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-
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 patients (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 ºC. 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-PGFα (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-PGFα 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 (SEV A, 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 illuminescence-values of the
Table 1. Patient data for evaluated corpus cavernosum (CC) samples. *ascertained by anamnestic evaluation; †ED for more than 2 years.

| Reason for surgery                        | n  | Age (mean) (years) | Erectile function | 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               | 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           |
BH₄ study: therapeutic potential for ED

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 luminescence 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 ≤ 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          |
| Body weight (kg)    | 64-101         |
| Mean: 41.6 ± 11.9   |
| 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-µm 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 showed no non-specific bindings of the used 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
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 balance in the production of NO and super-oxide anion.

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.

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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 |
| --- | --- | --- | --- | --- | --- |
| **Base** |  | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | 95% Cl | P-value | Mean ± SD | Mean ± SD | 95% Cl | P-value |
| 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** |  | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | 95% Cl | P-value | Mean ± SD | Mean ± SD | 95% Cl | P-value |
| 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 | –15.1–24.7 | 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 |  |  |  |  |  |  |
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.

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