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Determination of nitric oxide metabolites by means of the Griess assay and gas chromatography–mass spectrometry in the cavernous and systemic blood of healthy males and patients with erectile dysfunction during different functional conditions of the penis

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Abstract Recent research implicated that the relaxation of cavernous arterial and trabecular smooth muscle – the crucial event in penile erection – is initiated by the release of nitric oxide (NO) from nerve terminals within the cavernous tissue as well as from the endothelia that line the lacunar spaces and the intima of penile arteries. The present study was undertaken to determine whether plasma levels of the NO metabolites nitrate (NO_3^-) and nitrite (NO_2^-) in the systemic and cavernous blood of male subjects change during different penile conditions, and whether there is a difference in the NO_3^- and NO_2^- levels of normal males and patients with erectile dysfunction (ED). Twenty-four potent adult male volunteers and 15 patients with ED were exposed to visual and tactile erotic stimuli in order to elicit penile tumescence and, in the group of healthy volunteers, rigidity. Whole blood was aspirated from the corpus cavernosum and the cubital vein, and NO_3^- and NO_2^- levels were determined in plasma aliquots by means of the Griess reaction and a method combining gas chromatography and mass spectrometry (GC-MS). The mean systemic and cavernous plasma NO_3^-/NO_2^- level in blood samples obtained from the healthy volunteers was 25–31 μM when determined by means of the Griess reaction and 37–41 μM when measured by GC-MS. Both approaches revealed that NO_3^-/NO_2^- levels in the peripheral and cavernous blood do not change appreciably during developing erection, rigidity and detumescence. Moreover, no significant differences were found between NO_3^-/NO_2^- plasma levels in the systemic and cavernous blood samples taken from the normal subjects and patients during penile flaccidity, tumescence and detumescence. Our results may reflect the fact that NO metabolism in the corpora cavernosa in the phases of penile tumescence and rigidity may account for only a minor fraction of local levels of NO_3^- and NO_2^-, which may also derive from exogenous sources. Moreover, the basal levels of NO metabolites in the blood flushing the lacunar spaces of the cavernous body in the state of developing erection could conceal any release of NO that may occur within the penile tissue. Thus, we conclude that the quantification of NO metabolites by means of advanced detection methods, such as GC-MS, is of no use in the workup of ED.

Key words Nitric oxide metabolites · Erectile dysfunction · Griess reaction · Gas chromatography · Mass spectrometry

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Introduction

Penile erection results from a complex interaction between neurovascular, psychologic and physiologic events involving multiple biochemical signals derived from several neurotransmitters and vasoactive agents [1, 2]. Upon sexual stimulation, the activation of non-adrenergic, non-cholinergic nerves innervating the cavernous smooth muscle and the cavernous blood vessels results in the synthesis and release of nitric oxide (NO), the main relaxant neurotransmitter in the penis [6, 8]. The endothelia that line the lacunar spaces and the

intima of the penile arteries also contribute to the local release of NO [12]. NO diffuses into the trabecular smooth muscle cells and promotes the synthesis and accumulation of cyclic guanosine monophosphate (cGMP) by the activity of the enzyme guanylyl cyclase. This leads to a reduction in the cytosolic calcium (Ca^{2+}) concentration, enhanced cavernous and vascular smooth muscle relaxation, and finally penile erection. After inducing the formation of cGMP, NO is rapidly converted into nitrite (NO_2^-) and nitrate (NO_3^-).

Because the NO- cGMP pathway plays such a prominent role in normal erectile function, therapeutic efforts for the treatment of impotence have been directed towards preserving or enhancing this pathway [10, 13, 17]. Nevertheless, it has never been documented whether erectile dysfunction (ED) is due to an impairment in the synthesis or release of NO. One possible way to confirm or refute this hypothesis would be to determine the levels of the NO metabolites NO_2^- and NO_3^- in the cavernous and systemic blood of males both before and during developing and sustained penile erection. Over the past few years NO_2^- and NO_3^- have attracted much interest because they have been shown to be stable metabolites of endothelium-derived relaxing factor NO [5, 7].

To date, only a single study comparing the plasma concentrations of NO metabolites in the penile and brachial venous blood both before and during penile erection has been conducted; no appreciable changes were revealed [9]. That study was based on a basic chemiluminescence method to quantify the concentrations of NO_2^- and NO_3^-, and one can speculate as to whether this protocol was sufficient to ensure satisfactory accuracy and sensitivity in the detection of even small differences in NO_2^- and NO_3^- levels. The authors concluded that alternative methods are required to determine the release of NO in the cavernous body [9].

The present study was performed to determine the plasma levels of NO metabolites NO_3^- and NO_2^- in systemic and cavernous blood samples collected from healthy males and from a group of patients with ED. Blood was collected during different functional conditions of the penis (flaccidity, tumescence/rigidity, detumescence) by means of two different experimental approaches: the well-established Griess reaction and a method combining gas chromatography and mass spectrometry (GC-MS) using ^{15}N-labeled analogs. The latter method was recently described to allow the accurate quantification of even small amounts of NO_2^- and NO_3^- in biological fluids including human plasma and urine [14–16].

Material and methods

Blood collection

Twenty-four healthy adult males aged 19–44 years (mean age 26 years) with normal erectile function and 15 patients (mean age 52 years) suffering from ED were enrolled in the study after written informed consent was obtained. Among the group of patients, the mean duration of ED was 87 months. Concomitant diseases to the ED were cardiac insufficiency ($n = 4$), high blood pressure ($n = 6$), diabetes ($n = 4$), neurologic dysfunction ($n = 6$), alcohol abuse ($n = 2$), and hypogonadism ($n = 2$). The study was approved by the Ethics Committee of Hannover Medical School. Each subject completed a comprehensive questionnaire relating to medical history and sexual behavior. All volunteers were put on a standardized low-nitrate diet at least 24 h prior to entry into the study. Subjects were placed in a supine position with the upper part of the body in a 20–40° upright position. A 20-gauge (G) intravenous indwelling cannula (Vasofix Braunüle, B. Braun, Melsungen, Germany) was inserted into the left cubital vein, and a 19-G butterfly needle (Abbott Laboratories, Sligo, Ireland) was inserted percutaneously into the left corpus cavernosum. Following a rest period, blood was drawn from the cubital vein and the corpora cavernosa during the phase of penile flaccidity. Volunteers were then exposed to sexually explicit video movies and engaged in self-stimulation of the glans penis. During the phase of penile tumescence, blood was aspirated simultaneously from the corpora cavernosa and from the cubital vein. Blood was also drawn from the cubital vein and the cavernous body of the healthy males in the state of penile rigidity. After the patients and the healthy subjects had developed penile tumescence or erection, respectively, sexual stimulation was terminated. Blood was then drawn from the corpora cavernosa and the cubital vein in the phase of detumescence. For blood collection, S-Monovettes (Sarstedt, Nümbrecht, Germany) were used containing 1.6 mg (K^+) ethylenediamine tetraacetic acid (EDTA)/ml whole blood. Tubes were kept on ice immediately after blood collection. Blood samples were centrifuged at 4 °C for 10 min at 3,000 rpm (Cryofuge 5000, Heraeus-Christ, Osterode, Germany). Plasma was then aspirated and stored at −80 °C until analysis.

Determination of plasma NO_2^-/NO_3^- levels by the Griess reaction

Griess assays were performed in accordance with previously described protocols [3, 4]. Briefly, plasma was deproteinized by the addition of 1 M perchloric acid. Following centrifugation, the supernatant was neutralized by the addition of a mixture of 0.1 M Tris + 0.4 M potassium hydroxide (KOH), and diluted with 0.1 M HEPES buffer, pH 7.4. In order to determine the total NO_2^- and NO_3^- levels, NO_3^- was reduced for 30 min at room temperature by the addition of 0.3 U/ml nitrate reductase and 0.1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) prepared in 20 mM of Tris buffer (pH 7.6). The residual NADPH was then removed by incubation of the mixture with 1.2 U/ml glutamate dehydrogenase, 0.1 M ammonium chloride (NH_4Cl) and 1.5 mM α-ketoglutarate. Following a 15-min incubation, 500 µl of the samples was mixed with 500 µl Griess reagent. This was followed by an incubation period of 10 min at 37 °C, after which time the absorbance was measured at 543 nm using a microplate reader. A standard curve generated using an aqueous potassium nitrite (KNO_2) and potassium nitrate (KNO_3) solution from 0.1 to 100 µM was used as a reference.

Determination of plasma NO_2^-/NO_3^- levels by GC-MS

Derivatization procedure

Plasma aliquots (1 ml) were spiked with the internal standard, i.e. $^{15}NO_3^-$ (98 atom% at ^{15}N, Sigma-Aldrich Chemie, Deisenhofen, Germany), at a final concentration of 40 µM. In addition, 1-ml aliquots of a 5% (w/v) NH_4Cl buffer (pH 8.8) were also spiked with $^{15}NO_3^-$ at a final concentration of 40 µM. One hundred microliter aliquots of the plasma and the buffer samples were diluted with 900 µl of the same buffer. Accurately weighed cadmium powder (10 mg) was then added to the samples; reduction was achieved by shaking for 90 min at room temperature. The suspensions were centrifuged at 800 *g* for 5 min. Four hundred microliters of acetone and 10 µl 2,3,4,5,6-pentafluorobenzyl (PFB) bromide (Aldrich Chemie, Steinheim, Germany) were added to the supernatants, and the reaction mixtures were allowed to stand at 50 °C for 60 min.

Acetone was then removed under nitrogen and the reaction products were extracted by vortex-mixing with toluene (0.8 ml) for 1 min. Aliquots (0.5 μl) were injected into the GC-MS facility in the splitless mode.

GC-MS conditions

GC-MS analyses were performed on a Hewlett-Packard MS Engine 5989A directly connected to a gas chromatograph 5890 series II (Hewlett-Packard Co., Greeley, Colo., USA). A fused-silica capillary column Optima 17 [30 m × 0.25 mm inner diameter (ID), 0.25 μm film thickness; Macherey-Nagel, Düren, Germany] was used. Helium (70 kPa) and methane (200 Pa) were used as carrier and reagent gases, respectively, for negative-ion chemical ionization. The following oven temperature program was used: 2 min at 70 °C, then increased to 100 °C at a rate of 5°/min, followed by an increase to 310 °C at a rate of 30°/min. Constant temperatures of 180 °C, 280 °C and 200 °C were maintained at the ion source, interface and injector, respectively. Electron energy and electron current were set at 230 eV and 300 μA, respectively. Selected ion detection at *m/z* 46 for NO_2^- and 47 for $^{15}NO_2^-$ was performed. The plasma concentrations of NO_2^-/NO_3^- were calculated as previously described [16].

Quality control samples

Plasma samples were analyzed alongside three quality control samples [quality control (QC) 1, QC2 and QC3] within three runs (A, B, C) using pooled human plasma containing 49.8 ± 1.2 μM NO_2^-/NO_3^-. The QC1 sample was analyzed without NO_3^-, while the QC2 and QC3 samples were spiked with 20 and 40 μM of NO_3^-, respectively. Plasma samples obtained from the volunteers were analyzed once, while QC plasma samples were assayed in duplicate. Precision [RSD (relative standard deviation)] for QC1, QC2 and QC3 as well as accuracy for QC1 and QC2 were determined.

Statistical analysis

Only NO_2^-/NO_3^- concentrations in plasma aliquots obtained from blood samples simultaneously drawn from the cubital vein and the cavernous body of the volunteers were analyzed and subjected to statistical analysis. For comparison of NO_2^-/NO_3^- levels in the systemic and penile blood samples taken from one group, the Student's *t*-test for paired samples was performed using SPSS 7.5 for Windows (SPSS, Chicago, Ill., USA). Comparison of NO_2^-/NO_3^- levels determined in systemic as well as in penile blood samples taken from the normal males and the patients was performed using analysis of variance (ANOVA).

Results

Plasma levels of NO_2^-/NO_3^- in the systemic and cavernous blood of healthy subjects during different penile conditions

Blood collection from the corpora cavernosa in the flaccid state of the penis was possible in 14 out of 24 volunteers. All of them developed penile tumescence and rigidity. Blood sampling from the cavernous body took place in 15 out of 24 volunteers in the phase of penile detumescence.

Determination of NO_2^-/NO_3^- by the Griess reaction revealed no differences before and during penile tumescence and erection in either the cavernous or the peripheral blood taken from the healthy volunteers. Mean NO_2^-/NO_3^- levels were 26–31 μM. In the phase of detumescence, NO_2^-/NO_3^- levels in the penile blood were found to be significantly lower than those detected in the systemic blood ($P = 0.02$). Table 1 summarizes the results of the NO_2^-/NO_3^- assays using the Griess reaction. When determined by the GC-MS method, mean plasma levels of NO_2^-/NO_3^- were in the range 37–40.5 μM. No significant changes in NO_2^-/NO_3^- were detected in the phases of penile flaccidity, tumescence, rigidity and detumescence. Moreover, there were no significant differences in NO_2^-/NO_3^- levels in the cavernous and peripheral blood in the respective stages. NO_2^-/NO_3^- plasma levels as determined by GC-MS were significantly higher than those assessed by the Griess assay (see Table 2).

GC-MS analyses of NO_2^-/NO_3^- plasma levels in the systemic and cavernous blood taken from a group of patients during different penile conditions

Blood collection from the flaccid penis was possible in 13 out of 15 patients; 12 out of 15 patients developed tumescence. None of them reached the state of rigidity. Blood sampling from the cavernous body was performed in 10 out of 15 patients in the phase of penile detumescence.

GC-MS analyses revealed a significant increase ($P < 0.005$) in NO_3^-/NO_2^- levels in the systemic circulation of the patients in the phase of penile tumescence which was followed by a decrease during the phase of detumescence ($P < 0.005$). In the penile blood, NO_3^-/NO_2^- levels decreased from flaccidity to tumescence and detumescence. Significant differences were found in NO_3^-/NO_2^- levels of the systemic and penile blood in the phases of flaccidity ($P < 0.005$) and tumescence ($P = 0.005$). During flaccidity, NO_3^-/NO_2^- levels were more elevated in the cavernous compartment, whereas during detumescence, plasma levels in the cavernous body were just slightly higher than those registered in the systemic blood. Nevertheless, comparison of the mean

Table 1 Plasma levels of NO_2^-/NO_3^- (μM) determined by the Griess reaction in peripheral and cavernous blood samples obtained from healthy volunteers during different functional conditions of the penis. *Asterisks* indicate significant difference in NO_2^-/NO_3^- levels ($p = 0.02$) determined by Student's *t*-test for paired samples

Penile condition	Blood source	No. of blood collections	Total NO_2^-/NO_3^- (μM)
Flaccid	Cubital vein	14	30 ± 13.8
	Penis	14	29 ± 12.7
Tumescent	Cubital vein	24	29 ± 15.0
	Penis	24	26.4 ± 13.0
Rigid	Cubital vein	24	26 ± 12.2
	Penis	24	25 ± 12.2
Detumescent	Cubital vein	15	31.3 ± 13.2*
	Penis	15	27.8 ± 13.4*

Table 2 Comparison of plasma levels of NO_2^-/NO_3^- (μM) as determined by GC-MS in peripheral and cavernous blood samples collected from healthy males and a group of patients with erectile dysfunction (ED) during different functional conditions of the penis. *Asterisks* indicate significant difference ($p = 0.005$) in NO_2^-/NO_3^- levels determined in the systemic and cavernous blood of the patients

Penile condition	Blood source	No. of blood collections		Total NO_2^-/NO_3^- (μM)	
		Normal males	Patients	Normal males	Patients
Flaccid	Cubital vein	14	13	38 ± 12.7	33 ± 10.7*
	Penis	14	13	37 ± 16.7	40.3 ± 9.7*
Tumescent	Cubital vein	24	12	38 ± 11.5	44.5 ± 11*
	Penis	24	12	37 ± 15	40 ± 8.2*
Rigid	Cubital vein	24	0	36.7 ± 11.4	–
	Penis	24	0	37.5 ± 12.9	–
Detumescent	Cubital vein	15	10	40.2 ± 12.8	33.5 ± 8.7
	Penis	15	10	40.5 ± 11.2	35.4 ± 9.8

plasma levels of NO metabolites in the systemic and cavernous blood of the healthy subjects and patients during penile flaccidity, tumescence and detumescence revealed no significant differences. The results are given in Figs. 1 and 2 and in Table 2.

Table 3 summarizes the results from the QC samples processed in the GC-MS method. The data demonstrate the high accuracy and precision of the GC-MS method with regard to quantification of NO_2^-/NO_3^- in human plasma, and underline the validity of the NO_2^-/NO_3^- levels measured in the plasma of the volunteers' blood samples.

Discussion

Penile erection is strictly dependent on the balance of vascular and trabecular smooth muscle constriction and relaxation. It has been fairly well demonstrated that relaxation of cavernous smooth muscle is under the control of a variety of endothelial-derived relaxing factors, among which NO has been identified, both in vitro and in vivo, as the most important mediator of penile erection. To date, it has not been thoroughly evaluated whether an impairment in the local synthesis and release of NO might be a cause of ED. Thus, evaluation of the plasma levels of NO metabolites in the systemic and cavernous blood of healthy males during different penile conditions appears essential. To date, studies determining and comparing NO metabolites in the systemic and cavernous blood of male subjects are sparse. Moriel et al. [9] determined the serum levels of NO_2^- and NO_3^- in the systemic and cavernous blood of healthy male volunteers who were exposed to visual erotic stimuli and demonstrated that these parameters do not change

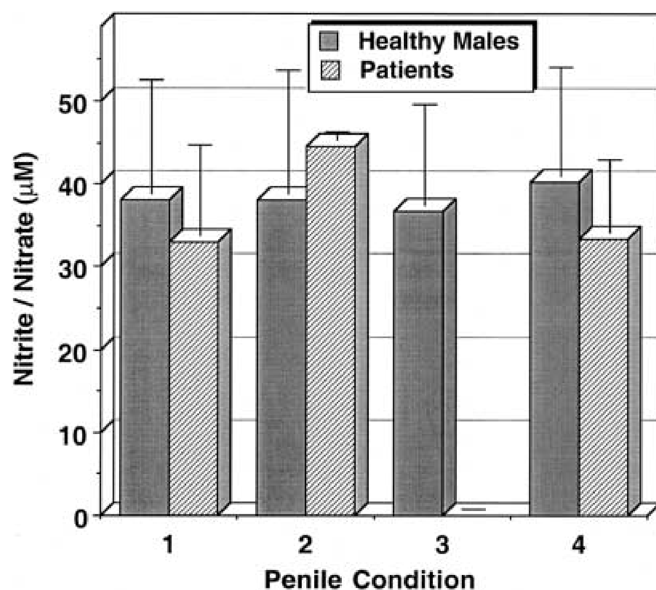

Fig. 1 Course of nitric oxide (NO) metabolites determined by gas chromatography and mass spectrometry (GC-MS) in systemic blood samples obtained from healthy males and patients during different functional conditions of the penis (flaccidity = 1, tumescence = 2, rigidity = 3, detumescence = 4)

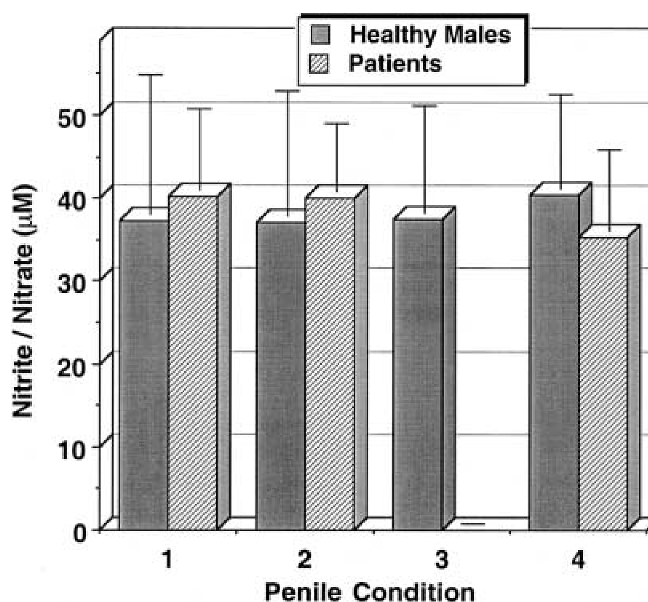

Fig. 2 Course of NO metabolites determined by GC-MS in cavernous blood samples obtained from healthy males and patients during different functional conditions of the penis (*1* flaccidity, *2* tumescence, *3* rigidity, *4* detumescence)

Table 3 Accuracy and precision of the GC-MS method in quality control (*QC*) samples (*RSD* relative standard deviation)

QC sample	Accuracy (%)				Precision (RSD, %)			
	Run A	Run B	Run C	(Mean ± SD)	Run A	Run B	Run C	Mean ± SD
QC1		not applicable			0.31	1.25	8.04	3.214 ± 4.24
QC2	100	110	105	(105 ± 5)	0.10	0.88	0.45	0.477 ± 0.391
QC3	98.8	99.6	98.6	(99 ± 0.5)	0.39	0.71	0.42	0.507 ± 0.177
Mean ± SD	99.4 ± 0.8	104.8 ± 7.4	101.8 ± 4.5	(102 ± 4.2)	0.267 ± 0.150	0.947 ± 0.277	2.97 ± 4.39	1.395 ± 1.57
Mean ± SD		102 ± 4.6				1.394 ± 2.515		

during and immediately following an erection. Rossi et al. [11] demonstrated that the levels NO_2^-/NO_3^- were significantly lower in the penile venous blood of men suffering from psychogenic impotence. Unfortunately, that study suffers from the absence of a control group of normal healthy male subjects. To determine levels of NO_2^- and NO_3^-, these authors used protocols based on modifications of the original Griess assay that have clearly been shown to be only partially applicable to biological fluids. This is due to the presence of a large number of interfering substances such as albumin and reduced thiols with different chemical structures and physicochemical properties [15]. The present study utilized both the Griess assay and a GC-MS protocol to ensure the accurate measurement and comparison of NO_2^-/NO_3^- in whole plasma samples.

Irrespective of the analytical method used to quantify plasma levels of NO metabolites, our data revealed no appreciable changes in NO_2^-/NO_3^- levels in the systemic and cavernous blood of healthy subjects before, during and after penile erection. These results concur with the data of Moriel et al. [9]. Therefore, it seems unlikely that the differences in NO_2^-/NO_3^- levels detected between the systemic and cavernous blood by the Griess assay during the detumescence phase are of any physiologic importance. When discussing the results of our study, consideration should be given to the fact that changes in penile hemodynamics due to systemic arterial inflow into the cavernous compartment during the tumescence and rigidity phases may mask any local release of NO that might occur within the penile erectile tissue. Moreover, cavernous NO metabolism during sexual arousal may account for only a minor fraction of circulating NO_2^- and NO_3^-, which mainly derive from exogenous sources, such as food and gut bacteria, and from basal release throughout the vascular tree.

GC-MS analyses revealed appreciable changes in plasma levels of NO metabolites in the systemic and cavernous blood taken from the patients during different penile conditions. Moreover, significant differences in the plasma levels of NO_2^-/NO_3^- in the systemic and cavernous blood were detected during the penile flaccidity and tumescence phases. When discussing these results, it should be borne in mind that the group of patients enrolled in the study was quite heterogeneous with regard to the underlying causes of ED. It should also be emphasized that we found no significant differences in plasma levels of NO_2^-/NO_3^- in the systemic and cavernous blood of patients and healthy subjects during the respective penile stages.

The present study demonstrates that determination of NO_2^-/NO_3^-, even with an advanced, sensitive, reliable, highly accurate and precise approach such as GC-MS, is not a useful way to detect the local release of NO within the cavernous erectile tissue during penile tumescence and rigidity. This may be due to the fact that stable metabolites of NO are not produced in substantial quantities during penile tumescence and erection. In conclusion, the quantification of NO metabolites, even

by means of an advanced contemporary detection method, is of no use in the experimental workup of ED.

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