International Journal of Engineering Science Invention
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org Volume 2 Issue 12 ǁ December. 2013 ǁ PP.08-13

Hepatotoxicity Studies of Linamarin in Low Protein Diet

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ABSTRACT: *Linamarin is an extremely toxic compound. Although, a lot of research work has been carried out on linamarin toxicity but little has been done on the evaluating linamarin toxicity with regards to protein diets. Therefore, this research work is aimed at evaluating linamarin toxicity in low protein diet in experimental animals. Fourteen healthy albino rats of average weight of 150g grouped into two: low protein diet (LPD) group fed with 8% low protein diet for one month followed by I.P. administration of 16.7mg/kg of linamarin obtained from cassava extract; and protein-free diet (PFD) group fed with protein-free diet for one month followed by I.P. administration of 16.7mg/kg of linamarin as control. The blood and liver tissue were collected 24 hours after I.P. linamarin administration and the serum was obtained. The liver function test results showed a significant increase in alkaline phosphatase activity (LPD 135.2±5.04; PFD 69.0±5.96), alanine transaminase activity (LPD 9.8±0.4; PFD 7.4±0.8), and aspartate transaminase activity (LPD 11.0±0.8; PFD 7.8±0.7). The serum level of bilirubin showed increase in indirect level (unconjugated) bilirubin level (LPD 0.73±0.02; PFD 0.49±0.03) and direct (conjugated) bilirubin level (LPD 0.21±0.02; PFD 0.16±0.01). There is reduction in the concentration of cyanide in linamarin (LPD 0.0020±0.0004; PFD 0.0034±0.0003) and in free cyanide (LPD 0.0024±0.0003; PFD 0.0031±0.0001) but an increase in concentration of thiocyanate (LPD 0.0088±0.0006; PFD 0.0066±0.0003) was observed. The histopathological examination of the liver showed mild vacuolar degeneration for LPD and very mild diffuse vacuolar degeneration for PFD. Increase in alkaline phosphatase, alanine transaminase and aspartate transaminase activities in the serum is an indication that linamarin is toxic to the liver in low protein diet. The histopathological lesions observed tend to support the toxicity of cyanide entities. Hence, consumption of cassava products should be done with great care especially in low income earners that consume low protein diet.*

KEYWORDS: *linamarin, thiocyanate, diet, protein, liver*

I. INTRODUCTION

Cyanogenesis is the ability of some plants to synthesize cyanogenic glycoside, which when enzymically hydrolyzed, release cyanohydric acid (HCN), known as prussic acid [1, 2, 3]. The occurrence of cyanogenic glucosides is wide spread in the plant kingdom and is present in many plants and foods; in the seeds of many stoned fruits (apricots, almonds and prunes to mention a few) as well as many seeds of grasses and legumes [4, 5]. Most glycosides remain inactive until they are hydrolyzed in the gastric tract by specialized bacteria which then releases an aglycone (phenols, terpenes, steroids and quinones) that has the active effect [3, 6]. These compounds could be phenol, sulphur or alcohol based and many of them like the cyanogenic glycosides are extremely toxic. Amygdalin (D-mandelonitrile-β-d-glucoside-6-β-D-glucoside) has been found in about 1000 species of plants, including cassava (tapioca, manioc), sweet potato, corn, cabbage, linseed, millet, and bamboo, in pits of stone fruits, such as cherries, peaches, and apricots, and in apple seeds [1, 7, 8, 9].

After ingestion, linamarin can be hydrolysed by either cassava linamarase or an endogenous β-glucosidase to yield D-glucose and ACH [10, 11, 12]. Liberation of hydrogen cyanide from cyanogenic glycosides occurs usually after ingestion and hydrolysis by the glycosidases of the intestinal microflora and, to a lesser degree, by glucosidases of the liver and other tissues [2, 13]. However, hydrolysis may also occur during the preparation of the food, which may account for the short interval between ingestion and the appearance of signs of poisoning in some accidents [7, 6, 14].

Glycosides are widespread in plants and are extremely varied in action, effect and medicinal application. Many edible plants contain cyanogenic glycosides, whose concentrations can vary widely as a result of genetic and environmental factors, location, season, and soil types [2, 13]. Cassava tubers vary widely in their cyanogenic glycoside content, although most varieties contain 15-400 mg cyanide/Kg fresh weight [9].

Occasionally varieties of cassava tubers contain 1300-2000 mg cyanide/Kg fresh weight, and cassava leaves contain 1000-2000 mg cyanogenic glucosides/Kg on a dry matter basis [7, 15]. Fermentation of cassava pulp for 96 hours during garri production reduced the hydrogen cyanide content by 50%; soaking of sliced cassava for 24 hr, 40%; and sun-drying, some 15% [7, 16].

Consumption of food containing cyanogenic glycosides has been linked to several different diseases affecting mainly the nervous system, such as tropical ataxic neuropathy in Nigeria, spastic paraparesis (called mantakassa in Mozambique and konzo in the Democratic Republic of Congo) in Cameroon, Central African Republic, Mozambique, Tanzania, and the Democratic Republic of Congo (formerly Zaire), as well as retrobulbar neuritis and optic atrophy associated pernicious anaemia [1, 5, 17, 18].
Therefore, this work compared linamarin hepatotoxicity in animals fed on low protein diet with that of protein free diet.

II. MATERIALS AND METHODS

Chemicals and Reagents

Sodium fluoride, potassium iodide, ammonium alum, ferric ammonium citrate, cupric sulfate, calcium carbonate, calcium citrate, calcium carbonate, magnesium carbonate, magnesium chloride, potassium chloride, potassium phosphate dibasic and sodium chloride were obtained from Sigma Chemicals, St. Louis, USA. Ethylacetate, methanol, diethylether, methanol and chloroform were obtained from British Drug House (BDH) Chemicals Ltd., London. All other chemicals used were of analytical grades.

Sample Collection and Preparation

Cassava root plants were harvested from Abadina farm, University of Ibadan, Nigeria and were used for this study. Diced cubes of parenchymal tissues (30-100g) homogenized at room temperature in 160ml of 0.1M orthophosphoric acid for 15 seconds at low speed is followed by 2 minutes operation at high speed in a warring blender. The resultant liquid was filtered using cheese cloth. The homogenizer vessel rinsed with 0.1M orthophosphoric acid which is filtered in the same way. The filtrate (linamarin) was then transferred to a screw capped bottle and stored at 4^0C after it has been measured.

Preparation of and Administration Feeds in Animals

Protein free diet and low protein diet were prepared according to AIN Standard [19]. Low protein diet feed composition: protein (fish meal) 8%, corn starch 78%, salt mixture 4%, vegetable oil 16% and protein free diet composition corn starch 70%, alphacel 15%, vegetable oil 10%, salt mixture 4%, cod liver oil 1%.
14 Albino Winstar rats were used for this experiment and were divided into two sets. One set was fed with protein free diet and the other set was fed with low protein diet (8% protein) for 35 days after which some are intubated and transferred into metabolic cage and other for cannulation.

I.P. Administration of 16.7mg/Kg Linamarin

Rats were anesthetized by injecting them with a 25% urethane aqueous solution (1.5g/Kg). A small mid-line abdominal incision was made, exposing the bile duct at junction with the duodenum and injected 16.7mg/Kg linamarin. The bile was then cannulated following the method of Boyland et al. [20].

Procedure for Sacrificing the Animals and Collection of Sample

Twenty four (24) hours after the I.P. administration of 16.7mg/Kg linamarin, the animals were anaesthesized using diethylether and the blood collected by cardiac puncture into serum bottles. The blood sample was allowed clot and then centrifuged at 5000rpm for 5 minutes and the red blood cell and serum were separated. The plasma was kept in a clean specimen bottles placed in ice bucket and refrigerated at -4^0C until they were used for assay. The rat is dissected starting from the central abdominal region upward to expose the internal region and organs. The liver in each rat were collected by dissection fixed in 10% formal saline.

Laboratory Analyses

Alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase activities were determined with commercial kits using Kodac Autoanalyser machine. Direct and indirect bilirubin concentration was determined automatedly as described by Simmons [21]. The presence of linamarin, free cyanide and thiocyanate were determined as described by Cooke [22] and Bowler [23] respectively. The liver tissue was histological examined when mounted on clean slide at 3µm and stained in haematoxylin and eosin.

Data Analysis

The data were processed and analyzed using the statistical package SPSS Version 17.0. The data were summarized with descriptive statistics: Mean (M)±Standard Error of Mean (±SEM) and Student's t-test was used to evaluate differences between data.

III. RESULTS

The result showed significant increase in the activities of alkaline phosphatase, alanine transaminase and aspartate transaminase in animals fed with low protein diet when compared with those fed with protein free diet as shown in Table 1.

Table 1: Alkaline phosphatase, alanine transaminase and aspartate transaminase activities in Rats fed with Low and Free Protein Following I.P. Administration of 16.7mg/Kg.

Diet	No of Animals	Alkaline Phosphatase	Alanine Transaminase	Aspartate Transaminase
Low Protein Diet	7	135.2±5.04	9.8±0.4	11.0±0.8
Protein Free Diet	7	69.0±5.96	7.4±0.8	7.8±0.7
T		8.481	2.683	3.010
DOF		12	12	12
P		0.000*****	0.020*****	0.011*****

Values are expressed as Mean ± SEM (Standard Error of Mean). Data were subjected to paired sample T-test at P < 0.05 of SPSS version 17 software. ***** Superscript indicates significant difference.

Bilirubin concentration also showed a significant increase in indirect (unconjugated) bilirubin but no difference in direct (conjugated) bilirubin in animals fed with low protein diet when compared with those fed with protein free diet as shown in Table 2.

Table 2: Serum direct and indirect bilirubin levels in Rats fed with Low and Free Protein Following I.P. Administration of 16.7mg/Kg.

Diet	No of Animals	Direct (conjugated bilirubin (mg/dl)	Indirect (unconjugated bilirubin (mg/dl)
Low Protein Diet	7	0.21±0.02	0.73±0.02
Protein Free Diet	7	0.16±0.01	0.49±0.03
T		2.236	6.656
DOF		12	12
P		0.045*	0.000*****

Values are expressed as Mean ± SEM (Standard Error of Mean). Data were subjected to paired sample T-test at P < 0.05 of SPSS version 17 software. ***** Superscript indicates significant difference.

The result also showed significant difference in the concentration of linamarin (glucosidic cyanide) and thiocyanate but no difference in free cyanade (non glucosidic cyanide) in animals fed with low protein diet when compared with those fed with protein free diet as shown in Table 3.

Table 3: Concentration of Cyanide Content and Thiocyanate in Serum in Rats fed with Low and Free Protein Following I.P. Administration of 16.7mg/Kg.

Diet	No of Animals	Linamarin (glucosidic cyanide (mg/HCN)	Free cyanide (non glucosidic cyanide) (mg/HCN)	Thiocayanate SCN (mg/ml)
Low Protein Diet	7	0.0020±0.0004	0.0024±0.0003	0.0088±0.0006
Protein Free Diet	7	0.0034±0.0003	0.0031±0.0001	0.0066±0.0003
T		-2.800	-2.214	3.280
DOF		12	12	12
P		0.016*****	0.047*	0.007*****

Values are expressed as Mean ± SEM (Standard Error of Mean). Data were subjected to paired sample T-test at P < 0.05 of SPSS version 17 software. ***** Superscript indicates significant difference.

The liver histopathological examination showed that animals fed with low protein diet undergo mild vacuolar degeneration whereas those of protein free diet undergo very mild diffuse vacuolar degeneration as shown in Table 4.

Table 4: Histopathology of Liver

Diets	**Result**
Low Protein Diet	Mild Vacuolar Degeneration
Free Protein Diet	Very mild diffuse vacuolar degeneration

IV. DISCUSSION

Cassava, the third most important food source in the tropics [24], produce two cyanogenic glycosides, linamarin and a small amount of lotaustralin or methyl linamarin [7]. These cyanogenic glycosides are hydrolysed in the presence of the enzyme linamarase to a cyanohydrin which breaks down further to hydrogen cyanide.

The amount of residual cyanide calculated on the average for cassava mash is 14.68 ± 0.66 mg/kg. There was a drop in residual cyanide from an average of 14.68 ± 0.66 to 9.87 ± 0.64 mg/kg for garri as a result of the action of linamarase during fermentation.

Ingestion of cyanide from high cyanide (bitter) cassava may occasionally cause death [24, 25], exacerbates goitre and cretinism and causes tropical ataxic neuropathy in older persons [26, 27]. According to Farombi [28], in Uganda, cassava were contaminated with aflatoxins (>100 ppb).

A great deal of interest has been shown in establishing the relationship between nutrient deficiencies and hepatic drug metabolism in vitro and in vivo. The effect of protein deficiency on drug metabolism has been reported by various workers [29, 30, 31]. It has been shown that nutritional deficiencies generally cause lowered rates of xenobiotic metabolism [32, 33].

The liver plays a major role in the disposition of majority of drugs. This is to the presence of several drug-metabolizing enzyme systems; including a group of membrane bound mixed function oxidative enzymes, mainly cytochrome P_{450} system [34]. The ability to respond to lexicological or pharmacological effects by an animal depends on the activity of the hepatic microsomal enzyme system which is related in considerable degree to dietary or nutritional status [35].

Sodium nitrate reduces blood cyanide levels by causing the formation of hemoglobin, to which cyanide binds with higher affinity than it does to the cytochrome oxidase enzyme [36]. Binding of cyanide to methemoglobin liberates cytochrome oxidase, which is necessary for aerobic cellular respiration. Sodium thiosulfate binds the cyanide ion to form thiocyanates, which are much less toxic cyanide and are excreted by the kidneys. In the U.S, hydroxocobalamin, a precursor of Vitamin B12 is now in use as an antidote for victims of cyanide poisoning [1]. The effect of low protein diet is shown in a very interesting fashion in the finding that carbon tetrachloride is less toxic to animals on a low protein diet; the enzyme responsible are at a low level and therefore little of the toxic agent is produced [30, 37].

In the tropics of Africa, there is a high incidence of protein energy malnutrition (PEM). Cassava which is a major food in this area of the world was analysed after sun-drying to contain 4.21% of crude protein [24, 27]. PEM is a spectrum of diseases arising from a deficiency of dietary protein especially in childhood. Kwashiorkor, one form of PEM, results from both the quantitative and qualitative deficiency of dietary protein with adequate caloric. This disease in experimental animals presents an ideal of nutritional status in the toxicity and carcinogenicity of environmental chemical [38, 39].

In this study, the liver function test results showed a significant increase in alkaline phosphatase activity, alanine transaminase activity, and aspartate transaminase activity. The serum level of bilirubin showed increase in indirect level (unconjugated) bilirubin but no difference in direct (conjugated) bilirubin level. Concentration of cyanide content and thiocyanate in serum also showed difference in linamarin and thiocyanate concentrations but no difference in free cyanide concentration.

Histopathological study revealed various degree of hepatocellular vacoulation indicating liver damage. The histopathological lesions observed tend to support the toxicity of cyanide entities.

The results indicate that in vitro the liver degrades linamarin. These results suggest that linamarin is toxic to the liver as shown in the results. Only in rats fed free protein diet that there is delay or prolonged metabolism of linamarin which is associated with tropical ataxic neuropathy, spastic paraparesis.

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