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Biochemical Studies of Iron Fortified Gari Fed to Phenyl Hydrazine-induced Anaemic Rats

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ABSTRACT

The blood chemistry, hematological and oxidative stress parameters of anaemic rats fed iron-fortified gari was investigated. Yellow gari, popular Nigerian fermented cassava gruel, was fortified with Ferrazone® (NaFeEDTA). Twenty albino rats were divided into Groups 1, 2, 3 and 4. Groups 1 and 2 were made anaemic by phenylhydrazine administration and fed with or without fortified gari, respectively for 30 days, while groups 3 and 4 were non-anaemic and fed with or without fortified gari respectively for 30 days. Groups 2 and 4 were used as the controls for the anaemic and non-anaemic rats, respectively. The blood chemistry, hematology, antioxidant enzyme activities and brain mineral composition were analysed. Histological examination of the brain, liver and heart of rats were assayed. There was no significant change in plasma levels of creatinine, triglyceride, glucose and brain levels of reduced glutathione and malonyldialdehyde of rats fed with or without iron-fortified gari. The hemoglobin and haematocrit levels were significantly increased after consumption of iron-fortified gari. The aspartate aminotransferase, alkaline phosphatase activities and bilirubin level of rats fed with iron fortified gari were significantly decreased, while alanine aminotransferase activity increased significantly. There was a significant decrease in the superoxide dismutase and catalase activities between the iron-fortified fed rats and the controls. There were no significant differences in the cellular architecture of the brain liver and heart of anaemic rats fed iron fortified gari. It could be suggested that iron fortification of gari using Ferrazone® NaFeEDTA contributed to protect and preserve the integrity of the brain, liver and heart, blood chemistry and antioxidant enzymes.

Key words: Iron-fortified gari, Ferrazone® NaFeEDTA, serum enzymes, chemistry, heamatology, minerals, tissue histology, anaemic rats

INTRODUCTION

Iron deficiency is the most prevalent micronutrient deficiency in the world today. It affects millions of individuals throughout lifecycle, particularly infants and the pregnant women, but also older children, adolescents and women of reproductive age (Andango *et al*., 2007; DeMaeyer and Adiels-Tegman,1985). In developing countries, the prevalence of iron deficiency is high and is due mainly to a low intake of bioavailable iron and may also co-exists with malnutrition, vitamin A deficiency, folate deficiency and infection (Fleming, 1982; INACG, 1993; Cook *et al*., 1997; Juliano, 1993; Bhaskaram, 2001).

Iron is required for the development of vital tissues such as brain, for transporting and storing oxygen in hemoglobin and myoglobin. Iron deficiency anaemia is the most severe type of iron deficiency (Lozoff and Georgieff, 2006; Allen *et al.*, 2006). It can result in low resistance to infection, impaired psychomotor development, impaired cognitive function in children, poor academic performance, fatigue, fetal resorption, low productivity and increased risk of maternal mortality (Bothwell and MacPhail, 2004; IZNCG, 2004; Appel *et al.*, 2001).

Gari is a staple, ready-to-eat product of cassava (*Manihot esculenta*) (Udofia *et al.*, 2007; Davidsson, 2003; Davidsson *et al.*, 2002). It is prepared in different shades of colour, taste, hand feel and consumed as fufu with soup, sipped with salt or sugar and milk, nuts or dried with nuts. Most Nigerians cherish gari since it is easy to prepare, provides mostly cheap energy including other nutrients at marginally nutritional significance (Ernesto *et al.*, 2000; Akerele, 1967; Asonye, 2001). Palm oil may be added to cassava mash before it is roasted, this practice is believed to reduce the effect of HCN in the product (Uvere, 2004). It also used for combating vitamin A deficiency and to impact the golden colour which influence choice and appetite. Traditionally, cassava mash for white gari is given longer fermentation time to reduce HCN to safe levels (FSANZ, 2004; Damardjati *et al.*, 1993).

In the continuous search for solution to the problem of malnutrition in its various forms, mainly among the people of the developing countries, views have been expressed of the need to improve the nutritive quality of our local food through better processing and enrichment. Gari is one of such basic foods worthy of attention. It is widely consumed in the West and Central Africa and forms a staple food for majority of the people in the Southern part of Nigeria. Fortification of gari may be a self-effective, sustainable way to improve iron status in developing countries. It can be achieved by addition of a suitable fortificant, such as using Ferrazone® (NaFeEDTA), either on a large-scale to centrally processed gari, or on a small-scale in communities (Philar and Johnson, 2005). It is a popular West African granular, staple food traditionally made from fermented, gelatinized fresh cassava tubers (Ebuehi *et al.*, 2005).

The present study was carried out using phenylhydrazine to induce anaemia in Sprague Dawley rats. Freshly prepared gari was fortified using Ferrazone® (NaFeEDTA) and fed to the anaemic rats for 30 days. The aim of the study therefore is to conduct biochemical studies of the iron fortified gari fed to phenylhydrazine induced- anaemic rats.

MATERIALS AND METHODS

Freshly prepared yellow gari in December, 2009, was purchased from a local market in Mushin Local Government Area, Lagos State, Nigeria. The iron supplement used for the study was Ferrazone® (NaFeEDTA), manufactured by AkzoNobel Chemicals, The Netherlands. A commercial rat chow containing 21% protein and water was given to the rats *ad libitum* throughout the duration of the study.

Iron fortification of gari: Iron fortification of gari was carried out using the method of Chitpan *et al.* (2005). One kilogram of freshly prepared yellow gari was soaked in one litre of Ferrazone® solution containing 0.057 g uniformly mixed in one litre of deionized water. 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 gari, full fat soya, soya bean meal, palm kernel meal, bone meal, salt, mineral and vitamin premixes. Two

different diets were formulated, namely the iron fortified gari and unfortified gari. 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). The iron fortified gari or unfortified gari provided the carbohydrate content in the dietary formulation.

Induction of anaemia: Anaemia was induced in rats by intraperitoneal administration of phenylhydrazine 60 mg kg^{-1} body weight daily for 2 days, according to Roque *et al.* (2008). A phenyl hydrazine hydrochloride solution in 0.1 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. Confirmatory test using 3 rats was carried in a trial experiment, after which the plasma hemoglobin level was determined to verify that the rats were anaemic.

Animal feeding and treatment: Forty virgin male Sprague Dawley rats (146.31±8.12 g) were used for the study. They were randomly divided into four groups with each group containing ten rats. Group A consists of anaemic rats fed unfortified gari and water and Group C consists of non-anaemic rats fed iron-fortified gari and water *ad libitum*. Group D comprises rats fed unfortified gari and water *ad libitum* for 30 days.

Collection of blood and tissues: After 30 days of feeding the rats with the respective diets, they were starved over night and then decapitated. Blood samples were taken by cardiac puncture into tubes containing Na-EDTA for haematological 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 and used for blood chemistry assay.

The liver, brain and heart of rats were excised. These organs were used for enzyme and histological analyses.

Blood chemistry: The plasma levels of bilirubin, creatinine and triglycerides were determined using Synchron CX5 autoanalyzer. The plasma protein concentration was determined by the method of Lowry *et al.* (1951).

Haematological assay: Haematological profile, such as haemoglobin level, hematocrit (packed cell volume, PVC), red blood cell and white blood cell counts, were determined using Synchron CX5 auto analyzer.

Enzyme assay: Biochemical analyses of plasma were analyzed for the activities of alanine aminotransferarase, aspartate aminotransferase and alkaline phosphatase. The anti-oxidant enzymes' assays were carried out. The activities of serum aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2) and alkaline phosphatase (EC 3.1.3.1) were assayed at 37°C, according to the recommended principles (Steffensen *et al.*, 1997) using commercial kits manufactured by Baehringer, Mannherim, Germany and Roche, Switzerland.

Electrolytes' assay: The mineral composition of Na^+, K^+ and Fe^{2+} ions in the brain and plasma of the anaemic and non-anaemic rats fed iron fortified gari were determined using atomic absorption spectrophotometer (AAS).

Histological assay: Histological examination of the brain and heart of the anaemic and non-anaemic rats fed fortified and non-fortified gari 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.

RESULTS

The haemotological indices of anaemic rats fed with iron fortified gari are presented in Table 1, there were no significant ($p>0.01$) differences before and after in the PCV, Hb level, WBC and RBC counts of anaemic and non-anaemic rats fed iron-fortified gari for 30 days. Similarly, no significant ($p>0.01$) differences existed before and after in the PCV, Hb level, WBC and RBC counts of anaemic and non-anaemic rats fed with iron-fortified gari.

The electrolytes' levels of Na^+, K^+ and Fe^{2+} in the brain and plasma of anaemic and non-anaemic rats were shown in Table 2, there were significant ($p<0.01$) differences in brain and plasma Na^+, K^+ and Fe^{2+} of anaemic and non-anaemic rats. The brain and plasma Na^+, K^+ and Fe^{2+} of anaemic rats were significantly higher than in non-anaemic rats.

The results of the anti-oxidant enzymes activities and liquid peroxidation of anaemic and non-anaemic rats fed iron-fortified or without iron-fortified gari for 30 days are presented in Table 3, the superoxide dismutase and catalase activities of the anaemic rats fed iron-fortified gari were significantly higher than in non-anaemic rats, but with no significant ($p>0.01$) difference in

Table 1: Haematological indices of anaemic rats fed with or without iron fortified gari for 30 days

	Iron fortified gari		Without iron-fortified gari	
Indices	Anaemic	Non-anaemic	Anaemic	Non-anaemic
PCV(%)				
Before	41.9±2.65	34.6±2.19	38.5±1.90	37.6±4.28
After Hb(g dL^{-1})	45.6±1.87	39.5±4.08	42.6±1.03	40.2±1.76
Before	9.4±0.62	8.5±0.31	10.3±0.6	9.4±0.71
After	10.3±0.46	10.3±0.82	13.5±0.7	12.8±0.52
WBC (x10^3 µL)				
Before	6.7±0.18	6.8±0.14	11.4±2.1	10.9±1.40
After	6.5±0.40	7.1±0.53	10.8±3.6	9.2±0.42
RBC (x10^6 µL)				
Before	6.8±0.15	6.4±0.47	6.5±0.18	6.2±0.10
After	6.4±0.73	6.6±0.30	6.2±0.33	6.0±0.34

Table 2: Electrolytes level in brain and plasma of anaemic and non-anaemic rats fed with or without iron fortified gari[1,2]

	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 gari	158.5±7.46[a]	26.03±7.58[a]	91.32±8.40[a]	146.24±17.42[a]	11.53±0.73[a]	115.85±9.46[a]	8.76±0.69[a]	1.58±1.24[a]
Without iron fortified diet	102.80±11.70[b]	23.8±1.45[a]	83.51±7.46[b]	143.74±9.57[a]	24.95±1.30[b]	94.57±5.94[b]	11.68±1.16[b]	6.87±1.05[b]

[1]Values are experimented as Mean±S.D from three determinations; [2]Value carrying different superscripts vertically are significantly different ($p<0.01$)

Table 3: Anti-oxidant enzymes activities and liquid peroxidation of anaemic and non-anaemic rats fed iron-fortified or without iron-fortified gari for 30 days

Anti-oxidant enzymes activities/MDA level of rats	Iron-fortified gari		Without iron-fortified gari	
	Anaemic	Non-anaemic	Anaemic	Non-anaemic
Superoxide dismutase activity (unit mg^{-1})	4.13±0.75	20.96±0.82	25.30±1.67	18.39±1.20
Catalase activity (unit mg^{-1})	14.52±1.37	66.85±3.78	67.90±3.52	58.38±4.47
Glutathione level (µmol mg^{-1})	1.44±0.20	1.67±0.35	2.30±0.37	1.92±0.19
Malonyldialdehyde (MDA) level (µmol mg^{-1})	5.42±0.13	3.71±0.06	4.51±0.36	4.92±0.21

Table 4: Blood chemistry of anaemic and non-anaemic rats fed without iron fortified gari[1,2]

Parameters	Anaemic rat	Non-anaemic rat
Aspartate amino transferase (U L^{-1})	32.60±4.17[a]	21.35±1.47[b]
Alanine amino transferase (U L^{-1})	74.11±1.25[a]	70.67±1.20[a]
Alkaline phosphatase (U L^{-1})	32.64±2.69[a]	70.14±3.62[b]
Total bilirubin (mg dL^{-1})	58.67±0.92[a]	72.68±4.03[b]
Direct bilirubin (mg dL^{-1})	12.30±0.47[a]	12.08±0.71[a]
Creatinine (mg dL^{-1})	5.14±0.42[a]	5.03±0.62[a]
Cholesterol (mg dL^{-1})	52.81±1.36[a]	56.47±0.39[a]
Triglyceride (mg dL^{-1})	91.06±7.05[a]	102.5±6.92[b]
Total protein (mg dL^{-1})	45.26±2.38[a]	92.67±3.14[b]
Glucose (mg dL^{-1})	72.5±6.04[a]	75.64±3.67[a]

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

the levels of glutathione and malonyldialdehyde. The activities of superoxide dismutase and catalase of anaemic rats fed without iron-fortified gari were significantly higher than in non-anaemic rats; while no significant differences existed in the reduced glutathione and malonyldialdehyde levels.

The blood chemistry of anaemic and non-anaemic rats fed without iron-fortified gari were presented in Table 4, the activities of aspartate aminotransferase, alkaline phosphatase and total bilirubin were lower in anaemic rats than in non-anaemic rats. However, the levels of triglyceride, protein and total bilirubin were lower in anaemic rats than in non-anaemic rats, while the aspartate aminotransferase activity was higher in anaemic rats and in non-anaemic rats.

The blood chemistry results of anaemic and non-anaemic rats fed iron fortified gari for 30 days are shown in Table 5, the activities of alanine aminotransferase and total bilirubin level were significantly ($p<0.01$) higher in anaemic rats than in non-anaemic rats.

The photomicrographs of brain and heart of anaemic and non-anaemic rats fed with or without iron fortified gari are shown in Fig. 1-4, 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.

Table 5: Blood chemistry of anaemic and non-anaemic rats fed iron fortified gari 1, 2 for 30 days

Parameters	Anaemic rat	Non-anaemic rat
Aspartate amino transferase (U L^{-1})	24.66±0.88[a]	29.05±0.86[a]
Alanine amino transferase (U L^{-1})	71.33±2.40[a]	52.60±1.76[a]
Alkaline phosphatase (U L^{-1})	24.66±0.88[a]	27.38±0.64[a]
Total bilirubin (mg dL^{-1})	59.33±0.67[a]	65.3±0.64[a]
Direct bilirubin (mg dL^{-1})	10.06±0.58[a]	13.68±0.89[a]
Creatinine (mg dL^{-1})	5.07±0.19[a]	5.04±0.80[a]
Cholesterol (mg dL^{-1})	48.673±0.53[a]	44.15±0.42[a]
Triglyceride (mg dL^{-1})	83.12±1.26[a]	81.6±1.49[a]
Total protein (mg dL^{-1})	45.81±0.76[a]	46.9±0.73[a]
Glucose (mg dL^{-1})	73.48±2.69[a]	71.14±2.14[a]

[1]Values are experimented as Mean±SD from three determinations; [2]Value carrying different superscripts horizontally are significantly different ($p<0.01$)

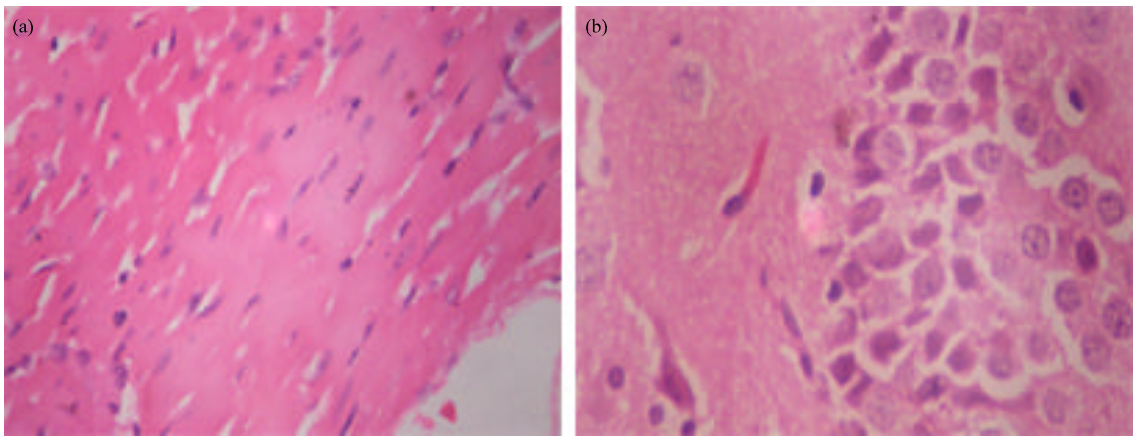

Fig. 1: Photomicrographs of the brain of anaemic rats fed with or without iron fortified gari (x400); (a) fortified and (b) unfortified

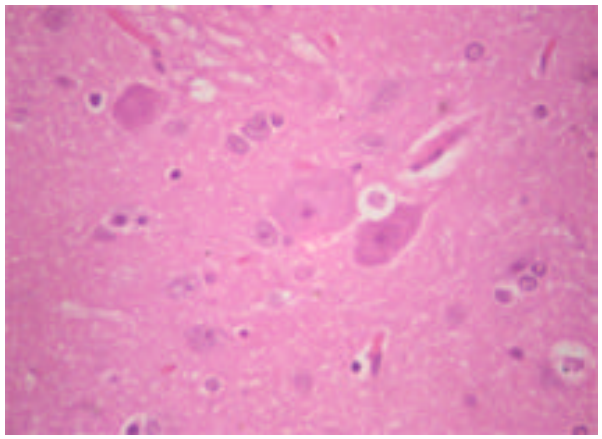

Fig. 2: Photomicrographs of the brain of non- anaemic rats fed with or without iron fortified gari (x400)

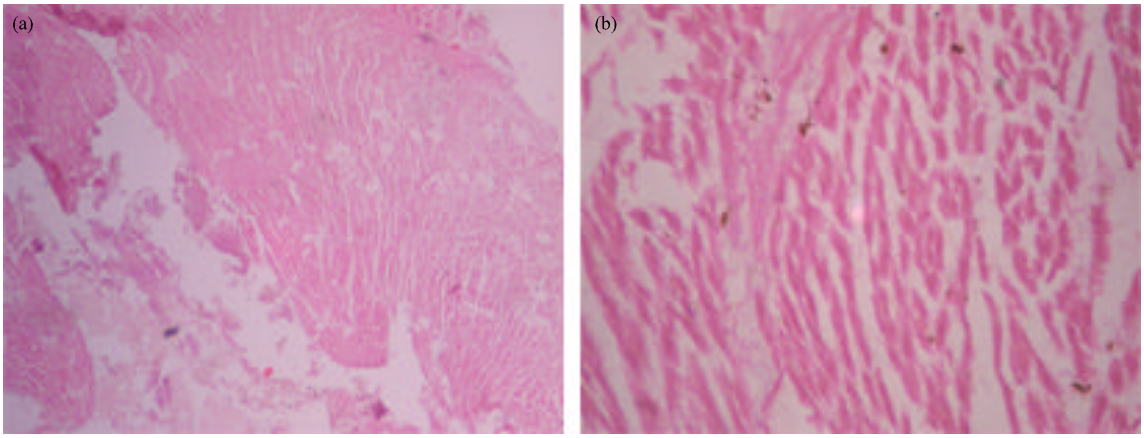

Fig. 3: Photomicrographs of the heart of anaemic rats fed with or without iron fortified gari (x400); (a) fortified and (b) unfortified

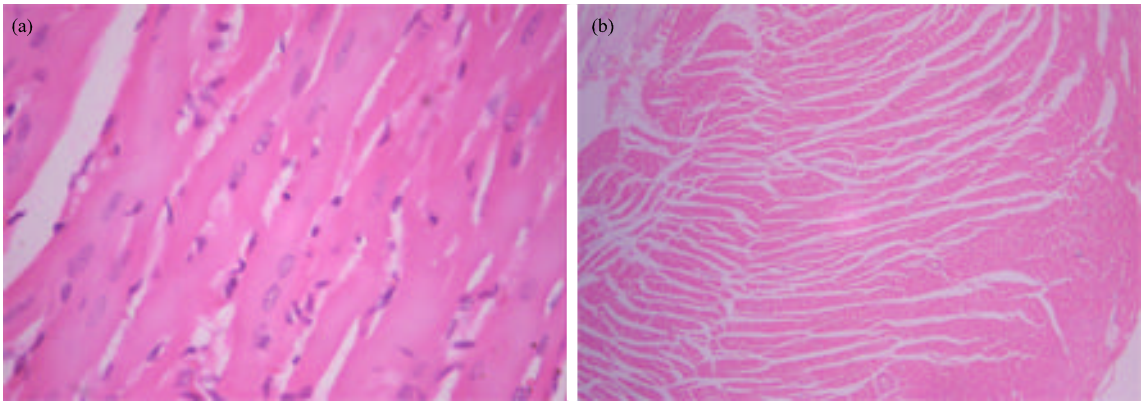

Fig. 4: Photomicrographs (x400) of the heart of non-anaemic rats fed with or without iron fortified gari; (a) fortified and (b) unfortified

DISCUSSION

The phenyl hydrazine induced anaemic rats had significantly ($p<0.01$) reduced plasma hemoglobin levels compared with the non-anaemic rats. This is indicative of iron deficiency anaemia as shown by the inadequate hemoglobin in the red blood cells. This finding concurs with the previous reports by Haas and Brownlie (2001).

The anaemic rats suffered hair loss or alopecia, were apathetic and lethargic. This could be due to inability of the blood which carries oxygen and nutrients to the various body tissues and to the hair follicles (body cells), resulting to a less effective circulatory system. The significant increase in blood Hb levels of the non-anaemic rats fed fortified diet suggest that Ferrazone® (NaFeEDTA) improved the bioavailability of iron of the fortificant and intrinsic iron of the rats and consequently increases blood haemoglobin concentration. These observations are in agreement with previous reports of Layrisse and Martinez -Torres (1977) and MacPhail *et al.*, (1994).

Ebuehi and Oduwole (2010) previously reported that iron fortification of rice with Ferrazone® did not cause any organoleptic changes (Hurrell, 1997; Hinton *et al.*, 2000). Rats fed iron-fortified

diet had significantly higher Hb and haematocrit levels in both anaemic and non- anaemic rats, which is indicative of an enhanced iron absorption. The WBC count had no significant change in comparison with the control. This supports that iron fortification do not affect immunological function as opposed to previous reports by Beard and Connor (2003) and Barton *et al.* (2000).

There were significant increase in the activities of ALT, AST and ALP of anaemic rats fed without iron-fortified diet as compared with rats fed iron-fortified diet. This finding suggest a possible hepatic damage in the anaemic rat and that the extent of damage was more severe in rats fed without iron-fortified gari. The ALT and AST are enzymes involved in amino acid metabolism and they are used as markers in liver diseases (Ebuehi and Asonye, 2007).

The elevated activity of the ALP in the anaemic rats fed without iron fortified diet as compared to rats fed iron-fortified gari diet could be described to a greater severity in bone mineralization and diseases (Vallee and Auld, 1990).

The total and free bilirubin levels in anaemic rats fed with iron-fortified gari significantly decreased as compared to their levels in rats fed without iron-fortified gari. This indicates that iron fortification of gari reduced iron deficiency anaemia because of the reduction in RBC count and Hb level. The insignificant change in the plasma creatinine level in the anaemic and non-anaemic rats fed with or without iron-fortified gari, suggests that Ferrazone®-fortified gari did not cause any kidney damage or impair kidney function of the rats.

There was significant decrease in the plasma cholesterol and total protein levels of anaemic rats fed with or without fortified diet. This may also suggest that fatty liver degeneration and production in protein synthesis were severe in anaemic rats fed without iron-fortified diet as compared to rats fed the fortified diet. This finding concurs with previous report of Floch *et al.* (1969).

The reduction in the activities of antioxidant enzymes namely catalase, superoxide dismutase and reduced glutathione level in the anaemic rats fed Ferrazone® fortified-gari as compared to rats without iron-fortified diet, indicate that oxidative stress was diminished. In other words, iron- fortification may contribute in scavenging free radicals from the tissues by these enzymes and thereby maintain the integrity of the cell-membranes, protecting it from cellular damage. This present observation is in contrast to the results of Kuratko (1998), who reported that iron rich diet resulted in lower oxidative stress than in those fed less dietary iron.

Food fortification has been considered the best long-term strategy for the prevention of iron deficiency anaemia, but there are technical problems related to the choice of a suitable iron compound. The functional effects of iron deficiency anaemia result both from a reduction in the circulating Hb and in the iron – containing enzymes and myoglobin. The amount of bioavailable iron is dependent both on iron intake and absorption (Kuratko, 1998). Previously, Ebuehi and Akande (2009) reported that brain specific activity of SOD and GSH level of zinc adequately fed rats were significantly reduced as compared to rats fed zinc deficient or control diet.

An inverse relationship was established in plasma sodium and potassium levels in the anaemic rats fed with or without iron-fortified gari. For example, plasma and brain sodium levels were significantly higher in anaemic rats fed iron-fortified gari, while plasma and brain potassium levels were significantly lower in anaemic rats fed iron-fortified gari. Data of the study suggest that Ferrazone® NaFeEDTA fortification of gari may not impair the synthesis of oligodendrocytes which require sodium for their myelination. Defective myelination is thought to be one mechanism by which iron deprivation impair neurological function (Thompson *et al.,* 2001). Additionally, iron is a catalytic element in the synthesis of neurotransmitters, such as dopamine and serotonin. (Thompson *et al.*, 2001).

The histopathological findings of the brain and liver tissues of anaemic rats fed with or without non-fortified gari do not reveal any obvious cellular abnormality. For instance, the brain tissue shows pale staining amorphous fibrillary background containing neuronal cell bodies and glial cells. Areas of lamination are seen and the meninges are not congested. Perivascular clearing or halos are seen around blood vessels indicating mild to moderate cerebral edema. Edema could be as a result of high level of sodium (hypernatremia) in the brain (Qureshi and Suarez, 2000).

In conclusion, data of the present study indicate that fortification of gari using Ferrazone® NaFeEDTA fed to rats curtailed oxidation stress, but did not impair histopathological changes in brain and liver tissues and blood chemistry. Therefore, Ferrazone® NaFeEDTA could be used as a suitable iron fortificant for gari, a popularly consumed cassava fermented meal by Nigerians and African descent, to alleviate iron-deficiency anaemia.

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