British Journal of Nutrition (2006), **95**, 1088–1093 DOI: 10.1079/BJN20061764

Antioxidant activity of vitamin B_6 delays homocysteine-induced atherosclerosis in rats

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(Received 19 July 2005 – Revised 30 January 2006 – Accepted 30 January 2006)

Elevated plasma homocysteine is a risk factor for atherosclerotic disease. In the present study, we have examined whether the oxidative stress due to a low level of vitamin B_6 accelerates the development of homocysteine-induced atherosclerosis in rats. First, the effect of homocysteine thiolactone intake (50 mg/kg per d) on vascular integrity, lipid peroxide concentration, endothelial NO synthase (eNOS) expression and biochemical profiles was examined at day 1, day 21 and day 42 (five rats per group). The histochemical staining of the rat aorta showed no change at day 1 and day 21, but the subendothelial space was observed to be enlarged in rat aorta at day 42 with exposure to homocysteine thiolactone. Expression of eNOS was observed in rat aorta at day 42, but not at day 1 and day 21. Serum lipid peroxide concentration and biochemical profiles including glucose cholesterol and triacylglycerol showed no change at any day. Second, the effect of homocysteine thiolactone intake in the presence and absence of vitamin B_6 on vascular integrity was examined at day 1 and day 14 (five rats per group). Aortic lesions were observed in vitamin B_6-deficient rat aorta at day 14 but not in vitamin B_6-supplemented rats. The expression of eNOS was also observed in vitamin B_6-deficient rat aorta at day 14. Serum lipid concentrations of the vitamin B_6-deficient group significantly increased compared with concentrations of the vitamin B_6-supplemented group, though serum concentration of homocysteine did not change between both groups. These results suggest that the oxidative stress caused by a low level of vitamin B_6 accelerates the development of homocysteine-induced atherosclerosis in rats.

Antioxidants: Atherosclerosis: Homocysteine: Vitamin B_6

Atherosclerosis is the major cause of heart attack and stroke, which constitute the most prevalent diseases (Ross, 1999). The first step of atherosclerosis involves endothelial dysfunction as proposed in 'the response-to-injury hypothesis' by Ross (1993). The factors causing endothelial dysfunction include shear stress, oxidised LDL and oxidative stress. When endothelial dysfunction occurs, the endothelial cells become active and secrete adhesion molecules, cytokines, chemokines and growth factors through induction of transcription factor NF-κB. Subsequently, the adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 mediate leucocytes rolling and adhesion to the endothelium. The migration of leucocytes from the blood vessels to the endothelium is promoted by chemokines such as monocyte chemotactic protein-1. The intimal macrophages and smooth muscle cells which are stimulated and transformed by cytokines engulf oxidised LDL and form fatty streaks. Ultimately, these fatty streaks develop into fibrous plaques, which rupture and cause thrombosis that may lead to acute coronary syndrome and myocardial infarction (Hansson, 2001).

Though hypercholesterolaemia has been suggested to be an important risk factor for atherosclerosis, most of the patients with heart attack myocardial infarctions show normal serum concentrations of cholesterol. Another factor associated with the formation of atherosclerotic lesions is hyperhomocysteinaemia (McCully, 1969). Recently, many studies have correlated associated high plasma or serum concentration of homocysteine with an increased risk of atherosclerosis (Eikelboom *et al.* 1999; Hankey & Eikelboom, 1999; Brattstrom & Wilcken, 2000; Ueland *et al.* 2000; Hofmann *et al.* 2001). Hyperhomocysteinaemia is a risk factor also in men. There seems to be a strong synergistic effect between hyperhomocysteinaemia and all traditional vascular risk factors (Taylor *et al.* 1991; Graham *et al.* 1997; van den Bosch *et al.* 2003). Furthermore, plasma homocysteine is a strong

Abbreviation: eNOS, endothelial NO synthase.

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predictor of both cardiovascular and non-cardiovascular mortality in a general population aged 65–72 years old (Vollset *et al.* 2001). As homocysteine is a sulfhydryl-containing amino acid, and is derived from the metabolism of dietary methionine, the reactive oxygen species generated during oxidation of homocysteine may damage endothelial cells and promote accumulation of platelets (Harker *et al.* 1976). Because vitamin B_{12} and folate act as cofactors for the re-methylation of homocysteine and vitamin B_6 for trans-sulfation to cysteine, low levels of these vitamins may cause hyperhomocysteinaemia and increase the risk for atherosclerosis (Verhoef *et al.* 1996, 1997; Siri *et al.* 1998). Interestingly, Robinson *et al.* (1995) reported that low pyridoxal 5′-phosphate confers an independent risk for coronary artery disease. Actually, it has been reported that folate and vitamin B_6 levels may be important in the primary prevention of CHD (Rimm *et al.* 1998).

On the other hand, many determinants of plasma homocysteine including genetic, physiological, and lifestyle factors, various diseases, and drugs are suggested (Refsum *et al.* 2004). Many of these factors cause a change in homocysteine concentrations by altering the function or blood concentration of the B vitamins (Ubbink, 1994), in particular folate and cobalamin, and/or by influencing renal function or, more rarely, by influencing enzyme activities.

Vitamin B_6 is an essential water-soluble vitamin required for normal growth and development (Tryfiates, 1980). Its physiologically active form, pyridoxal 5′-phosphate, is derived from inactive precursors and functions as a cofactor in numerous enzymic reactions of amino acid metabolism. Apart from its function as a cofactor, antioxidant activities of vitamin B_6 are reported (Ehrenshaft *et al.* 1999; Stocker *et al.* 2003; Chen & Xiong, 2005). Pyridoxine seems to quench singlet oxygen at a rate comparable with that of vitamins C and E, two of the most highly efficient biological antioxidants (Ehrenshaft *et al.* 1999). Furthermore, Ullegaddi *et al.* (2004) reported that B-vitamin supplementation may have antioxidant and anti-inflammatory effects in stroke disease independent of a homocysteine-lowering effect. Thus vitamin B_6 seems to be involved in active-oxygen resistance. Based on these reports it is proposed that the antioxidant activity of circulating vitamin B_6 will suppress the progression of homocysteine-induced atherosclerosis independently from the action of homocysteine itself.

In the present study, we have examined whether the oxidative stress due to a low level of vitamin B_6 accelerates the development of homocysteine-induced atherosclerosis in rats.

Materials and methods

Animals and diets

Young 3-week-old male rats (40–50 g) (Wistar; Kyudo, Kumamoto, Japan) were used. The control group (n 5) had free access to standard chow (CE-2; CLEA Inc., Tokyo, Japan) and tap water. The experimental group (n 5) was orally given homocysteine thiolactone (Sigma, St Louis, MO, USA) in tap water (50 mg/kg body weight per d) for 42 d (Fig. 1 (A)). Furthermore, to examine whether a low level of vitamin B_6 affects atherosclerosis, 3-week-old male rats (40–50 g) were subjected to experiments as shown in Fig. 1 (B). One group (n 10) was fed with control diets for 4 weeks, and another group (n 10) was fed with vitamin B_6-free diets (Oriental Yeast Co., Ltd, Tokyo, Japan) to produce the vitamin B_6-deficient rats. After 2 weeks of feeding, five rats of each group were given homocysteine thiolactone in distilled water and others were given only distilled water for 2 weeks. Rats were kept at room temperature (23°C) under 12 h light–dark cycles. Body weight, food intake and amount of drinking water were recorded every other day. Rats were killed at the end of the experiments and liver was taken for the examination of glutamate–oxaloacetic transaminase and glutamate–pyruvate transaminase activities (units/mg protein). Blood was taken from the abdominal aorta and the serum was collected. The aorta was removed for histological studies. The present study was approved by the Kagoshima University Animal Committee (Japan) and the rats were maintained in accordance with the Kagoshima University guidelines for the care and use of laboratory animals.

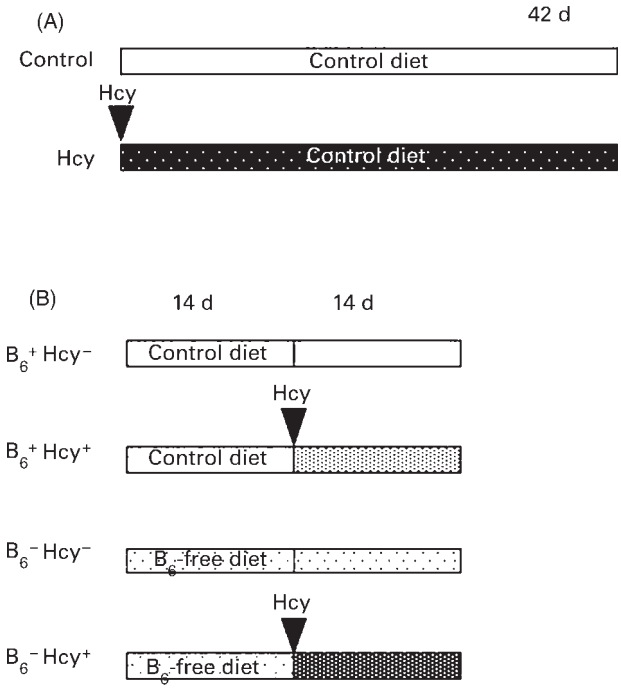

Fig. 1. Experimental protocols. (A) The control group was given standard chow and distilled water. The homocysteine (Hcy) group was given standard chow and Hcy (50 mg/kg body weight per d) in distilled water for 42 d. (B) The B_6^+ group was given the control diet and the B_6^- group was given a vitamin B_6-free diet for 28 d. Additionally, the Hcy^+ group was orally administrated Hcy (50 mg/kg body weight per d) in distilled water at day 15 for 14 d. The Hcy^- group was given distilled water.

Biochemical analysis

Serum biochemical data, glucose, triacylglycerol and total cholesterol were analysed to examine the nutritional condition of experimental animals. The serum concentration of triacylglycerol was estimated by a triglyceride G-test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The serum glucose and total cholesterol were determined using SPOTCHEM™ (Arkray Inc., Kyoto, Japan). The activities of glutamate–oxaloacetic transaminase and glutamate–pyruvate transaminase of liver tissue were analysed using the methods

of Okada & Ochi (1971). Total protein was measured with BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL, USA). The serum level of homocysteine was measured by homocysteine microplate (Substrate Trapping Enzyme) assay kit (Diazyme Laboratories, San Diego, CA, USA). The serum lipid peroxide concentration was determined by detection of thiobarbituric acid-reactive substances (TBARS). Thiobarbituric acid reagent (1 ml) was added to a tube containing 20 μl serum and 10 μl of 5 % butylated hydroxytoluene in ethanol. The tube was vortexed and heated at 100°C for 45 min. After centrifugation at 650 ***g*** for 10 min, the supernatant fraction was filtered (Millex-HV, 0·45 μm; Nihon Millipore Ltd, Tokyo, Japan) and the concentration of thiobarbituric acid-reactive substances was examined using a HPLC LC-10AD (Chromatopac, Shimadzu, Kyoto, Japan) attached C18 ODS column. The wavelength used for excitation was 515 nm and for emission 553 nm.

Histochemical staining

Aortic tissues derived from rats were fixed in 10 % formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan), embedded in paraffin, and sections (5 μm thick) were stained with haematoxylin and eosin. Ten different sections from aortic tissues of each rat were collected for the quantification of atherosclerotic lesions. The size of the lesion of a particular location was calculated from the average of the area quantified from the sections and expressed as a percentage of the total area investigated.

Immunohistochemistry for endothelial nitric oxide synthase

The tissue was deparaffinised (xylene 3 ×, 100 % ethanol 2 ×, 90 % ethanol, 80 % ethanol, 70 % ethanol, deionised water 3 ×) and heated in a microwave oven at 500 W for 15 min in a 10 mM-sodium citrate buffer (pH 6·0). After cooling the slides in the buffer for 20 min, the tissue was washed with deionised water for 5 min and incubated with 0·3 % H_2O_2 in 100 % methanol for 30 min. After washing with PBS (pH 7·4), the tissue was incubated with a blocking solution of 1·5 % normal goat serum for 1 h at room temperature. Then the slides were incubated with polyclonal antibody to endothelial NO synthase (eNOS) (2 μg/ml; Affinity BioReagents Inc., Golden, CO, USA) overnight at 4°C. After washing with PBS, biotinylated goat anti-rabbit immunoglobulins (Dako Japan Co., Ltd, Kyoto, Japan) were applied and incubated for 30 min at room temperature. The presence of eNOS was detected using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). Then the sections were visualised with 3,3′-diaminobenzidine tetrahydrochloride containing 0·05 % H_2O_2. Finally, the sections were washed, counterstained, dehydrated, cleared in xylene and mounted. Ten different sections in each rat were analysed to define the eNOS expression.

Statistical analysis

Data are presented as mean values and standard deviations. The statistical analysis used Student's *t* test for comparison of the subendothelial region and eNOS-positive region, and the Kruskall–Wallis test was used for comparison of the concentration of homocysteine and thiobarbituric acid-reactive substances. Analyses were performed using STATISTICA 4.5 software (Statsoft Japan Inc., Tokyo, Japan) on a Windows platform.

Results

Rats treated with homocysteine for 42 d showed the same growth curve as control rats. Also the biochemical data of serum such as glucose, triacylglycerol and cholesterol were almost similar in the two groups (Table 1). On the other hand, the histochemical staining of endothelium showed that subendothelial space enlargement increased in the homocysteine group after 42 d, probably due to the accumulation of lipid (Fig. 2 (A)), but the aorta from control rats did not show any change ($P < 0{\cdot}001$; Student's *t* test; Fig. 2 (B)). In addition, the immunohistochemical analysis for eNOS showed the positive staining in the endothelial cells of B_6-deficient rats given homocysteine. The expression of eNOS increased in the aorta from rats treated with homocysteine but not in control rats (Fig. 2 (C)).

To study vitamin B_6-mediated vascular effects *in vivo*, rats were fed B_6-free diets. After 2 weeks of the feeding, five rats of each group were given homocysteine thiolactone in distilled water and the others were given only distilled water for 2 weeks. The body weight of B_6-deficient rats decreased from 2 weeks after feeding B_6-free diets, characteristic of B_6-deficient rats (body weight of B_6-supplemented rats, 220·5 (SD 12·8) g; B_6-deficient rats, 175·8 (SD 15·4) g) as suggested by Coburn *et al.* (1981). Moreover, glutamate–oxaloacetic transaminase and glutamate–pyruvate transaminase activities in rat liver, which are the B_6 enzymes, decreased significantly in the liver of B_6-deficient rats (glutamate–oxaloacetic

Table 1. Serum concentrations of glucose, triacylglycerol (TG) and cholesterol in homocysteine (Hcy)-treated and control rats

(Mean values and standard deviations)

			Day 21				Day 42			
	Day 1		Control		Hcy		Control		Hcy	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Glucose (mg/l)	660	52	750	102	706	85	658	112	724	132
TG (mg/l)	1480	102	1460	187	1580	163	1500	152	1600	175
Cholesterol (mg/1)	386	92	426	116	356	84	456	98	400	124

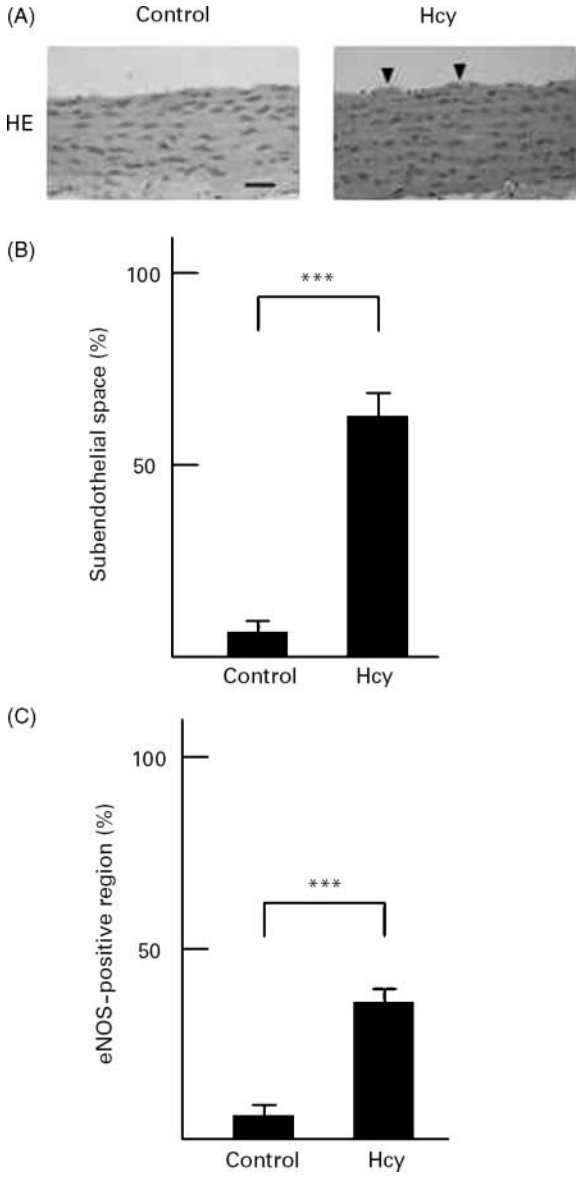

Fig. 2. Homocysteine (Hcy)-induced atherosclerosis-like alterations in rat aorta. (A) Rats were killed after 42 d, aorta were removed, and stained with haematoxylin–eosin (HE). ▼, Subendothelial space due to accumulation of lipid (× 400; ▬, 50 μm). (B) Percentage of subendothelial space. (C) Percentage of endothelial NO synthase (eNOS)-positive region. Values are means and standard deviations. ***Mean values were significantly different ($P<0{\cdot}001$; Student's *t* test).

transaminase activity was 13·8 (SD 1·2) for control liver and 8·7 (SD 0·7) for B_6-deficient liver; glutamate–pyruvate transaminase activity was 4·1 (SD 1·1) for control liver and 1·43 (SD 0·6) for B_6-deficient liver). Also the serum concentrations of glucose, triacylglycerol and cholesterol did not appear to differ significantly among the four groups (Table 2).

The histochemical staining of the aortic tissue from B_6--deficient rats treated with homocysteine showed that the subendothelial space increased after 14 d, whereas B_6-deficient rats treated with only distilled water and control rats treated with distilled water or homocysteine showed no change ($P<0{\cdot}001$; Student's *t* test; Fig. 3 (A) and (B)). Furthermore, the immunohistochemical detection for eNOS showed the positive staining in the endothelial cells of B_6-deficient rats given homocysteine (Fig. 3 (C)). B_6-deficient rats treated with homocysteine showed the appearance of atherosclerosis-like alterations after 14 d, which was earlier than the period of 42 d when the atherosclerosis-like alterations appeared in the homocysteine-treated rats.

There was no statistical difference in serum homocysteine level among the B_6-supplemented groups and the B_6-deficient groups, though the level of serum homocysteine was higher in homocysteine-treated rats ($P<0{\cdot}05$; Kruskall–Wallis test; Fig. 4). However, the serum lipid peroxide levels in B_6--deficient and homocysteine-treated rats were higher compared with those of control rats ($P<0{\cdot}05$; Kruskall–Wallis test; Fig. 5). These results suggest the earlier expression of endothelial dysfunction due to high levels of lipid peroxide caused by vitamin B_6 deficiency.

Discussion

Homocysteine has been reported as an important risk factor of atherosclerosis (McCully, 1969; Hankey & Eikelboom, 1999; Hofmann *et al.* 2001; Adachi *et al.* 2002). Its oxidation generates reactive oxygen species (Loscalzo, 1996), increases cytokine production (van Aken *et al.* 2000), up regulates adhesion molecules (Silverman *et al.* 2002), and induces endothelial dysfunction (Chambers *et al.* 1999). However, the mechanism by which homocysteine induces atherosclerosis remains unclear.

On the other hand, the concentration of homocysteine is regulated by the B vitamins B_6, B_{12} and folate. Since vitamin B_{12} and folate act as cofactors for the re-methylation of homocysteine and vitamin B_6 for trans-sulfation to cysteine, low levels of these vitamins may cause hyperhomocysteinaemia.

Table 2. Serum concentrations of glucose, triacylglycerol (TG) and cholesterol in B_6-supplemented and -deficient rats

(Mean values and standard deviations)

	B_6-supplemented				B_6-deficient			
	Control		Hcy		Control		Hcy	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Glucose (mg/l)	860	62	780	164	796	95	924	112
TG (mg/l)	1524	186	1652	194	1730	203	1695	159
Cholesterol (mg/1)	428	64	404	102	398	76	389	111

Hcy, homocysteine.

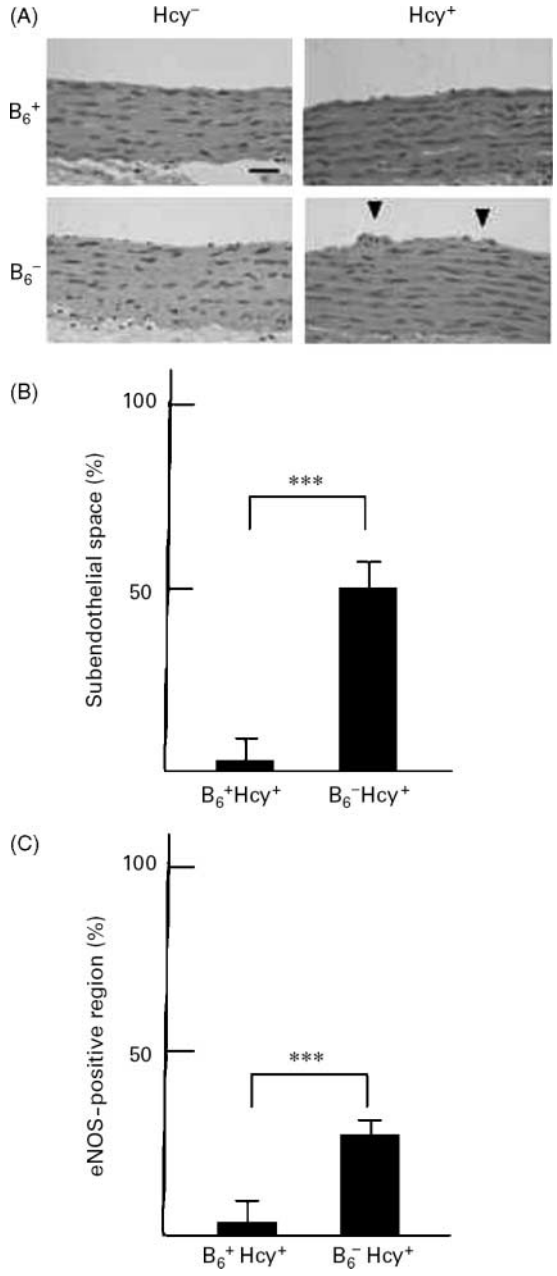

Fig. 3. Vitamin B_6 deficiency-accelerated atherosclerosis-like alterations. (A) Rats were killed at day 14 of the treatment, and tissues from rat aorta were stained with haematoxylin–eosin. ▼, Subendothelial space (× 400; ▬, 50 μm). (B) Percentage of subendothelial space. (C) Percentage of endothelial NO synthase (eNOS)-positive region. Values are means and standard deviations. ***Mean values were significantly different ($P<0{\cdot}001$; Student's *t* test).

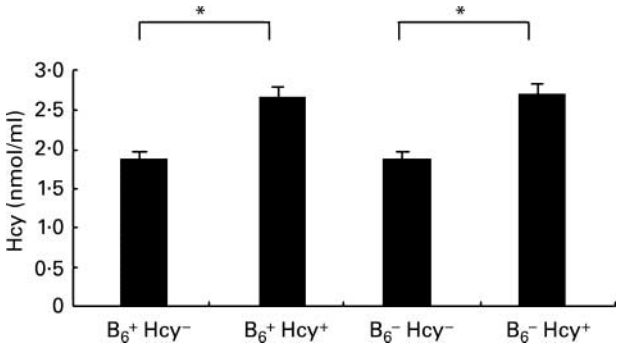

Fig. 4. Concentration of serum homocysteine (Hcy). The level of serum Hcy was examined as described on p. 1090. Values are means, with standard deviations indicated by vertical bars. *Mean values were significantly different ($P<0{\cdot}05$; Kruskall–Wallis test).

However, 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.

In the present study, we examined the effect of the antioxidant activity of vitamin B_6 on the occurrence of hyperhomocysteinaemia. Homocysteine-treated rats showed the structural alteration of endothelium after 42 d, whereas B_6-deficient rats treated with homocysteine showed the same effect after just 14 d. The level of serum homocysteine did not show any change but the level of serum lipid peroxide was increased in B_6-deficient rats. Serum homocysteine levels did not appear to change within a period of 14 d. Miller *et al.* (1992) reported that there was no difference in homocysteine concentrations between deficient and control animals after 6 weeks of the dietary regimen. These results suggest that the oxidant stress due to B_6 deficiency accelerates atherosclerosis. Indeed, Ehrenshaft *et al.* (1999) reported that pyridoxine quenches singlet oxygen at a rate comparable with that of vitamins C and E, two of the most highly efficient biological antioxidants. Interestingly, the findings of Bellamy *et al.* (1999) could be explained by folate itself having an antioxidant effect that opposes homocysteine-induced oxidative changes.

Furthermore, Barua *et al.* (2003) have reported that reactive oxygen species induce the up regulation of eNOS *in vitro*. The increase of eNOS expression observed in the present study is consistent with their findings and may support the decrease of antioxidant activity in B_6-deficient rats.

In conclusion, B_6 deficiency induced the oxidant stress which accelerates atherosclerosis and the antioxidant activity of vitamin B_6 appears to suppress homocysteine-induced atherosclerosis.

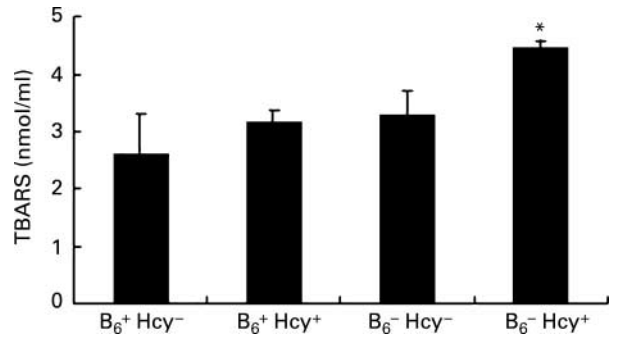

Fig. 5. Concentration of serum lipid peroxide determined by detection of thiobarbituric acid-reactive substances (TBARS). The level of serum peroxide level was examined as described on p. 1090. Values are means and standard deviations. *Mean value was significantly different from those of the $B_6^+Hcy^-$, $B_6^+Hcy^+$ and $B_6^-Hcy^-$ groups ($P<0{\cdot}05$; Kruskall–Wallis test).

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