Systemic and Vascular Oxidation Limits Efficacy of Oral Tetrahydrobiopterin Treatment in Patients with Coronary Artery Disease

Running title: Cunnington, et al.; Oral BH4 in coronary artery disease

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Journal Subject Code: [95] Endothelium/vascular type/nitric oxide
Abstract:

**Background** - The endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH4) plays a pivotal role in maintaining endothelial function in experimental vascular disease models and in humans. Augmentation of endogenous BH4 levels by oral BH4 treatment has been proposed as a potential therapeutic strategy in vascular disease states. We sought to determine the mechanisms relating exogenous BH4 to human vascular function, and determined oral BH4 pharmacokinetics in both plasma and vascular tissue in patients with coronary artery disease (CAD).

**Methods and Results** - Forty-nine patients with CAD were randomized to receive low-dose (400mg/d) or high-dose (700mg/d) BH4, or placebo, for two to six weeks prior to coronary artery bypass surgery. Vascular function was quantified by magnetic resonance imaging before and after treatment, along with plasma BH4 levels. Vascular superoxide, endothelial function and BH4 levels were determined in segments of saphenous vein (SV) and internal mammary artery (IMA). Oral BH4 treatment significantly augmented BH4 levels in plasma and in SV (but not IMA) but also increased levels of the oxidation product dihydrobiopterin (BH2), which lacks eNOS cofactor activity. There was no effect of BH4 treatment on vascular function or superoxide production. Supplementation of human vessels and blood with BH4 *ex vivo* revealed rapid oxidation of BH4 to BH2 with predominant BH2 uptake by vascular tissue.

**Conclusions** - Oral BH4 treatment augments total biopterin levels in patients with established CAD, but has no net effect on vascular redox state or endothelial function, due to systemic and vascular oxidation of BH4. Alternative strategies are required to target BH4-dependent endothelial function in established vascular disease states.

**Clinical Trial Registration Information** - clinicaltrials.gov; Identifier: NCT00423280 (http://www.clinicaltrials.gov).

**Key words:** tetrahydrobiopterin, nitric oxide synthase, endothelial function, coronary artery disease
Introduction

The endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH4) plays a pivotal role in maintaining normal endothelial function in experimental models of vascular disease \(^1,2\), and in human blood vessels \(^3\). When BH4 is limiting, eNOS becomes ‘uncoupled’ producing reactive oxygen species (ROS) rather than nitric oxide (NO) \(^4,5\). Under conditions of oxidative stress, BH4 can be readily oxidized to dihydrobiopterin (BH2), and eventually biopterin (B), which lack eNOS cofactor activity. Recently, higher BH4 levels in vascular tissue obtained from patients undergoing coronary artery bypass graft surgery (CABG) have been shown to be associated with increased NO bioavailability and decreased ROS production \(^6\). Furthermore, alterations in vascular redox status resulting from genetic variation in BH4 synthesis implicate a causal role for endogenous BH4 in modulating human endothelial function \(^7\). Thus, uncoupled eNOS, resulting from diminished BH4 in vascular disease states, is an important source of ROS production in the human vasculature.

Given the importance of endogenous BH4 levels on vascular endothelial function, administration of exogenous BH4 is a potential therapeutic strategy in vascular disease states. Indeed, a number of studies in human subjects with either overt coronary artery disease (CAD) \(^8\) or vascular risk factors \(^9-11\) have demonstrated that administration of exogenous BH4 by intra-arterial infusion leads to rapid improvements in endothelial function. However, these studies used very high BH4 doses and only tested short-term effects. In forearm plethysmography studies plasma BH4 levels are typically elevated to between 50-100 \(\mu M\) \(^12\), more than 1000-fold higher than physiological plasma BH4 levels of 10-50 nM \(^6\). These rapid and short-term supraphysiological BH4 doses may be confounded by non-specific effects, including direct antioxidant effects on the vessel...
Furthermore, intra-arterial infusion is not a relevant therapeutic approach to improve endothelial function in patients with CAD. A recent trial of chronic oral BH4 treatment demonstrated an improvement in endothelial function in subjects with hypercholesterolemia \(^1\). However, the mechanisms relating oral BH4 treatment to changes in vascular BH4 and eNOS coupling in vascular disease states are unknown, and the applicability to patients with established CAD is uncertain. Accordingly, we conducted a randomized, placebo-controlled trial of oral BH4 treatment in patients with CAD in order to determine the mechanisms relating exogenous BH4 treatment to human vascular function. We determined how oral BH4 treatment modified levels of biopterins in both plasma and vascular tissue, and the effect of oral BH4 treatment on vascular superoxide production and endothelial function in patients with CAD, both \textit{in vivo} and \textit{ex vivo}.

### Methods

**Study design and subjects**

Forty-nine patients with multi-vessel CAD undergoing elective CABG surgery at the John Radcliffe Hospital, Oxford, were randomised in a double-blind, parallel design to receive sapropterin dihydrochloride (6R-BH4) at either 700 mg/d (high-dose), or 400 mg/d (low-dose), or matching placebo for 2–6 weeks prior to surgery (clinicaltrials.gov identifier: NCT00423280). The study drug and randomization plan were provided by BioMarin Pharmaceutical Inc., Novato, California. **Figure 1** outlines the study protocol. Study medication was taken at 10p.m., at least one hour following the evening meal.

Patients aged >18 years who were scheduled for elective CABG surgery were included. Exclusion criteria were: recent acute coronary syndrome (<4 weeks); heart failure requiring...
diuretic therapy with evidence of severe left ventricular systolic dysfunction; emergency CABG surgery; newly diagnosed diabetes mellitus (<4 weeks); body weight >130kg; renal impairment (serum creatinine >2.0 mg/dL); hepatic impairment (serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) >2x upper limit of normal); contraindications to MRI scanning, e.g. pacemakers; pregnant women, or those planning pregnancy; terminal illness; known hypersensitivity to 6R-BH4; and patients already enrolled in another clinical trial.

In total, 247 potential subjects were screened. 41 subjects were excluded as they were known to fulfil at least one of the exclusion criteria. The remaining 206 subjects were invited to participate, of whom 51 agreed. Many eligible subjects could not be included for the sufficient treatment period because of the planned clinical scheduling of their CABG surgery, and/or the logistic complexity of returning for MRI scans at baseline and before surgery. One patient was unable to tolerate the initial MRI scan and was therefore not randomized. One patient dropped out due to declining CABG surgery. Thus, 49 subjects completed the study. Demographic characteristics are presented in Table 1. The study protocol was approved by the Oxfordshire Research Ethics Committee, and each participant gave written informed consent.

MRI quantification of vascular function

Vascular function was quantified by high-resolution MRI at baseline and at the end of the treatment period. Images of the aorta and carotid arteries were used to determine arterial distensibility and aortic pulse wave velocity (PWV) as indices of vascular stiffness. Arterial distensibility was determined in the ascending aorta, proximal descending aorta, distal descending aorta, and both common carotid arteries using a high resolution gradient-echo pulse sequence on a 1.5 Tesla MR scanner (Siemens Sonata, Erlangen, Germany), as described
A velocity-encoding gradient for phase-contrast MRI was applied to determine
PWV in the aorta\textsuperscript{14}.

Brachial artery flow-mediated dilatation (FMD) was used as a measure of endothelial
function. Using cross-sectional images of the brachial artery piloted in three dimensions, FMD
was defined as the maximal percentage change in luminal area from baseline following five
minutes forearm ischemia; endothelium-independent dilation was similarly quantified following
administration of 200 mcg sublingual glyceryl trinitrate (GTN).

**Tissue and plasma samples**

Paired samples of saphenous vein (SV) and internal mammary artery (IMA) were
obtained from each patient at the time of CABG surgery and transferred to the laboratory within
30 minutes in ice-cold Krebs Henseleit buffer. Blood samples were obtained at baseline and
immediately before surgery. Blood was centrifuged at 4000 rpm for 5 minutes; plasma was
collected and stored at -80°C. Plasma samples for BH4 quantification were collected and stored
in the presence of the antioxidant dithioerythritol (DTE, 1 mM), to prevent oxidation.

**Quantification of plasma and vascular biopterins**

BH4, BH2, and B levels in plasma and vascular tissue were each determined separately
by high-performance liquid chromatography (HPLC) followed by electrochemical (for BH4) and
fluorescent (for BH2 and B) detection, as described previously\textsuperscript{6}. Levels of biopterins were
expressed as pmol/g of tissue for vessels and nmol/L for plasma.

**Vasomotor studies**

Endothelium-dependent and -independent relaxations were assessed in SV rings using
isometric tension studies. Four rings from each vessel were pre-contracted with phenylephrine
(3x10\textsuperscript{-6} M), following which endothelium-dependent relaxations to acetylcholine (ACh, 10\textsuperscript{-10} to
$10^{-6}$ M) were quantified. Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, $10^{-10}$ to $10^{-6}$ M) were evaluated in the presence of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100 $\mu$M).

**Quantification of vascular superoxide production**

Vascular superoxide production was measured from paired segments of intact SV and IMA using lucigenin-enhanced chemiluminescence, as validated previously\textsuperscript{15}. Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 30 minutes in oxygenated (95% O2/5% CO2) Krebs-HEPES buffer (pH 7.4) at 37°C. Lucigenin-enhanced chemiluminescence was measured with low-concentration lucigenin (5 $\mu$M). NOS-derived superoxide production was determined by the difference in superoxide production after 30 minutes pre-incubation with L-NAME (100 $\mu$M).

**Ex vivo incubations of vessels and blood**

To further assess the vascular uptake of exogenous biopterins, SV rings from a further six CABG patients were incubated for 30 minutes in oxygenated Krebs-HEPES buffer at 37°C in either buffer alone, or in the presence of BH4 (100 nM, Schircks Laboratories, Jona, Switzerland), BH4 plus DTE (1 mM), or BH2 (100 nM, Schircks Laboratories). Samples of incubation media and vessel rings were stored at -80°C prior to HPLC analysis for quantification of BH4, BH2 and B.

To assess whether exogenous BH4 is present in the vascular endothelium, SV rings from 6 further patients were incubated either alone (n=2 rings per patient), or with BH4 plus DTE (n=2 rings per patient), under the same reagent concentrations and conditions as the experiment above. After incubation, endothelium was removed from one ring by gentle luminal abrasion, as described previously\textsuperscript{6}. Vessel rings were stored at -80°C prior to HPLC analysis.
To further evaluate the oxidation of endogenous and exogenous BH4, whole blood and plasma (separated from whole blood following centrifugation) from three healthy volunteers was incubated either alone, or with supplementary BH4 (50 nM), BH4 plus DTE (1 mM), or BH2 (50 nM). The whole blood samples were placed on rollers during incubation to prevent separation of cellular content from plasma. Samples of plasma (either incubated as plasma, or following separation from whole blood) were taken at baseline and following four hours incubation, and stored at -80°C prior to HPLC analysis.

**Statistical analysis**

All variables were tested for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed variables were log-transformed for analyses. Normally distributed variables are presented as mean±SEM.

According to the original power calculations (19 patients per group), the study would be able to detect a 4.7% difference in the change of FMD between any of the two active treatment groups vs placebo, with an assumed normally distributed SD of the change= 4, $\alpha=0.025$ and power 90%. However, the study recruited 19 patients in the placebo group, 14 in the 400mg/d group, and 16 in the 700mg/d group. Therefore, *post hoc* analysis showed that the true statistical power to detect the same differences was 83% for the 400mg/d group vs placebo, and 86% for the 700mg/d group vs placebo.

For MRI and plasma variables in the clinical trial we used two-factor ANOVA with “time” x “treatment group” interaction to compare the effect of treatment on these variables between groups. When a significant difference was observed between groups, then individual comparisons were performed using the Bonferroni *post hoc* test.
For tissue variables, and the _ex vivo_ experiments, we used one- or two-factor ANOVA (as stated in the figure legends) followed by Bonferroni _post hoc_ test to compare variables between groups.

For the organ bath experiments, the effect of treatment on vasorelaxation in response to ACh or SNP was evaluated using two-way ANOVA for repeated measures (examining the effect of “ACh or SNP concentration” x “treatment group” interaction on “vasorelaxations”), in a full factorial model.

All tests were two-tailed and p<0.05 was considered significant. All the statistical tests were performed using SPSS 19.0.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Effect of oral BH4 treatment on plasma biopterin levels**

Both low- and high-dose oral BH4 treatment elevated plasma BH4 levels approximately 2.5-fold compared to placebo (p<0.001; [Figure 2A](#)). There was a similar significant elevation in plasma BH2 ([Figure 2B](#)) and B. Accordingly, total biopterin levels (the sum of BH4, BH2 and B) were elevated by treatment ([Figure 2C](#)), but the ratio of reduced to oxidized biopterins (BH4/(BH2+B)) was not significantly altered by oral BH4 treatment ([Figure 2D](#)).

**Effect of oral BH4 treatment on vascular biopterin levels**

In SV, BH4 levels were increased twofold by both low- and high-dose oral BH4 treatment compared to placebo (p<0.001; [Figure 3A](#)), with a similar significant increase in BH2 and B. In IMA, however, there was no significant difference in BH4 (p=0.334), BH2 (p=0.134)
or B (p=0.082) in BH4-treated patients compared to placebo. In both SV and IMA there was no difference in the BH4/(BH2+B) ratio between placebo and BH4-treated patients (Figure 3B).

**Effect of oral BH4 treatment on vascular superoxide production, endothelial function and arterial stiffness**

There was no significant difference in either total vascular superoxide production or NOS-derived superoxide production between placebo- and BH4-treated subjects in either SV (p=0.70) or IMA (p=0.12) (Figure 4). Quantification of vascular function by MRI at baseline and after treatment with either placebo or BH4 revealed no effect of oral BH4 treatment on brachial FMD (p=0.325), aortic or carotid distensibility, or aortic PWV (Table 2). Correspondingly, samples of saphenous vein harvested at the time of CABG surgery for *ex vivo* organ bath studies showed no significant difference in endothelium-dependent relaxation to ACh, or endothelium-independent relaxation to SNP, between treatment groups (Figure 5).

**Oxidation and uptake of biopterins in human vessels and blood *ex vivo***

Following the observation that oral BH4 treatment resulted in significant increases in circulating BH4 and BH2, but no effect on biopterin redox status in either plasma or vascular tissue, we conducted *ex vivo* experiments to investigate the fate of exogenous BH4 in blood, and the uptake of exogenous BH4 and BH2 in human vascular tissue. SV samples from a further six subjects (not enrolled in the clinical trial) were collected during CABG surgery and incubated with BH4 (with or without the antioxidant DTE) or BH2. We incubated vessels in low concentration BH4 (50 nM), reflecting the plasma levels of BH4 achieved after oral BH4 therapy, rather than the very high concentrations used in previous studies.

Incubation of vessels with exogenous BH4 led to rapid and complete depletion of BH4 from the incubation medium, associated with a marked accumulation of BH2. However, the
addition of DTE significantly increased residual BH4 concentration (p<0.001; Figure 6A). In vascular tissue, incubation with exogenous BH4 significantly increased BH2 levels compared to control (p<0.05; Figure 6C), but only significantly increased BH4 levels in the presence of DTE (p<0.05, Figure 6B). Incubation with BH2 resulted in a larger increase in tissue BH2 but no increase in tissue BH4. Incubation in exogenous BH4 resulted in a significant reduction in tissue BH4/(BH2+B) ratio compared to control (p<0.01), even in the presence of DTE (p<0.05, Figure 6D).

In the subsequent experiment, removal of the endothelium from control vessel segments led to a reduction of vascular BH4 and total biopterins by ~75% (Figure 6E-F). In vessels incubated with BH4 plus DTE, vascular BH4 was significantly increased (p<0.05). After removing endothelium from these vessels, BH4 was reduced by ~85%, demonstrating that the majority of exogenous BH4 is present in the endothelium; however, as an absolute measure, BH4 remained significantly higher than in the control de-endothelialized vessel, suggesting that a significant minority of vascular biopterins measured in SV after incubation with BH4 is also due to BH4 uptake into vascular smooth muscle cells, or captured in the vascular adventitia and extracellular space (Figure 6E-F).

We next investigated the fate of endogenous and exogenous biopterins in whole blood and plasma, supplemented with BH4 (with or without the antioxidant DTE) or BH2. Endogenous BH4 levels in whole blood remained unchanged after four hours incubation. In contrast, the addition of exogenous BH4 significantly increased BH2 in human blood, even at baseline, indicating rapid BH4 oxidation (Figure 7A). Exogenous BH4 was totally depleted after four hours. The addition of DTE attenuated the rapid oxidation of exogenous BH4 (p<0.05), but had no effect on residual BH4 levels after four hours incubation (Figure 7A).
In contrast to whole blood, when plasma was incubated for four hours there was a significant reduction in endogenous BH4 (p<0.05), with evidence of oxidation to BH2 (Figure 7B). The effect of addition of exogenous BH4 mirrored that seen in whole blood, i.e. rapid oxidation to BH2 with attenuation by DTE at baseline but not after four hours. Taken together, these observations demonstrate that exogenous BH4 is rapidly oxidised to BH2 in human blood or blood vessels \textit{ex vivo}, resulting in little or no increase in BH4 levels, but an increase in BH2 and a decrease in the ratio of reduced to oxidized biopterins.

\textbf{Discussion}

In this study we demonstrate that oral BH4 treatment has no effect on measures of endothelial function, arterial stiffness or oxidative stress in patients with established CAD, either \textit{in vivo}, or using \textit{ex vivo} assays on vascular tissue obtained at the time of CABG surgery. The design of our study allows, for the first time in humans, detailed mechanistic evaluation of the pharmacokinetics and pharmacodynamics of oral BH4 treatment in both blood and vascular tissue, in order to explain these observations. Since BH4 has been proposed as a potential treatment to improve endothelial function in vascular disease states, our results demand fundamental reconsideration of strategies to harness BH4-mediated pathways as therapeutic targets in human cardiovascular disease.

We first demonstrate that treatment with oral BH4 in subjects with CAD is indeed sufficient to significantly elevate plasma BH4 levels. This is despite the fact that blood samples taken at the time of CABG surgery were effectively trough levels, i.e. 12–16 hours following the last dose. However, this elevation in plasma BH4 is tempered by similar rises in plasma BH2 and B, such that the ratio of reduced to oxidized biopterins (BH4/ (BH2+B)) in plasma remains
unchanged following treatment. In vessels there are different findings in venous and arterial tissue. Changes in SV mirror those seen in plasma, but in IMA the smaller absolute changes and greater variability in biopterin levels did not reveal a significant effect of oral BH4 treatment. If data from the low- and high-dose BH4 groups are combined to perform an analysis of ‘BH4 therapy’ versus placebo, the increase in BH2 (but not BH4) in IMA in the combined treatment group reaches statistical significance (19.3 pmol/g vs 11.8 pmol/g, p=0.03). Thus, oral BH4 therapy in subjects with CAD is more effective at elevating arterial BH2 levels than BH4 levels.

The explanation for this discrepancy between venous and arterial tissue is not clear. Biopterin transport is not fully understood, especially in humans and in intact tissues. Previous work has indicated that biopterin transport is cell-type dependent, and that both direct uptake (as BH4) and conversion to BH2 followed by recycling via DHFR are possible mechanisms. The first explanation, therefore, is that there is a genuine biological difference between human veins and arteries in biopterin uptake. If BH4 enters the vessel via passive diffusion from the lumen, then slow venous blood flow in lower limb veins, in comparison to high-pressure arterial flow, may promote uptake. If BH4 enters the vessel via the salvage pathway then it is possible that DHFR is regulated differently between SV and IMA.

The alternative possibility is that the difference observed between SV and IMA is not due to a different biological mechanism of transport between these tissue types. Our data do show a small but statistically non-significant increase in a BH4 in IMA (and a slightly larger accumulation of BH2); however, the variability in BH4 was greater in IMA than in SV, and therefore our sample size is too small to exclude a very small effect of BH4 therapy on arterial BH4 levels.
Despite the changes in BH4 seen after oral BH4 treatment, the BH4/(BH2+B) ratio remains unchanged in both SV and IMA. This finding reveals important insights into the significance of BH2 levels in human vessels. Recent data from cultured endothelial cells suggest that the intracellular levels of BH2, and more specifically the ratio between reduced and oxidized biopterins, is important in regulating eNOS coupling. Our data are consistent with the studies using purified eNOS enzyme which demonstrated that BH2 can act as a competitive inhibitor of BH4 and promote eNOS uncoupling. In the present study, there is no effect of oral BH4 therapy on total or NOS-derived superoxide in either SV or IMA, despite a twofold increase in absolute BH4 levels in SV. Furthermore, there is no effect of BH4 therapy on endothelium-dependent relaxation in SV rings. Taken together, these findings suggest that BH4/(BH2+B) ratio is a more important determinant of eNOS function than absolute BH4 levels in human vessels. Accordingly, pharmacological strategies that alter the balance between reduced and oxidized biopterins in favor of BH4 may be more effective at improving endothelial function than strategies that have either no effect, or indeed an opposite effect, on this ratio. Both folates – via scavenging of peroxynitrite – and vitamin C – via increased recycling of BH3+ radical to BH4 – have potential to be co-administered with BH4 to achieve this shift in the intracellular oxidative balance. Indeed, chronic oral folic acid treatment has been shown to augment vascular BH4 levels in patients with CAD.

The effects of oral BH4 treatment on endothelial function in patients with CAD may also be limited by uncertainty over how exogenous biopterins may enter the vascular endothelium. Studies in mice have suggested that oxidation of BH4 to BH2 and subsequent recycling back to BH4 by dihydrofolate reductase (DHFR) is necessary to augment intracellular BH4; accordingly, this elevation in BH4 can be attenuated by inhibition of DHFR with methotrexate.
In the present study we see evidence of significant oxidation of exogenous BH4 to BH2 in plasma. Thus, it is possible that co-administration of BH4 with an antioxidant (to prevent this oxidation in plasma) may result in a paradoxical reduction in drug uptake into the endothelium. Our *ex vivo* experiments using whole blood and vascular tissue shed further light on these important aspects of BH4 homeostasis. Using supplementary doses of exogenous BH4 to raise BH4 levels equivalent to those that are achievable through oral administration (at the doses used in the clinical trial), we show that there is a rapid oxidation of BH4 to BH2 when exogenous BH4 is added to human blood, even in healthy volunteers. Co-administration of an antioxidant (DTE) attenuates the observed oxidation in the initial stages following addition to blood, but has no effect following a prolonged incubation (four hours), during which all exogenous BH4 is totally depleted. In contrast, endogenous levels of BH4 in whole blood are unchanged following incubation, suggesting that endogenous and exogenous BH4 may be oxidized at different rates, possibly due to an interaction with the cellular content of blood.

When vessel rings are incubated in exogenous BH4 for 30 minutes, there is no increase in tissue levels of BH4, but a significant increase in BH2, resulting in a lower tissue BH4/(BH2+B) ratio. This finding is explained by the rapid oxidation of BH4 to BH2 in the incubation media under the experimental conditions at 37°C in organ chambers, resulting in a predominance of BH2 rather than BH4 for the majority of the duration of the control experiment. Co-administration with DTE significantly attenuates the oxidation of BH4 in the incubation media. The combination of BH4 and DTE is able to increase tissue BH4 levels, and more specifically endothelial BH4 levels, but importantly also results in a greater increase in tissue BH2 compared to incubation with BH4 alone, again resulting in a lower BH4/(BH2+B) ratio compared to
control. Finally, incubation with BH2 alone results in a large increase in tissue BH2, but no change in tissue BH4.

Taken together, these results suggest that both exogenous BH4 and BH2 can be taken up into human vascular tissue, but accumulation of tissue BH2 does not seem to result in an increase in tissue BH4. The strategy of BH4/antioxidant co-administration, to maintain a favourable BH4/BH2 ratio in circulating blood, might be hypothesized to increase intravascular BH4/BH2 ratio, however our results do not support this. Although DTE affords initial protection of exogenous BH4 from oxidation, this effect appears to be short-lived. More importantly, co-administration of BH4 and DTE still results in an unfavourable ratio of reduced to oxidized biopterins in tissue compared to control, despite increasing absolute BH4 levels, suggesting that in vessels from patients with established CAD there may be continued oxidation of BH4 to BH2 following uptake into the vessel wall, or incomplete regeneration of BH4 from BH2 via the salvage pathway catalyzed by dihydrofolate reductase (DHFR). Indeed, recent studies suggest an important role for DHFR in maintaining intracellular BH4 levels, especially in conditions where BH4 levels are limiting, or in response to increased oxidative stress where DHFR activity is downregulated.

It is important to acknowledge that the ex vivo blood and vessel models have a number of limitations. First, in the vessel incubation experiments, it has been previously described that high concentration BH4 can generate superoxide anion when exposed to Krebs buffer, potentially leading to increased BH4 oxidation. Second, exogenous BH4 in blood ex vivo may be oxidized differently compared with circulating blood in vivo; additionally, factors such as the partial pressure of oxygen in these models (no added oxygen in the blood experiments, and hyperoxia in the organ chamber experiments) may exert a significant effect on the rate of BH4 oxidation.
Furthermore, these models do not take into account oxidation of exogenous BH4 prior to entering the bloodstream, e.g. in the gut. Importantly, the concentrations of BH4 and BH2 measured in blood or plasma samples supplemented with known concentrations of exogenous BH4 or BH2 were very similar to the expected sum of endogenous levels plus the added exogenous levels, despite the samples undergoing deproteinisation prior to HPLC analysis. This would suggest that no exogenous biopterins were removed by deproteinisation, and that exogenous BH4 is therefore not protein-bound in peripheral blood. However, the potential interaction of biopterins with plasma proteins or other biomolecules is clearly of potential importance for understanding biopterin biology and pharmacokinetics.

The findings of the present study contrast with those of the only previous study of oral BH4 treatment, which demonstrated an improvement in endothelial function (forearm blood flow response to ACh) in middle-aged subjects with hypercholesterolemia. That study differed from our study in a number of respects. First, the dose of 400 mg BH4 was administered twice daily, potentially reducing the variation in plasma peak-trough concentrations compared to the once-daily regimen of the current study. Accordingly, vascular measurements will have been performed closer to peak plasma concentrations. Second, subjects were younger, had no evidence of cardiovascular disease, and had only one risk factor (hypercholesterolemia), and thus were likely to have lower systemic oxidative stress than subjects in the current study. This may be important, as systemic oxidative stress may be pivotal in determining the degree of oxidation of BH4 to BH2 in plasma, with a corresponding effect on vascular uptake. Third, these younger subjects without established CAD were not taking medications known to improve endothelial function, e.g. HMG-CoA reductase inhibitors (statins) and angiotensin-converting enzyme (ACE) inhibitors, and thus there may be greater ‘eNOS reserve’ in these subjects compared to
CABG patients who already take multiple secondary prevention therapies. Indeed, statin therapy alone appears to have significant beneficial direct effects on BH4 bioavailability, through upregulation of guanosine triphosphate cyclohydrolase-1 (GTPCH, the rate-limiting enzyme in BH4 synthesis) expression and activity, and eNOS-mediated endothelial function in CAD patients, independent of cholesterol lowering\textsuperscript{30,31}. Thus, the findings of the current study can only be applied to the specific population studied which represents the most diseased end of the atherosclerosis spectrum; BH4 monotherapy may have therapeutic potential in earlier stages of endothelial dysfunction.

In conclusion, we demonstrate that oral BH4 treatment in patients with CAD significantly elevates BH4 levels in blood, but this effect is significantly limited by systemic oxidation of exogenous BH4 to BH2, which lacks eNOS co-factor activity. Accordingly, the ratio of reduced to oxidized biopterins in blood and vascular tissue is unchanged by exogenous BH4 treatment, resulting in no net effect on eNOS coupling, endothelial function or vascular superoxide production. Targeting BH4 remains a rational therapeutic strategy in cardiovascular disease, but future studies should be directed towards interventions that can favourably alter the endogenous BH4/BH2 ratio in human vascular endothelium, either via a selective increase in absolute BH4 levels, prevention of BH4 oxidation, or increased BH4 recycling. In particular, the effect of antioxidant co-administration to prevent systemic and vascular oxidation of exogenous BH4 warrants further attention.

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safety monitoring and pharmacovigilance for the study. The University of Oxford was the trial Sponsor, and took sole responsibility for the conduct and data of the study. No author has received funding directly from, nor has any financial or other related interest in, BioMarin Pharmaceuticals Inc. Work in the authors’ laboratory is supported by the British Heart Foundation (Programme Grant RG/07/003/23133) and by the NIHR Biomedical Research Centre. RPC is a Wellcome Trust Senior Research Fellow in Clinical Science.

Conflict of Interest Disclosures: None

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Table 1: Demographic characteristics of study participants

<table>
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<th></th>
<th>Placebo</th>
<th>6R-BH4 400 mg/d</th>
<th>6R-BH4 700 mg/d</th>
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<td>14 (13:1)</td>
<td>16 (14:2)</td>
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<td>Treatment duration, days (SEM)</td>
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<td>Cholesterol, mg/dL</td>
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<tr>
<td>Median</td>
<td>135</td>
<td>170</td>
<td>147</td>
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<tr>
<td>25th-75th percentiles</td>
<td>127–174</td>
<td>143–205</td>
<td>127–162</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
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</tr>
<tr>
<td>Median</td>
<td>46</td>
<td>50</td>
<td>42</td>
</tr>
<tr>
<td>25th-75th percentiles</td>
<td>39–54</td>
<td>35–62</td>
<td>39–58</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
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</tr>
<tr>
<td>Median</td>
<td>106</td>
<td>106</td>
<td>115</td>
</tr>
<tr>
<td>25th-75th percentiles</td>
<td>88–124</td>
<td>88–212</td>
<td>80–195</td>
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<tr>
<td>HbA1c, %</td>
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<tr>
<td>Median</td>
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<td>6.2</td>
<td>5.6</td>
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<tr>
<td>25th-75th percentiles</td>
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<td>5.6–6.9</td>
<td>5.3–6.4</td>
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<td>Diabetes mellitus, n (%)</td>
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<td>5 (36)</td>
<td>4 (25)</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>13 (68)</td>
<td>11 (79)</td>
<td>11 (69)</td>
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<tr>
<td>Family history, n (%)</td>
<td>6 (32)</td>
<td>7 (50)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>MI, n (%)</td>
<td>9 (47)</td>
<td>6 (43)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
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<td>Current or recent smoker</td>
<td>2 (11)</td>
<td>2 (14)</td>
<td>4 (25)</td>
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<td>Ex-smoker &gt;1 year</td>
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<td>10 (71)</td>
<td>10 (63)</td>
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<tr>
<td>Never smoked</td>
<td>4 (21)</td>
<td>2 (14)</td>
<td>2 (13)</td>
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<td>Extent of CAD, n (%)</td>
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<tr>
<td>2-vessel</td>
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<td>1 (7)</td>
<td>7 (44)</td>
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<td>3-vessel</td>
<td>16 (84)</td>
<td>13 (93)</td>
<td>9 (56)</td>
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<td>Medication, n (%)</td>
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<td>Aspirin</td>
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<td>11 (79)</td>
<td>14 (88)</td>
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<td>4 (29)</td>
<td>5 (31)</td>
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<td>Statin</td>
<td>18 (95)</td>
<td>13 (93)</td>
<td>16 (100)</td>
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<tr>
<td>ACE inhibitor or ARB</td>
<td>16 (84)</td>
<td>11 (79)</td>
<td>9 (56)</td>
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<tr>
<td>β-blocker</td>
<td>16 (84)</td>
<td>10 (71)</td>
<td>9 (56)</td>
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<tr>
<td>Calcium channel blocker</td>
<td>7 (37)</td>
<td>6 (43)</td>
<td>6 (38)</td>
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<td>Diuretic</td>
<td>4 (21)</td>
<td>2 (14)</td>
<td>2 (13)</td>
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<tr>
<td>Nitrate</td>
<td>6 (32)</td>
<td>6 (43)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Nicorandil</td>
<td>2 (11)</td>
<td>0 (0)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Insulin</td>
<td>2 (11)</td>
<td>1 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Oral hypoglycemic</td>
<td>3 (16)</td>
<td>4 (29)</td>
<td>2 (13)</td>
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</table>
Table 2: Effect of oral BH4 treatment on MRI indices of arterial stiffness

Aortic and carotid distensibility and aortic pulse wave velocity (PWV) were measured by MRI at baseline and following treatment with oral BH4 or placebo. Values are expressed as mean±SEM. p-values represent two-way ANOVA for repeated measures with “time” x “treatment group” interaction.

<table>
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<tr>
<th></th>
<th>Baseline</th>
<th>Post-treatment</th>
<th>Δ</th>
<th>Baseline</th>
<th>Post-treatment</th>
<th>Δ</th>
<th>Baseline</th>
<th>Post-treatment</th>
<th>Δ</th>
<th>p</th>
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<td>Distensibility, 10⁻³ mmHg⁻¹</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Ascending aorta</td>
<td>1.66 (0.31)</td>
<td>1.54 (0.25)</td>
<td>-0.23 (0.23)</td>
<td>1.79 (0.22)</td>
<td>2.09 (0.45)</td>
<td>0.28 (0.26)</td>
<td>2.04 (0.37)</td>
<td>2.33 (0.38)</td>
<td>0.05 (0.30)</td>
<td>0.40</td>
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<tr>
<td>Proximal descending aorta</td>
<td>2.09 (0.33)</td>
<td>2.17 (0.36)</td>
<td>0.09 (0.12)</td>
<td>2.47 (0.20)</td>
<td>2.62 (0.34)</td>
<td>0.11 (0.23)</td>
<td>2.73 (0.39)</td>
<td>3.01 (0.37)</td>
<td>0.28 (0.29)</td>
<td>0.77</td>
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<tr>
<td>Abdominal aorta</td>
<td>2.69 (0.42)</td>
<td>2.87 (0.42)</td>
<td>0.18 (0.22)</td>
<td>3.00 (0.41)</td>
<td>3.28 (0.43)</td>
<td>0.28 (0.33)</td>
<td>3.32 (0.47)</td>
<td>3.18 (0.46)</td>
<td>-0.14 (0.36)</td>
<td>0.57</td>
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<tr>
<td>Right carotid artery</td>
<td>3.01 (0.25)</td>
<td>2.71 (0.24)</td>
<td>-0.30 (0.24)</td>
<td>3.20 (0.36)</td>
<td>4.48 (0.69)</td>
<td>1.12 (0.59)</td>
<td>4.05 (0.57)</td>
<td>3.83 (0.37)</td>
<td>-0.30 (0.47)</td>
<td>0.27</td>
</tr>
<tr>
<td>Left carotid artery</td>
<td>3.59 (0.31)</td>
<td>3.26 (0.40)</td>
<td>-0.53 (0.33)</td>
<td>3.62 (0.33)</td>
<td>4.39 (0.86)</td>
<td>0.68 (0.83)</td>
<td>3.53 (0.42)</td>
<td>3.84 (0.54)</td>
<td>-0.30 (0.47)</td>
<td>0.27</td>
</tr>
<tr>
<td>Aortic PWV, m/s</td>
<td>6.57 (0.37)</td>
<td>6.41 (0.37)</td>
<td>-0.16 (0.46)</td>
<td>6.33 (0.40)</td>
<td>7.19 (1.17)</td>
<td>0.73 (1.21)</td>
<td>7.23 (0.72)</td>
<td>6.83 (0.47)</td>
<td>-0.40 (0.59)</td>
<td>0.58</td>
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</table>
Figure Legends:

**Figure 1.** Study scheme

**Figure 2.** Effect of oral BH4 treatment on plasma biopterins. Levels of plasma biopterin species were quantified at baseline and following treatment with oral BH4 or placebo. Treatment with oral BH4 400 mg/d or 700 mg/d resulted in a significant increase in plasma BH4 levels compared to placebo (Panel A), but also a significant increase in plasma BH2 (Panel B) and biopterin (B) (data not shown). Accordingly, total biopterins (tBio, the sum of BH4 and BH2 and biopterin) were significantly elevated by BH4 treatment (Panel C), but there was no change in the ratio of reduced to oxidized biopterins (BH4/(BH2+B)) (Panel D). Values are expressed as mean±SEM of log transformed values. *p<0.001 vs. placebo group for change from baseline; p values calculated using two factor ANOVA with “time” x “treatment group” interaction, followed by Bonferroni post hoc test (2 comparisons per panel: BH4 400mg/d and BH4 700 mg/d vs placebo).

**Figure 3.** Effect of oral BH4 treatment on vascular biopterins. Samples of saphenous vein (SV) and internal mammary artery (IMA) were collected at the time of CABG surgery for quantification of biopterin species. In SV, treatment with oral BH4 resulted in a significant increase in tissue levels of BH4 and BH2 (Panel A) and biopterin (data not shown) compared to placebo. In IMA there were no significant differences between treatment groups (Panel A). In both vessel types, treatment with oral BH4 did not alter the tissue ratio of reduced to oxidized biopterins (BH4/(BH2+B)) (Panel B). Values are expressed as mean±SEM. *p<0.001 vs placebo; two-factor ANOVA showed a significant individual effect of “vessel type” and “treatment
group” on both BH4 (p<0.0001 for both) and BH2 (p<0.001 for both), but there was no significant interaction of “vessel type” x “treatment group” for either BH2 (p=0.380) or BH4 (p=0.053). This analysis was followed by Bonferroni post hoc test for individual between-groups comparisons, for each vessel type (SV or IMA) and each biopterin species (BH4 or BH2 or BH4/(BH2+B) ratio) separately (2 comparisons per variable: BH4 400mg/d and BH4 700 mg/d vs placebo).

**Figure 4.** Effect of oral BH4 treatment on vascular superoxide production and eNOS coupling. Samples of saphenous vein (SV) and internal mammary artery (IMA) were collected at the time of CABG surgery for quantification of superoxide production by lucigenin-enhanced chemiluminescence in the presence and absence of the NOS inhibitor L-NAME. There was no effect of BH4 treatment on total vascular superoxide production in SV or IMA (Panel A), and no effect on L-NAME-inhibitable superoxide (Panel B). Values are expressed as median (25th to 75th percentiles) and range. Groups were compared using one-way ANOVA of log-transformed values.

**Figure 5.** Effect of oral BH4 treatment on endothelial function. Isometric tension studies were performed on saphenous vein rings to quantify vasorelaxation to acetylcholine (ACh) and sodium nitroprusside (SNP) ex vivo. Oral BH4 treatment had no significant effect on endothelium-dependent relaxation to ACh (Panel A) or endothelium-independent relaxation to SNP (Panel B). Endothelial function in vivo was determined using MRI to quantify brachial artery flow-mediated dilatation (FMD). There was no significant effect of BH4 treatment on brachial FMD (Panel C). Values are expressed as mean±SEM. Data in panels A-B were analysed using two-way ANOVA for repeated measures (examining the effect of “ACh or SNP
concentration” x “treatment group” interaction on “vasorelayations”), in a full factorial model. The data in Panel C were analysed by two-factor ANOVA (examining the effect of “time” x “treatment group” interaction on brachial FMD).

**Figure 6.** *Ex vivo* incubation of saphenous vein rings with exogenous BH4 or BH2. Panels A-D. Saphenous vein rings (n=6 subjects) were incubated for 30 minutes under the following experimental conditions: Control, buffer alone; BH4, BH4 100 μM; BH4+DTE, BH4 100 μM plus dithioerythritol (DTE, antioxidant) 1 mM; BH2, BH2 100 μM. Samples of incubation medium were stored at the beginning and end of the experiment. Panel A: Exogenous BH4 in the incubation medium was totally depleted by the end of the experiment; the addition of DTE provided partial protection from oxidation. Panels B and C: Incubation with BH4 significantly increased tissue BH2 levels compared to control, but only increased tissue BH4 levels in the presence of DTE; incubation with BH2 resulted in a larger increase in tissue BH2 but no increase in tissue BH4. Panel D: Incubation in exogenous BH4 resulted in a significant reduction in tissue BH4/(BH2+B) ratio compared to control, even in the presence of DTE. *p<0.05 vs control; ¶p<0.05 vs BH4 alone; $p<0.05 vs BH4+DTE. Panels E-F: In a further experiment, SV tissue was obtained from a further 6 patients. Two rings from each subject were incubated with BH4 and DTE, with a further two control rings incubated without BH4/DTE. Endothelium was removed from one of each pair of rings. Endothelial denudation led to a reduction of vascular BH4 (Panel E) and total biopterins (tBio, Panel F) by ~75%. In vessels incubated with BH4+DTE, vascular BH4 was significantly increased. After removing endothelium from these vessels, BH4 was reduced by ~85%, demonstrating that the majority of both endogenous and exogenous BH4 is present in the endothelium. *p<0.05 vs. endo(+)/BH4(-); †p<0.05 vs. endo(+)/BH4(+); ≠p<0.05 vs. endo(-)/BH4(-). Values are expressed as mean±SEM. Panel A:
The levels of BH4 and BH2 in the medium pre- and post-incubation were compared between “Control”, “BH4” and “BH4+DTE” groups using one-way ANOVA followed by Bonferroni post hoc test for individual between-groups comparisons (3 comparisons). Panels B-F: Variables in each panel were compared using one-way ANOVA followed by Bonferroni post hoc test for individual between-groups comparisons (for 6 comparisons in panels B-D and 4 comparisons in panels E-F).

**Figure 7.** Fate of endogenous versus exogenous BH4 in whole blood and plasma. Samples of whole blood (Panel A) and plasma (Panel B) from healthy volunteers (n=3) were incubated for 4 hours under the following experimental conditions: **Control**, blood or plasma alone; **BH4**, supplementary BH4 50 nM; **BH4+DTE**, supplementary BH4 50 nM plus dithioerythritol (DTE, antioxidant) 1 mM; **BH2**, supplementary BH2 50 nM. In the whole blood samples, plasma was separated at baseline and after 4 hours. Plasma biopterins were then quantified in all samples. In whole blood there was no significant difference in endogenous BH4 or BH2 after 4 hours of incubation, whereas in plasma alone endogenous BH4 was oxidized to BH2. Exogenous BH4 was completely oxidized in both whole blood and plasma after 4 hours; DTE afforded protection from oxidation at baseline, but not after 4 hours. *p<0.05 vs control; ¶p<0.05 vs BH4 alone; $p<0.05$ vs BH4+DTE; §p<0.05 vs baseline. Both pre- and post-incubation BH4 and BH2 levels were compared separately using one-way ANOVA, followed by Bonferroni *post hoc* test for individual between-groups comparisons (6 comparisons). The change in BH4 pre- vs post-incubation was tested only in the control groups by using a paired t-test.
Patients awaiting CABG surgery

Vascular MRI (FMD, arterial distensibility)  
Blood sampling  
Randomization

Placebo  
BH4 400mg/d  
BH4 700mg/d  
2-6 weeks

Repeat vascular MRI  
Blood sampling  
CABG surgery

Vascular tissue sample collection:  
Saphenous vein (SV)  
Internal mammary artery (IMA)

Ex vivo assays:  
Vasomotor studies  
Superoxide ± L-NAME  
Tissue biopterins
1.5
Baseline Post-treatment

A
Log[Plasma BH4] (nmol/L)

B
Log [Plasma BH2] (nmol/L)

C
Log [Plasma tBio] (nmol/L)

D
Plasma BH4/(BH2+B)

Placebo BH4 400 BH4 700
Panel A: Graph showing relaxation (%) in response to Log[ACh] for Placebo, BH4 400, and BH4 700.

Panel B: Graph showing relaxation (%) in response to Log[SNP] for Placebo, BH4 400, and BH4 700.

Panel C: Bar graph showing Brachial FMD (%) for Baseline and Post-treatment for Placebo, BH4 400, and BH4 700.
BH4 or BH2 in incubation medium (nmol/L)

Pre-incubation

Post-incubation

Vascular BH4/(BH2+B)

Control BH4 BH4+DTE BH2

Vascular BH4 (of control)

Control BH4 BH4+DTE BH2

Vascular tBio (of control)

Control BH4 BH4+DTE BH2

Endothelium

BH4+DTE

Pre-incubation Post-incubation
Systemic and Vascular Oxidation Limits Efficacy of Oral Tetrahydrobiopterin Treatment in Patients with Coronary Artery Disease

Colin Cunnington, Tim Van Assche, Cheerag Shirodaria, Ilias Kylintireas, Alistair C. Lindsay, Justin M. Lee, Charalambos Antoniades, Marios Margaritis, Regent Lee, Ruha Cerrato, Mark J. Crabtree, Jane M. Francis, Rana Sayeed, Chandi Ratnatunga, Ravi Pillai, Robin P. Choudhury, Stefan Neubauer and Keith M. Channon

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