Asian Pacific Journal of Nursing

Journal homepage: www.mcmed.us/journal/apjn

A STUDY OF ANTIOXIDANT AND HEPATO-RENAL SYSTEMS IN FEMALE RATS DOSED WITH COUNTERFEIT NEUROBION

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ABSTRACT

Thiamine, pyridoxal and cyanocobalamin, constituents of neurobion are vital biomolecules, known for various biochemical roles that they play in the body. While they are mainly obtained through the diet, supplements have been identified as another good source. Since more than 25% of therapeutic drug or supplements available in Nigeria are counterfeit, the possibility of taking fake neurobion remains high. The effects of fake neurobion on hepatic and renal cells are therefore being investigated in rats. Eighteen female Wistar rats (230 g) constituted three experimental groups namely groups 1, 2, and 3, which received 30 mg/kg of fake neurobion, genuine neurobion® and distilled water (control) respectively. The route of administration was per oral. Hepatic and renal damage were assessed using indices such as activities of ALT, AST, GGT, ALP and levels of total protein, albumin, bilirubin, globulin, urea, creatinine and uric acid. Glutathione S transferase, glutathione reductase, superoxide dismutase, glutathione peroxidase, catalase, and MDA, as well as reduced and oxidized glutathione were assessed to determine the degree of oxidative stress. Histologic examinations of hepatic and renal tissues were carried out using hematoxylin-eosin staining technique. Data obtained were analyzed using ANOVA. At $p \leq 0.05$, differences were considered significant. Results showed that all the hepatic and renal markers were not significant different, the histology results are in complete agreement with the biochemistry results, with all examined tissues showing no visible lesions. Except for glutathione and glutathione peroxidase that were significantly different, all other markers of oxidative stress were not significantly changed. The data obtained from this study suggest inability of fake drug to induce appreciable oxidative stress, and this may be the basis of absence of liver and renal damage.

Key words: Hepato-nephrotoxicity; oxidative stress; fake drug; Wistar rat.

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Article Info

Received 15/03/2014; Revised 20/03/2014
Accepted 30/03/2014

INTRODUCTION

Vitamins such as thiamine mononitrate, pyridoxalHCl, and cyanocobalamin are organic compounds required as nutrients in small quantities by an organism to maintain health [1]. Such organic chemical compounds are called vitamins because they cannot be synthesized at required level by an organism, and must be obtained from the diet [2]. Even in mammals the term vitamin is relative, this depends not only on the circumstances but also on theparticular organism. For example, another vitamin that is not incorporated into neurobion i.e. the ascorbic acid is considered a vitamin for humans, although for other animal species it is not [3]. Those animal species are known to be capable of synthesizing vitamin C.

Vitamins are classified not according to their structures but by their biological and chemical activities. Although each of these organic acids is referred to as vitamin, in most cases they are a group of vitamer compounds which show the biological activity that are related to a particular vitamin [3]. For example, vitamin B_6 is known to occur in 3 different forms i.e. pyridoxal, pyridoxine and pyridoxamine; pyridoxal phosphate though is the active form. While many agents are known to considered toxic. Therefore, not many studies are found that addressed possible harmful effects of vitamin

formulations in animals especially when consumed within the range of recommended daily allowance but since a wide range of fake and adulterated products have been proven to contain many harmful chemicals, this fake vitamin formulation (neurobion) obtained from National Agency for Food and Drug Administration and Control (NAFDAC) was administered to female Wistar rats so as to determine its impact on hepatorenal and the antioxidant defense systems.

MATERIALS AND METHODS

Treatment

The study was executed in conformity with established national and international laws and Guidelines for Care and Use of Laboratory Animals in Biomedical Research Institutes of Health (revised 1985). The albino rats (230 g) used for the study were obtained from Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan (Nigeria). The rats were kept in cages at ambient temperature of 25±2°C and a 12 h light, 12 h dark cycle. Feed and water supply was without any form of restriction.

Of the eighteen rats used for the study, 12 were randomly divided into 2 groups of 6 rats each. For each of the rats, the route of administration was by gastric gavage. The rats in the first group were treated with 30 mg/kg BW of fake vitamin tablet (Neurobion®) while those in the second group received genuine tablet. The genuine drug was manufactured by Merck Marker (Pvt) Ltd (7, jail road Quetta), under license of Merck GaADamstadt Germany. The remaining 6 rats served as the control and were administered with distilled water. The fake vitamin tablet (Neurobion®) utilized for the study was supplied by NAFDAC, Western region office in Ibadan, whereas the original product was supplied by a reputable Pharmacy. The duration of the experiment was for a period of 21 days. Each of the rats was administered with its respective treatment regimen through the oral route.

Clinical Chemistry

Twenty-four hour after the last administration, collection of blood from the rats took place between 10.00 and 12.00; the method of collection was by retro-orbital bleeding. The serum activities and levels of the following indices of hepato-renal function were determined; alkaline phosphatase, γ-glutamyl transferase, alanine aminotransferase and aspartate aminotransferase (ALP & γ-GT, ALT, AST). Others were bilirubin and total protein and albumin. While serum activity of alkaline phosphatase (ALP) was by the method of Mc Comb and Bowers [4] those of AST & ALT were by the method of Bergmeyer et al. [5]. Serum levels of bilirubin and albumin were quantified using modified Jendrassik-Groff [6] & standard bromocresol methods respectively. Total protein was estimated through Biuret's method. Serum concentrations of creatinine and urea were determined by the Jaffé reaction and diacetylmonoxime oxidase method respectively. Hitachi® 902 automated machines (Roche Diagnostic, Germany) was used for these estimations.

Levels and activities of antioxidant indices

Serum activities of superoxide dismutase, glutathione peroxidase, catalase and levels of MDA were determined using the methods of Misra and Fridovich [7]; Rotruck et al. [8]; Sinha [9]; and Ohkawa et al. [10] respectively. On the other hand, reduced and oxidized glutathione were estimated using the methods of Prins and Loos [11] and Owen Joshua and Butterfield [12]respectively. While activity of glutathione S transferase was assessed by the method of Habig et al. [13], glutathione reductase activity was measured using the method of Zhou & Freed [14].

Statistical analysis

Data were subjected to statistical analysis using SPSS package version 15 to obtain mean ± SD (standard deviation). The degree of significant difference among three groups was determined by employing analysis of variance (ANOVA). $P \leq 0.05$ was considered significant.

RESULTS

Administration of female Wistar rats with fake or genuine neurobion resulted in significant differences only in the levels of total protein and globulin but none of the other hepato-renal indices like alkaline phosphatase, γ-glutamyl transferase, alanine aminotransferase and aspartate aminotransferase as well as bilirubin, albumin, creatinine, urea and uric acid were significantly changed as revealed in Table 1. In Table 2, while reduced glutathione was significantly different, oxidized glutathione and malondialdehyde were not significantly different. On the other hand, of all the antioxidant enzymes, only the activities of glutathione peroxidase were significantly different, those of superoxide dismutase, glutathione reductase, glutathione S transferase and catalase were not significantly altered as shown in Table 3. The photomicrographs of both Figures 1 and 2showed no visible lesion for all the tissues (liver and kidney) of not only the control and genuine drug treated rats but also of fake drug administered rats.

Table 1. Serum levels or activities of hepato-renal indices of Wistar rats dosed with genuine and fake neurobion

Parameter	Control	Fake drug	Genuine drug	F-value	P-value
Gamma-glutamyl transferase (IU/L)	50.54±7.19	50.33±3.57	53.44±3.58	0.807	0.462
Alkaline phosphatase (IU/L)	68.21±11.76	69.07±1.03	65.91±2.77	0.382	0.688
Total protein (g/L)	72.40±4.10	81.32±2.08	75.11±1.95	0.625	0.046*
Albumin (g/L)	38.03±3.04	41.17±1.26	40.40±3.22	2.665	0.097

Globulin (g/dL)	34.37±5.09	40.15±1.20	34.57±4.46	0.280	0.039*
Bilirubin (µmol\L)	17.12±2.77	19.38±1.31	20.41±3.63	2.640	0.099
Alanine aminotransferase (IU/L)	28.26±2.54	29.80±4.31	30.16±4.00	0.520	0.603
Aspartate aminotransferase (IU/L)	32.53±2.80	34.19±1.55	33.99±3.22	0.845	0.446
Uric acid (mmol\L)	146.46±11.14	143.74±8.42	149.05±10.73	0.954	0.404
Creatinine (µmol\L)	24.07±2.99	25.29±2.15	24.25±3.45	0.897	0.425
Urea acid (mg/dL)	28.61±2.17	29.33±4.30	27.32±3.02	1.397	0.273

Results are expressed as mean ± standard deviation. *P < 0.05 is considered significant. n=6.

Table 2. Serum concentrations of malondialdehyde, reduced glutathione, and oxidized glutathione of rats administered with fake and genuine neurobion.

Groups	GSH (mol/ml)	GSSG (mol/ml)	MDA (nmol/ml)
Controls	2.10±0.22	0.09±0.04	19.27±2.85
Fake drug	1.50±0.19	0.08±0.03	20.96±3.42
Genuine drug	2.09±0.21	0.09±0.03	20.41±2.41
F-value	30.99	0.310	0.617
P-value	0.018*	0.737	0.551

Results are expressed as mean ± standard deviation. *p <0.05 is considered significant. Abbreviations: GSH-reduced glutathione; GSSG-oxidized glutathione; GSH/GSSG- reduced/oxidized glutathione ratio; MDA- malondialdehyde. n=6.

Table 3. Serum activities of antioxidant enzymes of rats administered with fake and genuine neurobion.

Groups	Gln Red (U/mg protein)	CAT (µmol H_2O_2 consumed/(min·mg protein))	Gln-px (µmol GSH consumed/(min·mg protein)	GST (U/mg protein)	SOD (U/mg protein)
Controls	56.92±3.14	3.72±0.10	10.35±0.61	0.62±0.04	15.24±2.49
Fake drug	55.21±1.67	3.80±0.11	8.78±0.40	0.60±0.04	16.33±1.38
Genuine drug	52.35±5.00	3.69±0.19	9.98±0.48	0.59±0.03	17.22±1.37
F-value	2.765	1.265	10.413	0.825	2.068
P-value	0.090	0.102	0.045*	0.454	0.155

Results are expressed as mean ± standard deviation. Abbreviations: SOD-superoxide dismutase; CAT- catalase; Gln-Per-glutathione peroxidase; Gln Red- glutathione reductase; GST- glutathione S transferase. n=6.

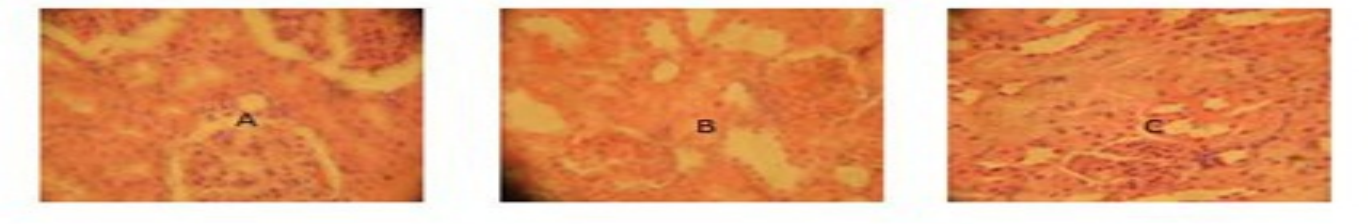

Figure 1: Photomicrographs of kidney of rats dosed with fake neurobion (A), genuine neurobion (B) and control (C) showing no visible lesion.

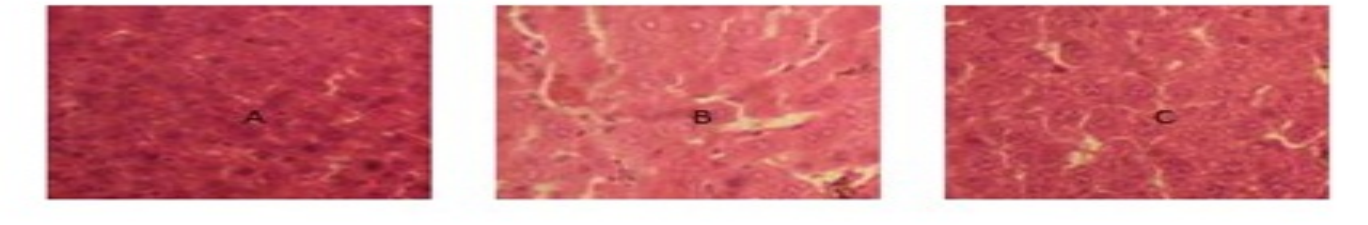

Figure 2: Photomicrographs of liver of rats dosed with fake neurobion (A), genuine neurobion (B) and control (C) showing no visible lesion.

DISCUSSION

Reactive oxygen species (ROS) are byproducts of normal metabolismandplay significant role in cell

signaling and homeostasis. These species are produced during the normal metabolism of oxygen via diverse enzymatic pathways. ROS can be generated from many different sources either exogenous or endogenous-derived molecules. The mitochondrion is one of the main sources of ROS within the cell, where the superoxide radical is produced as a byproduct of normal oxidative phosphorylation. Generation of reactive oxygen species can be from the activities of NADPH and lipoxygenase. Once released, reactive oxygen species can react with nitric oxide leading to the generation of reactive nitrogen species [15].

These species include oxygen radicals and reactive non-radicals. Chemically reactive molecules containing oxygen are termed ROS, many of which indeed are radicals i.e. due to the presence of unpaired electrons, although the non-radical hydrogen peroxide (H2O2) is also highly reactive in nature. There are a number of processes that control cellular levels of ROS, since their reactive properties is capable of causing damage to key cellular components including DNA, protein, and lipid. Oxidative stress occurs when the cellular antioxidant capacity is exceeded. Pleiotropic harmful effects of oxidative stress are observed in numerous disease states and are also implicated in a variety of drug-induced toxicities [15]. Since the liver is rich in enzymes that biotransform xenobiotics, in most cases the toxic presentation of drug administration is liver related.

Chronic liver diseases are already a major public health problem worldwide and their prognosis greatly depends on the extent of their progression which in some cases can even lead to fibrosis [16]. The liver, an important organ in the body, is essential for life because of its vast array of biochemical and metabolic functions. Some of the specific biochemical and metabolic functions include elimination from the body of substances that would otherwise be injurious if allowed to accumulate, and excretion of drug metabolites among others [17]. Severe alteration in mitochondrial function in the liver from drug exposure induces hepatic necrosis and cystolytic hepatitis that can progress into liver failure [18]. When the liver is damaged, it may not be able to perform its functions optimally causing the accumulation of toxins in the body faster than the liver can process. That such might not have taken place in rats administered with fake neurobion is evident from the result of serum bilirubin. Serum bilirubin levels in fake drug-administered rats were comparable and not significantly different from those of control rats and rats administered with genuine products.

Results of this study confirmed that fake neurobion used for this study, at a dose of 30 mg/kg body weight did not produce significant hepatotoxicity as evident by non-significant differences in serum level of albumin and activities of plasma ALT and AST following drug administration. This suggests that the synthetic ability of the liver was not compromised; albumin is a key component of serum proteins synthesized in the liver. Decrease in the plasma albumin level following drug administration is usually linked to the destruction of hepatic protein synthesizing sub-cellular structures. The integrity of membrane of liver parenchymal cells was also not altered, since there was no leakage of these hepatic enzymes into the extracellular fluid. AST is found in two isoforms in the body, one located in the cytoplasm and the other in the mitochondria. The presence of these enzymes outside the cell usually suggests damage to the hepatic cell. Not only these two enzymes (AST & ALT) but both alkaline phosphatase and γ-glutamyl transferase were also not affected through exposure to fake Neurobion as their activities were not significantly different in fake drug administered rats compared with control.

Although albumin was not significantly different, globulin and total protein were significantly increased in rats administered with fake drugs compared with the two other groups. The significant increase in globulin may be an indication that some other organs were affected, in which inflammatory processes were involved but in such a situation albumin level would have been significantly lower. Albumin is a negative acute-phase protein and the set-up of the experiment was not acute in nature. Neurobion was administered daily for duration of 21 days. The fact that albumin was not significantly lower probably rules out acute phase response with accompanied inflammatory reactions. On the other hand, these results suggest a probable modulation of genes that code for one or some of the proteins that constitute serum globulin fraction.

Certain chemicals have been reported to induce oxidative stress by forming chemical-derived radicals that can not only deplete the antioxidant defenses but also react directly with biomolecules. That other tissues apart from the liver or some other processes might have been compromised is evident by the results of the indices of oxidative stress, glutathione and glutathione peroxidase were significantly different compared with control. While free radical (FR) processes play an extremely important role in cell activity, the involvement of free radical is also a key mechanism in cellular pathology and apoptosis. FR processes are controlled by the antioxidant system (AOS). The enzymatic antioxidants have been suggested as first-line protection against the damaging effects of free radicals, nonenzymatic antioxidant also play some roles. Glutathione peroxidase detoxifies organic and inorganic peroxides by using reduced glutathione (GSH). Both glutathione and glutathione peroxidase were significantly lower in rats administered with fake drug. Another enzyme, glutathione reductase which is responsible for the regeneration of oxidized glutathione (GSSG) was not significantly different.

Lipid peroxidation has been reported as a significant perquisite to the loss of cell function under chemical-induced oxidative stress conditions [19]. According to Orrenius et al. [20] and Geeraerts et al. [21], peroxidation attack on microsomal membranes causes calcium release and uncontrolled activation of calcium-dependent proteases and lipases. The attack on mitochondrial membranes causes changes in permeability as well as induces a disruption of cellular energetics [22]. The histology results of both kidney and liver of no visible lesion seem to support the results of both blood biochemistry as well as other oxidative stress markers like MDA, GST, SOD and CAT.

The data obtained from this study suggest inability of fake drug to induce appreciable oxidative stress, as evidenced by the non-significant difference in MDA level (lipid peroxidation product) coupled with non-significant change in the endogenous antioxidants GST, SOD and CAT. It seems the decrease in glutathione level and GPx activity did not cause an increased accumulation of superoxide, H2O2 and hydroxyl radicals which could have stimulated lipid peroxidation. Reduction in the activity of glutathione peroxidase may be due to rapid consumption and exhaustion of storage of this enzyme in combating free radicals generated during the development of hepatotoxicity.

CONCLUSION

That reduced activity of glutathione peroxidase due to the direct toxic effect of fake drug administration probably might have led to significant oxidative stress affecting other components of antioxidants defense system (GST, CAT, SOD) if the exposure had persisted is a possibility. Therefore exposure of this fake drug to experimental animals in a chronic setting is being suggested to fully evaluate its toxic effects.

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