Lipid peroxidation in liver of vitamin B-6 deficient rats

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Vitamin B-6 deficient diet fed rat showed increased lipid peroxidation reactions in liver when compared to that of control. Liver homogenate of vitamin B-6 deficient rats showed enhanced susceptibility to lipid peroxidation upon incubation with NADPH or ascorbate or t-butyl hydroperoxide. Vitamin B-6 deficient liver showed increased levels of lipids, oxalate, calcium, iron, and copper, and decreased levels of antioxidants, ascorbic acid, α-tocopherol, reduced glutathione, total thiol groups, and antioxidant enzymes, glutathione peroxidase, catalase, and glucose 6-phosphate dehydrogenase. Further, vitamin B-6 deficient liver showed enhanced level of hydroxyl radicals and hydroperoxides when compared to that of control liver. The decreased antioxidant protection against free radicals may have led to increased levels of hydroxyl radicals and hydroperoxides which in turn may have led to increased levels of diene conjugates, thiobarbutric acid reactive substances, and lipofuscin-like pigments in liver homogenate. These observations may be an indirect secondary effect of the general disruption of metabolism associated with vitamin B-6 deficiency.

Keywords: vitamin B-6 deficiency; liver; lipid peroxidation; antioxidants

Introduction

Vitamin B-6 in the form of pyridoxal 5'-phosphate is involved in a number of metabolic reactions. Pyridoxine deficiency has been shown to lead to fatty liver,[1] hypercholesterolemia,[2] accumulation of total lipids, mainly of triglycerides and cholesterol ester in liver,[3] decrease in taurocholate conjugate in bile,[4] increased liver 3-hydroxy 3-methylglutaryl CoA reductase activity,[5] reduction of glycogen, glucose, and alanine in liver,[6] and low insulin-like activity in the serum and pancreas.[6,7] Decrease in activity of pyridoxal phosphate dependent enzymes,[8] serine dehydrase,[9] aspartate aminotransferase,[10] and tyrosine aminotransferase[11] and increased biosynthesis of oxalic acid by glycolic acid oxidase[12] have been reported in vitamin B-6 deficient animals.

Although there are many reports on the relation between pyridoxine and lipid metabolism, the actual role of pyridoxine in lipid metabolism is not yet clearly understood. Lipid peroxidation reaction, a type of oxidative degeneration of polyunsaturated fatty acids, has been linked with altered membrane structure and enzyme inactivation.[13]

There are several reports on lipid peroxidation in liver of vitamin A, vitamin B-2, and vitamin E deficiencies,[14-16] but there is no such report in vitamin B-6 deficiency. This study presents increased lipid peroxidation in liver of vitamin B-6 deficient rats.

Materials and methods

Male albino Wistar rats weighing about 130–140 g were divided into two groups. The experimental group was fed with vitamin B-6 deficient diet[17] (diet containing 25% vitamin-free casein,* 23% sucrose, 28.7% starch, 15% ground nut oil, 2.2% vitamin mixture,† and 6.1% salt mixture‡). The control group was pair-

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*Vitamin-free casein obtained from ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH, USA.

†Pyridoxine deficient vitamin mixture (in mg/kg diet): ascorbic acid 990; inositol 110; choline chloride 1,650; riboflavin 22.0; thiamine chloride 0.4; niacin 99.2; calcium pentothenate 66.2; biotin 0.44; p-aminobenzoate 110; folic acid 1.98; cyanocobalamine 0.03; menadione 49.60; α-tocopherol acetate 110; retinol 99.2, and ergocalciferol 0.5.

‡Salt mixture (in g/kg diet): $CaCO_3$ 18.0; K_2HPO_4 19.5; $CaHPO_4$ 3.6; NaCl 10.08; $FeSO_4 \cdot 7H_2O$ 1.5; $MgSO_4 \cdot 7H_2O$ 7.5; KI 0.015; $ZnCO_3$ 0.22; $CuSO_4 \cdot 5H_2O$ 0.018; $MnSO_4 \cdot H_2O$ 0.138; and $Na_2SeO_3 \cdot 5H_2O$ 0.01.

Table 1 Body weight and activities of AST, ALT, GAO, and LDH in liver of control and vitamin B-6 deficient rats

Particulars	Control	Vitamin B-6 deficient
Body wt, initial (g)	132.90 ± 15.50	135.50 ± 16.80
Body wt, at sacrifice (g)	160.20 ± 19.70	134.80 ± 15.40[a]
Liver wt/100 g body weight (g)	3.33 ± 1.02	3.24 ± 0.95
Liver protein (mg/g liver)	188.60 ± 15.50	185.00 ± 16.00
Plasma pyridoxal 5'-phosphate (PLP) (nM/l)	38.53 ± 2.97	20.83 ± 2.42[c]
Blood pyridoxal 5'-phosphate (nM/l)	254.30 ± 20.80	172.80 ± 14.55[c]
Aspartate amino transferase (μM of oxaloacetate produced/hr/mg protein)	0.14 ± 0.01	0.12 ± 0.01
Alanine amino transferase (μM of pyruvate produced/30 min/mg protein)	0.22 ± 0.02	0.16 ± 0.02[c]
Glycolate oxidase (units) (1 unit = nM of glyoxalate utilized/min at 37° C)	2.64 ± 0.68	3.90 ± 0.70[b]
Lactate dehydrogenase (units) (1 unit = enzyme required to produce one μM of pyruvate per min at 37° C)	0.47 ± 0.04	0.51 ± 0.08

Values are means ± SD from 6 rats.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

fed with the diet supplemented with pyridoxine hydrochloride (10 mg/kg diet).

The diet was prepared according to the original procedure of Thomas and Kriskey[17] with slight modification. They supplemented the diet with 5% vegetable oil and 10% hydrogenated oil with 30 mg of pyridoxine/kg diet and feed for the growing rats. For our study, the diet was supplemented with 15% peanut oil (15% saturated and 85% unsaturated) and 10 mg of pyridoxine hyrochloride/kg diet. Though AIN recommends 6–7 mg vitamin B-6/kg diet for growing rats, there is a lot of variation in literature in the supplementation of vitamin B-6 in the diet 5 mg,[18] 12 mg,[19] and 46 mg/kg diet.[20]

At the end of 12 weeks, blood was withdrawn by heart puncture using heparin as anticoagulant, rats were sacrificed, and liver was removed and rinsed in ice-cold water. A known amount of tissue was homogenized in 0.1 M Tris-HCl buffer, pH 7.4 to get a 10% homogenate. Lipid peroxidation was determined by the method of Devasagayam.[21] The NADPH-induced system in a total volume of 2 mL contained 0.2 mL of tissue homogenate, 50 μM $FeCl_3$, 5 mM ADP, 1 mM KH_2PO_4, and 0.4 mM NADPH in 0.15 M Tris-HCl buffer, pH 7.4. For ascorbate-induced system lipid peroxidation, the assay system (2.0 mL) contained 0.2 mL of tissue homogenate, 50 μM $FeSO_4$, 1 mM KH_2PO_4, 0.4 mM ascorbic acid in 0.15 M Tris-HCl buffer, pH 7.4. PLP or vitamin B-6 induced lipid peroxidation was determined as above in the absence of ascorbate and ferrous sulphate. For t-butyl hydroperoxide (tBH)-induced system in a total volume of 2.0 mL contained 0.5 mM t-BH in 0.15 M Tris-HCl buffer, pH 7.4. The release of thiobarbutric acid reactive substances (TBARS) was determined after incubation at 37° C for 30 min.[22] Lipofuscin-like pigments[23] and diene conjugates[24] were measured in the samples from the incubation mixture. Plasma was separated and pyridoxal phosphate was determined.[25]

Total lipids, cholesterol,[26] phospholipids,[27] free fatty acids,[28] protein,[29] oxalate,[30] lactate dehydrogenase (LDH),[31] superoxide dismutase (SOD),[32] and catalase[33] were measured. Ascorbic acid,[34] α-tocopherol,[35] reduced glutathione,[36] total thiol groups,[37] hydroxyl radicals,[38] hydroperoxides,[39] glutathione peroxidase,[40] glucose 6-phosphate dehydrogenase,[41] xanthine oxidase,[42] glycollate oxidase[43] aspartate aminotransferase, and alanine aminotransferase[44] were estimated in liver homogenate of control and vitamin B-6 deficient rats.

Five hundred mg of frozen homogenized sample was wet-ashed with trace element grade nitric acid-perchloric acids as described by Mustafa and Medeiros.[45] The residue remaining was transferred to a known volume with deionized-distilled water (for Ca, 1% lanthanum chloride was used for the dilution) and analyzed for calcium, iron, and copper concentrations with a Perkin-Elmer Flame Atomic Absorption Spectrophotometer.

Results

Vitamin B-6 deficient rats did not gain body weight (*Table 1*). A significant reduction in PLP was observed in plasma and blood of vitamin B-6 deficient rats when compared to that of control ($P < 0.001$). A significant decrease ($P < 0.01$) in ALT activity without change in AST activity was observed in vitamin B-6 deficient liver. The oxalate synthesizing enzyme, GAO, was increased drastically ($P < 0.01$), while LDH was unaffected in vitamin B-6 deficient rat liver, compared to that of control rat liver.

Table 2 presents the lipid peroxidation and susceptibility to lipid peroxidation in liver homogenate of control and vitamin B-6 deficient rats. It is interesting to note that basal thiobarbutric acid reactive substances (TBARS) and diene conjugates were increased in vitamin B-6 deficient liver. Upon stimulation of lipid peroxidation by NADPH or ascorbate or t-BH, vitamin B-6 deficient rat liver showed enhanced formation of TBARS, lipofuscin-like pigments, and diene conjugates, when compared to that of control. The degree

Table 2 Lipid peroxidation in liver homogenate of control and vitamin B-6 deficient rats

Particulars	Thiobarbutric acid reactive substances (nM TBARS/mg protein)		Lipofuscin-like pigments (relative fluorescence units/mg protein)		Conjugated dienes (absorbance in OD at 233 nM/mg protein)	
	Control	Vitamin B-6 deficient	Control	Vitamin B-6 deficient	Control	Vitamin B-6 deficient
Basal level (0')	1.48 ± 0.34	1.93 ± 0.17[a]	0.24 ± 0.03	0.23 ± 0.03	0.64 ± 0.06	0.69 ± 0.04[a]
Lipid peroxidation without cofactors	2.56 ± 0.48	3.85 ± 0.74[b]	0.25 ± 0.02	0.28 ± 0.03[b]	1.15 ± 0.15	1.36 ± 0.18[a]
with PLP	2.50 ± 0.50	3.87 ± 0.75[b]	0.24 ± 0.02	0.26 ± 0.04	1.08 ± 0.13	1.25 ± 0.15
with vitamin B-6	2.72 ± 0.55	3.95 ± 0.70[b]	0.25 ± 0.03	0.26 ± 0.03	1.20 ± 0.20	1.39 ± 0.22
with NADPH-induced system	6.75 ± 1.02	10.83 ± 1.28[c]	0.40 ± 0.04	0.60 ± 0.05[c]	1.40 ± 0.28	1.98 ± 0.30[b]
with ascorbate-induced system	25.70 ± 1.85	42.80 ± 2.45[c]	0.75 ± 0.08	0.96 ± 0.07[c]	1.95 ± 0.22	2.70 ± 0.20[c]
with t-BH-induced system	3.85 ± 0.98	6.73 ± 1.25[c]	0.30 ± 0.03	0.39 ± 0.05[b]	1.78 ± 0.38	2.90 ± 0.36[c]

Values are means ± SD from 6 rats.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

Table 3 Levels of lipids in liver of control and vitamin B-6 deficient rats

Particulars	Control	Vitamin B-6 deficient
Total lipids	52.07 ± 5.30	67.16 ± 6.10[c]
Cholesterol	6.02 ± 1.30	8.95 ± 1.60[b]
Phospholipids	37.57 ± 3.50	48.30 ± 4.00[c]
Free fatty acids	3.25 ± 1.05	4.98 ± 1.20[a]

Values are means ± SD from 6 rats and are expressed as mg/g wet tissue.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

Table 4 Levels of oxalate, calcium, copper, and iron in liver of control and vitamin B-6 deficient rats

Particulars	Control	Vitamin B-6 deficient
Oxalate (mg/g wet tissue)	1.05 ± 0.20	1.97 ± 0.32[c]
Calcium (μg/g wet tissue)	27.28 ± 2.50	39.15 ± 3.08[c]
Copper (μg/g wet tissue)	6.30 ± 1.05	7.60 ± 1.27[a]
Iron (μg/g wet tissue)	221.10 ± 10.50	247.70 ± 13.80[b]

Values are means ± SD from 6 rats.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

of stimulation was in the order: ascorbate > NADPH > t-BH. However, addition of PLP or vitamin B-6 had no effect on the lipid peroxidation reaction suggesting that the increased susceptibility to lipid peroxidation in vitamin B-6 deficiency was not associated with the lower concentration of vitamin B-6 or PLP. This clearly showed that vitamin B-6 deficiency had no specific direct effect on lipid peroxidation.

Since lipids are the substrates for the lipid peroxidation reactions, the effect of vitamin B-6 deficiency on the levels of various lipids was determined and is presented in *Table 3*. Total lipid concentration was 52 mg/g in normal liver, and this level was significantly increased to 67.2 mg/g in vitamin B-6 deficiency. Similarly, the concentrations of cholesterol, phospholipids, and free fatty acids were significantly increased ($P < 0.01$, $P < 0.001$, and $P < 0.05$, respectively) in vitamin B-6 deficient liver compared to control. This suggested that the enhanced lipids in vitamin B-6 deficiency leads to more formation of lipid peroxidation products.

Table 4 presents the concentrations of promoters of lipid peroxidation such as iron, copper, oxalate, and calcium in control and vitamin B-6 deficient rat liver. The concentrations of both oxalate and calcium were significantly increased ($P < 0.001$) while iron and copper increased moderately ($P < 0.05$) in vitamin B-6 deficient liver.

Increased susceptibility to lipid peroxidation is the result of decreased protection by antioxidants. Hence, the levels of antioxidants, antioxidant enzymes, and free radical producing systems were studied, and the values are given in *Table 5*. Vitamin B-6 deficient liver showed significant decrease in the levels of ascorbic acid ($P < 0.05$), α-tocopherol ($P < 0.05$), reduced glutathione ($P < 0.01$), and total thiol groups ($P < 0.05$), when compared to control. Decrease in the activities of glutathione peroxidase, catalase, and glucose 6-phosphate dehydrogenase ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) were observed in vitamin B-6 deficient liver. However, there was no significant difference in superoxide dismutase activity. The free radical producing enzyme, xanthine oxidase, was increased in liver of vitamin B-6 deficient rat. It is interesting to note that the concentrations of hydroxyl radicals and hydroperoxides were elevated in vitamin B-6 deficiency.

Discussion

Body weight loss is associated with vitamin B-6 deficiency. Since vitamin B-6 functions as co-enzyme in

Table 5 Values of antioxidants, antioxidant enzymes, and free radical producing systems in liver of control and vitamin B-6 deficient rats

Particulars	Control	Vitamin B-6 deficient
Antioxidants		
Ascorbic acid[1]	2.01 ± 0.64	1.15 ± 0.59[a]
α-tocopherol[1]	1.48 ± 0.32	0.95 ± 0.30[a]
Reduced glutathione[1]	6.79 ± 0.98	4.98 ± 0.86[b]
Total thiol groups[1]	15.86 ± 2.17	13.05 ± 2.06[a]
Antioxidant enzymes		
Superoxide dismutase[2]	5.95 ± 0.43	6.02 ± 0.30
Glutathione peroxidase[3]	9.94 ± 1.05	8.00 ± 1.25[a]
Catalase[4]	203.70 ± 11.87	178.00 ± 10.05[b]
Glucose 6-phosphate dehydrogenase[5]	15.05 ± 1.97	10.52 ± 1.67[c]
Free radical producing system		
Xanthine oxidase[5]	2.80 ± 0.27	3.21 ± 0.28[a]
Hydroxyl radicals[6]	2.85 ± 0.32	3.40 ± 0.40[a]
Hydroperoxides[7]	1.42 ± 0.22	1.96 ± 0.28[b]

Values are means ± SD from 5 rats and are expressed as [1]μg/mg protein; [2]units/mg protein (1 unit = the amount of enzyme that inhibits the autoxidation reaction by 50%); [3]μg of reduced glutathione utilized/min/mg protein; [4]μM of H_2O_2 consumed /min/mg protein; [5]units/mg protein (1 unit = the amount of enzyme that brings about a change in OD of 0.01/min); [6]nM of formaldehyde formed/min/mg protein; and [7]μg of t-BH/mg protein.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

transamination reactions, the decrease in the activity of ALT is associated with decreased availability of vitamin B-6. This is reflected by the reduction in the concentration of PLP in plasma of vitamin B-6 deficient rats. This leads to increasing the glyoxylate pool and thereby increasing the GAO activity, resulting in hyperoxaluria which is a characteristic feature of vitamin B-6 deficiency.[2] Weight loss, decrease in ALT activity, and increase in GAO activity have been reported in vitamin B-6 deficiency.[46]

The enhanced lipid peroxidation and susceptibility to lipid peroxidation by enzymatic and non-enzymatic systems in vitamin B-6 deficient liver may be due to either (a) increased substrate and promoters of lipid peroxidation, or (b) decreased antioxidant protection. Lipids are one of the substrates for lipid peroxidation reactions. The observed increased levels of lipid peroxidation products in vitamin B-6 deficiency may be due to the elevated levels of lipids. Similar results of enhanced lipid peroxidation have been reported in hyperlipidemia.[47] As PLP or vitmain B-6 has no direct effect on susceptibility of lipid peroxidation reaction, the increased lipid peroxide formation in vitamin B-6 deficient liver is thought to be associated indirectly with vitamin B-6 deficiency. The accumulation of lipids in vitamin B-6 deficient liver reported in this study may not be associated with the high fat content of the diet (15%). When our data of control liver are compared with the data of Okada and Iwami,[5] who fed the rats with the diet containing 20% casein and 8% oil for 6 weeks, no differences are observed in liver wt/100 g body wt and percent of total lipid content. Therefore, our observation supports the earlier reports that vitamin B-6 deficiency causes accumulation of lipids.[48-50]

Several promoters such as iron and copper have been shown to promote lipid peroxidation.[51] In addition, oxalate has been shown to stimulate lipid peroxidation in rats as a result of either feeding sodium glycolate or administration of sodium oxalate.[52,53] Further, our earlier studies have shown accumulation of oxalate, iron, calcium, and copper in kidney subcellular fractions in vitamin B-6 deficiency.[54] Thus, the elevated peroxidation reaction observed in vitamin B-6 deficiency may be associated with the increased levels of promoters.

α-Tocopherol, ascorbic acid, and reduced glutathione levels are found to be significantly low in vitamin B-6 deficiency, an interesting observation. These three antioxidants are interrelated with each other for recycling processes. Recycling of tocopheroxyl radicals to tocopherol is achieved by reaction with ascorbic acid.[55] The dehydroascorbic acid formed in the above reaction is reduced to ascorbic acid by a non-enzymatic reaction with reduced glutathione.[56] McCay et al.[57] have shown the presence of a labile glutathione-dependent factor, which cycles the tocopheroxyl radical back to tocopherol. However, the role of ascorbic acid in restoring tocopherol levels in animal tissues is not clear. Wefers and Sies[58] have shown that ascorbic acid produces a lag in lipid peroxidation in liver microsomes which are dependent on the normal levels of tocopherol in the membrane. By contrast, Sterrenberg et al.[59] have found no evidence that the tocopherol content of liver microsomes affects the antioxidant effects of ascorbic acid on iron mediated lipid peroxidation. If recycling of tocopheroxyl radicals to tocopherol is a major mechanism for maintenance of tissue tocopherol levels, deficiency of ascorbic acid is expected to result in depletion of tissue tocopherol. Since we have observed a significant decrease in ascorbic acid level, recycling of tocopheroxyl radicals to tocopherol may have been hindered, resulting in elevated lipid peroxidation reactions. To our knowledge, this is the first report to show a simultaneous decrease in the level of both tocopherol and ascorbic acid in vitamin B-6 deficiency. Reduced glutathione maintains cell membrane sulfhydryl groups and other structural proteins in the stable form. NADPH is required for GSH generation (via glutathione reductase) supplied by glucose 6-phosphate dehydrogenase.[60] The decreased activity of G6PD observed in vitamin B-6 deficiency may decrease the generation of NADPH and thereby decrease the reduction of oxidized glutathione to reduced glutathione.

Accumulation of H_2O_2 is highly toxic to cells. Catalase and glutathione peroxidase are involved in the elimination of H_2O_2. Several workers have pointed out that glutathione peroxidase is more important than catalase in removing H_2O_2[60,61] and in addition helps in removing lipid peroxides in mammalian cells.[62] The decreased activity of glutathione peroxidase in vitamin

B-6 deficiency may be correlated to decreased availability of its substrate, reduced glutathione. Catalase requires NADPH for its regeneration from inactive form[63] and, hence, the decreased catalase activity in vitamin B-6 deficiency may be due to low NADPH availability. Further catalase has been shown to be inhibited by oxalate,[52] and the observed decreased activity in vitamin B-6 deficiency may also be associated with the enhanced level of oxalate. In addition, GAO has been shown to produce H_2O_2 as one of the products[64] and, hence, the elevated GAO in vitamin B-6 deficiency accentuates H_2O_2 production. The accumulation of H_2O_2 formed by the various reactions leads to increasing the formation of hydroxyl radicals either with iron by Fenton type reaction or with iron in presence of superoxide by Haber Weiss reaction. This is supported by the fact that xanthine oxidase, which produces superoxide anions,[64] is increased in vitamin B-6 deficiency. The enhanced levels of hydroxyl radicals increase diene conjugates, hydroperoxides, lipofuscin-like pigments, and TBARS in vitamin B-6 deficiency. Due to the decreased antioxidant levels, the above reactions are not neutralized and, hence, show enhanced susceptibility to lipid peroxidation in presence of promoters of lipid peroxidation. These observations suggest that the increased lipid peroxidation may be associated with indirect secondary effects of the general disruption of metabolism associated with vitamin B-6 deficiency. This is supported by the fact that the functions of other vitamins are influenced by deficiency of vitamin B-6, resulting in change of enzymatic activities.[65]

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