

Chronic treatment with tetrahydrobiopterin reverses endothelial dysfunction and oxidative stress in hypercholesterolaemia

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ABSTRACT

Background: Reduced availability of tetrahydrobiopterin (BH₄), an essential cofactor of nitric oxide (NO) synthase (NOS), decreases NO production and increases reactive oxygen species. Both mechanisms contribute to atherosclerotic vascular disease. Although acute supplementation of BH₄ improves endothelial dysfunction, the effect of chronic BH₄ in humans is unknown.

Objective: To investigate the effect of chronic BH₄ supplementation on endothelial function and oxidative stress in hypercholesterolaemia.

Design: Randomised double-blind, placebo-controlled trial.

Setting: University Hospital.

Patients: 22 hypercholesterolaemic patients (low-density lipoprotein (LDL) >4.5 mmol/l) were randomised to 4 weeks of oral BH₄ (400 mg twice daily) or placebo. Age-matched healthy volunteers served as controls.

Main outcome measures: Endothelium-dependent and -independent vasodilatation was assessed by venous occlusion plethysmography. To elucidate the mechanisms of BH₄ effect, NO release and superoxide anion (O₂⁻) production were measured in human aortic endothelial cells exposed to native LDL (2.6 mmol cholesterol/l).

Results: BH₄ plasma levels were significantly increased by oral supplementation. NO-mediated vasodilatation to acetylcholine was reduced in patients compared with controls and restored by BH₄. No effect of BH₄ on endothelium-independent vasodilatation was seen. Furthermore, 8-F₂ isoprostane plasma levels, a marker of vascular oxidative stress, were reduced by BH₄. In LDL-treated endothelial cells, BH₄ levels and NO release were reduced and O₂⁻ production increased compared with control cells. Exogenous BH₄ normalised NO and O₂⁻ production.

Conclusions: In hypercholesterolaemia, endothelial dysfunction and oxidative stress can be reversed by chronic oral treatment with BH₄. Thus, BH₄ availability is essential for maintaining NO synthesis and low O₂⁻ production by endothelial NOS *in vivo*, and may provide a rational therapeutic approach to prevent cardiovascular disease.

In the past decade, the endothelial L-arginine-nitric oxide (NO) pathway has reached the clinical arena and NO is now regarded as a marker of cardiovascular health. Indeed, loss of NO bioavailability, due to reduced synthesis by nitric oxide synthase (NOS) or scavenging by oxidative species, is a cardinal feature of endothelial dysfunction that precedes the development of overt atherosclerosis and is an independent predictor of adverse cardiovascular events.¹ Tetrahydrobiopterin (BH₄) is an

essential cofactor for all three NOS isoforms and for the aromatic amino acid hydroxylases, which are key enzymes in the biosynthesis of several neurotransmitters, including catecholamines and serotonin.² In children, inborn errors in the metabolism of BH₄ lead to cofactor deficiency, hyperphenylalaninaemia and neurological impairment.³ Supplementation with this cofactor is an established treatment for BH₄-responsive hyperphenylalaninaemia, which includes BH₄-responsive phenylalanine hydroxylase deficiency and defects in the synthesis of BH₄.⁴ However, an important role for BH₄ in the cardiovascular system, as a regulator of NOS activity, has emerged only recently.^{2,5}

All major risk factors for atherosclerotic vascular disease, including hypercholesterolaemia, have been associated with loss of NO bioavailability and endothelial dysfunction.¹ The underlying defect involves both reduced NO synthesis and increased superoxide anion (O₂⁻) production. Several oxidase systems (eg, NADPH oxidase) are potential sources of O₂⁻ and, hence, contribute to oxidative stress.⁶ However, endothelial NOS (eNOS) itself can generate O₂⁻ under certain pathophysiological conditions.⁷ The importance of BH₄ in the catalytic process of L-arginine oxidation and NO synthesis is well established.^{2,7} When intracellular BH₄ levels are low, electron transfer within the active site of NOS becomes uncoupled from L-arginine oxidation; instead molecular oxygen is reduced to form O₂⁻.⁷ Such eNOS-dependent generation of O₂⁻ has been implicated in a variety of experimental and clinical vascular disease states, including hypercholesterolaemia,⁸ diabetes mellitus,⁹ cigarette smoking,¹⁰ hypertension,¹¹⁻¹³ ageing¹⁴ and overt atherosclerosis.¹⁵ Moreover, excess O₂⁻ reacts with NO leading to the formation of the powerful oxidant peroxynitrite (ONOO⁻), which may be toxic to endothelial cells through the direct nitrosylation of lipids or proteins, and DNA damage.¹⁶

Several recent studies have suggested that acute, mostly parenteral supplementation of BH₄ may restore NO-mediated endothelial function.^{8,10,15,17-20} However, no clinical studies on the long-term cardiovascular effects of chronic BH₄ treatment have yet been performed. These data are critical for the development of BH₄ supplementation as a new approach to prevent initiation and progression of cardiovascular disease in clinical practice.

Endothelial function

Accordingly, we prospectively studied 22 patients with hypercholesterolaemia and sought to determine whether chronic oral pharmacological doses of BH₄ might improve endothelial function and oxidative stress. In addition, to understand the cellular mechanisms of BH₄ supplementation in hypercholesterolaemia, we performed a series of *in vitro* experiments using human endothelial cells.

PATIENTS AND METHODS

Patients

Twenty-two patients, mean age 57.1 years, with hypercholesterolaemia defined by LDL-cholesterol >4.5 mmol/l were studied (table 1). None had a history of cardiovascular disease nor clinical evidence of atherosclerosis on physical examination. Exclusion criteria were age <20 years and >75 years, premenopausal status, hypertension (blood pressure >140/90 mm Hg), diabetes, smoking, lipid-lowering treatment within 2 weeks before study entry. A matched control group consisting of nine volunteers with normal lipid levels was also included for comparison of baseline endothelial function (table 1).

Study protocol

Hypercholesterolaemic patients were randomised to receive either 400 mg BH₄ (Schircks Laboratories, Jona, Switzerland) twice daily, or a matching placebo (saccharose 550 mg twice daily) in a double-blind fashion for a duration of 4 weeks. To ensure the biochemical stability of BH₄, patients were instructed to store the drug at -20°C. Assessment of forearm blood flow (FBF) by venous occlusion plethysmography and measurement of F₂ isoprostanes and BH₄ plasma levels were carried out at baseline and after 4 weeks of treatment. Further standard laboratory measurements including lipid levels were analysed at the same time points. In the control group, FBF and laboratory analysis were performed as described above but only once. The study complies with the Declaration of Helsinki and was approved by the local ethics committee of the University Hospital Zürich. All patients gave their written informed consent before study entry.

Assessment of forearm blood flow

FBF was assessed using venous occlusion plethysmography (EC4; Hokanson Inc, USA) with calibrated mercury-in-silastic strain gauges as described elsewhere.²¹ In brief, all measurements were performed at the same time in the morning and patients were instructed to refrain from drinking alcohol or caffeine and from eating for 12 hours before the examination. For assessment of endothelium-dependent vasodilatation, acetylcholine (Ach; Miochol E, Ciba Vision, Switzerland) was infused into the brachial artery of the non-dominant arm at increasing concentrations of 0.15, 0.45, 1.5, 4.5 and 15 µg/100 ml forearm volume per minute for 5 minutes each. Sodium nitroprusside (SNP; Nipruss, Schwarz Pharma, Germany) was infused at concentrations of 1, 3 and 10 µg/100 ml/min for 3 minutes each to assess endothelium-independent vasodilatation. Resulting FBF (ml/min/100 ml forearm volume) was measured simultaneously in both arms, with the non-infused arm serving as control. Recordings took place at baseline and during the last minute of each drug infusion. A minimum of five consecutive single readings were obtained for each concentration and analysis was performed by a single reviewer who was blinded to the patients and study visits and to the treatment regimen. Results during intra-arterial infusions (infused/control arm) were compared with baseline readings (infused/control arm) and calculated as the percentage change in flow ratio from baseline.

Biochemical analysis

All blood samples were drawn into EDTA (1 mg/ml), separated within 1 hour after sampling and stored at -80°C until determination was performed.

Biopterin contents were determined by high-performance liquid chromatography. Total biopterin represents the sum of BH₄, 7,8-dihydrobiopterin and fully oxidised biopterin. Under acidic conditions BH₄ and 7,8-dihydrobiopterin are oxidised to biopterin. Plasma (100 ml) was acidified by addition of 20 ml of 1 M hydrochloric acid, and 50 ml of iodine solution (1% (wt/vol) iodine in 2% (wt/vol) potassium iodide) was added. Samples were mixed and incubated for 1 hour in the dark at

Table 1 Baseline characteristics

Characteristics	Control subjects	Hypercholesterolaemic subjects			p Value	
		Total	BH ₄	Placebo	Hyperchol. vs controls	BH ₄ vs placebo
Age (years)	54.4 (9.5)	57.4 (10.1)	60.8 (9.2)	53.9 (9.6)	0.49	0.14
Sex (M/F)	7/2	17/4	7/4	10/0		
Weight (kg)	76.7 (11)	77.7 (12.4)	74.9 (13.4)	80.9 (10.3)	0.83	0.32
BMI (kg/m ²)	24.0 (2.2)	25.7 (2.6)	25.7 (2.2)	25.8 (3.1)	0.11	0.9
BP systolic (mm Hg)	121.9 (12.6)	129.5 (8.1)	126.3 (8.4)	132.6 (6.3)	0.07	0.09
BP diastolic (mm Hg)	58.4 (7.5)	75.5 (3.9)	64.6 (6.3)	75.5 (3.9)	0.001	<0.001
HR (bpm)	66.8 (11.2)	66.6 (9.7)	61.6 (8.9)	71.5 (7.6)	0.96	<0.05
Haemoglobin (g/dl)	13.9 (1.2)	14.3 (1.0)	13.8 (0.8)	14.8 (1.0)	0.53	<0.05
Creatinine (µmol/l)	82.2 (11.9)	92.3 (13.7)	86.5 (11.8)	98.0 (13)	0.08	0.07
Glucose (mmol/l)	5.4 (0.7)	4.8 (0.3)	4.8 (0.2)	4.8 (0.3)	<0.05	0.71
Total cholesterol (mmol/l)	4.9 (0.8)	7.7 (1.0)	7.4 (0.8)	7.9 (1.2)	<0.001	0.15
HDL (mmol/l)	1.6 (0.4)	1.5 (0.3)	1.7 (0.3)	1.4 (0.3)	0.68	0.06
LDL (mmol/l)	2.8 (0.8)	5.3 (1.1)	5.1 (0.7)	5.5 (0.3)	<0.001	0.61
Triglycerides (mmol/l)	1.2 (0.6)	1.9 (0.9)	1.6 (0.7)	2.3 (0.8)	<0.05	0.05
AUC Acetylcholine (aU)	2029 (682)	1519 (550)	1473 (512)	1586 (600)	<0.05	0.7
AUC SNP (aU)	1527 (612)	1428 (600)	1368 (421)	1540 (699)	0.47	0.57

Data are mean (SD).

AUC, area under the curve; aU, arbitrary units; BH₄, tetrahydrobiopterin; BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; SNP, sodium nitroprusside.

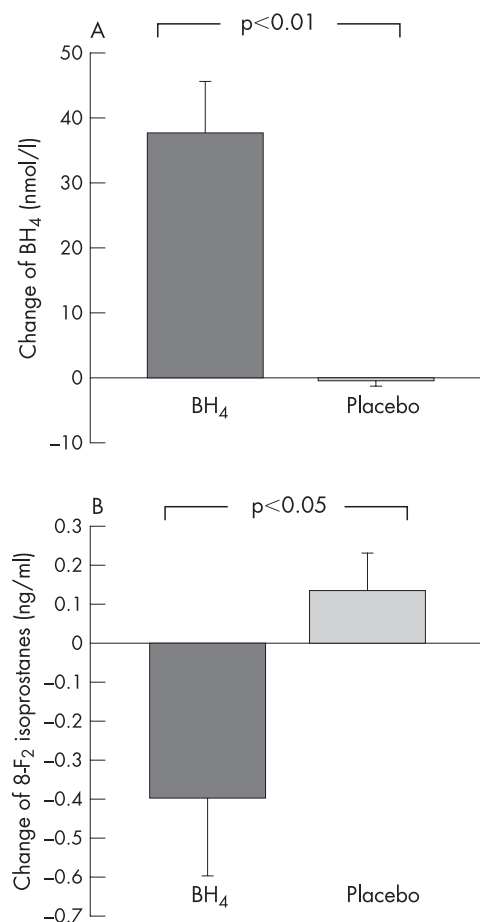


Figure 1 Changes in BH₄ and 8-F₂ isoprostane plasma levels after 4-weeks' treatment with tetrahydrobiopterin (BH₄) or placebo. (A) BH₄ levels were increased after oral BH₄ supplementation whereas no change occurred in placebo group. (B) 8-F₂ isoprostanes were significantly decreased by BH₄ compared with placebo. Data are mean (SEM).

room temperature. The reaction was stopped by adding 10 ml of 5% (wt/vol) ascorbic acid and 20 ml water. High-performance liquid chromatography of pterins was performed as described previously.²²

Plasma F₂ isoprostane was measured using an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor MI, USA).

Cell culture

Human aortic endothelial cells were obtained from Clonetics (San Diego, CA, USA) and grown in gelatin-coated flasks in optimised endothelial growth medium (Clonetics) supplemented with 10% fetal calf serum (Hyclone; Logam, UT, USA). Cells were detached by exposure to trypsin/EDTA for about 120 seconds in HEPES buffer saline, reseeded in collagen-coated 6 cm cell culture dishes. Cells were grown to confluency in humidified air (5% CO₂ at 37°C). Cells between passages 2 and 6 were used for experiments.

Low-density lipoprotein (LDL) isolation

The lipoproteins were prepared as follows: plasma from a pool of at least 20 000 donors was used as starting material. The density of the plasma was adjusted with NaBr to 1.063 g/ml, and the LDL/VLDL fraction was separated by preparative ultracentrifugation in a Centrikon T-2080 ultracentrifuge

(Kontron Instruments, Zürich, Switzerland) using a TFT 70.38 rotor (4°C, 16 hours, 55 000 rpm). The top layer containing LDL and very low-density lipoprotein (VLDL) was collected, the density adjusted to 1.019 g/ml, and LDL was separated from VLDL by ultracentrifugation under the same conditions as above. The bottom fraction containing LDL was dialysed against 0.9% NaCl, and stored under inert gas at 4°C. All preparative procedures were performed under sterile conditions. Cholesterol content was determined according to the method of Kattermann *et al*, using the CHOD-PAP method (Boehringer-Mannheim, Mannheim, Germany).

NO measurements

Direct in situ measurements of NO were carried out as described.¹¹ Immediately before NO measurements, the active tip of the L-shaped porphyrinic NO microsensor was placed directly on the surface of the endothelial cell monolayer. For maximal stimulation of eNOS calcium ionophore A23187 was injected into the cell culture dish to yield a final concentration of 1 µmol/l.

Measurement of O₂⁻ production

O₂⁻ production was measured as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c*. Briefly, human aortic endothelial cells were preincubated in Dulbecco's modified Eagle's medium without phenol red for 30 minutes at 37°C, and then cytochrome *c* (final concentration, 1 mg/ml) with or without SOD (final concentration, 500 U/ml) was added in a CO₂ incubator. After 60 minutes, the medium was removed from the cells, and the absorbance was read at 550 nm against a distilled water blank. Reduction of cytochrome *c* in the presence of SOD was subtracted from the values without SOD. The portion of O₂⁻-specific reduction of cytochrome *c* was between 20% and 35% according to the experiments. The optical density difference between comparable wells with or without SOD was converted to equivalent O₂⁻ production by use of the molar extinction coefficient for cytochrome *c* (21.0 × 10³ (mol/l)⁻¹ · cm⁻¹).

Statistical analysis

Clinical data and results are expressed as mean (SD). For cell culture experiments *n* indicates the number of experiments and data are expressed as mean (SEM). The ratio of blood flow in the infused arm compared with the control arm was calculated for each concentration of Ach and SNP, respectively. The response to Ach and SNP was then calculated as the area under the dose-response curve (AUC) and expressed in arbitrary units. A two-sided paired and unpaired Student *t*-test was used for intra- and intergroup comparison. Significance was accepted at *p* < 0.05.

RESULTS

Patient characteristics

Twenty-one patients and nine control subjects completed the study. One patient in the placebo group was excluded owing to unsuccessful arterial puncture at the second visit. Table 1 summarises the characteristics of the study group.

Adverse events

The study drug was well tolerated and no adverse events occurred in either treatment group.

Endothelial function

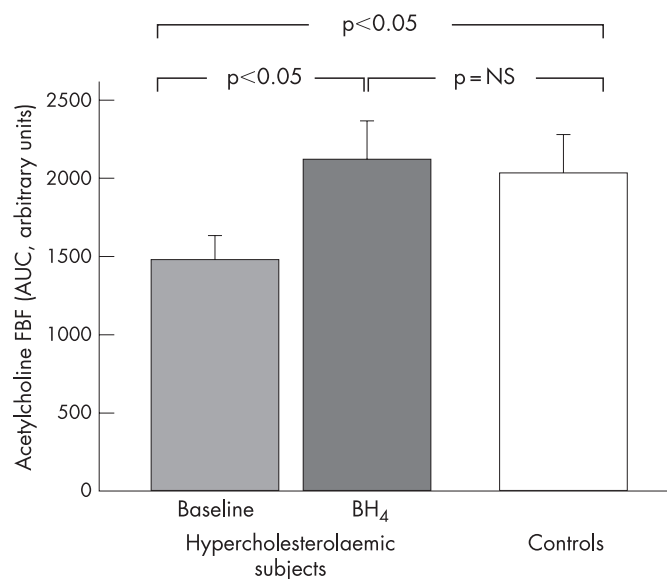


Figure 2 Effect of tetrahydrobiopterin (BH₄) supplementation on endothelial function. Forearm blood flow (FBF) response to acetylcholine was significantly impaired in hypercholesterolaemic subjects compared with controls and restored by a 4-week oral BH₄ supplementation. AUC, area under the curve. Data are mean (SD).

Laboratory variables

BH₄ plasma levels at baseline were similar between the two treatment groups (4.9 (2.3) nmol/l for the BH₄ treatment group vs 6.6 (2.7) nmol/l for placebo, *p* = NS). We observed a significant increase in plasma BH₄ levels in the BH₄ treatment group after oral supplementation (42 (26) vs 4.9 (2.3) nmol/l at baseline, *p* < 0.05), whereas no changes occurred in the placebo group (fig 1A). Chronic BH₄ treatment slightly but significantly reduced total cholesterol (7.4 (0.8) vs 7.0 (0.9) mmol/l, *p* < 0.05) and HDL cholesterol (1.7 (0.3) vs 1.5 (0.2) mmol/l, *p* < 0.05), but there was no effect on LDL cholesterol (5.1 (0.7) vs 4.9 (0.8), mmol/l, *p* = NS). Creatinine and glucose remained unchanged throughout the study.

Baseline 8-F₂ isoprostane plasma levels were similar between the two treatment arms (1.7 (0.8) and 1.8 (0.9) ng/ml, *p* = NS). Supplementation with BH₄ significantly decreased 8-F₂ isoprostane plasma levels compared with placebo (change from baseline: -0.4 ± 0.6 ng/ml vs $+0.13 \pm 0.4$ ng/ml, *p* < 0.05; fig 1B).

Effects on endothelial function and vascular measurements

The vasodilatory response to Ach was significantly impaired in hypercholesterolaemic subjects compared with controls (AUC: 1473 (512) vs 2029 (682); *p* < 0.05, table 1 and fig 2). In hypercholesterolaemic subjects, the response to Ach at baseline did not differ between the two treatment arms. Responses to SNP, as well as blood flows at rest in the non-infused arm were similar in all studied groups. BH₄ restored endothelium-dependent, NO-mediated vasodilatation (AUC: 1473 (512) vs 2116 (758), at baseline and after 4-weeks' treatment, respectively, *p* = 0.001, fig 2), but had no effect on endothelium-independent vasodilatation to SNP (AUC: 1368 (421) vs 1371 (441), before and after BH₄ treatment, respectively). In contrast, in the placebo group the response to Ach (AUC: 1586 (600) vs 1691 (638) at baseline and after 4-weeks' treatment, *p* = NS) as well as SNP remained unchanged. Heart rate and blood pressure did not significantly differ after BH₄ and placebo treatment (data not shown).

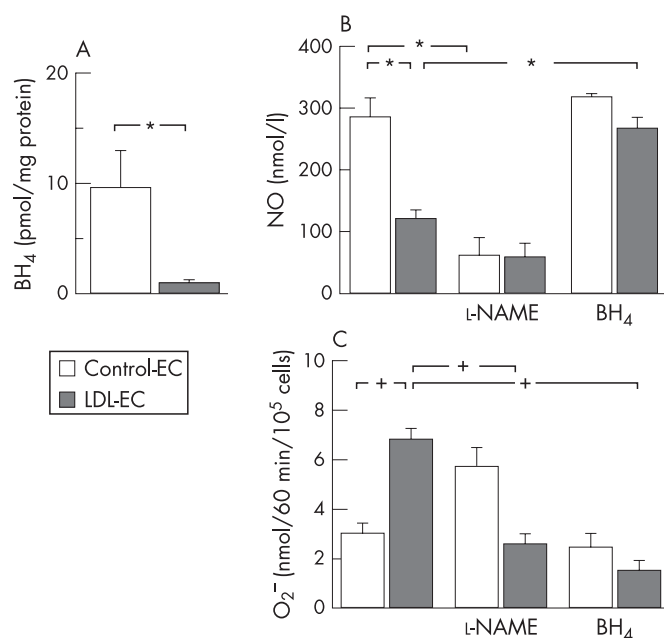


Figure 3 Intracellular tetrahydrobiopterin (BH₄) levels and changes in nitric oxide (NO) and superoxide anion (O₂⁻) production in control and human aortic endothelial cells incubated with native low-density lipoproteins (LDL) at 5.2 mmol cholesterol/l for 6 hours. Bar graphs showing (A) BH₄ levels; (B) NO release after stimulation with calcium ionophore A23187 (1 μmol/l); and (C) O₂⁻ production under basal conditions and after inhibition of eNOS by N^G-nitro-L-arginine-methyl ester (L-NAME, 3 × 10⁻⁴ mol/l) or in the presence of exogenous BH₄ (10⁻⁴ mol/l). EC, endothelial cells. Data are mean (SEM) (n = 3–8). **p* < 0.05; †*p* < 0.05.

Effects of LDL on release of NO and O₂⁻ in human aortic endothelial cells

To determine the mechanistic role of hypercholesterolaemia on the regulation of eNOS function by BH₄, human aortic endothelial cells were incubated with native LDL at 5.2 mmol cholesterol/l for 6 hours. Intracellular BH₄ levels were markedly lower in endothelial cells exposed to LDL than in control cells (9.9 (3.1) vs 0.9 (0.6) pmol/mg protein, *p* < 0.05, fig 3A). NO release and O₂⁻ production were measured in control cells and LDL-treated endothelial cells under basal conditions and after inhibition of eNOS by L-NAME (3 × 10⁻⁴ mol/l) or in the presence of exogenous BH₄ (10⁻⁴ mol/l). Basal NO release from LDL-treated cells was reduced compared with that from control cells (288 (29) vs 123 (12) nmol/l, *p* < 0.05, fig 3B). In contrast, in LDL-treated cells the basal production of O₂⁻ was higher than in controls (6.8 (0.4) vs 2.9 (0.5) nmol/60 min/10⁵ cells, *p* < 0.05, fig 3C). Importantly, in LDL-treated cells, O₂⁻ production was significantly decreased after incubation with L-NAME (2.6 (0.4) nmol/60 min/10⁵ cells, fig 3C), suggesting that increased O₂⁻ production in these cells was generated at least in part by uncoupled NOS. In contrast, preincubation of control cells with L-NAME resulted in an increase in O₂⁻ generation (5.8 (0.7) nmol/60 min/10⁵ cells, fig 3C), suggesting that in control cells NOS remained enzymatically coupled and a net producer of NO.

In the presence of exogenous BH₄, NO release was increased in LDL-treated cells (266 (4.5) nmol/l, fig 3B), and O₂⁻ production decreased to basal levels (1.5 (0.4) nmol/60 min/10⁵ cells, fig 3C). BH₄ supplementation did not affect NO or O₂⁻ production in control endothelial cells (figs 3B and C).

DISCUSSION

Hypercholesterolaemia is a major risk factor for the development of atherosclerosis. Early on, it impairs endothelial function, being an initial step in the pathogenesis of vascular disease. A key feature of endothelial dysfunction is reduced NO bioavailability caused both by scavenging of NO by oxidative species, and also decreased NO synthesis. Both mechanisms may be explained by eNOS enzymatic uncoupling, mediated by a relative deficiency of BH₄. To test this concept we investigated whether chronic oral treatment with BH₄ might restore NO bioavailability and endothelial function in subjects with hypercholesterolaemia. The key findings are: first, chronic BH₄ treatment leads to a marked eightfold increase in plasma BH₄ levels. Second, NO bioavailability, as determined by endothelium-dependent vasodilatation, is impaired in hypercholesterolaemic subjects compared with controls but restored to normal by BH₄ treatment. Importantly, this effect of BH₄ treatment on NO bioavailability is independent of any change in LDL cholesterol, and is associated with a reduction in systemic oxidative stress as assessed by 8-F₂ isoprostane plasma levels. Third, exposure of human aortic endothelial cells to LDL leads to reduced cellular BH₄ levels, reduced NO production, and increased superoxide production, at least in part from uncoupled NOS activity. Fourth, in human endothelial cells exposed to LDL, BH₄ supplementation can directly enhance NO production and reduce superoxide production. Taken together, these results indicate that chronic oral treatment with BH₄ is well tolerated and effective in normalising NO-mediated endothelial dysfunction in subjects with hypercholesterolaemia.

Acute administration of BH₄ has been shown to improve endothelial function in the brachial artery of smokers,¹⁰ patients with type II diabetes¹⁷ or hypercholesterolaemia⁸ and in the coronary arteries of patients with hypercholesterolaemia¹⁸ or angiographically overt coronary atherosclerosis.¹⁵ However, interpretation of these clinical studies is limited by the high systemic doses of sepiapterin or BH₄ used (often >100 fold in excess of physiological concentrations), which may increase NO bioactivity via non-specific antioxidant effects. When sepiapterin has been used to augment BH₄ concentrations in preclinical models of vascular disease, unexpected uncoupling of eNOS has been seen, possibly as a result of competition with BH₄ at the active site of the enzyme.²³ In addition, all the experimental interventions have been short term; there are no previous studies evaluating the effects as well as the tolerability of chronic low-dose BH₄ augmentation on NOS function in vascular disease, which is crucial for translation of this concept into clinical practice. The present study validates previous investigations, and importantly, demonstrates that chronic supplementation with low-dose BH₄ is feasible and effective and may therefore represent a new approach for the treatment of early cardiovascular disease.

In a clinical study it is not possible to determine whether BH₄ is pharmacologically active through a specific effect on eNOS coupling in the endothelium, or whether BH₄ acts simply as a non-specific antioxidant. To answer this question, we performed parallel experiments using human aortic endothelial cells to add mechanistic insight to our clinical data. When human aortic endothelial cells were exposed to oxidised LDL we observed a reduction in cellular BH₄ levels, together with a reduction in NO release and increased superoxide production. Inhibition of NOS by L-NAME reduced NO release in control endothelial cells, but not in LDL-exposed cells, indicating impaired NO synthesis after LDL exposure. In contrast, superoxide production from LDL-exposed cells was reduced by

L-NAME. This indicates that in LDL-treated endothelial cells superoxide production is at least in part generated by uncoupled NOS. Importantly, supplementation of LDL-treated cells with exogenous BH₄, analogous to our clinical study, restored their phenotype to that of control cells, supporting the concept that reduced intracellular BH₄ is responsible for NOS uncoupling. These observations are consistent with previous studies showing similar BH₄-dependent responses of NO and superoxide production in explanted vessels from patients with diabetes.²⁴

Oxidative stress is a common mechanism in the pathogenesis of vascular disease, and accelerated oxidative degradation of BH₄ may provide an explanation for the consistent beneficial effect seen in a variety of animal models and patients with endothelial dysfunction. This hypothesis is supported by observations that the antioxidant vitamin C can stimulate eNOS enzymatic activity by increasing intracellular concentration of BH₄.^{25 26} Indeed, the effect of vitamin C appears to be mediated by chemical stabilisation of BH₄.

Despite the striking results of our study, there are a number of potential limitations that remain to be dealt with in translating BH₄ treatment to wider clinical application. We measured plasma BH₄ levels in patients, but this may not reflect endothelial BH₄ concentration or oxidative state. Indeed, the mechanisms involved in BH₄ transport from plasma to endothelium, or the requirement for de novo BH₄ synthesis in the endothelium remain unclear. Current formulations of BH₄ are complex to deliver because the compound is readily oxidised at room temperature. Formulations with improved stability are needed before larger-scale studies can be undertaken, and the optimal doses and timing of BH₄ supplementation in vascular disease states are not yet defined. Finally, future large-scale clinical studies will be required to evaluate the effects of BH₄ treatment on vascular disease progression and clinical events. However, recent data suggest that long-term treatment with BH₄ in patients with hyperphenylalaninaemia is feasible and safe.^{3 4} World wide, several hundred patients are treated with the cofactor.²⁷ Some dose-dependent adverse reactions, including sleep disorders, polyuria and loose stools, were reported in a safety evaluation,²⁷ none of which were seen in this present study.

In conclusion, we show for the first time that in hypercholesterolaemia, endothelial dysfunction and oxidative stress can be normalised by chronic treatment with BH₄. Thus, long-term supplementation with BH₄ may provide a rational new approach to the prevention and treatment of cardiovascular disease.

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Competing interests: None.

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Endothelial function

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