

·Original Article·

Evaluation of tetrahydrobiopterin (BH₄) as a potential therapeutic agent to treat erectile dysfunction

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Abstract

Aim: Nitric oxide (NO)-mediated smooth muscle relaxation causes penile erections. The endothelial NO synthase (eNOS) coenzyme tetrahydrobiopterin (BH₄) converts eNOS-mediated catalytic activity from oxygen radical to NO production, improving endothelial function and vascular smooth muscle relaxation. **Methods:** Using quantitative immunohistochemistry, 8-isoprostane and nitrotyrosine concentrations were compared in cavernosal tissue from 17 potent and 7 impotent men, and the effect of single oral doses of BH₄ on penile rigidity and tumescence was investigated. The pharmacodynamic effect of single oral doses of BH₄ on penile rigidity and tumescence was investigated in a randomized, placebo-controlled, double-blind cross-over fashion in 18 patients with erectile dysfunction (ED) while receiving visual sexual stimulation. **Results:** 8-isoprostane content in endothelium and smooth muscle was significantly higher in impotent patient samples; the level of nitrotyrosine was unchanged in ED patients. Relative to placebo, a single dose of 200 mg BH₄ led to a mean increase in duration of > 60% penile rigidity (33.5 min [95% confidence interval (CI): 13.1–49.3] at base and 29.4 min [95% CI: 8.9–42.2] at tip). A 500-mg dose increased the relative duration of > 60% penile rigidity by 36.1 min (95% CI: 16.3–51.8) at the base and 33.7 min (95% CI: 11.4–43.9) at the tip. Treatments were well tolerated. **Conclusion:** BH₄ treatment is suggested to switch eNOS catalytic activity from super-oxide to NO formation, leading to a reduced formation of free radical reaction product 8-isoprostane without alteration of nitrotyrosine. The observed results make BH₄ a suitable candidate as an ED treatment through reconstitution of altered catalytic activity of the eNOS. (*Asian J Androl* 2006 Mar; 8: 159–167)

Keywords: tetrahydrobiopterin; nitric oxide; 8-isoprostane; nitrotyrosine; erectile dysfunction

1 Introduction

Erectile dysfunction (ED) is a sexual disorder in which diminishing tumescence and penile rigidity result in the

repeated inability to get or maintain an erection, restricting or preventing satisfactory sexual intercourse. Although previously considered a purely psychogenic phenomenon, ED is now suggested to be associated with aging [1], diabetes, neuropathy, vascular disease and smoking and recognized to have an organic etiology in the majority of cases [2]. The strong association with atherosclerosis and vascular risk factors has prompted the investigation of potential common vascular pathophysiological mechanisms. In this context, the preven-

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tion of ED seems to be of high importance for the prevention of vascular degeneration in other organ systems.

Penile erection is a hemodynamic process, involving increased arterial inflow and restricted venous outflow, coordinated by corpus cavernosum (CC) smooth muscle relaxation. Nitric oxide (NO), produced in CC nerves, smooth muscle and endothelium, plays a key role in the physiology of penile erection [3]. Endothelial NO synthase (eNOS) is one of the main sources of NO in CC tissue [4], and the NO synthase coenzyme tetrahydrobiopterin (BH₄) is an important factor in eNOS-dependent NO release. A reduction or lack of BH₄ leads to eNOS dysfunction, resulting in a switch from NO release to the formation of oxygen radicals [5]. Levels of functionally available NO are further reduced by the reaction of NO with free oxygen radicals, forming peroxynitrite [6].

Mounting evidence implicates vascular oxidative stress in the etiology of ED, principally through the reaction of a super-oxide anion with NO, resulting in the acute impairment of CC relaxation as well as long-term vasculopathy. Loss of NO bioavailability, as a result of reduced synthesis and increased scavenging by reactive oxygen species, is a cardinal feature of endothelial dysfunction in vascular disease states. Experimental studies show that augmentation of BH₄ concentrations in vascular disease by pharmacological supplementation, enhancement of its rate of *de novo* biosynthesis or by measures to reduce its oxidation enhances NO bioavailability. Therefore, BH₄ represents a potential therapeutic target in the regulation of eNOS function in vascular disease (Figure 1) [5].

Currently available therapeutic options for the treatment of erectile dysfunction do not prevent reduced or diminished NO release by eNOS. In addition, present-day therapies are unable to ameliorate ED in a consistent percentage of patients. BH₄ shows significant promise for development as a therapeutic agent to treat ED.

The objective of the present study was to analyze the levels of oxygen radical 8-isoprostane and nitrotyrosine, indicative of peroxynitrite levels in human CC tissue, in potent and ED patients, and to investigate the effect of systemically applied BH₄ on penile rigidity and tumescence in a population of ED patients.

2 Materials and methods

2.1 Patients and collection of tissue

Twenty-four human CC tissue specimens were obtained with informed consent from penile surgery pa-

tients (7 ED patients, 14 controls). Specimens were immediately fixed in 4% paraformaldehyde for 4 h and then rinsed in 0.1 mol/L phosphate-buffered saline (PBS) for 24 h. The tissue was stored for 12 h in a PBS-18% sucrose solution and then frozen at -80 °C. Patient background data are shown in Table 1.

2.2 Immunohistochemistry

All 24 tissue samples were analyzed. Prior to immunohistochemical examination, 7-μm tissue sections were prepared as described previously [7, 8]. Anti-8-isoprostane (Oxford Biomedical Research, Oxford, MI, USA) and anti-nitrotyrosine (Calbiochem, San Diego, CA, USA) antibodies were diluted 1:1500 and 1:400, respectively; as with the appropriate biotinylated secondary antibody (1:400; Dako, Glostrup, Denmark). Streptavidin-horseradish peroxidase detection (1:150; Amersham, Life Science, Little Chalfont, Buckinghamshire, UK) was applied for 1 h and developed for 3–10 min with 7.5 mg 3,3-diaminobenzidine tetrahydrochloride, 6.0 mg NH₄Cl, 0.06 mg glucose oxidase, 30 mg glucose and 3.9 mg NiSO₄ in 15 mL 0.1 mol/L phosphate buffer. Negative control sections were incubated without primary antibody and then with serum obtained from the species that the primary antibody was grown in.

2.3 Double immunofluorescence

The 7-μm tissue sections were incubated at room temperature with a cocktail mix of two primary antibodies: mouse monoclonal anti-PECAM-1 (1:300, Biogenex, USA) or mouse monoclonal anti-desmin (1:400, Dako, Denmark) with rabbit anti-nitrotyrosine antibody (1:400, Biomol, Germany) or goat anti-8-epi-PGFa₂ (8-isoprostane) antibody (1:1500, Oxford Biomedical, USA). Subsequent antibody detection was carried out with a cocktail mix of two secondary antibodies: CY2-conjugated anti-mouse IgG and CY3-conjugated anti-rabbit-IgG. The antibody detection for the goat anti-8-epi-PGFa₂ was performed using a biotinylated anti-goat antibody and subsequent CY3-conjugated streptavidin. The sections were washed in 0.05 mol/L Tris buffered saline and mounted with Entellan (SEVA, France).

2.4 TV-densitometry and statistics

For each patient, 8-bit images with a 40-fold magnification were generated using a Sony analog camera and analyzed using ImageJ software (NIH: <http://rsb.info.nih.gov/ij/>) to measure the illuminance-values of the

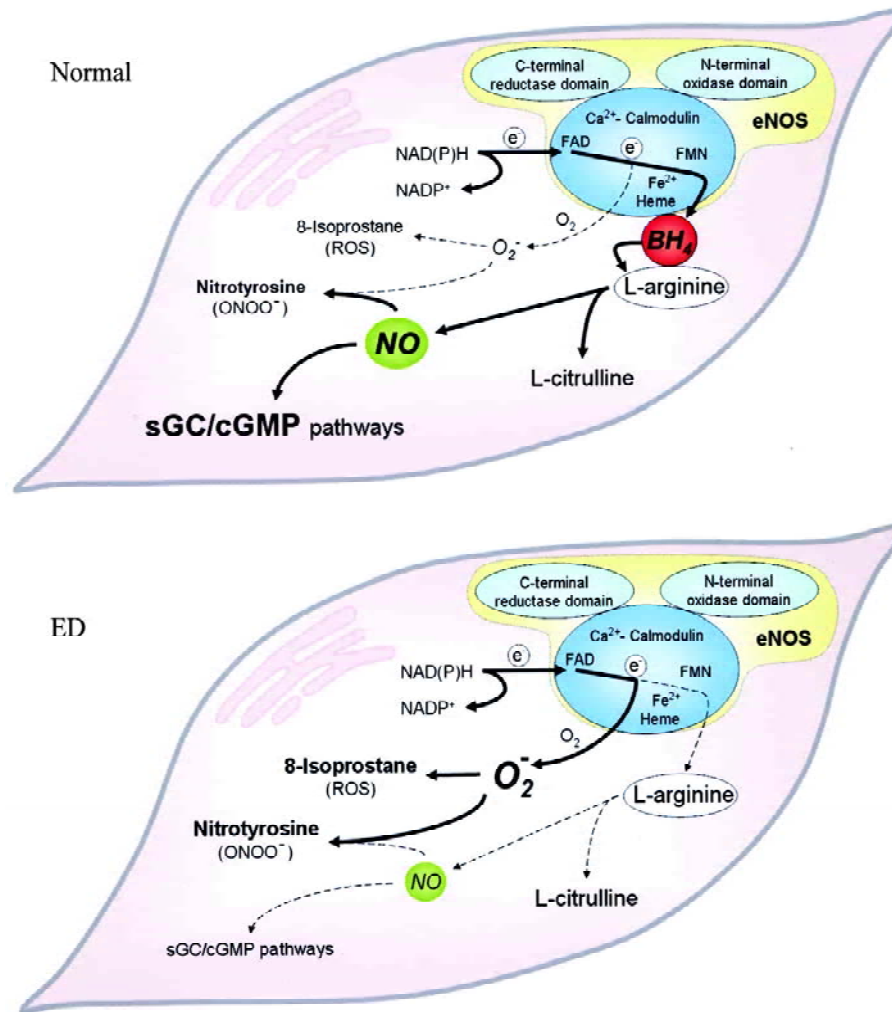


Figure 1. NO-mediated basis of penile erection. (A): During an erection, nitric oxide (NO) is produced by endothelial NO synthase (eNOS) coenzyme and released to the muscles of the corpus cavernosum (CC), activating the sGC/cGMP pathways. Resulting intracellular calcium depletion in the CC leads to relaxation of the muscles, blood flow in the penis increases and erection is initiated. The electron flow is conducted through tetrahydrobiopterin (BH_4) and is transferred to L-arginine which results in L-citrulline and NO formation. (B): A reduction or lack of nitric oxide synthase coenzyme BH_4 leads to eNOS dysfunction and a switch from NO release to the formation of oxygen free radicals. The electron flow to the L-arginine is impaired. Functionally available NO is further reduced by the reaction of NO with a free oxygen radical, leading to the formation of peroxynitrite. The increased formation of free radical reaction products results in an imbalance between NO release and oxygen free radical formation by eNOS [6], which leads to erectile dysfunction and erectile tissue injury [25].

Table 1. Patient data for evaluated corpus cavernosum (CC) samples. ^aascertained by anamnestic evaluation; ^bED for more than 2 years.

Reason for surgery	n	Age (mean) (years)	Erectile function ^a	Relevant factors
Penile deviations	14	16-63 (42)	yes	Treated via Nesbit's surgical method
Flexible hydraulic penile prosthesis implantation	7	27-66 (46)	no ^b	Severe venous leakage (n = 4) Diabetes, combined arterial-venous CC insufficiency (n = 2) Radical surgery bladder carcinoma (n = 1)
Transsexual operations (♂ → ♀)	3	33-57 (44)	yes	Normal erections reported in spite of long-term hormonal pre-treatment

immunohistochemistry stainings. The background value was set to 220 ± 5 units and was measured at least twice for each field. Five smooth muscle cell (SMC) areas in five different fields were examined for each tissue (one slice per tissue). The mean illuminance value of the SMC areas was subtracted from the mean background value for each field. Statistical analyses were performed using the *t*-test for independent groups with the analyzing software SPSS for Windows 10.0. $P \leq 0.05$ was considered significant.

2.5 Clinical study design

The influence of single, oral doses of placebo, 200 or 500 mg of tetrahydrobiopterin on penile rigidity and tumescence during visual sexual stimulation was evaluated objectively using a RigiScan device (Dacomed, Minneapolis, MN, USA). The present study had a single-center, randomized, placebo-controlled, double-blind, 3-way cross-over design with at least 5 d between each patient dosage. After baseline measurements, the patient ingested the treatment dosage and waited 20 min before viewing a 20-min sexually graphic video. Erection degree was measured during viewing, during a subsequent 20 min rest period and during three further 20 min viewing sessions.

2.6 Clinical study patients

Patients were recruited from the surrounding community through word of mouth, enrolled and randomized. The 18 volunteers were male, white, 21–62 years of age (mean 41.6 ± 11.9 years), of normal body weight (range 64–101 kg, mean 78.8 ± 7.9 kg; Broca-index 0.88–1.14; mean 1.00 ± 0.08), and diagnosed with ED as evaluated on the basis of a standardized questionnaire (international index of erectile function [IIEF]) [9]. The IIEF domain score of the patients ranged from 14 to 19 (median 17, mean 16), interpreted as moderate ED. All patients had had a self-reported penile erection in response to visual sexual stimulation in the 6 months prior to the study. Patients with no response or only weak tumescence were excluded. No control patients without ED were included in the study. Patient background data is summarized in Table 2.

Patients were excluded based on any of the following criteria: ED as a result of neurological or endocrine causes, an anatomical deformity such as severe penile fibrosis, spinal cord injury or radical prostatectomy, diabetes mellitus, any other relevant comorbidity, major psychiatric illness or concomitant

intake of any medication likely to interact with the study compound. Neurological impotence was diagnosed on the basis of a neurological examination; endocrine impotence was identified by checking testosterone levels. Patients were also excluded if blood pressure and heart rate were abnormal or other laboratory test results indicated a second or third degree AV (atrioventricular)-block, QRS duration >120 ms or QT interval >500 ms.

2.7 Treatment

The treatments under investigation were given as oral solutions containing either 200 mg or 500 mg of tetrahydrobiopterin or a placebo solution. Medication was given in the morning after a light breakfast, followed by 100 mL tap water.

2.8 RigiScan measurements during visual sexual stimulation

A RigiScan Ambulatory Rigidity and Tumescence Monitor (Dacomed, Minneapolis, MN, USA) were used to monitor penile rigidity and tumescence. Patients were connected to the RigiScan from 0.5 h before until 3 h after dosing, using a conventional set-up with a cuff around the base and another around the tip of the penis. Radial rigidity was determined automatically every 15 s by the device after internal calibration. Tumescence was calculated automatically by the device. Visual sexual stimulation consisted of sexually graphic videos (chosen from a selection by the patient). Patients were instructed to try to prevent ejaculation and to avoid manual stimulation during the entire session.

Table 2. Patient data for the study on the effect of BH₄ on penile rigidity and tumescence. The patients were volunteers from the surrounding community. No control patients were included.

Item	Value
Number	18
Gender	Male
Ethnic background	Caucasian
Age (years)	21–62
	Mean: 41.6 ± 11.9
Body weight (kg)	64–101
	Mean: 78.8 ± 7.9
Broca index	0.88–1.14
	Mean: 1.00 ± 0.08
Diagnosis	ED (based on IIEF [9])

The primary endpoint was the total duration of erections with greater than 60% rigidity, as calculated using the RigiScan software. Secondary endpoints were duration of > 80% rigidity, rigidity activity units (RAU) and tumescence activity units (TAU) [10]. The average erection rigidity, duration of event, circumference and average event tumescence were evaluated as ancillary criteria. All values were calculated separately for the base and the tip of the penis.

2.9 Safety surveillance and criteria of safety and tolerability

The volunteers spontaneously reported any adverse events, but they were also evaluated by non-leading questions while under close medical surveillance. Adverse events were defined as illnesses, subjective or objective signs or symptoms (including clinically significant changes in laboratory results) that appeared or worsened during the course of a study, independent of a relationship to study medication.

2.10 Data analysis

Relevant pharmacodynamic data were fitted to a general linear model with effects for sequence, subject within sequence, period and treatment by ANOVA. On the basis of the resulting variance estimates, point and 95% confidence interval (CI) estimates of the pair-wise mean treatment differences were calculated for exploratory purposes (without adjustment for multiplicity).

3 Results

3.1 Immunohistochemistry

As fingerprint for oxygen radical formation, 8-isoprostane was stained, whereas nitrotyrosine was detected as fingerprint for the formation of peroxynitrite, which is formed by the reaction of super-oxide with NO. Immunohistochemical staining was applied to 7- μ m tissue sections to identify the cellular localization of 8-isoprostane and nitrotyrosine in different cell compartments of CC tissue. All CC tissue specimens showed nitrotyrosine immunoreactivity in endothelium and smooth muscle cells of fibromuscular stroma without significant differences (Figure 2B), whereas 8-isoprostane immunoreactivity was mainly observed in samples from impotent patients (Figure 2A). Control stainings using serum from the species in which the primary antibody was grown in showed no non-specific bindings of the used

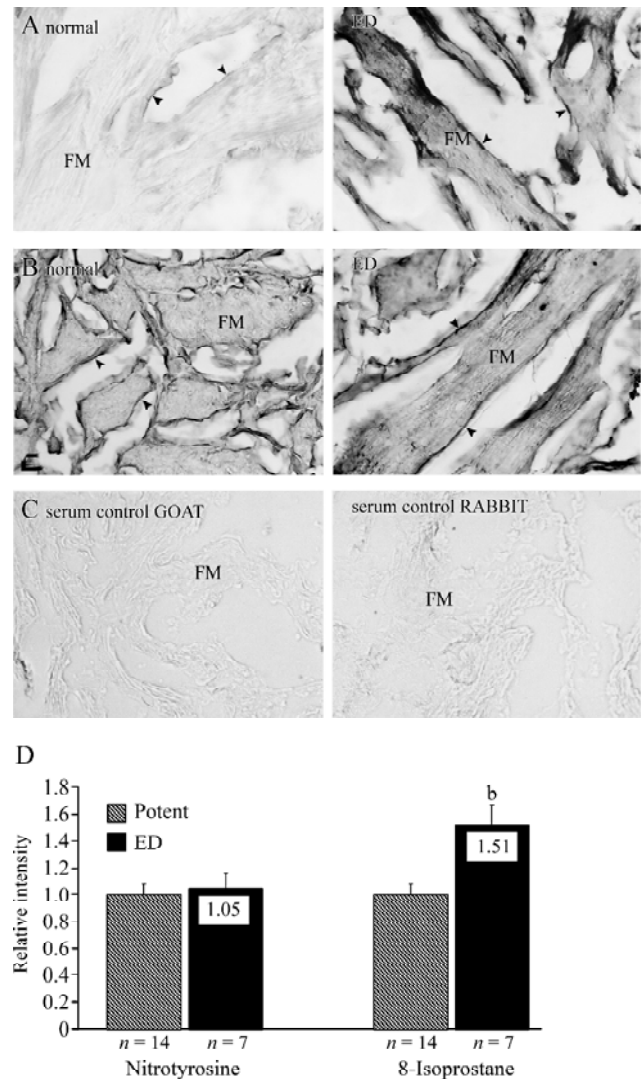


Figure 2. Immunohistochemical detection of 8-isoprostane (A) and nitrotyrosine (B). (A): Immunohistochemical staining to detect 8-isoprostane expression in 7- μ m sections of corpus cavernosum (CC) tissue from normal and erectile dysfunction (ED) patients. (B): Immunohistochemical staining to detect nitrotyrosine expression in 7- μ m sections of CC tissue from normal and ED patients. Increased 8-isoprostane and unchanged nitrotyrosine staining can be found in sinus endothelium (arrows) and fibromuscular stroma (FM) from ED patients. (C): Serum controls to test the primary antibody specificity have been performed. No unspecific labeling can be found. Magnification: $\times 630$. (D): Semiquantitative analysis of nitrotyrosine and 8-isoprostane expression in potent ($n = 14$) and ED ($n = 7$) patients. mean \pm SD, ^b $P < 0.05$.

secondary antibody (Figure 2C). All samples from impotent patients showed a level of 8-isoprostane expression that was significantly higher than that in potent pa-

tient samples (Figure 2D). Double staining experiments (Figure 3) demonstrate the co-localization of nitrotyrosine with smooth muscle cells (desmin) and endothelial cells (PECAM).

3.2 Rigidity and tumescence data

3.2.1 Patient disposition

The 18 patients were randomized and treated. All patients were available for evaluation of the pharmacodynamic effects of treatment and were included in safety analyses.

3.2.2 Pharmacodynamic effects

The results of RigiScan measurements on penile rigidity and tumescence during visual sexual stimulation following ingestion of placebo or BH₄ dosage are summarized in Table 3. BH₄ treatment resulted in significant improvements in erectile function using the primary endpoint of duration of > 60% rigidity. The RAU and TAU, which represent the product of degree of rigidity or tumescence, multiplied by the time, were statistically superior for the 200 mg dose over placebo for both the base and the tip. Response to the 500-mg dose was

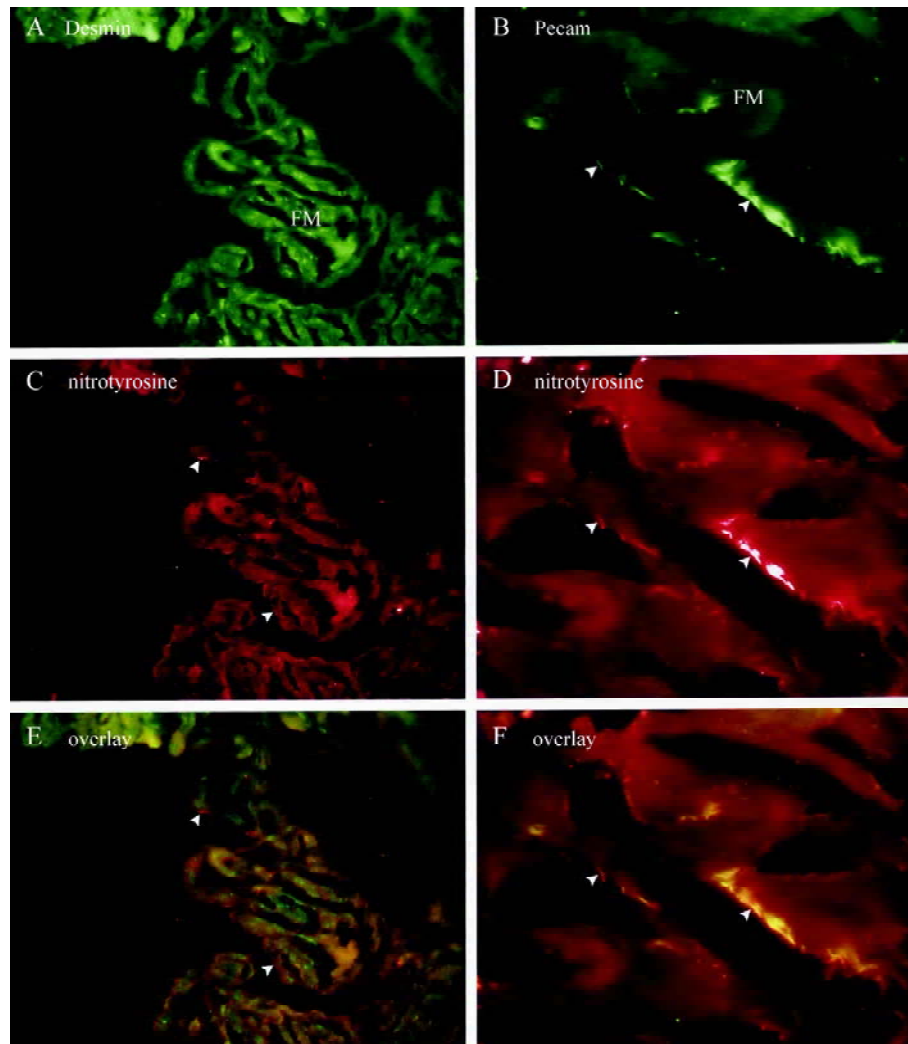


Figure 3. Fluorescence-immunohistochemical detection of desmin (A, E) and PECAM-1 (B, F) in comparison to nitrotyrosine (C–F) distribution. To show the smooth muscle or endothelial localization of nitrotyrosine, double-stainings have been performed. The results show the localization of the investigated antibodies to the fibro-muscular stroma (FM) (A, C, E) or the endothelial layer (arrows) (B, D, F). Magnification: $\times 200$.

numerically slightly better than the 200 mg dose and statistically better than the placebo. Secondary and ancillary endpoints (average event rigidity, average event tumescence, event duration and circumference) showed the same trend. Improvement in radial rigidity was also seen using the criterion of > 80% rigidity, suggesting that the improvements seen with BH₄ treatment in the present study might translate into improved sexual intercourse success rates.

These effects were not influenced by abnormal blood pressure or heart rate, as these were exclusion criteria.

3.3 Treatment safety and tolerability

Treatments were well tolerated. Two patients reported light headaches while taking 200 mg doses of BH₄ compared to one report of headaches in the placebo group. One patient taking 500 mg BH₄ reported light dizziness. No severe adverse events were reported.

4 Discussion

Within the past decade, there has been an explosion of new information on the physiology of penile erection and pathophysiology of ED, which is expected to lead to

new treatments and more effective drugs. To date, etiology-independent therapies comprise psychosexual therapy, vacuum constriction devices, intracavernosal and intraurethral therapy, penile prostheses and oral therapies. New oral agents (phosphodiesterase-5 inhibitors) have revolutionized the sex lives of millions of couples worldwide and freed countless men from the problems associated with other forms of ED therapy (e.g. the penile pain and scarring associated with intracavernosal injections). However, research looking for new treatments continues, such as gene-based therapies [11], novel delivery of known treatments (e.g. endothelial rehabilitation with phosphodiesterase-5 inhibitors) [12], injection of new vasoactive agents [13, 14], and new oral agents that target other factors in the NOS pathway, as described here.

Insufficient or impaired BH₄ metabolism results in increased super-oxide anion formation and reduced NO production [15]. BH₄ is an important cofactor, which couples the electron transfer from eNOS flavins to the *L*-arginine oxidation if BH₄ lacking super-oxide is formed by the oxygenase domain of the eNOS [6]. Therefore, decreased BH₄ availability might cause a shift in the bal-

Table 3. Arithmetic mean and standard deviation (SD) of primary and ancillary rigidity and tumescence criteria at the base and the tip of the penis for placebo and BH₄ (200 mg and 500 mg), and exploratory pair-wise treatment of primary rigidity and tumescence criteria.

	Rigidity and tumescence values at the base and tip of the penis								
	Placebo	200 mg	500 mg	200 mg vs. placebo			500 mg vs. placebo		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean	95% CI	P-value	Mean	95% CI	P-value
Base									
Time rigidity 60%–100% (min)	38.1 ± 26.4	71.6 ± 40.5	74.2 ± 44.1	33.5	9.4–48.1	<0.001	36.1	19.1–52.8	<0.001
Time rigidity 80%–100% (min)	19.6 ± 22.2	30.1 ± 28.5	37.6 ± 36.1	10.5	–4.1–27.4	0.156	18.0	3.4–31.8	0.023
Rigidity activity units	34.2 ± 24.2	53.9 ± 31.2	64.5 ± 39.7	19.7	5.8–33.7	0.007	30.3	16.3–45.1	<0.001
Tumescence activity units	19.3 ± 14.8	32.6 ± 19.1	39.4 ± 29.3	13.3	0.8–20.2	0.039	20.1	9.7–30.6	0.003
Average event rigidity (%)	62.9 ± 39.8	72.1 ± 29.3	74.1 ± 33.1						
Duration of event (min)	51.2 ± 26.9	74.1 ± 36.1	77.9 ± 41.1						
Circumference (cm)	8.0 ± 1.3	8.0 ± 1.0	8.1 ± 1.2						
Average event tumescence	8.9 ± 4.9	10.1 ± 3.2	10.7 ± 3.7						
Tip									
Time rigidity 60%–100% (min)	25.2 ± 22.3	54.6 ± 33.4	58.9 ± 46.1	29.4	9.8–46.3	<0.001	33.7	14.7–47.2	<0.001
Time rigidity 80%–100% (min)	8.9 ± 17.1	13.4 ± 17.5	31.5 ± 36.3	4.5	–8.1–18.7	0.431	22.6	7.6–30.6	0.006
Rigidity activity units	19.9 ± 19.9	39.0 ± 29.9	51.1 ± 40.3	19.1	5.8–33.1	0.007	31.2	16.9–44.2	<0.001
Tumescence activity units	12.4 ± 10.9	24.1 ± 16.4	25.1 ± 21.6	11.7	4.5–20.5	0.010	12.7	4.9–21.3	0.008
Average event rigidity (%)	36.1 ± 27.9	47.1 ± 29.9	48.2 ± 27.3						
Duration of event (min)	37.7 ± 29.8	67.2 ± 37.2	78.9 ± 42.4						
Circumference (cm)	6.8 ± 0.9	6.9 ± 1.2	7.2 ± 1.0						
Average event tumescence	6.8 ± 3.9	8.0 ± 2.9	8.1 ± 2.9						

ance between protective NO and toxic reactive oxygen species (ROS) [15]. Further reduction in the level of available NO occurs with the reaction of ROS with NO. One of the reaction products is peroxynitrite, which has a contractile effect on vascular smooth muscle cells (VSMC) [16]. The overproduction of super-oxide and its negation of NO are central to ED, both acutely by impairing VSMC relaxation and chronically by mediating endothelial dysfunction [17]. In different animal studies using diabetes and high cholesterol models the treatment with BH₄ or with precursors of the BH₄ could prevent the endothelial dysfunction by reduction of super-oxide formation and restoration of the catalytic activity of eNOS [18, 19]. Therefore, BH₄ treatment might lead to an improvement of erectile function by restoration of the endothelial function.

The production of oxygen radicals and peroxynitrite can be investigated in human tissue by detecting radical reaction products, such as 8-isoprostane and nitrotyrosine [20, 21]. The immunohistochemical analysis of 24 CC tissue samples from both potent and impotent patients in the present study showed significantly higher levels of oxygen radicals in samples from ED patients. In contrast, no difference in nitrotyrosine levels was observed; although, assuming constant NO concentrations, one might expect an increase in oxygen radicals to lead to increased peroxynitrite production. The lack of nitrotyrosine increase can be explained by a reduction of NO, which is necessary for peroxynitrite formation and subsequent nitration of tyrosine rests. This provides strong evidence for an imbalance in NO release and oxygen radical formation by eNOS in the CC tissue of patients with ED. The results suggest that clinical intervention to restore the balance between NO and oxygen radical formation can also improve erectile function.

Treatment strategies to prevent cavernosal degeneration and/or restore cavernosal function are widely considered to be a priority in ED research [22]. Aside from the reduced smooth muscle relaxation leading to impaired vasodilatation associated with a lack of BH₄, it has been suggested that dysfunctional eNOS, caused by insufficient BH₄, participates in oxidative injury, especially under pathological conditions, such as ischemia and reperfusion [5]. It is proposed that BH₄ could be used to treat ED by ameliorating the eNOS-dependent imbalance between NO release and oxygen radical formation in human CC tissue.

The present study demonstrates that BH₄ treatment produced a statistically significant increase in rigidity of the penis for 200 mg and 500 mg doses compared to the placebo when given in the presence of visual sexual stimulation. The observation that a higher dose only leads to limited increases in penile rigidity and tumescence has also been made for the ED agents apomorphine [23] and vardenafil [24], which might hint at a non-linear mechanism to relieve these ED symptoms.

The cross-over design of the present study adds considerable strength to the data and reduces data variability, because each patient acted as his own control. The patient group was pre-selected for patients who were known to respond to visual sexual stimuli, yet weak in the quality of their erections. The study was designed to maximize the detection of changes caused by the drug. The inclusion of fully unresponsive patients would have introduced more variability to the study; however, the extrapolation of these results to a larger patient population must, therefore, be applied with caution. The number of patients in this study is high enough to suggest that BH₄ might have a clinical effect on ED.

References

- 1 Shi JP, Zhao YM, Song YT. Effect of aging on expression of nitric oxide synthase I and activity of nitric oxide synthase in rat penis. *Asian J Androl* 2003; 5: 117–20.
- 2 Droupy S. [Epidemiology and physiopathology of erectile dysfunction]. *Ann Urol (Paris)* 2005; 39: 71–84.
- 3 Pickert RS, Powell PH, Zar MA. Nitric oxide and cyclic GMP formation following relaxant nerve stimulation in isolated human corpus cavernosum. *Br J Urol* 1995; 75: 516–22.
- 4 Bloch W, Klotz T, Sedlacek P, Zumbé J, Engelmann U, Addicks K. Evidence for the involvement of endothelial nitric oxide synthase from smooth muscle cells in the erectile function of the human corpus cavernosum. *Urol Res* 1998; 26: 129–35.
- 5 Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 1991; 266: 4244–50.
- 6 Alp NJ, Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* 2004; 24: 413–20.
- 7 Sommer F, Klotz T, Steinritz D, Schmidt A, Addicks K, Engelmann U, *et al.* MAP kinase 1/2 (Erk 1/2) and serine/threonine specific protein kinase Akt/PKB expression and activity in the human corpus cavernosum. *Int J Impot Res* 2002; 14: 217–25.
- 8 Mehlhorn U, Krahwinkel A, Geissler HJ, LaRosee K, Fischer

- UM, Klass O, *et al.* Nitrotyrosine and 8-isoprostane formation indicate free radical-mediated injury in hearts of patients subjected to cardioplegia. *J Thorac Cardiovasc Surg* 2003; 125: 178–83.
- 9 Rosen RC, Riley A, Wagner G, Osterloh IH, Kirkpatrick J, Mishra A. The international index of erectile function (IIEF): A multidimensional scale for assessment of erectile dysfunction. *Urology* 1997; 49: 822–30.
- 10 Benet AE, Rehman J, Holcomb RG, Melman A. The correlation between the new RigiScan plus software and the final diagnosis in the evaluation of erectile dysfunction. *J Urol* 1996; 156: 1947–50.
- 11 Kendirci M, Gur S, Sikka SC. Gene therapy for erectile dysfunction. *Front Biosci* 2005; 10: 2758–69.
- 12 Sommer F, Schulze M. Treating erectile dysfunction by endothelial rehabilitation with phosphodiesterase 5 inhibitors. *World J Urol* 2005; 23: 385–92.
- 13 Fu Q, Yao DH, Jiang YQ. A clinical comparative study on effects of intracavernous injection of sodium nitroprusside and papaverine/phentolamine in erectile dysfunction patients. *Asian J Androl* 2000; 2: 301–3.
- 14 Fu Q, Yao DH, Jiang YQ. Effect of sodium nitroprusside on hemodynamics of corpus cavernosum in Chinese. *Asian J Androl* 2001; 3: 311–3.
- 15 Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BSS, Karoui H, *et al.* Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998; 95: 9220–5.
- 16 Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002; 82: 47–95.
- 17 Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996; 40: C1424–37.
- 18 Shinozaki K, Nishio Y, Okamura T, Yoshida Y, Maegawa H, Kojima H, *et al.* Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. *Circ Res* 2000; 87: 566–73.
- 19 Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, *et al.* Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; 103: 1282–8.
- 20 Viera L, Ye YZ, Estevez AG, Beckman JS. Immunohistochemical methods to detect nitrotyrosine. *Methods Enzymol* 1999; 301: 373–81.
- 21 Morrow JD. The isoprostanes: Their quantification as an index of oxidant stress status *in vivo*. *Drug Metab Rev* 2000; 32: 377–85.
- 22 Andersson KE. Penile erectile function: Recommendations for future research. *Int J Impot Res* 2000; 12: S163–7.
- 23 Altwein JE, Keuler FU. Oral treatment of erectile dysfunction with apomorphine SL. *Urol Int* 2001; 67: 257–63.
- 24 Stark S, Sachse R, Liedl T, Hensen J, Rohde G, Wensing G, *et al.* Vardenafil increases penile rigidity and tumescence in men with erectile dysfunction after a single oral dose. *Eur Urol* 2001; 40: 181–8.
- 25 Jones RW, Rees RW, Minhas S, Ralph D, Persad RA, Jeremy JY. Oxygen free radicals and the penis. *Expert Opin Pharmacother* 2002; 3: 889–97.