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Plasma homocysteine and liver tissue S-adenosylmethionine, S-adenosylhomocysteine status in vitamin B_6-deficient rats

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Abstract. – OBJECTIVE: The aim of this study was to evaluate plasma homocysteine (Hcy), malondialdehyde (MDA), glutathione (GSH) levels, glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST) activities and liver tissue S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels in control and vitamin B6-deficient rats.

MATERIALS AND METHODS: Thirty-two male rats with a weight of 65-75 g were used for the experiment. The rats were divided into control (n=16) and vitamin B6-deficient groups. At the end of the experiment, the animals were anesthetized with ketamine-HCl (Ketalar, 20 mg/kg, i.p.), and the blood was collected by cardiac puncture after thoracotomy. Plasma Hcy, pyridoxal phosphate (PLP), liver SAM, SAH levels measured by an isocratic system with high performance liquid chromatography. Plasma GSH-Px, GST activities and GSH, MDA levels were carried out using a spectrophotometer.

RESULTS: Plasma Hcy, MDA, liver tissue SAH levels were significantly increased, whereas plasma GSH, PLP, liver tissue SAM levels, plasma GST, GSH-Px activities and SAM/SAH ratio were decreased compared to those of control group.

CONCLUSIONS: Vitamin B_6 deficiency causes an increase in plasma homocysteine levels. Thus, we think that vitamin B_6 supplementation could be used for therapeutic purposes in hyperhomocysteinemia condition.

Key Words:
Homocysteine, S-adenosylhomocysteine, S-adenosylmethionine, Vitamin B_6-deficiency, Pyridoxal-5-phosphate, Free radical.

Introduction

Hyperhomocysteinemia is a risk factor for atherosclerosis[1,2], which increases the effects of conventional risk factors such as arterial hypertension, hypercholesterolemia, and aging[3-5]. Normal human plasma contains total concentrations of homocysteine (Hcy) and its derivative disulfides close to 10 µmol/L, although there is some variation due to genetic factors, age, sex, menopausal status, and other physiological and lifestyle variables. Hyperhomocysteinaemia (defined by values of plasma Hcy > 15 µmol/L) is the presence of an abnormally elevated concentration of plasma or serum Hcy[6]. Hcy is a sulfur-cantaining amino acid. It forms various disulphides by oxidation at physiological pH, since the pKa of the thiol group is 8.9. All Hcy found in body is formed by demethylation of the essential amino acid methionine[7].

S-adenosylmethionine (SAM), the activated form of methionine, is the principal methyl group donor for a lot of methylation reactions. S-adenosylhomocysteine (SAH) is formed after demethylation, and irreversible hydrolysis leads to homocysteine. Hcy is metabolized by two major pathways. Hcy obtains a methyl goup from 5-methyl-tetrahydrofolate to form methionine in the remethylation pathway. It condenses with serine to form cystathionine in transsulfuration pathway. The pyridoxal-5-phosphate (PLP)-dependent enzyme cystathione-β-synthase catalyzes this irreversible reaction. Cysteine and ketobutyrate are formed by the action of γ-cystathionase, in the presence of PLP as a cofactor[8].

Vitamin B_6 has been shown to be important for normal cognitive function and in lowering the incidence of coronary heart disease among the elderly[9-12]. In addition, Vitamin B_6 supplementation

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has been shown to reduce diabetic complications and the occurrences of neurodegenerative diseases in varying degrees[9,13].

Vitamin B_6 refers to three primary forms of water-soluble vitamins: pyridoxine, pyridoxal phosphate, and pyridoxamine. Some studies have been reported antioxidant activities of vitamin B_6. Vitamin B_6-deficiency decreases the activity of γ-cystathionase markedly, resulting in the accumulation of cystathionine[14]. Methionine metabolism was found to be altered in rats fed a vitamin B_6-deficient diet, as indicated by accumulation of SAH in liver and accordingly a concomitant decrease in SAM/SAH ratio. The SAM to SAH ratio in liver cells is important in the regulation of transmethylation reaction[8,15].

Cells have developed different antioxidant systems and various antioxidant enzymes to defend themselves against free radical attacks. Glutathione-dependent antioxidant system consisting of reduced glutathione and an array of functionally related enzymes plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. Of these enzymes, glutathione peroxidase (GSH-Px) is a selenoprotein that reduces hydroperoxides as well as H_2O_2 while oxidizing glutathione. A number of potentially toxic electrophilic xenobiotics (such as certain carcinogens, bromobenzene, chlorobenzene) are conjugated to the nucleophilic glutathione by glutathione S-transferases (GSTs) present in high amounts in cell cytosol. GST can also catalyze reactions reducing peroxides like GSH-Px. Reduction of oxidized glutathione (GSSG) to glutathione (GSH)is mediated by the widely distributed enzyme glutathione reductase (GRD) that uses NADPH as the reductant[16,17].

In the present study, we aimed to investigate plasma GST, GSH-Px, Hcy, reduced GSH, malondialdehyde (MDA) pyridoxal phosphate (PLP) and liver tissue SAM and SAH levels in vitamin B_6-deficient rats, and relationship among these parameters in vitamin B_6-deficient rats.

Material and Methods

Animals

Thirty-two male rats (4 weeks old, Sprague-Dawley strain) with a weight of 65-75 g were used for the experiment. All animals received humane care compliance with the guidelines of Ataturk University Research Council's criteria. The composition of the diet was done as described[18]. After 4 weeks of feeding, the animals were anesthetized with ketamine-HCl (Ketalar, 20 mg/kg, i.p.), and the blood was collected by cardiac puncture after thoracotomy. Blood samples were collected in vacutainer tubes with K_3-EDTA as anticoagulant. They were centrifuged at 2000xg for 10 min and plasma was removed by a Pasteur pipette. Liver tissues were removed and washed out from contaminated blood with cold water, and homogenized 5-fold percloric acid (mol/L) solution by using a homogenizer (Omni Acceory Pack International omogenizer, Warrenton, VA, USA). The homogenate was centrifuged at 10,000 g for 20 min to remove debris. The plasma and homogenates were kept in –80°C until biochemical determinations.

Biochemical Measurements

Liver tissue SAM and SAH levels were measured by HPLC following the method of She et al[19]. The total plasma Hcy level was measured by high performance liquid chromatography (HPLC) pump (flow rate 1.7 mL/min), injector (injection volume 20 mL, analytical run time 4-6 min) and fluorescence detector (Ex: 385 nm, Em: 515 nm). Plasma PLP, the bioactive form of vitamin B_6, level was measured by HPLC pump (flow rate 1.3 mL/min), injector (injection volume 50 mL, analytical run time 12 min) and fluorescence detector (Ex: 370 nm, Em:470 nm) (HP 1100). Plasma GSH-Px, GST activities, GSH and MDA levels were measured as described, respectively[20-23].

Statistical Analysis

The findings were expressed as the mean ± SD. Statistical and correlation analyses were undertaken using the Mann-Whitney U-test and Spearman's rank correlation test, respectively. Significance (p) values less than 0.05 were considered significant. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL, USA).

Results

The results from vitamin B_6-deficient rats and control group the parameters measured in the study were summarized in Tables I and II, respectively. As seen from Tables, vitamin B_6-deficient rats caused a significant loss of body weight. Plasma Hcy, MDA, liver tissue SAH lev-

Table I. Mean ± SD of liver tissue S-adenosylmethionine and S-adenosylhomocysteine levels in control and vitamin B_6-deficient groups.

	Control group (n=16)	Vitamin B_6-deficient group (n=16)
S-adenosylmethionine (SAM) (nmol/g of liver)	163.7 ± 14.2	120.8 ± 5.1[a]
S-adenosylhomocysteine (SAH) (nmol/g of liver)	119.0 ± 6.5	176.2 ± 9.1[a]
SAM/SAH ratio	1.4 ± 0.2	0.7 ± 0.05[a]

[A]$p < 0.001$, vs. control group.

els were significantly increased, whereas plasma GSH, GST, GSH-Px activities, PLP levels, liver tissue SAM levels, and SAM/SAH ratio were decreased compared to those of control group.

Correlation analyses were shown in Table III. Serum Hcy level negatively correlated with serum PLP (r = –0.69, $p < 0.005$), GST and GSH-Px activities (r = –0.52, $p < 0.05$ and r = –0.54, $p < 0.05$, respectively) in vitamin B_6-deficient group while there was a positive correlation between serum Hcy and MDA levels in the group. There were significant negative correlation between GSH-Px and MDA and positive correlation between GST and GSH-Px in vitamin B_6-deficient group. However, no other correlation could be found among the parameters in controls group.

Discussion

Vitamin B6 deficiency is rare in humans, but occurs in association with low concentrations of other B-complex vitamins such as vitamin B_{12} and folate. For instance: populations most commonly at risk for vitamin B_6 deficiency include the elderly, alcoholics, renal patients undergoing dialysis, individuals with malabsorption conditions such as celiac disease, Crohn's disease or ulcerative colitis, and those with autoimmune disorders. Low vitamin B_6 levels have also been linked to various conditions including cardiovascular disease, cancer and cognitive function. Vitamin B_6 deficiency can lead to anemia, dermatitis, glossitis, depression, confusion, and weakened immune function[24]. Pyridoxal phosphate is, the biologically active form of vitamin B_6, a coenzyme in various metabolic pathways including degrading of Hcy.

Homocysteine has been emerged as a potential risk factor for premature cardiovascular disease, promoting many processes that play a role in vascular and endothelial cell damage[25,26]. Therefore, a progressive increase in the plasma Hcy concentration raises the risk of coronary artery disease[26,27]. Normal levels of Hcy in fasting conditions are of 5-15 μmol/L. In hyperhomocysteinaemia it may reach values of 16-30 μmol/L (moderate), 31-100 μmol/L (medium) or greater than 100 μmol/L (severe), values as high as 500 μmol/L being found in patients with homocystinuria[26,28]. The mechanisms underlying endothelial injury promoted either by Hcy alone or in combination with other risk factors are not understood well. *In vitro* studies provide evidence that supraphysiological Hcy levels have a

Table II. Mean ± SD of plasma homocysteine, GSH, MDA levels, GST, GSH-Px activities and initial and final body weight of control and vitamin B6-deficient groups.

	Control group (n=16)	Vitamin B_6-deficient group (n=16)
Hcy (μmol/L)	9.6 ± 2.8	18.6 ± 6.9[c]
PLP (μg/L)	104.9 ± 18.2	32.6 ± 7.0[c]
MDA (nmol/ml)	1.2 ± 0.6	2.2 ± 1.02[c]
GSH (nmol/L)	61.1 ± 6.9	52.5 ± 7.1[b]
GST (U/L)	18.5. ± 8.9	10.4 ±2.2[d]
GSH-Px (IU/L)	2.8 ± 0.58	2.3 ± 0.93[d]
Final body weight	183.8 ± 17.3	103.5 ±10.2[c]
Initial body weight	66.8 ± 2.6	67.4 ± 2.8

[b]$p < 0.01$, [c]$p < 0.001$, [d]$p < 0.0001$, vs. control group.

Table III. Spearman's rank correlation coefficients between plasma GSH-Px, GST activities, Hcy, PLP, MDA levels in vitamin B_6-deficient group.

	r =	p <
Hcy-PLP	–0.69	0.005
Hcy-GST	–0.52	0.05
Hcy-GSH-Px	–0.54	0.05
Hcy-MDA	0.53	0.05
GSH-Px-MDA	–0.87	0.001
GST-MDA	–0.77	0.001
GST-GSH-Px	0.57	0.05

direct toxic effect in endothelial cells, probably by free-radical generation through oxidation of the Hcy sulphydryl group[25,29]. Alvarez-Maqueda et al[26] found that Hcy significantly increased $O_2^{\bullet-}$ in a dose- and time-dependent manner. Lipid peroxidation is a well-established mechanism of oxidative damage caused by reactive oxygen species (ROS), and the measurement of MDA provides an important index of lipid peroxidation[22,30]. In our study, we found that MDA levels in plasma of vitaminB_6-deficient rats were significantly increased compared to those of control group. Increased MDA levels in vitamin B_6 deficient rats were increased in agreement with previous reports[22]. This confirms the presence of increased oxidative stress in vitamin B_6 deficient rats.

Furthermore, increased hydrogen peroxide (H_2O_2) production accompanies increased $O_2^{\bullet-}$release induced by Hcy. Different sources and pathways produce H_2O_2 in eukaryotic cells. For example, mitochondria, microsomes, peroxisomes and cytosolic enzymes are effective generators of ROS, which yield H_2O_2 after dismutation. In mitochondria, H_2O_2 is derived from the dismutation of $O_2^{\bullet-}$generated by minor side effects from the sequential reduction of $O_2^{\bullet-}$ via respiratory carriers[31].

In some studies, it has been reported that plasma total Hcy levels were significantly increased in vitamin B_6-deficient rats[32-34]. We found that vitamin B_6-deficiency led to a marked reduction of the plasma PLP level, accumulation of plasma Hcy in vitamin B_6-deficient rats. Our results are in agreement with these studies[32-34].

Overproduction of ROS generated from the oxidation of Hcy may cause endothelial injury and DNA damage. Free radicals including hydrogen peroxide can be generated upon oxidation of homocysteine, forming a disulfide linkage with free sulfhydryl group of albumin, cysteine or homocysteine, since reduced free Hcy contains a free sulfhydryl group. It is apparent that the plasma level of reduced free Hcy affects and enhances oxidative stress. The endogenous attack on DNA by ROS including hydrogen peroxide may generate many DNA adducts in human cells. Of the over 100 oxidative lesions in mammalian DNA, 8-hydroxyguanine is one of the most abundant lesion[31,35,36].

Important metabolic indicators of the cellular methylation capacity are the intracellular concentrations of SAM and SAH, as the substrate and product of essential cellular methyltransferase reactions[36]. A chronic increase in SAH, secondary to the homocysteine-mediated reversal of the SAH hydrolase reaction, may also have significant, indirect, pathologic consequences, despite the emphasis has been placed on the toxicity of increased Hcy and depressed SAM concentrations in metabolic pathology of various diseases[29,34,36,37]. Because, SAH can bind to and inhibit multiple cellular methyltransferases, an increase in SAH can lead to DNA hypomethylation and altered chromatin configuration, reduced membrane phosphatidylcholine concentrations, reduced protein and RNA methylation, and reduced neurotransmitter synthesis[31,38]. These alterations may lead to inappropriate gene expression, altered signal transduction, immune deficiency, and cytotoxicity[31,39]. Nguyen et al[15] and She et al[19] reported that an marked increase in SAH and a decrease SAM levels were in liver tissues of vitamin B_6-deficient rats. We found that vitamin B_6-deficiency for 4 weeks caused a marked reduction of the SAM/SAH ratio, accumulation of liver tissue SAH, reduction of SAM level in vitamin B_6-deficient rats. The increase in SAH, decrease in SAM levels and SAM/SAH ratio in rat liver induced by vitamin B_6 deficiency are in agreement with previous studies[15,19].

GSH shows antioxidant properties itself. GSH acts as an antioxidant molecule protecting sulphhydryl groups. Results demonstrated that GSH levels in plasma of vitamin B_6-deficient rats were significantly lower than in the control group. GSH synthesis depends on the availability of cysteine, which is synthesized by vitamin B_6-dependent enzymes cystathionine-β-synthase and cystathionine-γ-lyase from methionine. Cysteine synthesis appears to be reduced in vitamin B_6 deficient rats because of the altered activity of these enzymes, which leads to decreased GSH synthesis[22]. The results confirm this hypothesis.

We found that GSH-dependent enzyme activities (GSH-Px and GST) in plasma of vitamin B_6-deficient rats were significantly decreased when compared to those of control group. GSH has an important function both as a cosubstrate for GST and as a substrate for GSH-Px. The low GSH-Px activities may be directly explained by the low GSH content found in vitaminB_6-deficient rats, since GSH is a substrate of this enzyme, which has been mentioned above. Therefore, low GSH content implies low GSH-Px activity, which may increase the propensity for oxidative stress. In the study, decrease in GST activities in vitaminB_6-deficient rats may be the accumulation of the $O_2^{\bullet-}$ which inactivates that by reacting with the active site of this enzyme.

Examining correlation analysis results from Table III, it can be seen that plasma Hcy concentration negatively correlated with plasma PLP, GST, GSH-Px in vitamin B_6-deficient group. These negative correlations confirm the presence of increased oxidative stress in vitamin B_6 deficient rats. Robinson et al[40] reported that low PLP level confers an independent risk for coronary artery disease (CHD). Rimm et al[41] has also reported that folate and vitamin B_6 levels may be important in the primary prevention of CHD. Endo et al[14] reported that vitamin B_6 deficiency induced the oxidant stress which accelerates atherosclerosis and the antioxidant activity of vitamin B_6 appears to suppress homocysteine-induced atherosclerosis. They showed that the oxidative stress was caused by a low level of vitamin B6 accelerates the development of homocysteine-induced atherosclerosis in rats. Endo et al[14] also reported that as vitamin B_6 has antioxidant activity apart from its role as coenzyme, the antioxidant activity of vitamin B_6 may suppress the homocysteine-induced atherosclerosis independent of homocysteine action itself.

Selhub et al[42] showed an association between insufficient PLP status and carotid arteriosclerosis, though this reduced after adjustment for homocysteine. They also reported that the majority of persons with elevated plasma homocysteine concentrations have insufficient concentrations of folate, vitamin B_{12}, or vitamin B_6. In addition to, some studies have been demonstrated that innocuous regimens of vitamin supplementation (including folate, vitamin B_{12}, and vitamin B_6) effectively lower moderately elevated plasma homocysteine concentrations to the normal range[43,44].

Conclusions

We showed that vitamin B_6-deficiency caused a marked reduction of the plasma PLP, GSH levels, GST, GSH-Px activities, accumulation of plasma Hcy and liver tissue SAH levels and reduction of SAM level, SAM/SAH ratio, and an increase in plasma MDA level in vitamin B_6-deficient rats. The increase in plasma Hcy and liver tissue SAH levels and the reduction of SAM/SAH ratio may be due to a block in the methionine catabolism via the transsulfuration pathway. These may lead to inhibition of transmethylation reactions of DNA, RNA and protein, and result in liver damage. By increasing the formation of MDA, an indicator of lipid peroxidation, and reducing the GSH levels and GSH-dependent enzymes activities, vitamin B_6 deficiency causes an increase in plasma Hcy levels and oxidative stress. Thus, the results presented here suggest the vitamin B_6 supplementation can be reconsidered and be determined for therapeutic purpose in hyperhomocysteinaemia.

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