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Original Article

Improvement of endothelial function in uraemic patients on peritoneal dialysis: a possible role for 5-MTHF administration

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Abstract

Background. Hyperhomocysteinaemia is an independent risk factor for the development of atherosclerosis. Furthermore, homocysteine induces endothelial dysfunction by an increased inactivation of nitric oxide. In patients with chronic renal failure, the administration of folic acid or its metabolites reduces but does not normalize plasma homocysteine concentrations.

Methods. We examined the effect of oral treatment with 15 mg/daily of 5-methyltetrahydrofolate (5-MTHF) for 12 weeks, on homocysteinaemia and endothelial function in 19 patients undergoing peritoneal dialysis and compared them, for the same period of time, to a control group of patients on peritoneal dialysis. Endothelial function was evaluated by B-mode ultrasonography on the brachial artery. Flow-mediated dilation (FMD) was recorded during reactive hyperaemia produced by the inflation of a pneumatic tourniquet. Nitroglycerine-mediated dilation (NMD) was recorded after sublingual administration of glyceryl trinitrate. Finally, oxidative stress was assessed by evaluating the conjugated dienes plasma levels.

Results. Plasma homocysteine concentrations fell by 30% after oral treatment with 5-MTHF. Endothelial function improved significantly after oral 5-MTHF treatment ($13.8 \pm 1.2\%$ vs $11.4 \pm 1.4\%$; $P < 0.02$) while in the control group we observed a worsening of basal values from $12.1 \pm 2.66\%$ to $8.7 \pm 2.90\%$ ($P < 0.02$). The conjugated dienes plasma levels did not change either.

Conclusions. Our study demonstrated that 5-MTHF administration improves endothelial dysfunction in patients undergoing peritoneal dialysis. This effect appears to be independent of the reduction in homocysteine plasma levels.

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Introduction

Several studies have already shown a positive relationship between homocysteine and cardiovascular events in the general population [1]. Hyperhomocysteinaemia is frequently seen in patients with chronic kidney disease and is present in more than 90% of patients on dialysis treatment, independent of the vitamin B status [2]. Four prospective studies have observed that homocysteine is a marker of cardiovascular mortality and morbidity in haemodialysis patients [3–6]. However, no causal relationship between hyperhomocysteinaemia and cardiovascular outcomes was demonstrated. Some studies have reported an inverse relationship between homocysteine and cardiovascular risk [7,8]. Homocysteine, however, was also positively correlated with nutritional status, and malnutrition could have been a confounding factor.

Treatment with folic acid lowers, but does not normalize homocysteine plasma concentrations in dialysis patients. In healthy subjects, administration of folic acid prevents endothelial dysfunction produced by acute [9] or persistent hyperhomocysteinaemia [10]. Similar improvement of endothelium-dependent, flow-mediated vasodilatation is observed in patients with familiar hypercholesterolaemia, independent of homocysteine plasma levels [11]. Finally, folic acid improves endothelial function in patients with coronary artery disease [12]. The effect of folic acid on endothelial function in uraemic patients on dialysis treatment is still a matter of debate. While some studies showed no improvement of endothelial function in haemodialysis patients [13] or in peritoneal dialysis patients [14] by the administration of folic acid, in a previous study we found an improvement of endothelial function with the active metabolite of folic acid

5-methyltetrahydrofolate (5-MTHF) treatment, administered intravenously 15 mg/week, in patients undergoing acetate free biofiltration [15].

The aim of the present study was to evaluate whether 5-MTHF, administered orally for 12 weeks, could restore endothelial function and affect homocysteine plasma concentrations in peritoneal dialysis patients.

Subjects and methods

Patients

We studied a group of patients treated with 5-MTHF (treatment group) and a control group of patients. The first one consisted of 19 clinically stable patients (4 diabetics), 10 men and 9 women (mean age: 56 ± 3 years), with end-stage renal disease (ESRD; autosomal dominant polycystic disease in four cases, chronic pyelonephritis in one, diabetic nephropathy in 2, nephroangiosclerosis in 5, IgA nephropathy in 1, membranous glomerulonephritis in 1, amyloidosis in 1, focal glomerulosclerosis in 1, nephronophthisis in 1, 2 unknown), on peritoneal dialysis for a mean period of 17 ± 5 months. 10 patients were in continous ambulatory peritoneal dialysis (CAPD) with isotonic dialysate and icodextrin. Nine patients were in automated peritoneal dialysis (APD) with isotonic dialysate, hypertonic dialysate and icodextrin. The second group acted as controls and consisted of 8 patients (two diabetics), 5 men and 3 women (mean age: $66 + 2$ years) with ESRD [diabetic nephropathy in 2 cases, nephroangiosclerosis in 2, chronic pyelonephritis in 2, chronic glomerulonephritis in 1 and non-steroidal anti-inflammatory drug (NSAID) abusing in 1] on peritoneal dialysis for a mean period of $23.6 + 4.4$ months. 5 patients were in CAPD with isotonic dialysate and icodextrin. 3 patients were in APD with isotonic dialysate, hypertonic dialysate and icodextrin. All the patients were taking antihypertensive medications and the two groups were similar in terms of hypolipaemic treatment (27% in the treatment group and 37% in the control group were taking statins, $P = $ NS). All patients gave their informed consent to participate in the study.

Study design

According to the study design, patients first underwent a 12-month washout from folic acid and vitamin B12 supplementation (T1). The study group patients received treatment with oral administration of 15 mg 5-MTHF daily for 12 weeks (T2), while the controlled group did not receive any drug influencing plasma homocysteine concentration for the same period of time.

Plasma homocysteine and conjugate dienes were determined at T1 and T2. Endothelial function during reactive hyperaemia and after administration of glyceryl trinitrate was evaluated at the beginning and at the end of the study in both groups of patients.

Laboratory methods

After an overnight fast, blood was collected into tubes containing NaEDTA (0.1 mg/ml), and plasma was separated by low-speed centrifugation at $4°C$. The measurement of plasma lipids was performed by standard enzymatic techniques (HORIBA ABX for Cobas Mira Plus, Montpellier, France).

Plasma homocysteine, plasma folates and erythrocyte folate concentrations were determined as described elsewhere [15].

Conjugated dienes were assessed as follows: 200 μl of suspension was mixed with 3 ml of a solution of chlorophormium/methanol (2:1) and 200 μl of PBS. After 10 min of centrifugation at 3000 r.p.m 1.5 ml of chlorophormic phase was dried with N_2 and then, after a dilution with cyclohexane, learned at $\lambda = 234$ nm and $\lambda = 300$ nm. Adsorbance was multiplied with $2.95 \times 10^4 \, M^{-1} \, cm^{-1}$, obtaining molar concentration.

Endothelial function test

Endothelial function was evaluated non-invasively by B-mode ultrasonography (Biosound Au4 idea) as described elsewhere [15]. Briefly, each subject was requested to lie at rest for 10 min in a temperature-controlled room ($21°C \pm 1$), and the first scan of brachial artery in the left arm was taken.

This was followed by inflation of a standard pneumatic tourniquet placed around the upper arm at a pressure of 250 mmHg, followed by deflation after 4.5 min. Electrocardiography was monitored continuously during the study and measurements were taken at the end diastole. The reproducibility of the assay was determined by repeating the measurement on 15 subjects on consecutive days by the same observer. The coefficient of variation of the assay was 7%. During each test, vessel images were taken at rest and during reactive hyperaemia; flow-mediated dilation (FMD) was calculated 90–210 s after the deflation of a pneumatic tourniquet. Nitroglycerine-mediated dilation (NMD) was calculated as the percentage in variation between the basal diameter and the maximum diameter after sublingual administration or glyceryl trinitrate 0.3 mg.

Statistical analysis

Data were analysed using SPSS 13.0 for Windows (Chicago, IL, USA). Results are reported as mean $\pm$ SEM, unless otherwise stated. Group differences in continuous variables were determined by using a paired Student's t-test. Group differences or Pearson correlations with $P < 0.05$ were deemed statistically significant.

Results

No differences at baseline were seen in terms of patient characteristics and biochemical data between treatment and control group (Table 1). Biochemical results at T1 and T2 in the treated patients are shown in Table 2. At the beginning of the study, endothelial function showed a trend in the correlation with intraerythrocyte folates ($R = 0.51$, $P = 0.077$).

Plasma homocysteine concentrations were significantly higher at the end of the washout (37.3 ± 5.6 μmol/l) and decreased by about 30% after 5-MTHF oral treatment (to 20.71 ± 1.3 μmol/l; $P < 0.008$) (Figure 1). In the control group,

Table 1. Comparison of patient characteristics at baseline: treated group *vs* control group

	Treatment group T1	Control group T1	P
Sex (M/F)	10/9	5/3	NS
Age (years)	56 ± 3	66 ± 2	NS
Dialytic duration (months)	17 ± 5	23.6 ± 4.4	NS
Plasma homocysteine (µmol/l)	37.3 ± 5.6	44.3 ± 9.7	NS
Erythrocyte folates (nmol/l)	611 ± 122.7	ND	
Serum folate (ng/ml)	8.85 ± 1.17	6.23 ± 1	NS
Kt/V (SPVV)	2.2 ± 0.2	2.3 ± 0.1	NS
Vit. B12 (pg/ml)	604.2 ± 485.1	601.5 ± 111.9	NS
Albumin (g/dl)	3.5 ± 0.1	3.98 ± 0.1	NS
FMD (%)	11.5 ± 1.4	12.2 ± 2.6	NS
NMD (%)	21.2 ± 2.1	20.3 ± 2.04	NS
Total cholesterol (mg/dl)	205 ± 9.1	186 ± 4.5	NS
HDL-c (mg/dl)	44 ± 3.1	41 ± 4.2	NS
LDL-c (mg/dl)	128 ± 7	117 ± 8.4	NS
Triglycerides (mg/dl)	170 ± 21.3	160 ± 15.6	NS
Conjugated dienes (title)	172 ± 20.1	189 ± 30.2	NS
Haemoglobin (g/dl)	12 ± 0.4	12 ± 0.4	NS

ND, not determined; NS, not significant.

Table 2. Treated group characteristics at T1 and at T2

	Treatment group		P
	T1	T2	
Sex (M/F)	10/9		
Age (years)	56 ± 3		
Dialytic duration (months)	17 ± 5		
Plasma homocysteine (µmol/l)	37.3 ± 5.6	20.71 ± 1.3	$P = 0.008$
Erythrocyte folates (nmol/l)	611 ± 122.7	ND	
Serum folate (ng/ml)	8.85 ± 1.17	>45.4	$P = 0.00001$
Kt/V (SPVV)	2.2 ± 0.2	2.0 ± 0.4	NS
Vit. B12 (pg/ml)	604.2 ± 485.1	598.5 ± 66.2	NS
Albumin (g/dl)	3.5 ± 0.1	4.0 ± 0.1	NS
FMD (%)	11.5 ± 1.4	13.8 ± 1.2	$P = 0.016$
NMD (%)	21.2 ± 2.1	20.1 ± 2.9	NS
Total cholesterol (mg/dl)	205 ± 9.1	196 ± 8.8	NS
HDL-c (mg/dl)	44 ± 3.1	47 ± 3.2	NS
LDL-c (mg/dl)	128 ± 7	117 ± 8.4	NS
Triglycerides (mg/dl)	170 ± 21.3	160 ± 15.6	NS
Conjugated dienes (title)	172 ± 20.1	189 ± 30.2	NS
Haemoglobin (g/dl)	12 ± 0.4	12 ± 0.4	NS

ND, not determined; NS, not significant.

homocysteine plasma concentrations increased by 12% (from 44.3 ± 9.7 µmol/l to 49.8 ± 17.9 µmol/l).

Folate treatment did not modify plasma levels of conjugated dienes (189 ± 30 *vs* 172 ± 20 mg/dl, $P = $ NS) or lipid parameters.

Endothelial function in terms of the percent variation in vessel diameter induced by hyperaemia increased significantly after 5-MTHF oral treatment (13.8 ± 1.2 *vs* 11.5 ± 1.4%; $P = 0.016$) (Figure 2), while in the control group we observed a worsening of basal values from 12.2 ± 2.6% to 8.7 ± 2.9% ($P = 0.016$).
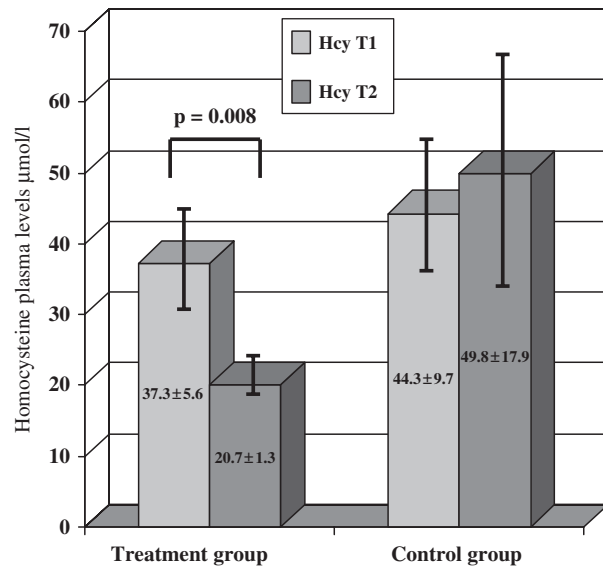
Fig. 1. Effect of treatment with 5-MTHF on plasma homocysteine concentration at T1 and T2 in the treated group and in the control group of patients.
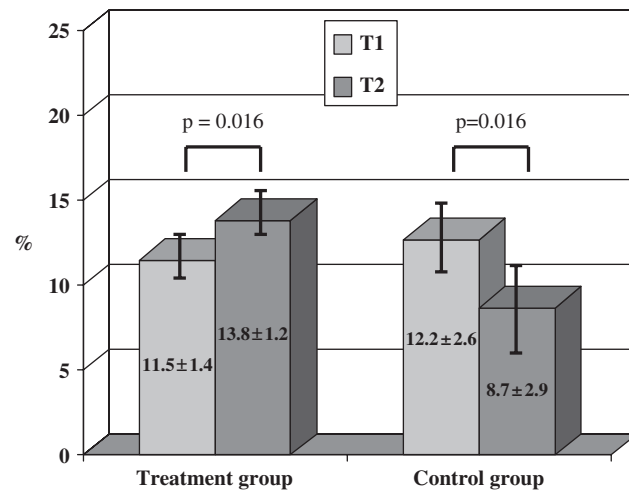
Fig. 2. Flow-mediated dilation (FMD) in peritoneal patients before and after 5-MTHF oral treatment and in the control group. The figure shows a significant impairment of FMD after the administration of 5-MTHF and a worsening of endothelial function in the control group of patients.

Non-endothelium-derived dilation by nitrate administration did not change in both groups of patients (21.2 ± 2.1 *vs* 20.1 ± 2.9%, $P = $ NS in the treated group and 20.3 ± 2.04 *vs* 20.5 ± 3.1% in the control group). We did not observe any relationship between homocysteine plasma concentrations and endothelial function.

Discussion

In haemodialysis and peritoneal dialysis patients, all the authors reported that folic acid or its derivatives,

administered orally at doses varying from 1 to 60 mg, all appear to reduce homocysteine plasma levels from 30% to 40% [16]. However, normalization of homocysteine plasma concentrations has never been demonstrated. In this study, we confirmed these results, observing a reduction of homocysteine by 30% from the basal values administering 5-MTHF. We also observed an improvement of endothelial function in our population of peritoneal dialysis patients without detecting any relationship between homocysteine plasma concentrations and endothelial function. Instead, we found a positive relationship between erythrocyte folate and FMD at baseline. Patients' clinical conditions and plasma albumin levels were not changed during the study.

Recently, Jiang and colleagues [17] found in mouse and human endothelial cells that hyperhomocysteinaemia impairs endothelial function and endothelial nitric oxide synthase (eNOS) activity, primarily through protein kinase C activation. They found that arterial relaxation in response to the endothelium-dependent vessel relaxant, acetylcholine or nitric oxide synthase (NOS) activator, was significantly impaired in cystathionine β-synthase null (CBS −/−) mice. However, the vascular smooth muscle cell (VSMC) response to the nitric oxide (NO) donor (SNAP) was preserved in CBS −/− mice. In addition, superoxide dismutase and catalase failed to restore endothelium-dependent vasodilatation. Hcy-mediated eNOS inhibition was associated with decreased e-NOS protein expression and increased threonine 495 phosphorylation of eNOS in human aortic endothelial cells (HAECs). Ultimately, a protein kinase C (PKC) inhibitor, GF109203X (GFX), reversed Hcy-mediated eNOS inactivation and threonine 495 phosphorylation in HAECs. Hyperhomocysteinaemia causes endothelial dysfunction and it is a marker of cardiovascular morbidity and mortality in uraemic patients [3–6]. Nevertheless, malnutrition could overcome it [18] and the beneficial effect of folic acid on cardiovascular outcome could be lost. The majority of clinical trials failed to show any improvement of cardiovascular risk by the administration of folic acid [19–21], only one showed a beneficial effect [22]. The role of folic acid in improving cardiovascular outcome in uraemia is not yet clear. The confounding results of these trials could be a consequence of the malnutrition present in a large portion of dialysis patients. In fact, in this condition, folic acid could be less effective in improving hyperhomocysteinaemia either for the low levels of homocysteine plasma concentration in malnourished patients and because malnutrition could overcome folic acid supplementation in worsening their cardiovascular outcome.

Also, a resistance to folic acid supplementation is described in uraemia. Folate of dietary origin, introduced in the form of polyglutamate, requires the presence of glutamyl carboxypeptidase for transformation to monoglutamate in the intestinal wall. This passes via the portal vein to the liver where it is first transformed into dihydrofolate, then tetrahydrofolate and finally into 5-10-MTHF. The methylenetetrahydrofolate reductase (MTHFR) enzyme reduces the latter to 5-MTHF. Thanks to enterohepatic circulation, 5-MTHF returns to the small intestine and, after absorption, is distributed to the tissues.

In uraemia, experimental and clinical data suggest the presence of plasma inhibitors that limit the activity of the conjugases responsible for transforming polyglutamates into monoglutamates [23] and for transmembrane transport of folic acid [24]. Although fragmentary, these data suggest that both oral and intravenous treatment with active metabolites of folic acid should be more effective than the use of folic acid itself. However, the use of the active forms of folic acid (5-MTHF and folinic acid) was no more efficient than folic acid in lowering plasma homocysteine concentrations [25–28].

The type of folic acid seems instead to be important in terms of endothelial function. In contrast with some studies [14,29,30] in which folic acid supplementation was ineffective in improving endothelial dysfunction in pre-dialysis, haemodialysis and peritoneal dialysis patients, we found a beneficial effect of metabolically active form of folic acid 5-MTHF in improving endothelial-dependent vasodilatation either in haemodialysis patients [15] or, now, in peritoneal dialysis patients: the use of 5-MTHF instead of folic acid could be responsible for the difference. Further studies will clarify this beneficial effect.

In uraemic patients, 5-MTHF could be the treatment of choice because beyond the lowering effect on homocysteine plasma levels, its peculiar methyl and hydrogen donation capability makes it a dual source for stabilization of the tetrahydrobiopterin (BH4) cofactor for the eNOS reaction with the additional function of being a local endothelial microenvironment antioxidant without affecting other parameters of oxidative stress, such as conjugated dienes.

Similar to tetrahydrobiopterin, 5-MTHF has been suggested to improve endothelial dysfunction directly interacting with NOS, promoting NO production [31]. It would supply the hydrogen and electrons to stabilize the requisite BH4 cofactor to run the eNOS reaction, resulting in the production of eNO and a decreased production of peroxynitrite by the endothelium [32] and could improve the electrotonically endothelium-dependent vasodilatation [33]. Restoration of the eNOS reaction by 5-MTHF supplementation has been demonstrated acutely by FMD studies in type 2 diabetes mellitus and coronary artery disease via mechanisms, which are largely independent of homocysteine lowering [34,35]. Improvement of oxidative stress by normalizing folic acid plasma levels could explain the beneficial effect of folic acid administration observed in numerous studies, while the reduction in homocysteine plasma levels should be considered as an efficacy marker of the ongoing treatment.

Cardiovascular mortality is still elevated in uraemic patients and it is related to oxidative stress and

inflammation. Further trials to study the effect of folic acid in reducing cardiovascular mortality in dialysis patients, should take into account the prevalent effect of malnutrition on hyperhomocysteinaemia, and the type of folic acid metabolite administered.

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