



Tetrahydrobiopterin Improves Endothelial Function in Patients with Cystic Fibrosis

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1 **Tetrahydrobiopterin Improves Endothelial Function**
2 **in Patients with Cystic Fibrosis**

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ABSTRACT

38

39 Cystic fibrosis (CF) is a genetic disorder associated with vascular endothelial
40 dysfunction. Nitric oxide (NO) plays a major role in maintaining vascular function and
41 tetrahydrobiopterin (BH₄) is a critical determinant of NO bioavailability. Thus, the
42 purpose of this study was to investigate the effects of oral administration of BH₄ on
43 endothelial function in patients with CF.

44 **Methods:** 29 patients with CF (18 ± 8 yrs old) and 29 healthy matched controls were
45 recruited. Patients with CF participated in a randomized trial where they received a 5
46 mg/kg dose of oral BH₄ (BH₄-5; n=17) or a 20 mg/kg dose of oral BH₄ (BH₄-20; n=12).
47 On a separate visit, a subset of patients from each group were retested following a
48 placebo (PLC, n=9). Brachial artery flow-mediated dilation (FMD) was used to evaluate
49 vascular endothelial function and a plasma sample was obtained before and 3 h after
50 treatment. Cultured endothelial cells were treated with plasma to assess NO
51 bioavailability.

52 **Results:** Baseline FMD was lower in patients compared to controls (5.7 ± 3.4% vs. 8.4
53 ± 3.5% respectively, p = 0.005). No change in FMD was observed following PLC or BH₄-
54 5 (ΔFMD: -0.8 ± 0.0% and -0.5 ± 2.5%; p=0.273 and 0.132, respectively). Treatment
55 with BH₄-20, however, resulted in significant improvements in FMD (ΔFMD: 1.1 ± 1.4%)
56 compared to BH₄-5 (p=0.023) and PLC (p=0.017). Moreover, BH₄-20 significantly
57 decreased endothelial cell superoxide production and increased NO production.

58 **Conclusion:** These data suggest that a single oral dose of BH₄ at 20 mg/kg improves
59 vascular endothelial function in patients with CF, likely via increased endothelial NOS

60 coupling. These findings support the hypothesis that loss of BH₄ bioactivity contributes,
61 in part, to endothelial dysfunction in patients with CF.

62 **New & Noteworthy**

63 For the first time, the present study documents that a single dose of oral BH₄ can
64 improve vascular endothelial function in patients with CF, and our *in vitro* data suggests
65 this is via decreasing uncoupled NO. These data provide insight into the important role
66 of BH₄ bioactivity on vascular dysfunction and provide the foundation for further
67 investigation into the chronic effects of BH₄ treatment in patients with CF.

68

69

70 INTRODUCTION

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

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

84 Nitric oxide (NO), a potent endothelium-derived vasodilator produced by NO
85 synthase (NOS), plays a major role in maintaining normal vascular endothelial function
86 (16). Tetrahydrobiopterin (BH₄) is an essential cofactor for eNOS function and is a
87 critical regulator of NO production. Consequently, when BH₄ bioactivity is reduced,
88 eNOS can become uncoupled and results in production of superoxide rather than NO
89 (2). Indeed, BH₄ deficiency reduces NO bioavailability and contributes to a decrease in
90 endothelial function in various pathological conditions including pulmonary hypertension
91 (25), diabetes (14), and smoking (13). Conversely, ingestion of BH₄ has been show to
92 improve flow-mediated dilation (FMD), a non-invasive bioassay of NO bioavailability and

93 vascular endothelial function (10) in older adults (6), chronic smokers (32) and patients
94 with rheumatoid arthritis (19). Whether or not ingestion of BH₄ improves endothelial
95 function in patients with CF has yet to be investigated.

96 The vascular effects following oral administration of BH₄ are complex and appear
97 to be dose dependent (5, 23). Enzymatic reactivity of BH₄ and the subsequent influence
98 on NO-dependent endothelial vasodilation are highly sensitive to the surrounding
99 biochemical conditions, including overall redox state (33). Thus, identifying an
100 appropriate dose of BH₄ that can effectively increase NO production and improve
101 vascular endothelial function is of great interest in CF, a population that exhibits
102 elevated oxidative stress and vascular dysfunction(9). Accordingly, this proof of concept
103 study sought to test the hypothesis that oral administration of a high dose of BH₄ in
104 patients with CF would improve vascular endothelial function, compared to a low BH₄
105 dose or placebo.

106

107 **METHODS**

108 Participants. Patients with CF, aged 8-39 years old, were enrolled if they had a clinical
109 diagnosis of CF based on positive sweat tests and genotype analysis. Patients were
110 excluded if they 1) had a forced expiratory volume in 1 second (FEV₁) < 50 % predicted,
111 2) had a resting oxygen saturation (SpO₂) <85%, 3) self-reported to be a smoker, 4)
112 were diagnosed with pulmonary hypertension, 5) were pregnant or nursing at the time of
113 the investigation, 6) had a clinical diagnosis of cardiovascular disease, hypertension, or
114 CF related diabetes, or 7) were prescribed any vaso-active medications (e.g. nitrates,
115 beta blockers, ACE inhibitors, etc.). Demographically-matched healthy individuals were
116 recruited as controls (CON) to compare basal vascular function to the patients. All
117 participants and parents of minors provided written and verbal informed consent/assent
118 prior to participation. All study protocols were carried out according to Declaration of
119 Helsinki and not only approved by the Augusta University Institutional Review Board,
120 but also registered at Clinicaltrials.gov (NCT01772758).

121 Experimental Design. This proof of concept study was designed based on our pilot
122 study in 5 CF patients that tested the safety and feasibility of a low dose (5 mg/kg) of
123 oral BH₄ and found no BH₄-induced change in endothelial function. Based on this
124 preliminary result, we included a higher dose BH₄ group (20 mg/kg) for comparison.
125 Therefore, in the present randomized and placebo-controlled investigation (**Figure 1**),
126 patients received either a single low dose treatment of BH₄ (5 mg/kg [BH₄-5]; n=17) or a
127 high dose BH₄ treatment (20 mg/kg [BH₄-20]; n=12). On a separate day, a
128 demographically matched subset of patients from each treatment group were re-tested
129 following ingestion of a placebo (PLC; n=9). All participants reported to the Laboratory

130 of Integrated Vascular and Exercise Physiology (LIVEP) at the Georgia Prevention
131 Institute on two separate occasions: a preliminary day and an experimental day. The
132 preliminary day consisted of the informed consent process, body composition
133 assessments, and a baseline pulmonary function test. For the experimental days,
134 participants were asked to come to the LIVEP at 8 AM following an overnight fast, and
135 having abstained from moderate to vigorous physical activity for 24 hours prior to
136 arrival. All patients were instructed to adhere to the timing of their daily pulmonary
137 therapy and come to the lab following their morning airway clearance treatments and
138 inhaled medicines. Upon arrival to the LIVEP, vascular endothelial function was
139 assessed in both patients and controls at baseline (Pre) followed by a venous blood
140 draw. Then, patients received a treatment with either BH₄-5 or BH₄-20 (KUVAN[®],
141 BioMarin Pharmaceutical Inc. Novato, CA) dissolved in 120 ml of apple juice or a PLC
142 was administered. Three hours following treatment (Post), another blood sample was
143 obtained and vascular endothelial function was re-evaluated. The post-measurement
144 time point was chosen based on previous studies indicating that the peak plasma BH₄
145 concentration occurs around 3 hours (7). All treatments were dispensed by the Augusta
146 University Research Pharmacy. The details of the series were unknown by any of the
147 investigators or patients involved in the study.

148

149 Participant Characteristics and Clinical Laboratory Values. Participant testing included
150 standard anthropometric assessments of height, weight, calculation of body mass index
151 (BMI; kg/m²), and resting systolic and diastolic blood pressures. Oxygen saturation was
152 obtained at rest using an Onyx II fingertip sensor (Nonin Medical, Plymouth, MN).

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

158
159 Pulmonary Function Testing (PFT). Pulmonary function testing was performed using
160 closed circuit spirometry (ParvoMedics, Sandy, UT) to determine forced vital capacity
161 (FVC), forced expiratory volume in one second (FEV₁), FVC/FEV₁ and forced expiratory
162 flow (FEF₂₅₋₇₅) according to the American Thoracic Society standards(1). A minimum of
163 three reproducible trials were completed by each participant and the best of three
164 acceptable forced expiratory maneuvers was selected to represent the pulmonary
165 function values. The percent predicted data set was determined following spirometric
166 reference standards(26).

167
168 Endothelial Function. Endothelial function was assessed via the brachial artery flow-
169 mediated dilation (FMD) test in accordance with the tutorial on the ultrasound
170 assessment of FMD (12). Briefly, using a 12 MHz linear transducer, simultaneous B-
171 mode and blood velocity profiles (duplex mode) of the brachial artery were obtained
172 (Logiq 7, GE Medical Systems, Milwaukee, WI). A forearm occlusion cuff (D.E.
173 Hokanson, Bellevue, WA), placed immediately distal to the medial epicondyle, was
174 rapidly inflated to 250 mm Hg for 5 minutes (E-20 rapid cuff inflator, D.E. Hokanson,
175 Bellevue, WA) to induce arterial occlusion and subsequent reactive hyperemia of the

176 brachial artery. R-wave gating (Accusync 72, Accusync Medical Research Corporation,
177 Milford, CN) was utilized to capture end-diastolic arterial diameters for automated offline
178 analysis of brachial artery vasodilation (Medical Imaging Applications, Coralville, Iowa).
179 Hyperemic diameter and blood velocity were recorded every 4 seconds for the first 20
180 seconds and every 5 seconds for the remainder of the 2 minute collection period. Peak
181 diameter was determined by the highest 5 second average following cuff release
182 according to recommendations(12). FMD is expressed as a percent increase in peak
183 diameter from baseline diameter. Cumulative shear rate (area under the curve, s^{-1} ,
184 AUC) was determined using the trapezoidal rule, every four seconds for the first 20
185 seconds following cuff release, and every 5 seconds thereafter for the remainder of the
186 2 minute data collection period.

187

188 **Cultured Endothelial Cells**

189 To assess if treatment improved NOS coupling, cultured endothelial cells were
190 incubated with a subset of patients (n=6) plasma before and following BH₄-20 treatment
191 and superoxide and NO production were measured fluorometrically. Cryopreserved
192 human aortic endothelial cells (HAoEC) were purchased from PromoCell (Heidelberg,
193 Germany) and cultured on tissue culture-treated dishes (Corning Inc., Corning, NY) at
194 37°C in 5% CO₂ and 95% humidity. The recommended culture medium used was
195 endothelial cell growth medium (ECGM) MV2 (PromoCell) supplemented with 0.05
196 ml/ml heat-inactivated fetal calf serum, 5 ng/ml epidermal growth factor, 10 ng/ml basic
197 fibroblast growth factor, 20 ng/ml insulin-like growth factor, 0.5 ng/ml of vascular
198 endothelial growth factor 165, 1 µg/ml of ascorbic acid, 0.2 µg/ml of hydrocortisone

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

201

202 **In vitro Assessment of Uncoupled NOS**

203 To investigate mechanisms related to the change in FMD, HAoEC were cultured in
204 24-well plates (100,000 cells per well) for 24 hrs at 37°C in 5% CO₂ environment. After
205 cells were grown to 60-70 % confluency, they were then incubated for ~16h in ECGM-
206 MV2 medium supplemented with 20% (v/v) plasma taken before and after BH₄-20
207 treatment from a subset of patients with CF (n=6). After incubation, superoxide and NO
208 levels were assessed using the fluorescent probes dihydroethidium (DHE) and 4,5-
209 Diaminofluorescein diacetate (DAF-2A), respectively. Briefly, cells were washed two
210 times with PBS 1X then stimulated with angiotensin II (100 nM) for superoxide
211 measurements or methacholine (100 μM) for assessment of NO production. Cells were
212 then treated with either DHE (10 μM, Sigma) or DAF-2A (10 μM, EMD Millipore) and
213 incubated for 30 minutes at 37°C for detection of superoxide and NO, respectively. To
214 confirm specificity of signal detection, additional cells were pretreated with the
215 superoxide dismutase mimetic tempol (100 nM, Sigma) or the nonselective NOS
216 inhibitor L-nitroarginine methyl ester (L-NAME, 1mM, Sigma) for 30 minutes at 37°C
217 prior to stimulation and fluorescent probe application. All treatments were performed in
218 duplicate and reported as averaged values. 4-5 pictures were taken for each
219 well/treatment using Zeiss 780 Inverted Confocal microscope at 100X magnification (for
220 DHE excitation/emission maxima were 510/595 nm and for DAF-2A excitation/emission
221 maxima were 495/515 nm). The intensity of fluorescence was assessed using image J-

222 NIH software and was corrected to mm². All cell experiments were completed within
223 the same time of day, minimizing the potential confounding effects of diurnal variation.

224

225 Statistical Analyses.

226 The sample size was computed for detecting a significant difference in the change in
227 FMD% from Pre- to Post-BH₄ treatment based on our previous study that examined the
228 acute effect of BH₄ in another clinical population(29). We found that the change in
229 FMD% following BH₄ treatment was 2.1 ± 2.3% which yielded a sample size of 12 at a
230 significance level 0.05 with a power of 0.85.

231

232 Values are presented as mean ± SD unless otherwise noted. Differences in patient
233 characteristics between groups were determined using independent groups *t*-tests or
234 analysis of variance (ANOVA). Primary analysis for group differences in the FMD
235 response following treatment was performed using repeated-measures ANOVA or
236 analysis of covariance (ANCOVA) to adjust for baseline measures and to provide an
237 unbiased estimate of the mean group difference. Bonferroni correction was used for
238 post-hoc analysis when a significant main effect was found. Paired *t*-tests were used to
239 compare DHE and DAF pre and post BH₄-20 treatment. Effect sizes for FMD responses
240 after treatment are reported by Cohen's *d* values to represent small (*d*=0.2), medium
241 (*d*=0.5), and large effect sizes (*d*=0.8) (4). An alpha <0.05 was considered statistically
242 significant for all analyses. All analyses were performed using SPSS version 24.0 (IBM
243 Corporation, Somers, NY).

244

245 RESULTS

246 Participants Characteristics

247 Demographic and clinical characteristics of patients with CF and healthy controls are
248 presented in **Table 1**. Importantly, no significant differences were observed between
249 patient groups for any demographics, pulmonary function, or blood chemistry values (all
250 $p>0.05$). In addition, the pulmonary function variables represent a relatively healthy
251 patient cohort with mild to moderate disease severity (8). Some indices of lung function
252 as well as diastolic blood pressure, however, were significantly higher ($p>0.05$) in
253 controls compared to the patients. Although all blood chemistry variables were within
254 normal ranges, CRP was lower ($p=0.003$) and HDL was higher ($p=0.003$) in controls
255 compared to patients.

256

257 Basal Endothelial Function between CF Patients and Controls

258 The average baseline FMD of all patients combined ($n=29$) was significantly lower
259 compared with the demographically matched healthy control cohort ($5.7 \pm 3.4\%$ vs. 8.4
260 $\pm 3.5\%$ respectively, $p = 0.005$). In addition, the absolute change in artery diameter was
261 greater in controls (0.17 ± 0.01 vs. 0.26 ± 0.01 cm, $p=0.001$) while baseline diameter
262 (0.31 ± 0.05 vs. 0.30 ± 0.05 cm, $p=0.599$), peak diameter (0.32 ± 0.05 vs. 0.33 ± 0.05
263 cm, $p=0.536$), shear rate (55698 ± 27878 vs. 46971 ± 12444 s^{-1} , AUC, $p=0.129$), and
264 TTP (53 ± 23 vs. 43 ± 18 sec, $p=0.086$) were all similar between patients and healthy
265 controls.

266

267 **Effect of BH₄ on Endothelial Function in Patients with CF**

268 Pre- and post-treatment parameters of the FMD test following BH₄-5, BH₄-20 and PLC
269 are presented in **Table 2**. Importantly, pre- and post- shear rates following treatment
270 with BH₄-5, BH₄-20 or PLC were all similar ($p=0.143$, $p=0.517$, and $p=0.132$,
271 respectively).

272 All pre-treatment FMD parameters were similar between the three groups (all $p>0.05$). A
273 significant treatment by time interaction for FMD ($F_{(2, 33)}=7.51$, $p=0.017$) was observed
274 when controlling for pre-treatment values. Specifically, **Figure 2** illustrates a significantly
275 greater change in FMD following BH₄-20 treatment (1.1 ± 1.4 %, $p = 0.023$; $d=0.33$),
276 whereas no change was observed with either BH₄-5 (-0.51 ± 2.53 %, $p = 0.132$; $d=0.16$)
277 or PLC (-0.84 ± 0.02 , $p = 0.273$; $d = 0.45$).

278 Similarly, there was a significant treatment by time interaction for peak diameter (cm)
279 ($F_{(2,34)}=6.926$, $p=0.003$). While there was a trend for an increase in peak diameter
280 following BH₄-20 (0.004 ± 0.007 cm, $p=0.056$), it decreased following PLC and BH₄-5
281 (0.005 ± 0.009 and -0.005 ± 0.007 cm, $p=0.049$ and 0.004 respectively). In addition,
282 there was a significant treatment by time interaction for time to peak vasodilation (TTP;
283 s) ($F_{(2,34)}=4.594$, $p=0.017$). Specifically, no change was observed following BH₄-5 ($-3.8 \pm$
284 24.7 s, $p=0.574$) or BH₄-20 (5.0 ± 17.5 s, $p=0.537$); however, a significant decrease was
285 observed following PLC (-32.5 ± 43.34 p=0.002). No difference ($p>0.05$) in blood
286 pressure was observed between baseline and post treatment, respectively, following
287 BH₄-5 (SBP 108 ± 7 vs. 111 ± 6 mm Hg; DBP 61 ± 7 vs. 62 ± 8 mm Hg) and BH₄-20
288 (SBP 112 ± 15 vs. 111 ± 11 mm Hg; DBP 61 ± 8 vs. 61 ± 7 mm Hg).

289 No significant differences in baseline artery diameter (cm) ($F_{(2, 34)}=1.874$, $p=0.169$),
290 shear rate (s^{-1} , AUC) ($F_{(2, 34)}=1.545$, $p=0.228$), or absolute change in diameter (cm) ($F_{(2,$
291 $34)}=2.32$, $p=0.114$) were observed. In addition, no significant relationships were
292 observed between baseline FEV₁ and the change in FMD either from BH₄-5 ($r=0.066$;
293 $p=0.801$) or BH₄-20 ($r=0.092$; $p=0.776$).

294

295 Endothelial Cell Coupling of NOS3

296 Stimulated superoxide production was significantly lower ($p=0.01$) in endothelial cells
297 pre-incubated with post BH₄-20 plasma compared to endothelial cells treated with pre-
298 treatment plasma (Figure 3A). Inhibition of NOS (using L-NAME) significantly ($p<0.001$)
299 attenuated stimulated superoxide production in cells treated with plasma prior to BH₄-20
300 treatment (Figure 3A). NOS inhibition had no significant ($p=0.065$) effect on superoxide
301 production in cells incubated with plasma post-BH₄-20. Specificity of stimulated DHE
302 fluorescence for superoxide was confirmed with tempol (Figure 3A). Consistent with the
303 above, stimulated NO production tended to be greater ($p=0.10$) in endothelial cells
304 incubated with post BH₄-20 plasma compared to incubation with pre-BH₄-20 plasma,
305 although this did not reach statistical significance. Pre-incubation with the NOS inhibitor
306 L-NAME confirmed NOS-mediated NO production (Figure 3B).

307

308

309

310 **DISCUSSION**

311 Patients with CF exhibit systemic vascular endothelial dysfunction (24, 30);
312 however, the mechanisms have yet to be elucidated. The present study sought to test
313 the hypothesis that an acute treatment with BH₄ would improve vascular endothelial
314 function in patients with CF. For the first time, findings from the present study
315 demonstrate that a single dose of 20 mg/kg of BH₄ improves endothelial function in
316 patients with CF that is accompanied by a decrease in NOS-mediated superoxide
317 production, suggesting an improvement in NOS coupling. In contrast, no change in
318 endothelial function was observed following PLC or 5 mg/kg of BH₄. Our proof of
319 concept findings support the importance of BH₄ bioactivity as a potential mechanism
320 that may, in part, be responsible for vascular endothelial dysfunction in patients with CF
321 (24, 30).

322

323 **Endothelial Function in Patients with CF**

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

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

334 **BH₄ Improves Vascular Endothelial Function in Patients with CF**

335 For the first time, the present study demonstrates that a single dose of BH₄ can
336 improve vascular endothelial function in patients with CF, evidenced by a 1.1 %
337 absolute and a 17% relative increase in FMD following high dose treatment (**Figure 2**).
338 The FMD test represents a bioassay of NO bioavailability and assessment of
339 endothelial function. Although apparently small in magnitude, a 1% absolute increase in
340 FMD has been associated with a 10-13% reduction in risk of future cardiovascular
341 events and all-cause mortality (17, 36). In addition, this moderate improvement
342 (Cohen's $d=0.33$) in FMD may have significant implications for quality of life and survival
343 in this patient population through the improvement in other systemic manifestations of
344 CF, including exercise capacity. Future studies are warranted to determine the
345 therapeutic effect of chronic BH₄ treatment on both FMD and exercise capacity in CF.

346 The improvement in FMD observed in the present study is in line with previous
347 studies that have demonstrated an increase in endothelial function following acute
348 administration of oral BH₄ in different populations that exhibit systemic oxidative stress
349 (6, 18, 19, 32). Although the exact mechanisms by which oral BH₄ treatment increases
350 endothelial-dependent vasodilation in patients with CF are unclear, an increase in
351 endothelial NO production associated with improved eNOS coupling is likely to play an
352 important role. To support this hypothesis we performed *in vitro* studies examining the
353 ability of BH₄-treated patient plasma to modulate endothelial cell superoxide and NO
354 production (Figure 3). Findings from this cell culture experiment suggest an

355 improvement in NOS coupling and subsequent NOS-mediated NO production in
356 endothelial cells following BH₄-20 treatment. Importantly, the change in FMD observed
357 following treatment in the present study was independent of basal lung function. Conduit
358 artery FMD is shown to be primarily mediated by endothelial-derived NO (20), and BH₄
359 is a cofactor specific for eNOS that modulates NO synthesis in the vascular endothelium
360 (3). In fact, previous studies have demonstrated that BH₄ is able to increase NO
361 bioavailability (5, 15) and promote vasodilation without impacting endothelial-
362 independent vasodilatory mechanisms (18, 25). Moreover, results from animal studies
363 demonstrate that deficiency of endothelial BH₄ alone is sufficient to cause vascular
364 dysfunction even in the absence of atherogenic vascular disease (3). Collectively,
365 findings from the present study support the idea that insufficient bioactivity of BH₄ in the
366 endothelium contributes to endothelial dysfunction in CF (24). Although speculative, CF-
367 associated BH₄ oxidation by overproduction of vascular reactive oxidative species may
368 lead to decreased endothelial BH₄ bioavailability, which in turn may uncouple eNOS and
369 lead to a reduction in NO-mediated vasodilation. Impaired NO-dependent vasodilation
370 can impair blood flow regulation during exercise especially under conditions of elevated
371 basal oxidative stress. Exercise intolerance is an independent contributor of mortality in
372 CF. Therefore, understanding the mechanisms that contribute to vascular dysfunction in
373 CF may have important clinical implications, and future studies are certainly needed to
374 examine the mechanistic role of BH₄ on NO generation and the development of
375 vascular dysfunction in CF.

376 **A High Dose of BH₄ may be Needed to Improve Endothelial Function in Patients**
377 **with CF**

378 Enteral administration of BH₄, although unrelated to its vascular benefit, is
379 clinically indicated to treat phenylketonuria using a dose between 5-20 mg/kg. This wide
380 dosing range depends on 1) varying responsiveness related to genotype, 2) absorption,
381 and/or 3) metabolic states of BH₄ (23). Perhaps unsurprisingly, even the vascular effects
382 following oral administration of BH₄ appear to be complex and dose dependent (5, 23).
383 In the present study, we observed an improvement in vascular endothelial function only
384 following oral administration of BH₄ at a dose of 20 mg/kg; PLC or 5 mg/kg of BH₄ did
385 not alter the FMD response. In support, prevailing data in the literature have already
386 suggested a similar dose-dependent effect of BH₄ on the vasculature (21, 23, 25). A
387 daily dose of at least 400 mg of oral BH₄ significantly improves endothelial function in
388 patients with hypertension, whereas no effect was observed following a daily dose of
389 200 mg(25). The average amount of BH₄ given to the BH₄-5 group in the present study
390 was 265 ± 72 mg, which was similar to the previously demonstrated ineffective dose(25)
391 and may explain the null response.

392 A classic feature of CF is elevated systemic oxidative stress (9). In addition, BH₄
393 is easily oxidized by excessive free radicals. Therefore, a higher dose of BH₄ may have
394 been needed to balance multiple modulators of the eNOS uncoupling cascade, such as
395 BH₄ oxidation status, BH₄ clearance rate, and the ratio of BH₄ to BH₂ in the vascular
396 endothelium. Moreover, the progressive dose escalation designs that are often
397 performed during early stages of clinical trials with BH₄ supplementation (28) suggests
398 the importance of disease-specific investigation for the sake of safety and efficacy of the
399 use of BH₄ in clinical populations. Although it is uncertain whether the BH₄-associated
400 improvement in endothelial function can be maintained with chronic BH₄ administration

401 in patients with CF, further investigation is warranted to explore effective strategies for
402 maintenance of adequate levels of intracellular BH₄ bioavailability in patients with CF.
403 Nonetheless, the present study demonstrates that a treatment dose of 20 mg/kg of BH₄
404 produces a significant increase in endothelial dependent vasodilation that is
405 accompanied by an improved NOS3 coupling in patients with CF.

406

407 **Clinical Significance**

408 Vascular endothelial dysfunction plays an important detrimental role in many
409 pathological conditions. With respect to patients with CF, vascular dysfunction is a
410 contributor to exercise intolerance (24), a predictor of mortality that is independent of
411 lung function in this patient population (22). Improvements in both endothelial function
412 and NOS3 coupling following BH₄ treatment observed in the present study may
413 increase longevity of these patients due to 1) the overall improvement in cardiovascular
414 disease risk, and 2) directly or indirectly increasing blood flow regulation and
415 contributing to an improvement in exercise capacity. Although both scenarios will have
416 significant clinical implications on survival in patients with CF, further research is
417 warranted to test these hypothesis.

418

419 **Conclusion**

420 For the first time, the present study has shown that a single oral dose of 20 mg/kg of
421 BH₄ significantly improved vascular endothelial function in patients with CF. The
422 improvement in FMD was also accompanied by a significant decrease in endothelial cell

423 NOS-mediated superoxide production and an increase in NO supporting an
424 improvement in NOS coupling with BH₄ treatment. These findings indicates an
425 important role of BH₄ bioactivity in the regulation of endothelial dependent vasodilation
426 in CF. Further studies are needed to investigate the ultimate translational potential of
427 BH₄ in prevention and treatment of vascular dysfunction in patients with CF.

428

429 **Competing Interests**

430 None to declare.

431

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553 **FIGURE LEGEND**

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558 **Figure 1.** Study Flow Chart

559 **CF:** cystic fibrosis, **BH₄-5:** 5mg/kg of BH₄, **BH₄-20:** 20mg/kg of BH₄, **PLC:** placebo

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561

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

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567 **Figure 3.** Stimulated human aortic endothelial cell production of superoxide (panel A)
568 and nitric oxide (panel B) incubated with pre and post BH₄-20 patient
569 plasma. Representative fluorescent images of cells under basal, L-NAME, and Tempol
570 conditions for each probe are illustrated. *Significant from basal pre BH₄-
571 20. †Significant from corresponding basal value. #Significant from corresponding basal
572 and pre BH₄-20 L-NAME.

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574 **Table 1.** Characteristics and laboratory values for patient treatment groups (BH₄-4 and
 575 BH₄-20) and healthy controls (CON).

	BH₄-5	BH₄-20	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, **FEV₁**: Forced
 578 Expiratory Volume in one second, **FEF₂₅₋₇₅**: 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 BH₄-5 and BH₄-20 (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 BH₄ (BH₄-
 583 5), 20 mg/kg of BH₄ (BH₄-20), 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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