Tetrahydrobiopterin Improves Endothelial Function in Patients with Cystic Fibrosis

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Key Words: BH_4, Nitric Oxide Bioavailability, Flow-Mediated Dilation, Dose-Response, Cystic Fibrosis

Running Title: BH_4 improves FMD in CF

ABSTRACT

Cystic fibrosis (CF) is a genetic disorder associated with vascular endothelial dysfunction. Nitric oxide (NO) plays a major role in maintaining vascular function and tetrahydrobiopterin (BH_4) is a critical determinant of NO bioavailability. Thus, the purpose of this study was to investigate the effects of oral administration of BH_4 on endothelial function in patients with CF.

Methods: 29 patients with CF (18 ± 8 yrs old) and 29 healthy matched controls were recruited. Patients with CF participated in a randomized trial where they received a 5 mg/kg dose of oral BH_4 (BH_4-5; n=17) or a 20 mg/kg dose of oral BH_4 (BH_4-20; n=12). On a separate visit, a subset of patients from each group were retested following a placebo (PLC, n=9). Brachial artery flow-mediated dilation (FMD) was used to evaluate vascular endothelial function and a plasma sample was obtained before and 3 h after treatment. Cultured endothelial cells were treated with plasma to assess NO bioavailability.

Results: Baseline FMD was lower in patients compared to controls (5.7 ± 3.4% vs. 8.4 ± 3.5% respectively, p = 0.005). No change in FMD was observed following PLC or BH_4-5 (∆FMD: -0.8 ± 0.0% and -0.5 ± 2.5%; p=0.273 and 0.132, respectively). Treatment with BH_4-20, however, resulted in significant improvements in FMD (∆FMD: 1.1 ± 1.4%) compared to BH_4-5 (p=0.023) and PLC (p=0.017). Moreover, BH_4-20 significantly decreased endothelial cell superoxide production and increased NO production.

Conclusion: These data suggest that a single oral dose of BH_4 at 20 mg/kg improves vascular endothelial function in patients with CF, likely via increased endothelial NOS

coupling. These findings support the hypothesis that loss of BH_4 bioactivity contributes, in part, to endothelial dysfunction in patients with CF.

New & Noteworthy

For the first time, the present study documents that a single dose of oral BH_4 can improve vascular endothelial function in patients with CF, and our *in vitro* data suggests this is via decreasing uncoupled NO. These data provide insight into the important role of BH_4 bioactivity on vascular dysfunction and provide the foundation for further investigation into the chronic effects of BH_4 treatment in patients with CF.

INTRODUCTION

Cystic fibrosis (CF), the most common autosomal recessive disorder among Caucasians, is caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (27). The pathological consequences of this CFTR mutation in CF leads to multiple systemic complications including respiratory, gastrointestinal, endocrine, and musculoskeletal manifestations; all together contributing to early mortality (11, 31).

CFTR is expressed in the vascular endothelium (34). Therefore, it is reasonable to believe a mutant CFTR protein within the endothelium contributes to the existence of both micro- (30) and conduit- (24) vascular endothelial dysfunction in patients with CF. Endothelial dysfunction impairs adequate nutrient supply to active tissues in patients with CF (35), which may contribute to exercise intolerance, an independent predictor of mortality in this patient population (22). The underlying mechanism of endothelial dysfunction in CF; however, has yet to be elucidated.

Nitric oxide (NO), a potent endothelium-derived vasodilator produced by NO synthase (NOS), plays a major role in maintaining normal vascular endothelial function (16). Tetrahydrobiopterin (BH_4) is an essential cofactor for eNOS function and is a critical regulator of NO production. Consequently, when BH_4 bioactivity is reduced, eNOS can become uncoupled and results in production of superoxide rather than NO (2). Indeed, BH_4 deficiency reduces NO bioavailability and contributes to a decrease in endothelial function in various pathological conditions including pulmonary hypertension (25), diabetes (14), and smoking (13). Conversely, ingestion of BH_4 has been show to improve flow-mediated dilation (FMD), a non-invasive bioassay of NO bioavailability and

vascular endothelial function (10) in older adults (6), chronic smokers (32) and patients with rheumatoid arthritis (19). Whether or not ingestion of BH_4 improves endothelial function in patients with CF has yet to be investigated.

The vascular effects following oral administration of BH_4 are complex and appear to be dose dependent (5, 23). Enzymatic reactivity of BH_4 and the subsequent influence on NO-dependent endothelial vasodilation are highly sensitive to the surrounding biochemical conditions, including overall redox state (33). Thus, identifying an appropriate dose of BH_4 that can effectively increase NO production and improve vascular endothelial function is of great interest in CF, a population that exhibits elevated oxidative stress and vascular dysfunction(9). Accordingly, this proof of concept study sought to test the hypothesis that oral administration of a high dose of BH_4 in patients with CF would improve vascular endothelial function, compared to a low BH_4 dose or placebo.

METHODS

Participants. Patients with CF, aged 8-39 years old, were enrolled if they had a clinical diagnosis of CF based on positive sweat tests and genotype analysis. Patients were excluded if they 1) had a forced expiratory volume in 1 second (FEV_1) < 50 % predicted, 2) had a resting oxygen saturation (SpO_2) <85%, 3) self-reported to be a smoker, 4) were diagnosed with pulmonary hypertension, 5) were pregnant or nursing at the time of the investigation, 6) had a clinical diagnosis of cardiovascular disease, hypertension, or CF related diabetes, or 7) were prescribed any vaso-active medications (e.g. nitrates, beta blockers, ACE inhibitors, etc.). Demographically-matched healthy individuals were recruited as controls (CON) to compare basal vascular function to the patients. All participants and parents of minors provided written and verbal informed consent/assent prior to participation. All study protocols were carried out according to Declaration of Helsinki and not only approved by the Augusta University Institutional Review Board, but also registered at Clinicaltrials.gov (NCT01772758).

Experimental Design. This proof of concept study was designed based on our pilot study in 5 CF patients that tested the safety and feasibility of a low dose (5 mg/kg) of oral BH_4 and found no BH_4-induced change in endothelial function. Based on this preliminary result, we included a higher dose BH_4 group (20 mg/kg) for comparison. Therefore, in the present randomized and placebo-controlled investigation (**Figure 1**), patients received either a single low dose treatment of BH_4 (5 mg/kg [BH_4-5]; n=17) or a high dose BH_4 treatment (20 mg/kg [BH_4-20]; n=12). On a separate day, a demographically matched subset of patients from each treatment group were re-tested following ingestion of a placebo (PLC; n=9). All participants reported to the Laboratory

of Integrated Vascular and Exercise Physiology (LIVEP) at the Georgia Prevention Institute on two separate occasions: a preliminary day and an experimental day. The preliminary day consisted of the informed consent process, body composition assessments, and a baseline pulmonary function test. For the experimental days, participants were asked to come to the LIVEP at 8 AM following an overnight fast, and having abstained from moderate to vigorous physical activity for 24 hours prior to arrival. All patients were instructed to adhere to the timing of their daily pulmonary therapy and come to the lab following their morning airway clearance treatments and inhaled medicines. Upon arrival to the LIVEP, vascular endothelial function was assessed in both patients and controls at baseline (Pre) followed by a venous blood draw. Then, patients received a treatment with either BH_4-5 or BH_4-20 (KUVAN®, BioMarin Pharmaceutical Inc. Novato, CA) dissolved in 120 ml of apple juice or a PLC was administered. Three hours following treatment (Post), another blood sample was obtained and vascular endothelial function was re-evaluated. The post-measurement time point was chosen based on previous studies indicating that the peak plasma BH_4 concentration occurs around 3 hours (7). All treatments were dispensed by the Augusta University Research Pharmacy. The details of the series were unknown by any of the investigators or patients involved in the study.

Participant Characteristics and Clinical Laboratory Values. Participant testing included standard anthropometric assessments of height, weight, calculation of body mass index (BMI; kg/m^2), and resting systolic and diastolic blood pressures. Oxygen saturation was obtained at rest using an Onyx II fingertip sensor (Nonin Medical, Plymouth, MN).

Fasting concentrations of total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TRIG), and glucose were obtained using a Cholestech LDX point of care analyzer (Alere Inc., Scarborough, ME). Concentrations of high sensitivity C-reactive protein (CRP) were determined using standard clinical core laboratory techniques (Laboratory Corporation of America Holdings, Burlington, NC).

Pulmonary Function Testing (PFT). Pulmonary function testing was performed using closed circuit spirometry (ParvoMedics, Sandy, UT) to determine forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FVC/FEV_1 and forced expiratory flow (FEF_{25-75}) according to the American Thoracic Society standards(1). A minimum of three reproducible trials were completed by each participant and the best of three acceptable forced expiratory maneuvers was selected to represent the pulmonary function values. The percent predicted data set was determined following spirometric reference standards(26).

Endothelial Function. Endothelial function was assessed via the brachial artery flow-mediated dilation (FMD) test in accordance with the tutorial on the ultrasound assessment of FMD (12). Briefly, using a 12 MHz linear transducer, simultaneous B-mode and blood velocity profiles (duplex mode) of the brachial artery were obtained (Logiq 7, GE Medical Systems, Milwaukee, WI). A forearm occlusion cuff (D.E. Hokanson, Bellevue, WA), placed immediately distal to the medial epicondyle, was rapidly inflated to 250 mm Hg for 5 minutes (E-20 rapid cuff inflator, D.E. Hokanson, Bellevue, WA) to induce arterial occlusion and subsequent reactive hyperemia of the

brachial artery. R-wave gaiting (Accusync 72, Accusync Medical Research Corporation, Milford, CN) was utilized to capture end-diastolic arterial diameters for automated offline analysis of brachial artery vasodilation (Medical Imaging Applications, Coralville, Iowa). Hyperemic diameter and blood velocity were recorded every 4 seconds for the first 20 seconds and every 5 seconds for the remainder of the 2 minute collection period. Peak diameter was determined by the highest 5 second average following cuff release according to recommendations(12). FMD is expressed as a percent increase in peak diameter from baseline diameter. Cumulative shear rate (area under the curve, s^{-1}, AUC) was determined using the trapezoidal rule, every four seconds for the first 20 seconds following cuff release, and every 5 seconds thereafter for the remainder of the 2 minute data collection period.

Cultured Endothelial Cells

To assess if treatment improved NOS coupling, cultured endothelial cells were incubated with a subset of patients (n=6) plasma before and following BH_4-20 treatment and superoxide and NO production were measured fluorometrically. Cryopreserved human aortic endothelial cells (HAoEC) were purchased from PromoCell (Heidelberg, Germany) and cultured on tissue culture-treated dishes (Corning Inc., Corning, NY) at 37°C in 5% CO_2 and 95% humidity. The recommended culture medium used was endothelial cell growth medium (ECGM) MV2 (PromoCell) supplemented with 0.05 ml/ml heat-inactivated fetal calf serum, 5 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor, 20 ng/ml insulin-like growth factor, 0.5 ng/ml of vascular endothelial growth factor 165, 1 µg/ml of ascorbic acid, 0.2 µg/ml of hydrocortisone

(PromoCell) and 1% penicillin/streptomycin (ThermoFisher, Waltham, MA). Cells were not used beyond passage six and allowed to reach confluence before experiments.

In vitro Assessment of Uncoupled NOS

To investigate mechanisms related to the change in FMD, HAoEC were cultured in 24-well plates (100,000 cells per well) for 24 hrs at 37°C in 5% CO_2 environment. After cells were grown to 60-70 % confluency, they were then incubated for ~16h in ECGM-MV2 medium supplemented with 20% (v/v) plasma taken before and after BH_4-20 treatment from a subset of patients with CF (n=6). After incubation, superoxide and NO levels were assessed using the fluorescent probes dihydroethidium (DHE) and 4,5-Diaminofluorescein diacetate (DAF-2A), respectively. Briefly, cells were washed two times with PBS 1X then stimulated with angiotensin II (100 nM) for superoxide measurements or methacholine (100 μM) for assessment of NO production. Cells were then treated with either DHE (10 μM, Sigma) or DAF-2A (10 μM, EMD Millipore) and incubated for 30 minutes at 37°C for detection of superoxide and NO, respectively. To confirm specificity of signal detection, additional cells were pretreated with the superoxide dismutase mimetic tempol (100 nM, Sigma) or the nonselective NOS inhibitor L-nitroarginine methyl ester (L-NAME, 1mM, Sigma) for 30 minutes at 37°C prior to stimulation and fluorescent probe application. All treatments were performed in duplicate and reported as averaged values. 4-5 pictures were taken for each well/treatment using Zeiss 780 Inverted Confocal microscope at 100X magnification (for DHE excitation/emission maxima were 510/595 nm and for DAF-2A excitation/emission maxima were 495/515 nm). The intensity of florescence was assessed using image J-

NIH software and was corrected to mm^2. All cell experiments were completed within the same time of day, minimizing the potential confounding effects of diurnal variation.

Statistical Analyses.

The sample size was computed for detecting a significant difference in the change in FMD% from Pre- to Post-BH_4 treatment based on our previous study that examined the acute effect of BH_4 in another clinical population(29). We found that the change in FMD% following BH_4 treatment was 2.1 ± 2.3% which yielded a sample size of 12 at a significance level 0.05 with a power of 0.85.

Values are presented as mean ± SD unless otherwise noted. Differences in patient characteristics between groups were determined using independent groups *t*-tests or analysis of variance (ANOVA). Primary analysis for group differences in the FMD response following treatment was performed using repeated-measures ANOVA or analysis of covariance (ANCOVA) to adjust for baseline measures and to provide an unbiased estimate of the mean group difference. Bonferroni correction was used for post-hoc analysis when a significant main effect was found. Paired t-tests were used to compare DHE and DAF pre and post BH_4-20 treatment. Effect sizes for FMD responses after treatment are reported by Cohen's *d* values to represent small (*d*=0.2), medium (*d*=0.5), and large effect sizes (*d*=0.8) (4). An alpha <0.05 was considered statistically significant for all analyses. All analyses were performed using SPSS version 24.0 (IBM Corporation, Somers, NY).

RESULTS

Participants Characteristics

Demographic and clinical characteristics of patients with CF and healthy controls are presented in **Table 1**. Importantly, no significant differences were observed between patient groups for any demographics, pulmonary function, or blood chemistry values (all $p>0.05$). In addition, the pulmonary function variables represent a relatively healthy patient cohort with mild to moderate disease severity (8). Some indices of lung function as well as diastolic blood pressure, however, were significantly higher ($p>0.05$) in controls compared to the patients. Although all blood chemistry variables were within normal ranges, CRP was lower ($p=0.003$) and HDL was higher ($p=0.003$) in controls compared to patients.

Basal Endothelial Function between CF Patients and Controls

The average baseline FMD of all patients combined (n=29) was significantly lower compared with the demographically matched healthy control cohort (5.7 ± 3.4% vs. 8.4 ± 3.5% respectively, $p = 0.005$). In addition, the absolute change in artery diameter was greater in controls (0.17 ± 0.01 vs. 0.26 ± 0.01cm, $p=0.001$) while baseline diameter (0.31 ± 0.05 vs. 0.30 ± 0.05 cm, $p=0.599$), peak diameter (0.32 ± 0.05 vs. 0.33 ± 0.05 cm, $p=0.536$), shear rate (55698 ± 27878 vs. 46971 ± 12444 s^{-1},AUC, $p=0.129$), and TTP (53 ± 23 vs. 43 ± 18 sec, $p=0.086$) were all similar between patients and healthy controls.

Effect of BH_4 on Endothelial Function in Patients with CF

Pre- and post-treatment parameters of the FMD test following BH_4-5, BH_4-20 and PLC are presented in **Table 2.** Importantly, pre- and post- shear rates following treatment with BH_4-5, BH_4-20 or PLC were all similar (p=0.143, p=0.517, and p=0.132, respectively).

All pre-treatment FMD parameters were similar between the three groups (all p>0.05). A significant treatment by time interaction for FMD ($F_{(2, 33)}$=7.51, p=0.017) was observed when controlling for pre-treatment values. Specifically, **Figure 2** illustrates a significantly greater change in FMD following BH_4-20 treatment (1.1 ± 1.4 %, p = 0.023; *d*=0.33), whereas no change was observed with either BH_4-5 (-0.51 ± 2.53 %, p = 0.132; *d*=0.16) or PLC (-0.84 ± 0.02, p = 0.273; *d* = 0.45).

Similarly, there was a significant treatment by time interaction for peak diameter (cm) ($F_{(2,34)}$=6.926, p=0.003). While there was a trend for an increase in peak diameter following BH_4-20 (0.004 ± 0.007 cm, p=0.056), it decreased following PLC and BH_4-5 (0.005 ± 0.009 and -0.005 ± 0.007 cm, p=0.049 and 0.004 respectively). In addition, there was a significant treatment by time interaction for time to peak vasodilation (TTP; s) ($F_{(2,34)}$=4.594, p=0.017). Specifically, no change was observed following BH_4-5 (-3.8 ± 24.7 s, p=0.574) or BH_4-20 (5.0 ± 17.5 s, p=0.537); however, a significant decrease was observed following PLC (-32.5 ± 43.34 p=0.002). No difference (p>0.05) in blood pressure was observed between baseline and post treatment, respectively, following BH_4-5 (SBP 108 ± 7 vs. 111 ± 6 mm Hg; DBP 61 ± 7 vs. 62 ± 8 mm Hg) and BH_4-20 (SBP 112 ± 15 vs. 111 ± 11 mm Hg; DBP 61 ± 8 vs. 61 ± 7 mm Hg).

No significant differences in baseline artery diameter (cm) ($F_{(2, 34)}$=1.874, p=0.169), shear rate (s^{-1},AUC) ($F_{(2, 34)}$=1.545, p=0.228), or absolute change in diameter (cm) ($F_{(2, 34)}$=2.32, p=0.114) were observed. In addition, no significant relationships were observed between baseline FEV_1 and the change in FMD either from BH_4-5 (r=0.066; p=0.801) or BH_4-20 (r=0.092; p=0.776).

Endothelial Cell Coupling of NOS3

Stimulated superoxide production was significantly lower (p=0.01) in endothelial cells pre-incubated with post BH_4-20 plasma compared to endothelial cells treated with pre-treatment plasma (Figure 3A). Inhibition of NOS (using L-NAME) significantly (p<0.001) attenuated stimulated superoxide production in cells treated with plasma prior to BH_4-20 treatment (Figure 3A). NOS inhibition had no significant (p=0.065) effect on superoxide production in cells incubated with plasma post-BH_4-20. Specificity of stimulated DHE fluorescence for superoxide was confirmed with tempol (Figure 3A). Consistent with the above, stimulated NO production tended to be greater (p=0.10) in endothelial cells incubated with post BH_4-20 plasma compared to incubation with pre-BH_4-20 plasma, although this did not reach statistical significance. Pre-incubation with the NOS inhibitor L-NAME confirmed NOS-mediated NO production (Figure 3B).

DISCUSSION

Patients with CF exhibit systemic vascular endothelial dysfunction (24, 30); however, the mechanisms have yet to be elucidated. The present study sought to test the hypothesis that an acute treatment with BH_4 would improve vascular endothelial function in patients with CF. For the first time, findings from the present study demonstrate that a single dose of 20 mg/kg of BH_4 improves endothelial function in patients with CF that is accompanied by a decrease in NOS-mediated superoxide production, suggesting an improvement in NOS coupling. In contrast, no change in endothelial function was observed following PLC or 5 mg/kg of BH_4. Our proof of concept findings support the importance of BH_4 bioactivity as a potential mechanism that may, in part, be responsible for vascular endothelial dysfunction in patients with CF (24, 30).

Endothelial Function in Patients with CF

CF is caused by mutation of the CFTR gene that is expressed in a wide spectrum of cells including the vascular endothelium (27). Our group has previously provided evidence of both micro- and macro- vascular endothelial dysfunction in young patients with CF who presented with a relatively well-preserved spirometric function (24, 30). Importantly, the present cohort of patients also exhibit endothelial dysfunction when compared to a demographically matched healthy control group. Collectively, these findings not only suggest that CF-related systemic consequences may precede a marked decline in pulmonary function, but also indicate that endothelial dysfunction may

be an early indicator of systemic deterioration and/or clinical manifestation in patients with CF.

BH_4 Improves Vascular Endothelial Function in Patients with CF

For the first time, the present study demonstrates that a single dose of BH_4 can improve vascular endothelial function in patients with CF, evidenced by a 1.1 % absolute and a 17% relative increase in FMD following high dose treatment (**Figure 2**). The FMD test represents a bioassay of NO bioavailability and assessment of endothelial function. Although apparently small in magnitude, a 1% absolute increase in FMD has been associated with a 10-13% reduction in risk of future cardiovascular events and all-cause mortality (17, 36). In addition, this moderate improvement (Cohen's *d*=0.33) in FMD may have significant implications for quality of life and survival in this patient population through the improvement in other systemic manifestations of CF, including exercise capacity. Future studies are warranted to determine the therapeutic effect of chronic BH_4 treatment on both FMD and exercise capacity in CF.

The improvement in FMD observed in the present study is in line with previous studies that have demonstrated an increase in endothelial function following acute administration of oral BH_4 in different populations that exhibit systemic oxidative stress (6, 18, 19, 32). Although the exact mechanisms by which oral BH_4 treatment increases endothelial-dependent vasodilation in patients with CF are unclear, an increase in endothelial NO production associated with improved eNOS coupling is likely to play an important role. To support this hypothesis we performed *in vitro* studies examining the ability of BH_4-treated patient plasma to modulate endothelial cell superoxide and NO production (Figure 3). Findings from this cell culture experiment suggest an

improvement in NOS coupling and subsequent NOS-mediated NO production in endothelial cells following BH_4-20 treatment. Importantly, the change in FMD observed following treatment in the present study was independent of basal lung function. Conduit artery FMD is shown to be primarily mediated by endothelial-derived NO (20), and BH_4 is a cofactor specific for eNOS that modulates NO synthesis in the vascular endothelium (3). In fact, previous studies have demonstrated that BH_4 is able to increase NO bioavailability (5, 15) and promote vasodilation without impacting endothelial-independent vasodilatory mechanisms (18, 25). Moreover, results from animal studies demonstrate that deficiency of endothelial BH_4 alone is sufficient to cause vascular dysfunction even in the absence of atherogenic vascular disease (3). Collectively, findings from the present study support the idea that insufficient bioactivity of BH_4 in the endothelium contributes to endothelial dysfunction in CF (24). Although speculative, CF-associated BH_4 oxidation by overproduction of vascular reactive oxidative species may lead to decreased endothelial BH_4 bioavailability, which in turn may uncouple eNOS and lead to a reduction in NO-mediated vasodilation. Impaired NO-dependent vasodilation can impair blood flow regulation during exercise especially under conditions of elevated basal oxidative stress. Exercise intolerance is an independent contributor of mortality in CF. Therefore, understanding the mechanisms that contribute to vascular dysfunction in CF may have important clinical implications, and future studies are certainly needed to examine the mechanistic role of BH_4 on NO generation and the development of vascular dysfunction in CF.

A High Dose of BH_4 may be Needed to Improve Endothelial Function in Patients with CF

Enteral administration of BH_4, although unrelated to its vascular benefit, is clinically indicated to treat phenylketonuria using a dose between 5-20 mg/kg. This wide dosing range depends on 1) varying responsiveness related to genotype, 2) absorption, and/or 3) metabolic states of BH_4 (23). Perhaps unsurprisingly, even the vascular effects following oral administration of BH_4 appear to be complex and dose dependent (5, 23). In the present study, we observed an improvement in vascular endothelial function only following oral administration of BH_4 at a dose of 20 mg/kg; PLC or 5 mg/kg of BH_4 did not alter the FMD response. In support, prevailing data in the literature have already suggested a similar dose-dependent effect of BH_4 on the vasculature (21, 23, 25). A daily dose of at least 400 mg of oral BH_4 significantly improves endothelial function in patients with hypertension, whereas no effect was observed following a daily dose of 200 mg(25). The average amount of BH_4 given to the BH_4-5 group in the present study was 265 ± 72 mg, which was similar to the previously demonstrated ineffective dose(25) and may explain the null response.

A classic feature of CF is elevated systemic oxidative stress (9). In addition, BH_4 is easily oxidized by excessive free radicals. Therefore, a higher dose of BH_4 may have been needed to balance multiple modulators of the eNOS uncoupling cascade, such as BH_4 oxidation status, BH_4 clearance rate, and the ratio of BH_4 to BH_2 in the vascular endothelium. Moreover, the progressive dose escalation designs that are often performed during early stages of clinical trials with BH_4 supplementation (28) suggests the importance of disease-specific investigation for the sake of safety and efficacy of the use of BH_4 in clinical populations. Although it is uncertain whether the BH_4-associated improvement in endothelial function can be maintained with chronic BH_4 administration

in patients with CF, further investigation is warranted to explore effective strategies for maintenance of adequate levels of intracellular BH_4 bioavailability in patients with CF. Nonetheless, the present study demonstrates that a treatment dose of 20 mg/kg of BH_4 produces a significant increase in endothelial dependent vasodilation that is accompanied by an improved NOS3 coupling in patients with CF.

Clinical Significance

Vascular endothelial dysfunction plays an important detrimental role in many pathological conditions. With respect to patients with CF, vascular dysfunction is a contributor to exercise intolerance (24), a predictor of mortality that is independent of lung function in this patient population (22). Improvements in both endothelial function and NOS3 coupling following BH_4 treatment observed in the present study may increase longevity of these patients due to 1) the overall improvement in cardiovascular disease risk, and 2) directly or indirectly increasing blood flow regulation and contributing to an improvement in exercise capacity. Although both scenarios will have significant clinical implications on survival in patients with CF, further research is warranted to test these hypothesis.

Conclusion

For the first time, the present study has shown that a single oral dose of 20 mg/kg of BH_4 significantly improved vascular endothelial function in patients with CF. The improvement in FMD was also accompanied by a significant decrease in endothelial cell

NOS-mediated superoxide production and an increase in NO supporting an improvement in NOS coupling with BH_4 treatment. These findings indicates an important role of BH_4 bioactivity in the regulation of endothelial dependent vasodilation in CF. Further studies are needed to investigate the ultimate translational potential of BH_4 in prevention and treatment of vascular dysfunction in patients with CF.

Competing Interests

None to declare.

Funding

Supported in part by BioMarin Pharmaceutical Inc. (R.A.H.), American Heart Association (10SDG305006 to R.A.H.) and the Child Health Discovery Institute (R.A.H.).

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FIGURE LEGEND

Figure 1. Study Flow Chart

CF: cystic fibrosis, **BH_4-5**: 5mg/kg of BH_4, **BH_4-20**: 20mg/kg of BH_4, **PLC**: placebo

Figure 2. The change (Δ) in FMD(%) following BH4-20 compared to BH4-5 or PLC in patients with CF. Values are means ± standard error. * Significant difference ($p < 0.05$) from both BH4-5 and PLC.

Figure 3. Stimulated human aortic endothelial cell production of superoxide (panel A) and nitric oxide (panel B) incubated with pre and post BH_4-20 patient plasma. Representative fluorescent images of cells under basal, L-NAME, and Tempol conditions for each probe are illustrated. *Significant from basal pre BH_4-20. †Significant from corresponding basal value. #Significant from corresponding basal and pre BH_4-20 L-NAME.

574 **Table 1.** Characteristics and laboratory values for patient treatment groups (BH_4-4 and
575 BH_4-20) and healthy controls (CON).

	BH_4-5	BH_4-20	CON	p-value*
N	17	12	29	
Demographic				
Sex (M/F)	**7/10**	**6/6**	**13/16**	
Age (yrs)	17 ± 7	19 ± 8	22 ± 9	0.182
Height (cm)	158 ± 14	162 ± 11	165 ± 13	0.170
Weight (kg)	53 ± 14	57 ± 14	62 ± 21	0.285
BMI (kg/m^2)	20.8 ± 3.3	21.8 ± 3.8	22.1 ± 5.6	0.665
Body Fat (%)	22.2 ± 6.0	23.5 ± 4.1	22 ± 8.5	0.525
SBP (mm Hg)	109 ± 13	110 ± 11	113 ± 15	0.635
DBP (mm Hg)	60 ± 8	62 ± 8	68 ± 11	**0.015***
Pulmonary function				
FVC (L)	3.7 ± 1.3	3.8 ± 1.2	3.9 ± 1.1	0.711
FEV_1 (L)	2.8 ± 1.1	3.0 ± 1.1	3.3 ± 0.8	0.145
FEV_1/FVC (%)	74 ± 8	78 ± 10	85 ± 7	**0.001***
FEV_1 (% predicted)	87 ± 18	88 ± 20	98 ± 13	0.059
FEF_{25-75} (L/s)	2.5 ± 1.3	2.9 ± 1.5	3.6 ± 1.1	**0.011***
Blood chemistry				
CRP (mg/L)	2.3 ± 2.1	3.9 ± 3.5	0.9 ± 1.4	**0.003***
TC (mg/dL)	133 ± 39	150 ± 37	158 ± 27	0.066
HDL (mg/dL)	43 ± 15	48 ± 19	62 ± 18	**0.003***
LDL (mg/dL)	73 ± 28	78 ± 16	85 ± 24	0.194
TRIG (mg/dL)	96 ± 36	83 ± 22	76 ± 35	0.191
TC/HDL ratio	3.1 ± 1.0	3.3 ± 0.8	2.7 ± 0.8	0.165
Glucose (mg/dL)	93 ± 35	95 ± 19	88 ± 9	0.667

576 Values are mean ± SD. **BMI**: Body Mass Index, **SBP**: Brachial Systolic Blood Pressure,
577 **DBP**: Brachial Diastolic Blood Pressure, **FVC**: Forced Vital Capacity, **FEV_1**: Forced
578 Expiratory Volume in one second, **FEF_{25-75}**: Forced Expiratory Flow at 25-75%, **CRP**: C-
579 Reactive Protein, **TC**: Total Cholesterol, **HDL**: High-density Lipoproteins, **LDL**: Low-
580 density Lipoproteins, **TRIG**: Triglycerides

581 *Significant difference between healthy controls and both BH_4-5 and BH_4-20 ($p<0.05$).

582 **Table 2.** Parameters of the FMD test in patients with CF before (Pre) and after (Post) treatment with 5 mg/kg of BH_4 (BH_4-
583 5), 20 mg/kg of BH_4 (BH_4-20), and placebo (PLC).

Variable	**BH_4-5**		**BH_4-20**		**PLC**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Baseline diameter (cm)	0.31 ± 0.05	0.30 ± 0.05	0.30 ± 0.06	0.31 ± 0.06	0.33 ± 0.05	0.33 ± 0.05
Peak diameter (cm)	0.32 ± 0.05	0.32 ± 0.06	0.32 ± 0.05	0.33 ± 0.06	0.35 ± 0.05	0.34 ± 0.05
Shear rate (s^{-1},AUC)	58269 ± 33110	49548 ± 29841	52056 ± 18949	54460 ± 23250	52527 ± 25003	43242 ± 30749
Absolute change (cm)	0.016 ± 0.009	0.014 ± 0.007	0.018 ± 0.007	0.021 ± 0.006*	0.021 ± 0.012	0.018 ± 0.008
FMD (%)	5.3 ± 3.5	4.8 ± 2.8	6.3 ± 3.2	7.4 ± 3.4*	6.5 ± 4.1	5.7 ± 2.7
TTP (sec)	54.17 ± 33.16	47.50 ± 27.16	53.61 ± 21.33	59.72 ± 17.70	70.83 ± 35.26	41.39 ± 16.35*

584 Values are mean ± SD. **AUC**: Area under a Curve, **FMD**: Flow-Mediated Dilation, **TTP**: Time-to-Peak.

585 * Significant change from Pre-treatment values ($p < 0.05$)
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