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Association between Pyridoxal-5'-Phosphate Levels, Liver Homogenates and Serum Activities of Aminotransferases and De Ritis Ratio amongst Alcoholic Hepatitis Patients

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Authors' contributions

This work was carried out in collaboration among all authors. Author FJN suggested the conception of study. Authors FJN and IIE designed of study. Sample analysis was done by authors FJN and UOA. Authors FJN, EWO and IIE performed data analysis. Statistical analysis completed by authors FJN, EWO, MNS and UOA. Initial manuscript draft submitted by authors FJN, EWO, IIE, UOA and MNS. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The current study assessed the association between Pyridoxal-5'-phosphate (PLP) levels, liver and serum activities of aminotransferases (ALT and AST) and De Ritis ratio (DRR) in ten subjects with alcoholic hepatitis (test group -TG) and 10 healthy subjects without alcoholic hepatitis

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(control group-CG) with mean age of 41 years of both genders who were attending the University of Calabar Teaching Hospital, Calabar, Nigeria.
Study Design: The current study was a randomized experimental designed work.
Place and Duration of Study: The study carried out in Chemical Pathology Unit, Department of Medical Lab Sciences, University of Calabar, Nigeria between December 2014 and December 2016.
Methodology: Appropriate samples were collected from both TG and CG after 8-10 hours fasting and treated using standard procedures. PLP was determined by techniques of Lumeng and co-workers, 1981 (reagents from New England Nuclear, Massachusetts, USA). Hepatic protein levels and aminotransferase activities were determined by spectrophotometric methods, (reagents from Sigma- Aldrich Company, USA). Liver biopsy specimens were obtained with a Klatskin needle for histology.
Results: Before PLP supplementation of liver homogenates a significant decreased in PLP levels, increased liver ALT and decreased AST activities were observed in test group. After one month of pyridoxine supplementation, there was a significant increase ($p<0.02$) in plasma PLP level in all groups with decreased serum AST and increased serum ALT activities ,while liver ALT and AST activities increased significantly ($p<0.005$) resulting to a decrease serum DRR ($p<0.001$) in test group.
Conclusion: PLP depletion may be partially responsible for the low serum DRR which is reputed to be a typical potent, non-invasive, economical and dynamic prognostic biomarker of alcoholic hepatitis.

Keywords: Association; Pyridoxal-5'-phosphate; activities of aminotransferases; De Ritis ratio; alcohol hepatitis.

1. INTRODUCTION

The International Union of Biochemistry and Molecular Biology (IUBMB) defines an enzyme as a biological catalyst that speeds upthe rate of biochemical reactions [1,2], while the transaminases (also call aminotransferases) can be defined as group II enzymes found in cells of the human body , helping in the catalysis of transamination as well as other reactions [3]. These reactions are usually characterized by rapid reversible inter-conversion of amino acids and oxo-acids that involve transfer of the amino group (—NH2) of an α-amino acid to a carbonyl compound, commonly an α-keto acid (an acid with the general formula-RCOCOOH) [4]. According to the International Commission on Enzymes, there are two main types of aminotransferases of diagnostic significance [5] namely:-L- Aspartate transaminase (AST) or Aspartate aminotransferase (ASAT) formerly known as serum glutamic oxaloacetic transaminase -SGOT (EC 2.6.1.1), and Alanine transaminase (ALT) or alanine aminotransferase ALAT, formerly serum glutamic pyruvic transaminase (SGPT) (EC 2.6.1.2) [6]. AST ispredominantly found in both cytosol and mitochondria in several organs such as the liver, cardiac muscles, skeletal muscles, kidney, brain, pancreas, lung, leukocytes, and erythrocytes with highest concentration in the hepatic parenchyma [7] while ALT is a cytosolic enzyme which is present predominantly in the liver and it is used as a clinical marker of hepatic damage and hepatotoxicity [8]. ALT has two isoforms; ALT1 and ALT2 which are encoded by separate genes [9]. There has been development of novel enzyme specific ALT1 and ALT2 antibodies and the expression of ALT1 is highest in liver, skeletal muscle and kidney, low in heart muscle but not detectable in the pancreas [10]. High ALT2 activity is detected in the heart, skeletal muscle and no with no activity in liver or kidney [11]. In all amino transfer reactions, 2-oxoglutarate and l-glutamate serve as one amino group acceptor and donor pair and Pyridoxal-5′-phosphate (P-5′-P) functions as coenzyme [12]. All amino acids are involved in transamination reactions except Lysine and Threonine [13]. These reactions are usually rapid and reversible, but the equilibra of ALT and AST favors the formation of L-alanine and L-aspartate. The α -keto glutamate and L-glutamate couples serve as the amino group acceptor in the forward reaction and donor in the reverse reaction in all amino groups transfer reactions [14,15]. During transamination of ALT and AST, the serum pyridoxal –5 –phosphate (p-5-p) serving as the co-enzyme (forms a true prosthetic group) [16] binds to the apo-enzyme and accepts the amino group from the first substrate L-alanine or L-aspartate forming an enzyme-bound pyndoxamine-5-phosphate. This results to the first reaction product pyruvate or oxaloacetate

[17,18]. The co-enzyme, in its amino form, then transfers the amino group to the second substrate (α-keto glutamate) to form the second product (L-glutamate) respectively [19]. At the end the p-5-p is regenerated to its initial form via Schiff base [20]. Generally, only a small amount of the co-enzyme is required to saturate the apo-enzyme [21] and thep-5-p is usually present in normal serum, but where it is deficient, it can be supplied [22]. Pyridoxal-5'-phosphate (PLP) is the biologically active form of vitamin B6. It is necessary for the activity of both aminotransferases [23,24]. Pyridoxal-5'-phosphate deficiency is common in alcoholics with or without liver disease [25]. The mechanism involved in PLP deficiency is yet to be understood, but it cannot be explained by depressed liver aminotransferase activities of patients with alcoholic liver disease alone, because if this was so then one would expect that liver ALT is more sensitive to PLP depletion than AST and that levels of both aminotransferases would increase during PLP repletion, with the change in ALT being greater than the change in AST [26]. In a case exclusion of all factors that may cause depletion of alanine aminotransferases to undetectable levels should be is taken into consideration [27].Although much has done in this aspect, there is still no consensus why different responses of liver and serum ALT and AST activities has been documented [28]. However, serum activities of aminotransferases are only mildly increased in alcoholic patients with the onset of liver disease, despite the factthatthere may be histopathological evidence of acute hepatocellular injury [29]. In addition, the De Ritis ratio, that is aspartate aminotransferase (AST) to alanine aminotransferase (ALT) is usually greater than 2 which is one of clinical criteria and diagnostic indicators highly suggestive of alcohol-related hepatic pathology. It has been reported by [30] that low serum aminotransferase activities and high AST to ALT ratio of patients with alcoholic liver disease reflect similar changes in the liver activities of these enzymes when PLP levels are manipulated [30]. A study carried out by Ning and collaborators in 1966 found no change in liver ALT or AST activities when homogenates of human liver were incubated with PLP [31]. However, in 1976, Horder and collaborators reported an increase in liver and serum ALT activities when patients with chronic alcoholic liver disease were given oral pyridoxine supplementation [32]. A similar observation was made by Ludwig and co-researchers (1980) who reported a marked increase in AST activity when serum from patients with acute non-cholestatic liver disease was pre-incubated with pyridoxal phosphate [33]. Furthermore working with pyridoxine-deficient rats [34] observed an increase in liver and serum AST but no change in ALT levels when the animals were given pyridoxine. These discrepancies are difficult to rectify but these may reflect differences in baseline vitamin B6 status, severity of underlying liver disease and state of protein-calorie nutrition. Each of these conditions has been shown to influence pyridoxine metabolism, ALT, AST activities and De Ritis ratio [35,36].

1.1 Purpose and Objectives of the Current Study

The current study assessed the association between plasma PLP levels, liver and serum aminotransferases (AST and AST) activities and De Ritis ratio amongst patients with alcoholic hepatitis as compared to healthy subjects.

1.2 Justification of the Current Study

Findings from this study will help broaden our scope and understanding of the relationship betweenplasma pyridoxal phosphates (PLP) levels, activities of aminotransferases: L-Aspartate transaminase (AST) and Alanine transaminase (ALT) in liver tissues, serum samples and De Ritis Ratio in healthy state and liver pathology such as alcoholic hepatitis.

2. METHODS

2.1 Patient Population

Ten patients of both genders of mean age 41 years were diagnosed with acute alcoholic hepatitis using findings of ultrasound scan, liver biopsy, invasive and non –invasive tests, scoring tests, screening and confirmatory laboratory tests, and with clinical assessment of the stage and degree of the severity of the disease. Patients were excluded from further consideration if they had any signs or symptoms of significant acute gastrointestinal hemorrhage (Hemoccult positive stool or nasogastric aspirate and hematocrit (PCV) < 30%), acute pancreatitis, insulin-dependent diabetes mellitus; or any other significant infections or encephalopathy severe enough to impede oral alimentation. During the one month hospitalization, each patient received 2 Litres of a 5% glucose solution that had been

medicated with 4 mg pyridoxine/L, electrolytes, magnesium, and commercially available multivitamins intravenously administered each day. Patients were prescribed a dietary balance and standard intake profile and nutrition chart was kept for recording purpose. Patients were counseled and encouraged to eat the standard hospital diet which was available at any time they needed to eat (ad libitum feeding) and a daily account of their caloric intake and distribution of calories among the three major food groups' was recorded in the nutrition chart provided. Using appropriate sample containers, urine and stool for nitrogen balance were collected daily. Histologically normal biopsy specimens were collected from control group and sent for histology. Determination of Plasma PLP level was carried out by spectrophotometry. They were all confirmed free alcoholic hepatitis using the inclusive and exclusive criteria of this study.

2.2 Procedure

With the help ofa Klatskin needle which is a modification of the Menghini needle, biopsy specimens were obtained from the liver and about one-third of the collected specimen were separated and reserved for enzyme studies. The rest of the specimens were completely immersed in a plastic container filled with 10% formaldehyde serving as the fixative and sent immediately to the Histopathology Department for histological examination. The dimension of the weight of the samples for enzymes determination were 9-18 mg and were stored at -20°C in a solution of 1.0 ml of 0.25 M sucrose. AST and ALT activities were determined in these liver homogenates by the spectrophotometric method adopted by Bergmeyer and Bernt, 1965 [37] at 30°C, with the addition of PLP and without the prior addition of PLP to the reaction mixture in a final concentration of 0.1 mM. The activity of the aminotransferases were expressed as units per gram of liver protein. Hepatic protein concentration was determined using a spectrophotometric method adopted by Lowry, 1951 [38]. Thismethod uses a commercially prepared bovine albumin as a standard. About 10ml of blood samples for the determination of PLP levels collected from both groups after 8-10hrs of fasting into labeled sample bottles containing ethylenediaminetetra-acetate, (EDTA) and the separated plasma was stored at -70°C. Plasma PLP was determined by the PLP-dependent decarboxylation of L-[1- ^{14}C] tyrosine by the apo-enzyme of tyrosine decarboxylase, as described and adopted by Lumeng, 1981 [39]. L-[1-^{14}C] Tyrosine (55 µmCi/µmol) was purchased from New England Nuclear, Boston, Massachusetts. The apoenzyme of tyrosine decarboxylase, isolated from *Streptococcus faecalis*, was purchased from Sigma Chemical Company, Saint Louis, MO, United States of America and was further purified by precipitation between 60% and 85% saturation with ammonium sulfate, followed by Sephadex G-200 column chromatography, as described by [40]. The apoenzyme eluted in a single peak and fractions, which in the presence of saturating concentrations of PLP, resulted in the production of >1 mmol of $^{14}CO_2$ per milliliter per minute, were pooled for use. Each PLP determination was run in duplicate, with PLP standards ranging from 0.5 to 4.0 ng/ml. Serum aminotransferases were measured in the Chemical Pathology Laboratory Department of University of Calabar Teaching Hospital, Calabar. Nutrition status was assessed by determining nitrogen balance at the beginning and at the end of the hospitalization [41]. The severity of acute hepatocellular injury was determined by coding, grading and staging of the sections of the liver biopsy specimens, which were stained with hematoxylin and eosin, were viewed according the standard norms [42].

2.3 Statistical Analysis

Statistical analysis was computed using the Statistical Package for Social Students (SPSS) Statistics software version 20 from International Incorporation, Chicago, United States of America. The results were analyzed with the aid of cross tabulations to explore proportional associations between variables and Chi Squared X^2 test were used to explore proportional association between groups. The level of statistical significance was set at $p \leq 0.05$ (95% confidence interval). Comparisons among various age groups were done using Student's t-test and one way analysis of variance (ANOVA) and Coefficient of correlation (r). Alpha value of 0.05 was used. Data were expressed as plus or minus two standard deviation. The coefficient of linear regression was also calculated to establish the significance of correlations (16). Data are expressed as mean ± SEM.

3. RESULTS

Table 1 and Table 2 show the plasma Pyridoxal 5'-Phosphate levels, liver and serum aminotransferase activities and De Ritis ratio in patients with alcoholic hepatitis (test group) and healthy subjects (control group) respectively. Prior to the decision of admission and

hospilisation of these subjects in test group, a lower level of plasma PLP which was statistically significant in test group was observed when compared to the control group ($p<0.05$). Similarly, the liver De Ritis Ratio of the test group was significantly lower than the control group ($p<0.05$). Unexpectedly, there was a common serum De Ritis ratio of >5: 1for both the test group and control groups despite the fact that only the test group had elevated AST to ALT ratio. There was no correlation (r=0) between the level of plasma PLP and serum or liver aminotransferase activities with the severity of acute hepatocellular injury as graded and staged histopathologically. Neither was there any correlation with follow-up biopsy specimens. There was no correlation (r=0) between nitrogen balance, plasma PLP levels, liver or serum aminotransferase activities, and the severity of acute liver injury at the beginning or at the end of the study. Throughout the one month period of hospitalization, the test group received four-fold the required recommended therapeutic daily dosage of pyridoxine (8 mg/day) administered intravenously, plus 1-1.5mg pyridoxine/ 100 g protein in their diets. An increased of nitrogenbalance from 0.26 ± 1.9 to 3.85 ± 1.73 g/day ($p<0.05$) was observed and plasma PLP levels also increased significantly during the study period ($p <0.02$).

Table 3 Shows the effect of Pyridoxal-5'-Phosphate added in-vitro to the initial liver aminotransferases of patients with alcoholic hepatitis (test group). It was observed that plasma PLP levels increased in all patients except one patient and only 5 of the 9 patients from the test group had a final PLP level that was within the mean range ±SEM for plasma PLP values in the control group. The increase in plasma PLP was associated with a significant increase in activities of liver ALT activities ($p<0.005$). There was no significant change in the level of liver AST activities during the study. The De Ritis ratio in both the liver and the serum decreased significantly ($p<0.05$) and this decrease was predominantly due to the increase in ALT activities ($p<0.005$) that occurred, because the change in AST was negligible and statistically insignificant. This observation was in sharp contrast to the decrease in the serum De Ritis Ratio which was due to both an increase in ALT and a decrease in AST. The mean serum ALT increased from 53.1 ± 6.8 to 70.4 ± 15.6 IU/L whereas serum AST fell from 148.3 ± 19.1 IU/L initially to 95.6 ± 26.4 IU/L by the end of the month. When a portion of each patient's admission liver biopsy specimen was homogenized and incubated with PLP, the mean activity of ALT increased to about 38.2% ($p<0.001$), whereas there was no significant change in the mean AST activity.

4. DISCUSSION

Prior to admission and hospilisation, there was an initial depletion in the plasma levels of PLP inthetest group than the control group .This decrease could be attributed to the pathological injuries induced by the alcoholic hepatitis. The biochemical basis for the altered PLP metabolism in chronic alcohol abuse in alcoholics are due to cellular content of PLP which is determined only by the activities of PLP-synthesizing enzymes, activity of a phosphate-sensitive, membrane-associated, neutral phosphatase, which hydrolyzes phosphorylated B6 compounds. Acetaldehyde, but not ethanol, impaired the net formation of PLP from pyridoxal, pyridoxine, and pyridoxine phosphate by erythrocytes which is mediated by the phosphatase, resulting in an acceleration of the degradation of the phosphorylated B6 compounds in erythrocytes. This is expected and is in line with the overwhelming reports of previous findings by others researchers such as [43-46]. It has been established that in alcoholic liver disease there is a deficiency of dietary intake of vitamin B6 which is a potent coenzyme of PLP and low plasma PLP levels in this population are thought to result from combined effects of suboptimal dietary pyridoxal intake, impaired synthesis of PLP in the presence of ethanol metabolites or severe liver diseases and increased clearance of PLP [5 a, 18a, 47- 49]. It was observed that all except one patient in the test group demonstrated an elevated level of plasma PLP throughout the one month period of pyridoxine administration. This means that PLP level in the test group had a 120-fold increase during this period and with only 5 out of the 10 alcoholics achieved final PLP levels that were within the range of the control group. It has been observed that consistent and persistent subnormal plasma PLP levels in this setting of "dietary" pyridoxine excess suggest that decreased PLP synthesis, or increased PLP degradation, or both, are important contributing factors [50]. An alternative explanatory hypothesis for this observation could have been due to the fact that normalization of plasma PLP levels lags behind restoration of tissue PLP levels. This must be considered, despite recent evidence to the contrary (22a). Increases in

Table 1. Plasma Pyridoxal 5'-Phosphate Levels, liver and serum aminotransferase activities and De Ritis Ratio in male and female patients with alcoholic hepatitis (test group)[a]

		Hospitalization of patients with alcohol hepatitis									
		Male mean ±SEM (n=7)					Female mean ±SEM (n=3)				
		Plasma	Serum	Liver Homgenates			Plasma	Serum	Liver homogenates		
No of days	Age yrs	PLP[c] (ng/ml)	De Ritis Ratio (AST/ALT)	ALT (IU/g protein)[b]	AST (IU/g protein)[b]	De Ritis Ratio (AST/ALT)	PLP (ng/ml)	DeRitis Ratio AST/ALT	ALT (IU/g protein)[b]	AST (IU/g protein)[b]	De Ritis Ratio AST/ALT
1st	41	5.9 ± 2.2[d]	2.9±1.1	524 ±17[e]	99 ± 14[d]	0.18±0.8 [h]	5.3 ± 2.2[f]	2.3 ±1.1[i]	601±54[g]	207 ±25	0.3±11
30th		35 ± 8.0	0.4±2	442±98	221±25	0.5±.26	35 ± 6.0	0.5±1.1	451±60	299±24	0.6±0.4

PLP = pyridoxal 5' -phosphate; AST = aspartate aminotransferase; ALT = alanine aminotransferase. All values are expressed as mean ± SEM, SEM=standard error of the mean, n= total number of subjects a =Alcoholic hepatitis (non -viral hepatitis), b=International units per gram of homogenized liver protein determined in the absence of added PLP in vitro. c= Plasma PLP was determined in 10 patients with alcoholic hepatitis, whereas liver aminotransferases were determined in both serum and liver homogenates. d= significantly different from male and female mean valuesat p < 0.001. e=significantly different from control at p < 0.005. f= significantly different from day 1 value at p < 0.02. g =significantly different from day 1 value at p < 0.005. h =significantly different from day 1 value at p < 0.05. i= significantly different from day 1 value at p < 0.001. Normal range of PLP = 5 -50mcg/L, Normal range of AST = 5-40 IU/L, Normal range of ALT =7-56 IU/L

Table 2. Plasma Pyridoxal 5'-Phosphate levels, liver and serum aminotransferase activities and De Ritis Ratio in healthy subjects[a] (control group)

		Subjects without alcohol hepatitis									
		Male mean ±SEM (n=7)					Female mean ± SEM (n=3)				
		plasma	Serum	Liver Homogenates			plasma	Serum	Liver Homogenates		
No of Days	Mean Age	PLP (ng/ml)	DeRitis Ratio AST/ALT	ALT (IU/g protein)b	AST (IU/g protein)[b]	De Ritis Ratio AST/ALT	PLP (ng/ml)	De Ritis Ratio AST/ALT	Liver ALT (IU/g protein)b	Liver AST (IU/g protein)[b]	De Ritis Ratio AST/ALT
1st	40	24.6±2.3[c]	1.1±4.1	850 ± 85[d]	290 ± 28[e]	0.3±0.3	23.9 ±50[h]	1.1±4	790± 99[g]	179 ±50[i]	0.3±0.5
30th		36.1±9.8	1.0±3	770±90	320±44	0.4±0.4	35.0±9.7	1.0±5	754±91	279±22	0.3±0.2

PLP = pyridoxal 5'-phosphate; AST = aspartate aminotransferase; ALT = alanine aminotransferase. "All values are expressed as mean ± SEM, n= total number of subjects b=International units per gram of homogenized liver protein. Determined in the absence of added PLP in vitro c= Plasma PLP was determined in 10 healthy subjects (control group) with serum alanine aminotransferases (ALT) and aspartate aminotransferases (AST), d= significantly different between male and female control at p < 0.001 e=significantly different between ALT and AST at p < 0.005., f= significantly different from day 1 value at p < 0.02 g =significantly different from day 1 value at p < 0.005, h =significantly different from day 1 value at p < 0.05 i= significantly different from day 1; Normal range of PLP = 5 -50mcg/L, Normal range of AST = 5-40 IU/L, Normal range of ALT =7-56 IU/L

Table 3. Effect of Pyridoxal 5'-Phosphate added In-vitro to the Initial liver aminotransferases of patients with alcoholic hepatitis[a]

Added PLP	AST (IU/g protein)[b]	ALT (IU/g protein)[b]	De Ritis ratio	*p*-value
Absent	524 ± 17	99 ± 14	5.2±1.21	$P<0.05$[c]
Present	569 ± 33	137 ± 17	4.15±1.21	
% Change	8.6	38.2	0.23	$P<0.05$[c]

LP = pyridoxal 5'-phosphate; AST = aspartate aminotransferase; ALT = alanine aminotransferase
a = All values are expressed as Mean ± SEM. b = International units per gram of homogenized liver protein
c= significantly different from initial value at p <0.05

plasma PLP during the period of PLP supplementation were associated with normalization of liver ALT activities but with no significant change in the initially low liver AST activities . A similar, smaller, increase in liver ALT activities could be produced by incubating homogenates of the PLP-deficient patients' initial liver biopsy specimens with PLP, whereas no change in liver AST activities was observed. Follow-up liver biopsy specimens from the PLP-replete patients had significantly higher activities of ALT than did the initial biopsy specimens, and incubating these follow-up liver biopsy specimens with PLP provided no further increase in liver ALT activities. Similarly, incubation of liver biopsy specimens from controls with PLP produced no change in liver ALT activities. These findings contradict that of previous studies (30a) which documented no increase in the liver ALT of patients with alcoholic liver disease during incubation of liver tissue with PLP in vitro because the patients were not significantly deficient in PLP at the time the determinations were done. Current data suggest that PLP depletion is responsible for at least some of the decreased activity of liver ALT. Factors other than PLP availability are also important determinants of ALT activity.

5. CONCLUSION

Current study shown that PLP depletion may be partially responsible for the low serum (De Ritis ratio) which is reputed to be a typical screening indicator, a noninvasive, cost–effective and dynamic prognostic biomarker of alcoholic hepatitis .

AVAILABILITY OF DATA AND MATERIALS

Data sets generated and analyzed in this study are available from the corresponding author on request.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

After obtaining written consent, questionnaires were administered to patients hospitalized for a duration of one month in the medical wards of the University of Calabar Teaching Hospital Calabar.

ETHICAL APPROVAL

Ethical approval was obtained from University of Calabar Teaching Hospital Ethical Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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