

The Influence of Folate and Antioxidants on Homocysteine Levels and Oxidative Stress in Patients with Hyperlipidemia and Hyperhomocysteinemia

J. RACEK¹, H. RUSŇÁKOVÁ¹, L. TREFIL¹, K. K. SIALA²

¹*Institute of Clinical Biochemistry and Laboratory Diagnostics, Charles University Hospital, Plzeň, Czech Republic and* ²*Department of Internal Medicine, St. Helier Hospital, Surrey, United Kingdom*

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Summary

The aim of this study was to observe the effect of folate and antioxidants alone on homocysteine levels and oxidative stress markers, and to evaluate whether their co-administration promotes their effects. One hundred patients with hyperhomocysteinemia were randomized into four equal groups, which were then treated with folate, antioxidants or folate plus antioxidants for 2 months; group IV was a control group. Serum homocysteine, folate and oxidative stress markers were measured before the study, at the end of folate and/or antioxidants administration and 3 months later. Folate caused a significant decrease in homocysteine concentration. Antioxidants did not influence homocysteine concentration, but they improved the antioxidative defense (plasma antioxidant capacity and intraerythrocyte glutathione were increased) and partially prevented lipid peroxidation (malondialdehyde level was slightly decreased). Supplementation with folate had a similar effect on intracellular glutathione and plasma malondialdehyde. Simultaneous administration of folate and antioxidants did not show any additive effect with the exception of a slower decrease of folate concentration after its supplementation had been discontinued. Folate may be considered as an effective antioxidant in patients with hyperhomocysteinemia; this can be a result of decreased production of free radicals due to a reduced level of homocysteine. Its antioxidative effect cannot be promoted by co-administration of antioxidants.

Key words

Homocysteine • Folate • Antioxidants • Oxidative Stress • Atherosclerosis

Introduction

In 1932, de Vigneaud described homocysteine (Hcy) as a sulfur-containing amino acid and an intermediary product of methionine metabolism. It was studied for many years only in context with the hereditary

disease homocystinuria, which is characterized by high plasma Hcy concentrations, venous and arterial thrombosis, and a very rapid development of atherosclerotic changes. Later, it has been discovered that a mild hyperhomocysteinemia (HHcy) represents a risk factor of atherogenesis and thrombogenesis. Mainly

retrospective studies described the changes of Hcy concentration in patients with manifest atherosclerosis and in healthy controls (Graham *et al.* 1997, Šimon *et al.* 1996, Ueland *et al.* 1993). Metaanalysis of 27 studies (Boushey *et al.* 1995) confirmed the role of HHcy in the pathogenesis of vascular disease. Nygard *et al.* (1995) described a positive correlation of Hcy concentration with the age, cigarette consumption, total cholesterol, and the blood pressure and a negative correlation with physical activity. On the other hand, many other authors did not find most of these relations and described Hcy as an independent risk factor.

The cause of mild HHcy is usually a hereditary defect of any of the Hcy metabolic enzymes (cystathionine β -synthase, methylene tetrahydrofolate reductase) or depletion of folic acid and vitamins B₆ or B₁₂; the derivatives of these vitamins are known to act as coenzymes for the above mentioned enzymes.

Hcy influences the vessel wall through a complex series of the direct toxic effects on endothelial cells (Chambers *et al.* 1999, Lentz 1997) and a nitric oxide binding or production, which hinders vasodilatation (Zhang *et al.* 1998, Pruefer *et al.* 1999). Furthermore, Hcy induces a procoagulative state due to increased thromboxane formation and platelet aggregation, factor XII activation, inhibition of protein C activator, and the facilitation of lipoprotein (a) binding to fibrin. The direct formation of adhesive molecules and stimulation of smooth muscle cell proliferation in vessel walls has also been documented by Nappo *et al.* (1999).

The idea that HHcy can promote formation of free radicals (FR) is supported in the majority of cases with only indirect evidence. Thus Hcy concentrations positively correlate with the concentration of oxidized LDL (McCully 1993); patients with HHcy usually have an increased level of the end products of lipid peroxidation, for example malondialdehyde (Aukrust *et al.* 1997) or F₂-isoprostanes (Voutilainen *et al.* 1999), and an increased susceptibility to LDL oxidation (Blom *et al.* 1995). The toxic influence of Hcy to the endothelium can be blocked by antioxidative enzyme supplementation (Kim and Pae 1996). Finally, autooxidation of thiols leads to FR formation (McCully 1993).

Hcy is an atherogenic risk factor (Nygard *et al.* 1995, Šimon *et al.* 1996). Its adverse effect is linked with the overproduction of FR, which thanks to their ability to modify LDL particles represent an added risk factor (Ross 1996, Witzum 1994). Hcy levels can be decreased by folate administration (Brattstrom 1996), while the

production and effects of FR are influenced by antioxidants.

The aim of this study was: a) to confirm the effect of folate on Hcy, b) to evaluate, whether the decrease in Hcy level is followed by amelioration of oxidative stress – decreased lipid peroxidation and preserving the original antioxidant status, and c) to evaluate, whether antioxidants promote the effect of folate on Hcy levels and oxidative stress.

Methods

Patients

One hundred patients with HHcy (serum total homocysteine >15 μ mol/l), treated at the out-patients department for lipid metabolism disorders (Institute of Clinical Biochemistry and Laboratory Diagnostics, Charles University Hospital in Pilsen), average age of 57.3 years (variance 36-66 years), were randomized into four equal age- and sex-matched groups. These patients were supplemented for two months with:

Group I – folate (Acidum folicum, Léčiva, Prague, Czech Republic) 5 mg daily;

Group II – antioxidants: vitamin E (Vitamin E, Slovafarma, Hlohovec, Slovakia) 300 mg daily, β -carotene (Beta-carotene, Nature's Bounty, USA) 24 mg daily, vitamin C (Vitamin C, Léčiva, Praha, Czech Republic) 200 mg daily, troxerutine (Cilkanol, Léčiva, Praha, Czech Republic) 600 mg daily and selenium (Selenium, Nature's Bounty, USA) 50 μ g daily;

Group III – combination of folate and antioxidants in the above mentioned daily doses;

Group IV – the control group without any supplementation.

Basic characteristics of the groups including the initial parameters of lipid metabolism are summarized in Table 1. The diet and medication of the patients have not changed for three months before the study and during it. Blood for laboratory tests was collected before supplementation (month 0), after two months of supplementation (month 2), and three months after the supplementation was completed (month 5).

Laboratory analyses

The majority of the tests were conducted in the blood serum. Heparinized venous blood (sodium heparinate) was used for oxidative stress markers and K₃EDTA venous blood for determination of folate concentration in erythrocytes. The following analyses

were determined in all samples:

- Parameters of lipid metabolism in the serum (total cholesterol, triglycerides, HDL- and LDL-cholesterol, apolipoproteins A-I and B) by kits from Roche, Mannheim, Germany.
- Markers of oxidative stress: superoxide dismutase (SOD, Kit Ransod, Randox Laboratories, Crumlin, Great Britain) and reduced glutathione (GSH, kit GSH 400, OXIS, Portland, USA) in erythrocytes, glutathione peroxidase (GSHPx, kit Ransel, Randox Laboratories, Crumlin, Great Britain) in whole blood; plasma antioxidant capacity (AOC, kit Total Antioxidant Status, Randox Laboratories, Crumlin, Great Britain) and plasma malondialdehyde (MDA) as thiobarbituric acid-reactive substances (Jentzsch *et al.* 1996).
- Homocysteine in the serum and folate in erythrocytes (immunochemical methods with AxSym Analyzer,

Abbott, Abbott Park, IL, USA).

- Other standard laboratory tests: activities of aminotransferases AST and ALT, creatinine kinase activity, glucose, urea, creatinine and uric acid concentrations in serum by common photometric tests on Olympus 2700 Analyzer (Olympus, Tokyo, Japan).

Statistics

During statistical evaluation of the results, we compared the parameters before the study, at the end of a two-month supplementation and three months following its completion, i. e. month 2 vs. 0, 5 vs. 2 and 5 vs. 0, in each group separately. For the statistical evaluation, the paired-test according to Friedman was used. The changes during these periods (i. e. months 2 – 0, 5 – 2 and 5 – 0) were compared between the groups I, II, III and IV. The Wilcoxon non-paired test was used for this purpose.

Table 1. Basic characteristics of the groups and the initial parameters of lipid metabolism.

	Group of patients			
	I	II	III	IV
Age (years, min – max)	59.0 (37 – 76)	55.7 (38 – 74)	58.1 (37 – 71)	56.4 (35 – 74)
Number (men/women)	19/6	17/8	18/7	18/7
Total cholesterol (mmol/l)	6.62 ± 0.79	6.53 ± 1.12	6.77 ± 0.93	6.69 ± 1.12
HDL-cholesterol (mmol/l)	1.34 ± 0.21	1.36 ± 0.30	1.44 ± 0.25	1.39 ± 0.29
LDL-cholesterol (mmol/l)	4.49 ± 0.77	4.14 ± 0.92	4.52 ± 0.87	4.40 ± 0.95
Triglycerides (mmol/l)	2.58 ± 0.86	2.68 ± 1.44	2.58 ± 0.90	2.70 ± 0.95
ApoAI (g/l)	1.35 ± 0.17	1.38 ± 0.17	1.40 ± 0.20	1.36 ± 0.19
ApoB (g/l)	1.24 ± 0.21	1.29 ± 0.27	1.35 ± 0.20	1.28 ± 0.33

Data are expressed as mean ± S.D.

Results

The levels of Hcy, folate and oxidative stress markers (SOD, GSHPx, GSH, AOC and MDA) are summarized in Tables 2-5. Figures 1 and 2 show the course of GSH (principal intracellular antioxidant) and MDA (marker of lipid peroxidation) concentrations.

Two-month supplementation with folate or/and antioxidants had the following results:

Folate caused a significant decrease in Hcy concentration ($p < 0.001$), while antioxidants did not influence Hcy concentration but improved antioxidative defense of the patients (both plasma antioxidant capacity and intraerythrocyte GSH increased; $p < 0.05$ and $p < 0.001$ respectively). Furthermore, they partially prevented lipid

peroxidation, while MDA levels were slightly but significantly decreased ($p < 0.05$).

Supplementation with folate had a similar effect on intracellular GSH and plasma MDA as antioxidants. This finding can be interpreted as a result of decreased production of free radicals during the lowering of Hcy level. Folate can thus be considered as an effective antioxidant.

Three months after the end of folate supplementation, Hcy level again increased, but not to its initial value. Similarly, a drop of antioxidant administration led to an incomplete decrease of GSH concentration. MDA and antioxidant capacity values returned to their initial values within three months after finishing any supplementation.

The simultaneous administration of folate and antioxidants did not show any additive effect with the exception of a slower decrease of folate concentration after finishing its supplementation.

No changes in serum lipids, glucose and tests

showing liver or kidney function impairment were observed in any of the groups. The activity of antioxidative enzymes SOD and GSHPx had also constant activities during the whole study.

Table 2. Changes in laboratory parameters of 25 patients supplemented with folate

Parameter	Month 0	Month 2	Month 5
<i>Homocysteine (μmol/l)</i>	17.2 ± 2.4	12.9 ± 3.0 ***	14.6 ± 3.6 *
<i>Folate (μg/l ery)</i>	268 ± 86	506 ± 100 ***	268 ± 83 ***
<i>SOD (U/g Hb)</i>	1275 ± 73	1230 ± 81	1280 ± 86
<i>GSHPx (U/g Hb)</i>	52.7 ± 9.4	49.9 ± 10.4	49.6 ± 11.0
<i>GSH (mmol/l ery)</i>	1.60 ± 0.26	2.19 ± 0.22 ***	1.96 ± 0.20 +
<i>AOC (mmol/l)</i>	1.40 ± 0.08	1.39 ± 0.07	1.40 ± 0.08
<i>MDA (μmol/l)</i>	4.15 ± 0.63	3.29 ± 0.91 **	4.20 ± 0.90 **

Data are as mean ± SD. Significant changes in comparison with the previous blood sample (i. e. month 2 vs. 0 and month 5 vs. 2): * p<0.05; ** p<0.01; *** p<0.001, significant changes between month 5 and pre-study results (month 0): +p<0.05; ++ p<0.01).

Table 3. Changes in laboratory parameters of 25 patients supplemented with antioxidants

Parameter	Month 0	Month 2	Month 5
<i>Homocysteine (μmol/l)</i>	15.3 ± 3.1	15.9 ± 4.3	15.8 ± 3.6
<i>Folate (μg/l ery)</i>	252 ± 91	243 ± 70	246 ± 74
<i>SOD (U/g Hb)</i>	1267 ± 93	1252 ± 86	1266 ± 122
<i>GSHPx (U/g Hb)</i>	56.7 ± 10.1	58.8 ± 8.6	57.6 ± 8.7
<i>GSH (mmol/l ery)</i>	1.54 ± 0.19	2.21 ± 0.36 ***	1.93 ± 0.31 * ++
<i>AOC (mmol/l)</i>	1.35 ± 0.08	1.39 ± 0.08 *	1.36 ± 0.09 *
<i>MDA (μmol/l)</i>	3.55 ± 0.67	3.10 ± 0.72 *	3.74 ± 0.89 *

For legend see Table 2

Table 4. Changes in laboratory parameters of 25 patients supplemented with a combination of folate and antioxidants

Parameter	Month 0	Month 2	Month 5
<i>Homocysteine (μmol/l)</i>	16.6 ± 2.6	12.7 ± 2.7 ***	14.8 ± 3.2 * +
<i>Folate (μg/l ery)</i>	267 ± 77	487 ± 99 ***	408 ± 80 ++
<i>SOD (U/g Hb)</i>	1233 ± 82	1213 ± 93	1242 ± 91
<i>GSHPx (U/g Hb)</i>	53.7 ± 8.4	53.5 ± 8.9	54.7 ± 9.4
<i>GSH (mmol/l ery)</i>	1.64 ± 0.28	2.16 ± 0.38 ***	1.94 ± 0.27 ++
<i>AOC (mmol/l)</i>	1.34 ± 0.06	1.38 ± 0.05 *	1.34 ± 0.06 *
<i>MDA (μmol/l)</i>	3.79 ± 0.77	3.22 ± 0.55 *	3.97 ± 0.96 *

For legend see Table 2

Table 5. Changes in laboratory parameters of 25 patients without any supplementation

Parameter	Month 0	Month 2	Month 5
Homocysteine ($\mu\text{mol/l}$)	16.9 ± 2.5	16.3 ± 2.9	16.7 ± 3.1
Folate ($\mu\text{g/l ery}$)	259 ± 66	271 ± 88	261 ± 82
SOD (U/g Hb)	1244 ± 88	1281 ± 95	1264 ± 93
GSHPx (U/g Hb)	55.8 ± 7.4	54.5 ± 8.4	56.1 ± 8.2
GSH (mmol/l ery)	1.61 ± 0.33	1.58 ± 0.37	1.57 ± 0.29
AOC (mmol/l)	1.38 ± 0.06	1.40 ± 0.06	1.38 ± 0.07
MDA ($\mu\text{mol/l}$)	3.82 ± 0.88	3.75 ± 0.67	3.84 ± 0.92

For legend see Table 2

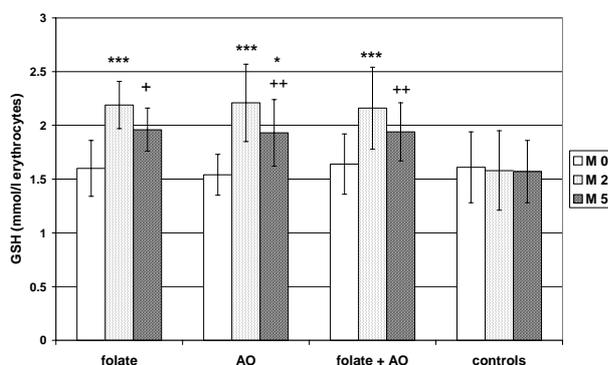


Fig. 1. Changes in GSH concentrations in erythrocytes in individual groups of patients with different supplement types. AO – antioxidants; M0, M2, M5 = month of the study; symbols describing the statistical significance are the same as in Table 2.

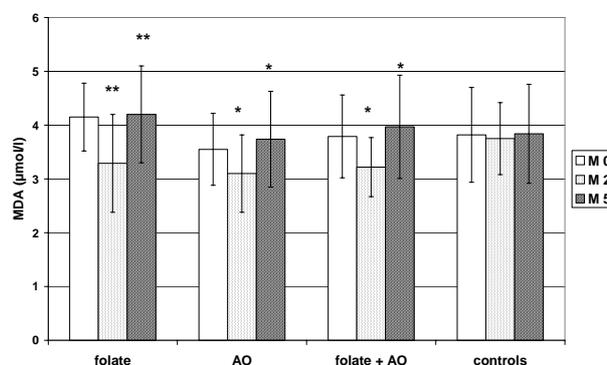


Fig. 2. Changes in plasma MDA concentrations in individual groups of patients with different supplement types (AO – antioxidants; M0, M2, M5 = month of the study; symbols describing the statistical significance are the same as in Table 2.

Discussion

Of the three vitamins, which are necessary for normal Hcy metabolism, depletion of folic acid is most frequently reported. Folic acid is far more effective as an Hcy-lowering agent compared with vitamins B₆ and B₁₂ that cause little, if any, reduction in Hcy (Chauveau *et al.* 1996, Dierkes *et al.* 1999). The daily dose of folate, which was used in clinical studies as a supplement, varied quite considerably. While some authors chose fortification with low doses of folic acid (Jacques *et al.* 1999, Schorah *et al.* 1998), others used a synthetic drug in a daily dose of less than 1 mg (Brattstrom 1996) to 10 mg (Chauveau *et al.* 1996, Landgren *et al.* 1995). In our study, we decided to administer the most frequently used supplementation dose of 5 mg (Fonseca *et al.* 1997).

As anticipated, folate supplementation resulted in a significant increase of its concentration within erythrocytes. Hcy concentration significantly decreased due to folate supplementation in accordance with the

findings of other authors (Beaulieu *et al.* 1999, Chauveau *et al.* 1996, Schorah *et al.* 1998). Antioxidant supplementation had no effect on Hcy concentration. Furthermore, there were no additive effects of antioxidants and folate with respect to an influence on Hcy levels. In the group supplemented with folate only, erythrocytic folate levels returned to their original levels within three months of supplementation. The addition of antioxidants resulted in a slower decline in these levels ($p < 0.05$); the cause of this is unclear.

As mentioned in the Introduction, it has been proposed that HHcy may cause endothelial dysfunction. This could be explained partly as a direct toxic effect on endothelial cells and partly as a result of FR overproduction. Free radicals enhance lipid peroxidation and oxidized LDL itself represents a source of further damage to endothelial cells, leading to the production of adhesion molecules. Toxic effects of Hcy are mediated especially by FR, although some studies do not support this hypothesis (Blom *et al.* 1995, Fonseca *et al.* 1997). It

has been proposed that homocysteine thiolactone is the substance, which may modify LDL particles. These homocysteine-modified LDL particles are then, as in the case of glycated or oxidized LDL, absorbed by macrophages resulting in the formation of foam cells (McCully 1993).

Evidence suggesting that HHcy patients are subjected to elevated levels of oxidative stress is mostly indirect. These subjects have been found to have unusually raised levels of lipid peroxidation products and these effects of Hcy can be blocked by AO. Increased concentrations of MDA have been described in persons following a methionine load, which is known to lead to HHcy (Domagala *et al.* 1997). Similarly, pigs with experimentally induced HHcy through repeated inhalation of nitrous oxide were found to have high MDA concentration in their myocardial tissue (Young *et al.* 1997). A positive correlation was found between F₂-isoprostanes and Hcy levels; isoprostanes are considered sensitive markers of lipid peroxidation (Voutilainen *et al.* 1999). Increased activities of antioxidative enzymes SOD and GSHPx in patients with HHcy may also be caused by FR overproduction (Moat *et al.* 2000). Hultberg *et al.* (1993) described co-operation of Hcy and copper ions in oxidative stress: their co-administration *in vitro* caused a more pronounced decrease of intracellular reduced glutathione than administration of any of these substances alone.

Toxic effects of both Hcy and Hcy in combination with Cu²⁺ ions or hydrogen peroxide on the culture of umbilical vein cells was found to be hindered by catalase and decreased by deferoxamine (Jones *et al.* 1994). Hcy promoted the mitogenic effect of platelet-derived growth factor BB in the culture of smooth muscle vascular cells; this effect was also inhibited by catalase (Nishio and Watanabe 1997). Catalase and SOD blocked Hcy neurotoxicity in an experiment with rat cerebellar cells in a tissue culture (Kim and Pae 1996). Administration of vitamins C and E to persons with mild HHcy hindered the activation of hemocoagulation and decreased adhesive properties of endothelium (Nappo *et al.* 1999). Similarly, vitamin C alone prevented endothelial cells impairment by high Hcy concentration after methionine load to healthy volunteers (Chambers *et al.* 1999). In our study, the administration of antioxidants caused an increase in plasma antioxidant capacity and GSH concentration. Activities of antioxidative enzymes SOD and GSHPx remained constant and, contrary to the results of Henning *et al.* (1999), we did not find their

increased activity. Nevertheless, their patients had chronic renal failure. In accordance with the improvement of antioxidative defense, we found a lower MDA level following supplementation with antioxidants as a result of a reduced lipid peroxidation degree. These findings could also be expected and are in agreement with the results of our studies with administration of antioxidants in other clinical situations characterized by overproduction of free radicals (Jeschke *et al.* 1995, Kadeřábková *et al.* 1997). The observation of a significant increase of GSH in erythrocytes and decrease of plasma MDA after supplementation with folate alone represents another interesting result. Co-administration of antioxidants and folate had no additive effect on oxidative stress markers or on Hcy levels.

The influence of folate on the pro-oxidative effect of Hcy has been studied by only a few researchers. Carmody *et al.* (1999) observed increased proliferation of smooth muscle cells of the vessel wall and an elevation in the DNA synthesis rate in these cells as a result of a raised Hcy concentration *in vitro*. The addition of folate significantly inhibited this proliferation; vitamins B₆ and B₁₂ did not show this effect. It is known that cell proliferation can be caused by an increased production of free radicals (Griendling and Ushio-Fukai 1998). In another experiment, folate improved endothelial function in subjects with HHcy (Brattstrom and Wilcken 2000). Laboratory markers of endothelial damage (soluble thrombomodulin and von Willebrand factor) decreased as a result of three-month administration of folate with vitamin B₆ (Constans *et al.* 1999). A vitamin cocktail containing 0.65 mg of folate caused a decrease in Hcy concentration and reduced LDL oxidability *in vitro* after 15 days; in this case it was not possible to evaluate the antioxidative effect of folate alone (Bunout *et al.* 2000). Three-months of folic acid administration had a positive effect on oxidative stress markers in hemodialysis patients (Bayés *et al.* 2001) – MDA concentration decreased by 40 % and the titer of antibodies against oxidized LDL decreased by 13 %. In our study folate administration had no effect on the activities of antioxidative enzymes and plasma AOC also remained constant. On the other hand, other oxidative stress markers improved similarly as in the case of antioxidant administration: the average increase of the principal intracellular antioxidant, reduced glutathione, was 36.9 % (p<0.001), average MDA decrease was 20.7 % (p<0.05). These changes indicate an improvement of antioxidative defense and a reduction of lipid

peroxidation. Three months after the end of folate administration, only a slightly increased concentration of glutathione remained, MDA concentration returned to its original value.

Both folate and antioxidants improved antioxidative defence and lowered lipid peroxidation in patients with hyperhomocysteinemia, while supplementation with folate only caused a significant decrease of Hcy concentration. Simultaneous administration of folate and antioxidants resulted in a slower decrease of folate concentration after finishing its supplementation. No

changes of plasma lipid spectrum and activities of antioxidative enzymes superoxide dismutase and glutathione peroxidase were observed in any of the groups.

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Reprint requests

Prof. MUDr. Jaroslav Racek, DrSc., Institute of Clinical Biochemistry and Hematology, Charles University Hospital, Alej Svobody 80, 304 60 Plzen, Czech Republic, e-mail: racek@fnplzen.cz