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The Effect of Folic Acid, Vitamin B_6 and Vitamin B_{12} on the Homocysteine Levels in Rabbits Fed by Methionine-Enriched Diets

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NARIN, F., NARIN, N., AKCAKUS, M., USTDAL, M., KARAKÜÇÜK, İ. and HALICI, C. *The Effect of Folic Acid, Vitamin B_6 and Vitamin B_{12} on the Homocysteine Levels in Rabbits Fed by Methionine: Enriched Diets.* Tohoku J. Exp. Med., 2002, **198** (2), 99-105 —— Atherosclerosis is an important cause of cardiovascular morbidity and mortality in recent years. Hyperhomocysteinemia is recognized as an independent risk factor for premature atherosclerosis and venous thrombosis. It is suggested that administration of folic acid, vitamin B_6 and vitamin B_{12} may decrease homocysteine levels. In our study, we induced hyperhomocysteinemia in rabbits by giving methionine and studied the effects of folic acid, vitamin B_6 and vitamin B_{12} on homocysteine levels. A total of 40 (20 female, 20 male New Zealand rabbits) were divided into four groups, each consisting of 10 rabbits. Methionine (100 mg/kg/day), methionine (100 mg/kg/day) plus vitamin B_6 (30 mg/kg/day), methionine (100 mg/kg/day) plus vitamin B_{12} (80 mg/kg/day) and methionine (100 mg/kg/day) plus folic acid (20 mg/kg/day) were given to the first, second, third and forth groups respectively. These rabbits were followed up for two months. We studied homocysteine levels on the 0, 20th, 40th and 60th days in all groups. In rabbits we induced hyperhomocysteinemia by giving methionine for 2 months. The decreases of homocysteine levels in the forth group were significant with respect to the second and third groups. Folic acid supplementation clearly resulted in a reduction of plasma homocysteine levels, whereas vitamin B_{12} was little effective and vitamin B_6 failed to show an effect. We conclude that even folic acid treatment alone may be sufficient for decreasing negative effects of homocysteine. ——— homocysteine; hyperhomocysteinemia; folic acid; vitamin B_6; vitamin B_{12}

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Extremely high levels of homocysteine (Hcy) were reported several decades ago in the plasma and urine of patients with inborn errors of methionine metabolism. Mental retardation, premature arteriosclerosis and thrombotic complications are the clinical features of the syndrome (McCully 1969). More recently, mild to moderate hyperhomocysteinemia has been reported as a risk factor for atherosclerosis among non-homocysteinuric subjects with vascular disorders (Jacopsen 1998).

Homocysteine is a sulfur containing amino acid that is formed during methionine metabolism. Metabolism of homocysteine is by pathways, which re-methylate it (and which require vitamin B_{12} and folic acid), or by a transsulphuration pathway, which requires vitamin B_6. The level of homocysteine in blood (and elsewhere) is a determined by how much methionine is eaten, mainly in protein (with about three times more methionine in animal than plant protein), and how much is metabolised (and metabolism may be affected by amounts of B vitamins and folate available (Abby et al. 1998; Scott et al. 1999).

The homocysteine theory of atherosclerosis is attractive because it might explain the effect of vitamin deficiencies such as vitamin B_6, B_{12} or folic acid and the overloading of the transsulfuration pathway by excessive consumption of methionine-rich animal proteins (Guttormsen et al. 1996; Hornberger 1998; Pietrzik and Brönstrup 1998).

A methionine-loading test is supposed to test, in particular, the capacity of homocysteine degradation via the transsulfuration pathway. Patients with vascular disease and elevated homocysteine concentrations have been considered to be heterozygotes for cystathionine synthase (the first enzyme in the transsulfuration pathway) and the methionine-loading test is often used to investigate the homocysteine status in these patients (Miller et al. 1994; den Hijer et al. 1996; van der Grieng et al. 1998).

Despite its simple elegance, however the methionine-loading test is not necessarily specific for heterozygotes. Cystathionine β-synthase requires vitamin B_6 for its activity. A positive methionine-loading test might not represent a heterozygous cystathionine β-synthase defect, but rather impairment of this enzymes activity as a result of vitamin B_6 deficiency. Also homocysteine metabolism does not consist solely of its catabolism through cystathionine synthesis, but also of remethylation to form methionine (Miller et al. 1994; den Hijer et al. 1996; van der Grieng et al. 1998). Nutritional deficiencies of vitamin B_{12} and folate and certain genetic enzyme defects (Ubbinks et al. 1993, 1996; Dalery et al. 1995; Rosenberg 1996; Selhub et al. 1996) that affect the metabolism of these vitamins are known to cause impairment of homocysteine remethylation and lead to abnormally elevated plasma homocysteine concentration. Little is known, though, about the methionine loading in these conditions.

The purpose of the present study was to assess the contribution of the methionine loading to the detection of hyperhomocysteinemia and to study the affect of the oral vitamin supplements (vitamin B_6, B_{12} and folate) as a therapeutic agent to normalize elevated circulating homocysteine concentrations in rabbits.

Subjects and Methods

Animals and diets

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Erciyes University. A total of 40 (20 female, 20 male) New Zealand rabbits were divided into four groups, each consisting of 10 rabbits. Methionine (100 mg/kg/day), methionine (100 mg/kg/day) plus vitamin B_6 (30 mg/kg/day), methionine (100 mg/kg/day) plus vitamin B_{12} (80 μg/kg/day) and methionine (100 mg/kg/day) plus folic acid (20 mg/kg/day) supplemented diets were given to the first, second, third and forth groups respective-

ly. These rabbits were followed up for two months. Weight gain and food consumptions were determined at weekly intervals. At the beginning (1st day), the 20th, 40th, and 60th days of the treatment rabbits were deprived of food overnight and blood samples for homocysteine determinations were collected from the marginal ear vein.

Homocysteine assay

Blood was collected by venepuncture in bottles containing EDTA from the rabbits and controls after a standard 12 hour fast and immediately centrifuged at 3000 g for 15 minutes. The plasma was stored at −70°C until analyzed. The sample, internal standard and phosphate buffered saline (PBS, pH 7.4) were mixed. Tris-2-carboxy-ethylphosphine (TCEP) in water-soluble was added to reduce the thiols and mixed sulfides and also to release thiols bound to protein. After incubation at room temperature for 30 minute, trichloroacetic acid was added to precipitate proteins. Next, the centrifuged supernatant was mixed with NaOH, borate buffer containing EDTA and SBD-F (ammonium-7-fluorobenzo-2-oxa-1.3-diazole-4-sulfonate) in the borate buffer. The sample incubated at 60°C in a water bath for 1 hour, to form fluorescent derivates. After cooling at 4°C, 10 μL of the samples, were injected to HPLC. HPLC was carried out on solvent delivery system and a fluoresans detector (385 nm excitation, 515 nm emission). 0.1 mol/liter acetic acid-acetate buffer, pH 5, 5 containing 30 ml/liter methanol was used to elute the constituents as mobile phase. Flow rate was 0.7 ml/minute. L-homocysteine calibrators (5-100 μLmol/L) were prepared in PBS, pH 7.4 and in pooled EDTA plasma (Pfeiffer et al. 1999). Hcy peaks which were seperated with analytic HPLC column were provided in 3.3 minute. Hcy concentrations were calculated according to peak levels provided from fluorescein detector. Variation co-efficient was 3.5%.

Statistical analysis

Statistical analysis was performed using a computer statistical package SPSS 8.0 for Windows. A p-values of <0.05 were regarded statistically significant. The significance of differences between the groups were assessed with One-Way ANOVA, the significance of differences within the groups were assessed with Repeated Measures ANOVA.

RESULTS

We compared the homocysteine levels within the groups

The homocysteine levels of the rabbits in all groups on the 20th, 40th, and 60th days of the treatment were significantly higher than that of the levels at the beginning of the treatment (on the 1st day) ($p<0.05$) (Table 1).

In the groups I, II and III, the homocys-

TABLE 1. *The homocysteine levels of the groups on the 0, 20th, 40th and 60th days*

Day	Group I	Group II	Group III	Group IV
0	9.906±4.226	9.513±4.075	8.355±4.091	8.615±5.227
20	23.728±1.886[a]	20.728±6.405[a]	23.936±10.660[a]	17.429±9.447[a,1]
40	44.485±4.176[a,b]	39.778±10.420[a,b]	46.536±19.010[a,b]	23.002±10.867[a,1,2,3]
60	104.250±53.320[a,b,c]	65.873±14.890[a,b,c,1]	84.922±17.350[a,b,c]	19.033±8.825[a,1,2,3]

[a]with respect to the day 0, it was statistically significant.
[b]with respect to the 20th day, it was statistically significant.
[c]with respect to the 40th day, it was statistically significant.
[1]with respect to the first group, it was statistically significant.
[2]with respect to the second group, it was statistically significant.
[3]with respect to the third group, it was statistically significant.

teine levels on the 40th, and 60th days were higher than that of the levels on the 20th day. And also in these groups, the homocysteine levels on the 60th day were higher than that of the levels at the 40th day ($p<0.05$) (Table 1).

In the group IV, the homocysteine levels on the 40th day were higher than that of the levels on the 20th day. We could not find significant differences with respect to the other days (Table 1).

We compared the homocysteine levels between groups

20th day. In the group I, the homocysteine levels were significantly higher than that of the group IV. There was no significant difference between other groups (Table 1).

40th day. In the groups I, II and III, the homocysteine levels were significantly higher than that of the group IV (Table 1).

60th day. In the first group, the homocysteine levels were significantly higher than that of the group II and IV. In the group II and III, the homocysteine levels were significantly higher than that of the group IV (Table 1).

DISCUSSION

Hyperhomocysteinemia was first hypothesized to be linked with atherosclerosis more than 25 years ago by Kilmer McCully 1969, when he observed extensive atherosclerotic disease in young patients who had elevated homocysteine concentrations as a result of inborn errors of metabolism. Patients may have ectopia lentis, skeletal abnormalities, osteoporosis, Marfanoid features, thromboembolism, premature arteriosclerosis and various degrees of mental retardation. Defective metabolism of the essential amino acid methionine, resulting in overt hyperhomocysteinemia or situational hyperhomocysteinemia (after a methionine load), has been established as an independent risk factor for atherosclerotic heart disease (Miller et al. 1994; den Hijer et al. 1996; van der Griend et al. 1998). Dietary intake and metabolism of folic acid, the nutrient most closely identified with neural tube defects, has been studied in depth for the past fifteen years. The information from these studies has illuminated the mechanisms of these congenital defects, and has lead to the discovery of connections with other nutrients related to homocysteine metabolism which may also be involved in negative pregnancy outcomes, including spontaneous abortion, placental abruption (infarct), pre-term delivery, and low infant birth weight (Alan et al. 1996).

Hyperhomocysteinemia has received increasing attention during the past decade, increased homocysteine levels have been implicated in a variety of other clinical conditions, including neural tube defects, spontaneous abortion, placental abruption, osteoporosis, renal failure, diabetic microangiopathy, neuropsychiatric disorders, and pre-menstrual syndrome (Bakker and Bradjes 1997). The prevalence of hyperhomocysteinemia in the general population is between 5% and 10%. However, rates may be as high as 25-30% in the mothers with pre-term delivery, 20-30% in patients with premature atherosclerosis and 25-30% in cases with neural tube defects (Bakker and Bradjes 1997).

Most studies conclude that hyperhomocysteinemia is an independent serious risk factor for premature atherosclerosis and venous thrombosis (Rosenberg 1996; Selhub et al. 1996; Bakker and Bradjes 1997; Abby et al. 1998; Scott and Sutton 1999). Methionine loading is a test used to identify heterozygous carriers for homocysteinuria (Folsom et al 1998; van der Griend et al. 1998). First used successfully to identify such heterozygotes in the early 1970s. In 1976, methionine-loading test was used for the first time to detect moderate hyperhomocysteinemia in man with cardiovascular disease (van der Griend et al. 1998). The importance of identifying heterozygotes for homocyteinuria, which may affect as many as 1 in 70 individuals, is reflected by studies demonstrating a

high prevalence of premature vascular disease associated with this condition. Boers et al. (1985) have shown heterozygotes to be at increased risk for premature peripheral and cerebral occlusive arterial diseases, where as Clarke et al. (1991) have shown that heterozygosity is an independent risk factor for coronary, and peripheral and cerebrovascular diseases.

The methionine-loading test is not necessarily specific for heterozygotes. Cystathionine β-synthase requires vitamin B_6 for its activity. A positive methionine-loading test might not represent a heterozygous cystathionine β-synthase defect, but rather impairment of this enzymes activity as a result of vitamin B_6 deficiency. Also; homocysteine metabolism does not consist of solely of its catabolism through cystathionine synthesis, but also at remethylation to form methionine (Smolin et al. 1983; Miller et al. 1994; Dunn et al. 1998; van der Griend et al. 1998).

Nutritional deficiencies of vitamin B_{12} and folate and certain genetic enzyme defects that affect the metabolism of these vitamins are known to cause an impairment of homocysteine remethylation and lead to abnormally elevated plasma homocysteine concentration (Boers et al. 1985; Ubbink et al. 1993; Bakker and Bradjes 1997; Dunn et al. 1998). Little is known, though, about the methionine loading in these conditions.

In our study, we induced hyperhomocysteinemia in rabbits by giving methionine and studied the affect of the oral vitamin supplements (vitamin B_6, B_{12} and folate) as a therapeutic agent to normalize elevated circulating homocysteine concentrations. Rats fed with vitamin B_6, B_{12} or folate enriched diets for 8 week were administered a gastric gavage of methionine (100 mg/kg body weight). In the group I (only methionine enriched diet), methionine loading resulted in significantly higher increases in circulating total homocysteine. Our findings were similar with the findings of Toborek et al. (1996).

Hyperhomocysteinemia can be acquired as the result of dietary deficiencies of folate, vitamin B_{12} and/or vitamin B_6. These nutrients are necessary cofactors for the optimal function of methylene tetrahydrofolate reductase and cystathionine β-synthase. Deficiencies in the absorption or transport of these vitamins can also cause hyperhomocysteinemia. Since homocysteine levels are not initially elevated in vitamin B_6 deficiency, hyperhomocysteinemia is an insensitive gauge of vitamin B_6 status. In most cases, however, an elevated homocysteine level is a sufficient marker of folate or vitamin B_{12} deficiency, whereas an elevated level of methylmalonic acid specifically suggests vitamin B_{12} deficiency. Thus, an elevated homocysteine concentration may indicate a deficiency in folate, vitamin B_{12} or, less commonly, vitamin B_6 (Ballal et al. 1997; Fallest-Strobl et al. 1997; van der Griend et al. 1997; Ward et al. 1997; Robinson et al. 1998). In several studies, it is emphasized that daily folic acid administration or combination with vitamin B_{12} resulted in significant reductions in plasma homocysteine (Miller et al. 1992; Ward et al. 1997). In an experimental study, Smith et al. (2001) showed that folate has inhibitory effect on intimal hyperplasia induced by a high-homocysteine diet in a rat carotid endarterectomy model.

In our study, there was no change in homocysteine levels in vitamin B_6 supplemented group (group II) compared to the group (group I) only receiving methionine. This findings support that vitamin B_6 is not effective in conditions with no vitamin deficiency. The homocysteine levels in the group III (vitamin B_{12} supplemented) were significantly lower than that of the group (group I) only receiving methionine on the 60th day but in this group homocysteine levels were significantly higher than that of group IV (folic acid supplemented). In group IV (folic acid supplemented), elevations of homocysteine levels were lower than that of the other groups during the ther-

apy and the results were significantly lower compared to the other groups. Miller et al. (1992) showed that vitamin B_{12} only was not sufficient to reduce homocysteine levels. Deutsch et al. (1998) and Brattström (1996) emphasized that folic acid treatment was more effective. Our findings were also similar.

Folic acid supplementation clearly resulted in a reduction of plasma homocysteine levels, whereas vitamin B_{12} was little effective and vitamin B_6 failed to show an effect.

The results of this study indicate that supplementation with physiologic doses of folic acid can reduce homocysteine levels. Since even moderate elevations in homocysteine concentrations are a risk factor for premature atherosclerosis and venous thrombosis, the reduction in homocysteine produced by supplementation might have long-term health benefits. Additional studies are needed to determine whether similar effects on homocysteine can be produced with dietary folate rather than folic acid supplements.

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