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Urate Synthesis and Oxidative Stress in Phenytoin Hepatotoxicity: The Role of Antioxidant Vitamins

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Abstract: Phenytoin is known to induce microsomal enzymes including xanthine oxidase which catalyzes uric acid synthesis with superoxides as byproducts, thus contributing to the oxidative stress of phenytoin hepatotoxicity. To investigate the role of antioxidant vitamins in ameliorating phenytoin induced hepatic changes through possible actions on xanthine oxidase activities as measured by urate concentration. Growing albino rats of Wistar strain were randomly divided into 8 groups of 7 rats each. Group 2, 3, 4, 5, 6, 7 and 8 were treated with phenytoin alone, phenytoin + folic acid, phenytoin + vitamin E, phenytoin + vitamin E + vitamin C, phenytoin + vitamin C, phenytoin + folic acid + vitamin E and phenytoin + vitamin E + vitamin C + folic acid respectively while animals in group 1 were given normal saline to serve as control. Serum concentrations of uric acid, albumin, total protein and the activities of aspartate and alanine aminotransferases (AST and ALT) and catalase were measured spectrophotometrically using appropriate commercial reagent kits. Result showed that administration of phenytoin alone caused significant ($p < 0.05$) increase in serum levels of globulin, uric acid, AST and ALT activities while the levels of albumin and catalase were reduced significantly ($p < 0.05$). Supplementation of phenytoin treatment with vitamins resulted in various degrees of protection. However, the elevated level of uric acid in serum was not significantly ($p < 0.05$) affected by any of the vitamins used and there was no significant correlation between the activities of aminotransferases and uric acid concentration in the vitamin treated animals as was observed between aminotransferases and catalase. The findings in this study suggest that antioxidant vitamins were able to ameliorate phenytoin hepatotoxic effects by improving oxidant radicals removal in the animals but would not inhibit further generation of the superoxides by xanthine oxidase activity and that xanthine oxidase may contribute significantly to the oxidative stress of phenytoin therapy.

Key words: Uric acid, hyperuricemia, oxidative stress, xanthine oxidase, antioxidants, phenytoin-hepatotoxicity

INTRODUCTION

Phenytoin is a hydantoin anticonvulsant drugs, used widely for the treatment of generalized or partial seizures associated with epilepsy. The complications of phenytoin therapy have become a subject of interest owing to its cellular toxicity (Gupta, 1999; Ekaidem *et al.*, 2007). Hepatotoxicity of phenytoin has been said to be accompanied with rashes, fever, lymphadenopathy and eosinophilia suggesting that the mechanism of phenytoin toxicity is probably related to hypersensitivity (Howard *et al.*, 1991; Vittorio and Muglia, 1995; Havill, 1998). Phenytoin hypersensitivity syndrome is not fully understood but it has been said to be unrelated to

drug dosage or serum concentration (Gupta, 1999). However, the cytotoxic and immunologic activities of the drug may be associated with its intermediate metabolites called arene oxides (Gennis *et al.*, 1991). These electrophilic reactive arene oxides derived by the action of cytochrome P450 enzyme on the drug may acrylate cellular macromolecular such as membrane lipids, proteins and DNA, thereby interfering with cellular functions. Oxidative stress results, when the amounts of phenytoin reactive arene oxides overwhelm the antioxidant capacity of the exposed individuals. This may be particularly common when phenytoin is administered in a much higher dose than the therapeutic level. Catalase, one of the frontline antioxidant enzymes and reduced glutathione may be

significantly depleted in this condition. Catalase is involved in the decomposition of superoxides and hydrogen peroxides generated in the body during cellular metabolism of nutrients for energy and drugs/xenobiotics for activation or elimination.

Phenytoin is also known to be potent microsomal enzymes inducer. The microsomal enzymes are involved in the metabolism for degradation and/or bioactivation of a number of drugs (McNamara, 2001). The mechanism of phenytoin hepatotoxicity, therefore, may also be associated with its ability to induce several microsomal enzymes including NADPH and xanthine oxidases. Oxidative stress may originate from excessive stimulation of NADPH or xanthine oxidase activities. Xanthine oxidase is recognized as the rate limiting enzyme in the pathway of purine metabolism. The enzyme is widely distributed in body, occurring in milk, small intestine, kidney and liver. It catalyzes the degradation of hypoxanthine to xanthine and then to uric acid, producing superoxide radicals as by products which are converted to hydrogen peroxide (Hille and Nishino, 1995). Xanthine oxidase can also catalyze the hydroxylation of a wide range of N-heterocyclic and aldehyde substrates and can produce nitric oxide under hypoxic conditions from organic and inorganic nitrates and nitrites (Zhang *et al.*, 1998; Doel *et al.*, 2000). Therefore, the enzyme is also involved in the generation of peroxy nitrite and nitric oxide.

Small elevation of serum uric acid, or mild hyperuricemia can be derived either from diet (such as proteins) rich in purine or from the impaired renal excretion of uric acid. As pointed out by Hayden and Tyagi (2004) that an increase of urate reabsorption can be induced from a decreased renal blood flow due to a decreased glomerular filtration rate or taking diuretic medication and results in a slight increase of serum uric acid. Because xanthine oxidase enzyme is not involved in this type of elevation of serum uric acid, no superoxide is produced. On the other hand, any local tissue ischemia (hypoxia) derived from any microvascular disease may also lead to an increased uric acid synthesis due to an increased RNA-DNA breakdown, resulting in an increased purine concentration and an increased substrate (xanthine) concentration for enzyme xanthine oxidase (Hayden and Tyagi, 2004). Under this condition, superoxide is also produced by xanthine oxidase enzyme. Hyperuricemia can also be derived from increased cell apoptosis and necrosis due to inflammation. Consequently increased xanthine oxidase activity is derived from increased concentration of breakdown products of RNA and DNA associated with cell apoptosis and necrosis. Because elevated level of

serum uric acid can be effectively reduced by allopurinol, an inhibitor of xanthine oxidase activity (Wu and Wu, 2008), therefore, the activity of xanthine oxidase enzyme is conceivably playing a critical role in the induction of hyperuricemia. It is important to note that it is the superoxide, not uric acid which is most likely the causal factor initiating the inflammatory pathway leading eventually to the development of oxidative and nitrosative stress and all the subsequent development of inflammatory diseases (Hayden and Tyagi, 2004).

High level of xanthine oxidoreductase have been found in the liver and phenytoin induced generation of super oxides, hydrogen peroxide and peroxy nitrites via., enhanced activities of this oxidoreductase may be one of the underlying mechanism of phenytoin hepatotoxic damage. The study therefore, investigates the influence antioxidant vitamins on uric acid synthesis, oxidative stress and markers of hepatotoxicity, with a view to highlighting the mechanism of phenytoin hepatotoxicity in experimental animal models.

MATERIALS AND METHODS

Growing albino rats of the Wistar strain (135-150 g) were obtained from the Animal House, Department of Biochemistry in the Faculty of Basic Medical Sciences, University of Uyo, Uyo-Akwa Ibom State. The animals were kept in the experimental section of the animal house, in a well ventilated standard laboratory condition of temperature and relative humidity. The animals were fed with normal rat formular (Pfizer Livestock Co. Ltd., Aba, Nigeria). Both the experimental and control animals had free access to both rat chow and water during the experimental period. The animals were randomly divided into 8 groups of 7 rats each. Group 1 animals served as the control and were gavaged normal saline (1.0 mL). Groups 2, 3, 4, 5, 6, 7 and 8 received phenytoin, phenytoin + folic acid, phenytoin + vitamin E, phenytoin + vitamin E + vitamin C, phenytoin + vitamin C, phenytoin + folic acid +vitamin E and phenytoin + vitamin E + vitamin C + folic acid, respectively.

Administration of phenytoin and antioxidant vitamins:

Commercially available phenytoin capsules were obtained from Parke-Davis Hoofreg, Vitamin C and folic acid tablets were obtained from Emzor Pharmaceuticals (Nig) Ltd, Lagos, Nigeria while Vitamin E (soft gel) was of Vibost Healthcare Limited, India. The choice of commercial products was to reflect the condition under which these medications are administered. The drugs were administered daily by oral intubation as follows,

Phenytoin: 5 mg kg⁻¹ body weight of rat, folic acid: 70 µg kg⁻¹ body weight of rat, vitamin C: 1.4 mg kg⁻¹ body weight of rat and Vitamin E: 10 IU kg⁻¹ body weight of rat. The treatments lasted for 4 weeks.

Collection and preparation of samples: The animals were anaesthetized with chloroform and sacrificed following guidelines of our Institutional Ethics Committee on animal studies. Blood samples were collected into plain sample tubes for sera preparation. The blood samples were allowed to clot at room temperature for 2 h. Sera were separated by centrifugation at 3000 g for 5 min using bench top centrifuge (MSE Minor, England). Sera were separated into plain sample tubes and stored in the refrigerator for analysis. All analysis on serum samples were completed within 24 h of sample collection.

Biochemical analysis: Serum proteins, uric acid and hepatic enzymes of clinical significance were measured in serum samples spectrophotometrically, using Randox kits (Randox Laboratory Ltd., Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY). Serum catalase activity was determined by the spectrophotometric assay using Bioxytech catalase kit (OxisResearch, Foster City, U. S. A.). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to the method of Reitman and Frankel (1957). Serum total protein was analyzed using biuret kit method (Tietz *et al.*, 1995). The absorbances of all the tests were determined using spectrophotometer (HAICH, DR 3000, Germany).

Statistical analysis: Statistical analysis was carried out by employing student's t-test and ANOVA *post hoc*, a probability of p<0.05 was considered significant.

RESULTS

Uric acid and serum catalase levels of rats on phenytoin therapy were estimated to investigate the effects of antioxidant vitamins on phenytoin related hepatotoxicity. Table 1 and 2 shows the influence of phenytoin and antioxidant vitamins supplement on some biochemical indicators of liver injury. The administration of phenytoin alone caused significant (about 100%) increases in AST (p = 0.00) and ALT (p = 0.000) when compared to control animals. Significant increase in serum globulin and decrease in serum total protein and albumin were also observed in the phenytoin treated rats. Serum concentration of uric acid was highly elevated (p = 0.000) while serum catalase activity was significantly reduced following phenytoin treatment. Supplementation of phenytoin treatment with antioxidant vitamins resulted in various degrees of protective changes against phenytoin induced toxicity. The serum activities of AST and ALT were significantly reduced by supplementation with folic acid, vitamin E and/or vitamin C when compared with groups that received no supplement. The phenytoin associated increase in globulin and decrease in albumin level and serum catalase enzyme activity, were reversed by antioxidant vitamin supplementations. However, the elevated level of uric acid in serum was not affected by any of the vitamins. Analysis of data (Fig. 1, 2 and 3) showed significant positive correlation between serum aminotransferases and serum globulin (r = 0.481, p = 0.000) and significant negative correlation between the aminotransferases and albumin (r = -0.773, p = 0.000) and catalase activities (r = -0.663, p = 0.000). No correlation between the transaminases and uric acid was observed in the treated groups of animals.

Table 1: Effects of antioxidant vitamins on phenytoin induced changes in serum proteins

Groups	Total protein	Albumin (g dL ⁻¹)	Globulin	Albumin/globulin ratio
Control	7.27±0.24	4.04±0.14	3.23±0.11	1.25±0.22
Phenytoin only	6.84±0.54	3.00±0.16*	3.84±0.56*	0.80±0.15*
Phenytoin + Folic acid	6.80±0.38	4.11±0.09	2.69±0.41	1.57±0.28
Phenytoin + Vitamin E	7.01±0.39	4.00±0.26	3.01±0.36	1.35±0.19
Phenytoin + Vit E+ Vit C	7.04±0.26	4.01±0.11	3.03±0.26	1.33±0.12
Phenytoin + Vitamin C	7.29±0.41	4.13±0.18	3.14±0.29	1.42±0.11
Phenytoin + Folic Acid + Vit C	7.17±0.30	4.30±0.16	2.87±0.39	1.53±0.29
Phenytoin + Folic Acid + Vit E + Vit C	7.30±0.25	4.02±0.20	3.27±0.26	1.24±0.14

Values are expressed as Mean±SD. *Significantly different values of test group compared to control at p<0.05

Table 2: Effects of antioxidant vitamins on phenytoin induced changes in serum aminotransferases, catalase and uric acid

Groups	AST (U L ⁻¹)	ALT (U L ⁻¹)	Catalase (µmol mg ⁻¹ protein)	Uric acid (mg dL ⁻¹)
Control	27.29±2.43	11.57±2.00	1.63±0.11	3.37±0.11
Phenytoin only	40.14±4.41**	24.57±3.90**	0.97±0.10**	5.49±0.37*
Phenytoin + Folic acid	19.71±5.44	9.71±2.14	2.06±0.37	5.54±0.28*
Phenytoin + Vitamin E	21.71±4.46	11.71±2.75	1.90±0.36	5.79±1.05*
Phenytoin + Vit E + Vit C	18.43±2.51	13.29±4.07	1.94±0.30	4.79±0.49*
Phenytoin + Vitamin C	15.14±1.77	10.86±1.57	1.84±0.27	5.01±0.46*
Phenytoin + Folic Acid + Vit C	17.71±2.14	12.29±3.00	1.89±0.28	5.34±0.68*
Phenytoin + Folic Acid + Vit E + Vit C	19.43±1.62	14.14±1.77	1.91±0.23	6.19±0.59**

Values are expressed as Mean±SD. ***Significantly different values of test group compared to control at p<0.05 and p<0.01, respectively

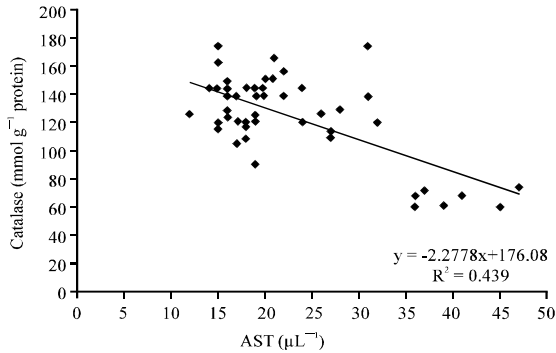


Fig. 1: Correlation graph of catalase versus AST

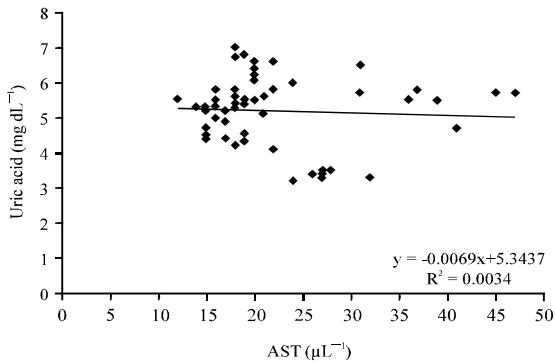


Fig. 2: Correlation graph of uric acid versus AST

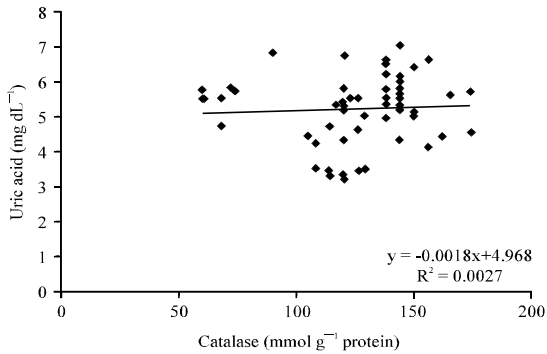


Fig. 3: Correlation graph of uric acid versus catalase

DISCUSSION

Immunological responses to the drug and excessive generation of reactive intermediates are thought to be the probable mechanisms of phenytoin toxicity. Although, clinical evidence of immunologic hypersensitivity to phenytoin have been demonstrated by the presence of rashes, fever, lymphadenopathy and eosinophilia (Howard *et al.*, 1991; Havill, 1998), molecular interactions of phenytoin or its reactive metabolic products with

tissues that could result in generation of inflammatory modulators and markers of tissue destruction such as uric acid, have never been demonstrated. Uric acid is a major product of purine catabolism, a reaction catalyzed by xanthine oxidase. Increased serum concentrations of uric acid have been associated with conditions of increased nucleic acid turnover as in cancers, infections, trauma, chemotherapy and inflammatory diseases (Wu and Wu, 2007, 2008; Conen *et al.*, 2004). In this study we demonstrated that xanthine oxidase, a microsomal enzyme known to generate Reactive Oxygen Species (ROS) and uric acid (Berry and Hare, 2004), might play a role in phenytoin induced liver injury. Recently evidence have shown that ROS and/or their products may modulate cell signaling pathways of inflammatory response such as transcription factor activation (NF-KB and NrF2) and apoptosis (Haddad *et al.*, 2000; Jacobi *et al.*, 2005) and that failure of inflammatory cell to undergo apoptosis has been implicated in persistence of the inflammatory response. Chronic exposure to oxidative stress could therefore be associated with resistance of inflammatory cells to apoptosis (D'Angio and Finkelstein, 2000; Ruggiero *et al.*, 2007).

Enzymes and protein makers of liver injury were measured in the experimental animals and controls. AST, ALT, uric acid and globulin were significantly raised while serum albumin and catalase activity were significantly reduced by the administration of phenytoin. These findings suggest hepatocellular damage by the drug. Aspartate and alanine aminotransferases are known to be elevated in serum during liver damage and/or during increased cellular proliferation. Increased synthesis of immunoglobulin and acute phase reactants following tissue injury may contribute to elevated level of globulin fraction of the serum proteins and impaired synthesis of albumin by the hepatocytes during phenytoin treatment may be responsible for decrease albumin level in the serum. Serum catalase activity in the rats receiving phenytoin becomes depressed probably because of over consumption of catalase by the excessive amount of reactive oxygen and arene oxides species. Decreased catalase and reduced glutathione levels had been reported following phenytoin toxicity (Navarova *et al.*, 2005). Increase in serum uric acid concentrations of rat receiving phenytoin therapy also support the likelihood of phenytoin induced tissue injury. Phenytoin is a known microsomal enzymes inducer and xanthine oxidase is one of them. This enzymes catalyzes the degradation of hypoxanthine to xanthine, then to uric acid and in the process produces superoxide radicals which are converted to hydrogen peroxide (Hille and Nishino, 1995).

These reactions also contribute to the oxidation state of individuals receiving phenytoin therapy in addition to the effects of phenytoin arene oxides.

Supplementation of phenytoin treatment with vitamins C and E and folic acid resulted in various degrees of protection against phenytoin-induced liver damage as evident by reduction in the levels of the liver enzymes, namely AST and ALT. However, the serum levels of uric acid in all phenytoin exposed animals were significantly higher than those of unexposed animals, thus suggesting that the protection offered by these antioxidant vitamin supplementations might not have been mediated by inhibition of induction of xanthine oxidase by phenytoin but may be related to vitamins' ability to quench the effects of the reactive oxygen species and phenytoin arene oxide intermediate on cellular macromolecules. This was further supported by the finding that catalase activities in animal receiving the vitamin supplementations were restored to values above that of control animals. Significant negative correlation was observed between the transaminases and serum catalase activities but was not however observed between transaminases activities and uric acid concentrations. This suggests that, although liver injury following phenytoin treatment increased proportionally to depressed catalase activities (oxidative stress) in the controls and vitamin treated animals, uric acid concentration was not affected by changes in transaminases activities following antioxidants supplement. Induction of xanthine oxidase enzyme by phenytoin which resulted in increased uric acid synthesis did not appear to be affected by antioxidants treatment, however, the reactive oxygen species generated by the enzyme reactions were quenched by the antioxidants thus preventing excessive oxidative cellular damage.

In vivo and *in vitro* studies indicate that phenytoin mediated liver injuries may involve, at least in part peroxidase catalysed bioactivation of phenytoin to a reactive free radical intermediate which, if not detoxified, may initiated oxidative stress leading to oxidation of liver cell lipid, protein and DNA molecules (Winn and Wells, 1997). Highly reactive oxygen species, such as hydroxyl radicals ($\cdot\text{OH}$) could be generated by the phenytoin radicals via Fenton reactions. *In vivo* catalytic iron may be found loosely bound to membrane lipids, DNA and phosphate complexes to enhance fenton reaction (Smith *et al.*, 2004). While OH^{\cdot} is generally thought to be a primary intermediate of *in vivo* damage, reactive oxygen species are ultimately responsible for cellular damage if these are not detoxified by cytoprotective enzymes or antioxidants. Lipid peroxidation and protein oxidation and degradation by these radicals generally lead to structural and functional changes (Kehrer and Lund, 1994). This study has shown that supplementation of phenytoin treatment with

antioxidant vitamins could ameliorate phenytoin related hepatic damage but however do not affect phenytoin induced uric acid synthesis by xanthine oxidase. The mechanism of phenytoin associated liver damage may therefore involve excessive generation of reactive species and that these oxidant species may be contributed significantly by the reactions of xanthine oxidase.

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