96	44.58±3.75
Ater	44.83±2.90	38.53±2.74	42.61±1.17	47.63±5.10
Hb (g dL^{-1})				
Before	8.51±0.12	13.86±1.94	8.63±1.14	12.84±1.90
After	13.36±1.67	16.32±1.50	13.19±1.91	14.27±1.21
WBC (×103 µL)				
Before	6.72±0.52	11.3±1.68	7.42±1.30	6.94±1.17
After	6.91±1.10	12.01±0.94	7.07±1.69	7.10±0.93
RBC (×106 µL)				
Before	6.54±0.69	6.43±0.58	7.04±0.85	7.25±0.92
After	7.03±1.12	6.19±0.72	6.94±1.09	7.40±1.10

The electrolytes' levels of Na^+, K^+ and Fe^{2+} in the brain and plasma of anaemic and non-anaemic rats fed with or without iron fortified rice are shown in Table 3. There was a significant ($p<0.01$) decrease in Na^+ , K^+ and increase in Fe^{2+} of the brain of anaemic rats compared with the non-anaemic rats fed with iron fortified rice while in the group fed without iron fortified rice there was a significant ($p<0.01$) decrease in K^+ and Fe^{2+} of the brain and plasma Fe^{2+} of the anaemic rat as compared with the non-anaemic rat. Although, there was a significant ($p<0.01$) increase in the plasma Fe^{2+} of both the anaemic and non-anaemic rats when compared to the brain Fe but that of the anaemic rat was higher than that of the non-anaemic rats.

The haematological indices of anaemic and non-anaemic rats fed with or without iron fortified Ofada rice are presented in Table 4. There was a significant ($p<0.01$) increase before and after in the PCV and Hb level of anaemic and non-anaemic fed with or without iron fortified rice and contrarily there was no significant ($p<0.01$) different in before and after the WBC and RBC of anaemic and non-anaemic rat fed with or without iron fortified rice.

The results of the antioxidant enzyme activities and lipid peroxidation of anaemic and non-anaemic rats fed iron fortified or without iron fortified rice for 30 days is presented in Table 5. The superoxide dismutase (SOD) activity and malonyldialdehyde (MDA) level of the anaemic rats fed fortified rice were significantly ($p<0.01$) lower than in non-anaemic rats but there was no difference in catalase (CAT) and reduced glutathione (GSH) activities. The activities of SOD and CAT of anaemic rats fed without iron fortified rice were significantly higher and a decrease in MDA level as compared with the non-anaemic rat, while no significant difference existed in GSH level.

Table 5: Antioxidant enzyme activities and lipid peroxidation of anaemic and non-anaemic rats fed with or without iron fortified diet for 30 days

	Iron fortified rice		Without iron fortified rice	
Parameters	Anaemic	Non-anaemic	Anaemic	Non-anaemic
Superoxide dismutase (units mg^{-1})	13.04±1.25	19.25±1.32	17.64±1.20	13.01±1.70
Catalase activity (units mg^{-1})	64.49±3.15	65.01±7.51	64.79±5.38	59.23±2.49
Reduced glutathione level (umol L^{-1})	1.37±0.26	1.48±0.22	1.34±0.26	1.12±0.04
Malonyldialdehyde level (µmol L^{-1})	3.12±0.11	4.36±0.52	2.76±0.19	4.03±0.28

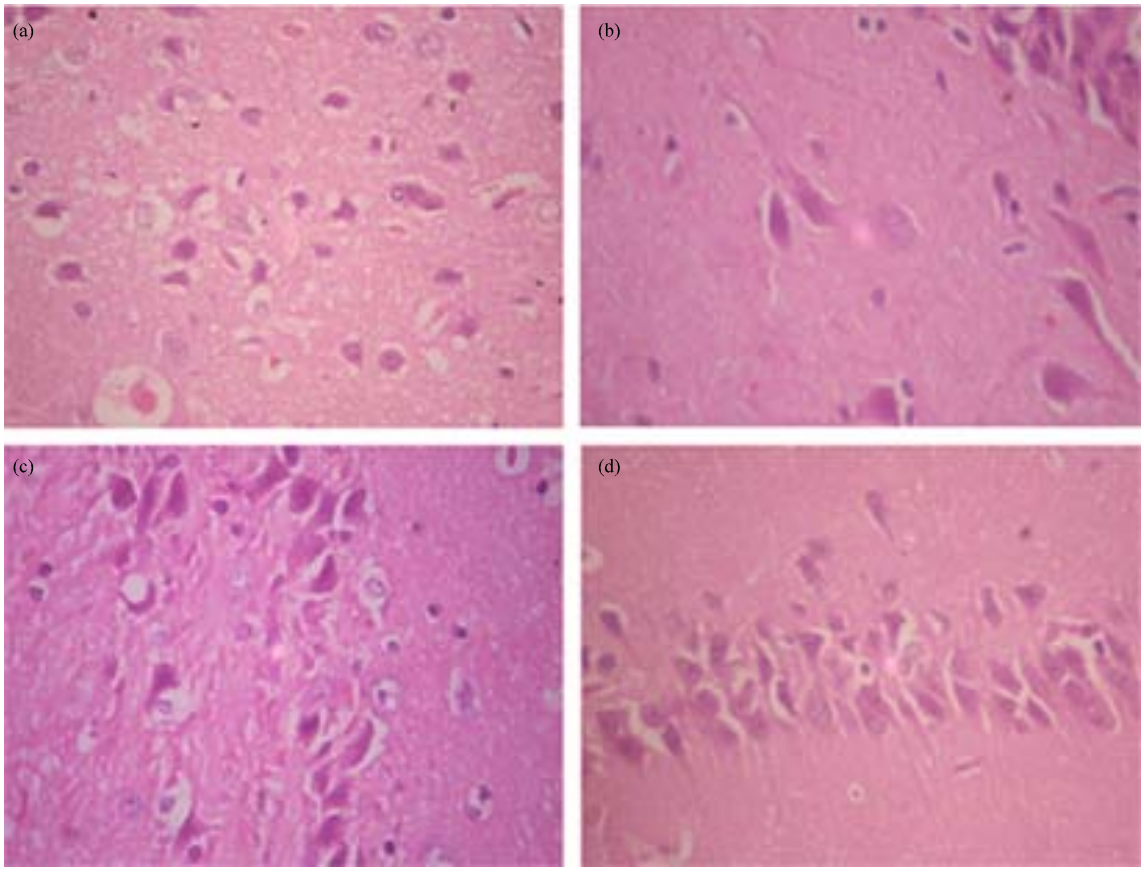

Fig. 1: Photomicrographs of brains of anaemic and non-anaemic rats fed with or without iron fortified rice (H and E x400); (a) brain of anaemic rat fed without Iron fortified diet; (b) brain of non-anaemic rat fed with iron fortified diet; (c) brain of anaemic rat fed with iron fortified diet and (d) brain of non-anaemic rat fed without iron fortified diet

The photomicrographs of brain and heart of anaemic and non-anaemic rats fed with or without iron fortified rice are shown in Fig. 1 and 2, respectively. The brain tissue was pale amorphous background containing neuron cells bodies and glial cells, the areas of lamination were seen. Pervascular clearing or halos are seen around blood vessels indicating mild to moderate cerebral edema. The heart cardiac muscle shows myocytes, which are arranged in interlacing fascicles, their nuclei are spindled and displayed eccentrically with eosinophilic cytoplasm. Delicate intervening of blood vessels are seen in between fascicles.

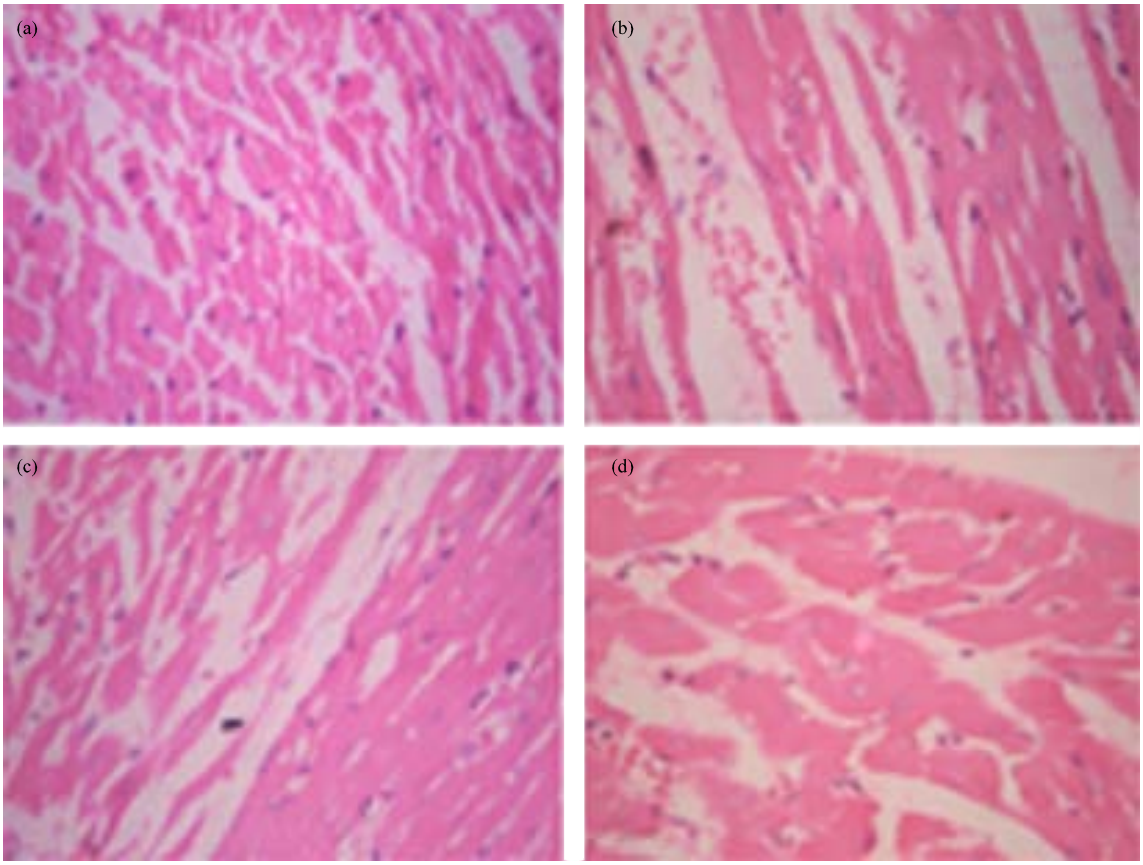

Fig. 2: Photomicrographs of heart anaemic and non-anaemic rats fed with or without iron fortified rice (H and E x 400); (a) heart of anaemic rat fed with Iron fortified diet; (b) Heart of non-anaemic rats fed with iron fortified diet; (c) heart of anaemic rat fed without Iron fortified diet and (d) heart of non-anaemic rats fed without iron fortified diet

DISCUSSION

Ferrazone® (NaFeEDTA) is an iron fortificant which was easy to apply to milled Ofada rice. Phenylhydrazine induces reactive oxygen species formation, peroxidation of lipids and oxidative degradation of spectrin in the membrane skeleton. The faeces of the rat fed iron fortified diet were darker than in the rats fed without iron fortified diet. This agrees with earlier study on iron deficiency and reduced work capacity. The fortified diet shows no noticeable change in the food in terms of colour, odour, appearance and organoleptic properties as compared with the control. This observation corroborates with earlier study that NaFeEDTA, appears to be the most appropriate iron fortificant for use in developing countries, though it is two-to threefold better absorbed than ferrous sulphate (Hurrell, 1997).

The blood chemistry result shows that total bilirubin, direct bilirubin and glucose increase significantly in anaemic rats fed with or without iron fortified diet as compared with the non-anaemic. Bilirubin is a major breakdown of product that results from old blood cells and increase in bilirubin shows that the liver is functioning well (Ebuehi and Asonye, 2007).

This may be due to the effect of the iron acting as blood enhancer and the blood enhancing cardiac function. Iron and calcium are known to enhance the qualities of blood, bones, teeth and

blood formation and also of cardiac function. They also play a predominant role in oxygen and electron transport. AST and ALT are enzymes necessary for energy production, decrease in their activities indicate that the liver is functioning well (Ebuehi and Asonye, 2007). The reduced cholesterol in this study may be caused by NaFeEDTA which has a lowering effect and acts by decreasing the capacity of serum to transport cholesterol (Ganz and Nemeth, 2006). The reduced serum creatinine level in anaemic rats fed with iron fortified diet may have resulted from the decreased synthesis or increased functional capacity of tubular excretion (Zilva *et al*., 1991).

The Fe status of anaemic and non-anaemic rat fed iron fortified diet was higher than in the rats fed without iron fortified diet which is in contrast with earlier reported findings that an increase in Fe status leads to a decrease in Fe absorption (Lynch *et al*., 1989; INACG, 2005). In addition, the results show that Fe was easily absorbed in plasma than in the brain for both groups. The large amount in plasma shows that increase in iron fortified diet could specifically be useful in the treatment of diseases such as anaemia, malaria bleeding and amenorrhea (Iwalewa *et al*., 2009). 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 (Nestel *et al*., 2006; WHO, 2001). The presence of iron, a metallic ion in Ferrazone® (NaFeEDTA) is essential for normal biochemical functioning and development of organs (Cook and Reusser, 1983; INACG, 2005). The diet with the metallic ions would contribute essential metal ions for metabolic activities in livestocks and in turn, humans (WHO, 2001).

We must not lose sight of the substantial effect of individual differences in iron status, which are controlled for and often corrected for when the iron bioavailability of meals or diets is evaluated. Whereas nonheme-iron absorption can vary by 2-5-fold because of dietary bioavailability, differences of 10-15-fold occur across a range of normal iron stores (Hunt and Roughead, 1999). For heme iron, the control of absorptive efficiency suggested by both cross-sectional and longitudinal observations is considerably more modest (Lynch *et al*., 1989; Martinez-Torres *et al*., 1979). Consistent with the results of Cook *et al*. (1997) individual iron status seems to be more influential than is bioavailability in determining nonheme-iron absorption and iron status may be as influential as is bioavailability in determining total iron absorption from a complete diet (Cook and Reusser, 1983).

The significant increase in the Packed Cell Volume (PCV), Haemoglobin (Hb) level and Red blood cell (RBC) of the anaemic rats agrees with previous work by Andango *et al*. (2007), who reported an increase in PCV and Hb level in iron fortified maize. Nath and Prasannan (1959) also reported in earlier works that there was an increase in Hb level using glucose. The data of the study indicate a slight decrease in superoxide dismutase (SOD) activity and malonyldialdehyde level (MDA) in anaemic rats fed with fortified diet as compared with the control which agrees with earlier study on brain activity of SOD and reduced glutathione (GSH) level of zinc adequate rats, which were significantly lowered as compared with the zinc deficient or control diet fed rats (Ebuehi and Akande, 2009; Ebuehi *et al*., 2009). There was a significant increase in SOD and catalase (CAT) activities in rats fed unfortified diet , which disagrees with the report that low CAT activity in rats belonging to the anaemic group is supported by the simultaneous inhibition of enzymes in the group (Nestel *et al*., 2006). The histological examination of the brain and heart tissues for anaemic and non-anaemic rats fed with or without iron fortified diet shows that they were no cellular abnormalities as compared with the control group.

The histopathological data of the rat brain and heart tissues revealed no cellular abnormalities in the consumption of fortified rice using Ferrazone® (NaFeEDTA), which are added information to the literature. In addition, there were no adverse biochemical damage reported from the data obtained from study in the blood chemistry, electrolytes, hematology and oxidative stress in the blood and tissues investigated, due to iron fortification using Ferrazone® (NaFeEDTA). Data of the study show that iron fortification of milled Nigerian Ofada rice using Ferrazone® (NaFeEDTA) alleviates anaemia in rats. Thus, iron fortification of staple food such as rice using Ferrazone® (NaFeEDTA), will be a major intervention to deliver iron in an absorbable form to a large population.

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