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Cavernosal tissue nitrite, nitrate, malondialdehyde and glutathione levels in diabetic and non-diabetic erectile dysfunction

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Summary

The aim of this study is to investigate the role of nitric oxide (NO) stabile end products, membrane lipid peroxidation and antioxidant defensive mechanism in diabetic erectile dysfunction (ED) and compare these parameters with non-diabetic ED groups. We examined the penile cavernosal tissues, obtained from 22 patients who had undergone surgery of penile prostheses implantation, for the nitrite, nitrate, malondialdehyde (MDA) and glutathione (GSH) levels. Eight patients were suffering from diabetic erectile dysfunction (ED) and 14 patients had non-diabetic ED. Nitrite and nitrate levels were lower; MDA and GSH levels were higher in the diabetic group. There were statistically significant differences between diabetic and non-diabetic groups amongst the nitrite ($p < 0.001$), nitrate ($p < 0.01$), MDA ($p < 0.001$) and GSH ($p < 0.01$) levels. Our data provide evidence that NO deficiency, possibly due to the membrane lipid peroxidation and defective antioxidant defensive mechanism, may contribute to the development of diabetic ED and thus is involved in the pathogenesis of ED in diabetic patients.

Keywords: diabetes mellitus, erectile dysfunction, glutathione, malondialdehyde, nitric oxide

Introduction

Penile erection is a haemodynamic event resulting from a complex interaction between the nervous system and cavernosal tissue (smooth muscle and endothelium) (Andersson & Wagner, 1995). There is an emerging consensus that in many patients, the aetiological basis of organic erectile failure may be related to primary changes in corporeal smooth muscle physiology/function (Christ, 1995). During the last decade, significant advances have been made in the understanding of male sexual dysfunction. Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS), was considered to be the major mediator of corporal smooth muscle cell relaxation (Truss *et al.*, 1994).

Diabetes mellitus (DM) is a disease of metabolic deregulation, mainly of glucose metabolism, accompanied by characteristic long-term complications. Erectile dysfunction (ED) is one of the most prevalent complications, increasing from 35 to 70% with age, and is virtually present in all men suffering from autonomic diabetic neuropathy. The mechanism of diabetic ED is the subject of current interest. Investigations in animal models and diabetic patients have

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shown impaired neurogenic and endothelium-dependent relaxation of the penile smooth muscle (De Tejada *et al.*, 1989).

In earlier studies, it was proposed that the endothelium is an insulin target tissue that exhibits an increase in the release of endothelium-derived NO in response to insulin. Insulin resistance in diabetic patients causes decreased endothelial NO synthase and defective vasodilatation (Baron, 1996). According to Cartledge *et al.* (2001a), diabetes impairs endothelial and neuronal NO-mediated cavernosal smooth muscle relaxation. It has also been shown that toxic and reactive free oxygen radicals have a major role in DM. A possible explanation that could account for diabetic ED resides in oxidative damage of cell membrane due to the auto-oxidation of glucose, non-enzymatic protein glycosylation (glycation) and peroxidation of unsaturated fatty acides (Godin *et al.*, 1988; Baynes, 1991; Kakkar *et al.*, 1995; Toleikis & Godin, 1995; Seftel *et al.*, 1997). Furthermore, advanced glycation end products (AGEs) which were generated because of high serum glucose levels seen in diabetes could be in the pathological processes responsible for the development of diabetic ED and they impair both the neuronal and endothelial production of NO (Cartledge *et al.*, 2001a).

As NO is rapidly degraded within 5–6 sec, stabile end products, nitrite and nitrate, have been used to evaluate NO levels in tissues (Bush *et al.*, 1992). Lipid peroxidation is thought to be an important biological sequence of oxidative cellular damage. Membrane lipid peroxidation may generate reactive carbonyl compounds such as malondialdehyde (MDA), an indicator of free oxygen radical-induced tissue damage (Tirmenstein & Reed, 1988; Basaga, 1990). Glutathione (GSH), a substrate of glutathione peroxidase defensive mechanism, is an inhibitor of lipid peroxidation. Thus it is a preventive mechanism for oxidative stress (Ursini *et al.*, 1982; Burk, 1983).

We examined the levels of nitrite, nitrate, MDA and GSH in penile cavernosal tissues of patients with diabetic or non-diabetic ED. The aim of our study is to evaluate the role of NO stabile end products, membrane lipid peroxidation and antioxidant defensive mechanisms in diabetic ED and compare these parameters with non-diabetic ED groups.

Materials and methods

In this study, human penile tissue was obtained from 22 patients who had undergone penile prostheses implantation surgery. Eight of the patients were suffering from diabetic ED and 14 had non-diabetic ED. Of the 14 patients, six had psychogenic ED and eight, Peyronie's disease. The mean age was 55 (43–67) and 59 (38–71) years for the diabetic and non-diabetic ED group, respectively.

Before penile implantation, all patients underwent a comprehensive and standardized workup for ED, including detailed history, physical examination, psychological assessment, routine blood tests including serum testosterone levels, pharmacopenile Doppler ultrasonography (PPDU) and nocturnal penile tumescence (NPT) test.

All patients had penile prostheses implantation under general anaesthesia. Specimens of penile tissues were obtained from both corpus cavernosum before dilatation with wedge resection. The specimens were stored at −70 °C. Tissue nitrite, nitrate, MDA and GSH levels were determined.

Nitrite analysis method

Cavernosal tissue specimens were homogenized with fivefold volume of phosphate-buffered saline (8 g sodium chloride, 0.2 g potassium chloride, 0.92 g Na_2HPO_4 diluted to 1 L with distilled water, pH: 7.5) and centrifuged for 5 min at 2000 ***g***. Following centrifugation, 0.25 mL of 0.3 M sodium hydroxide (1.2 g NaOH completed to 100 mL with distilled water) was added to 0.5 mL of supernatant. After a 5 min incubation at room temperature, 0.25 mL 5% zinc sulphate was added for deproteinization. The mixture was centrifuged for 20 min at 3000 ***g***. One millilitre of ethanol (0 °C) was added to 0.5 mL supernatant, incubated at 0 °C for 30 min and then centrifuged for 5 min at 14 000 ***g***. The final supernatant was used for nitrite determination (Sievers Model 280A NOA, Sievers Instruments Inc., USA). Standard solutions (25, 50, 75, 100 μM, and 1 and 10 mM) from a 100 mM stock solution, obtained by the dissolution of 69 mg sodium nitrite in 10 mL deionized water, were used for the standard curve. The concentrations were calculated from the supernatant of prepared tissue specimens by using this standard curve.

Nitrate analysis method

Total nitrate levels were measured with an NO analyzer (Sievers Model 280A). Tissue specimens were deproteinized with 1/2 volume of 96% ethanol. Saturated VCL_3 (Vanadium Chloride) solution in 1 M hydrochloric acid was filtrated and 5 mL of this reagent was treated with nitrogen gas for 10 min in the purge pipette. Circulating water bath which heats the reagent up to 95 °C and cold water condenser were applied to purge pipette. Hydrochloric acid vapour was drained with gas bubbler equipment containing 15 mL 1 M sodium hydroxide. The velocity of gas flow in the chemiluminescence detector was controlled with needle-valve apparatus allowing a 6 Torr pressure. The materials, which convert nitrate to NO via VCL_3 and HCL (Hydrogen Chloride) reagents, were injected into the purge pipette. NO product was drained from the reaction chamber and determined with ozone-inducted chemiluminescence. A standard curve was employed to calculate various levels of nitrate (10–100 μM).

Tissue MDA assay

Tissue lipid peroxidation status was investigated by measuring tissue MDA levels. Tissue specimens were homogenized with 9 mL 10% cold trichloroacetic acid (TCA)

for 1 g tissue. The resulting mixture was chilled on ice and then centrifuged for 8 min at 4000 ***g***. Following centrifugation, the supernatants were removed and 750 μL of the solution was added to 750 μL of 0.67% thiobarbituric acid (TBA). The samples were heated at 100 °C for 15 min, cooled at room temperature and centrifuged at 4000 ***g***. Absorbances were measured at 532 nm. The cavernosal tissue lipid peroxidation levels were expressed as MDA equivalence with 1.56×10^5/M coefficient (Casini *et al.*, 1986).

Tissue glutathione assay

Modified Ellman method was used for penile cavernosal tissue GSH analysis (Aykaç *et al.*, 1985). Following homogenization and centrifugation of cavernosal tissue specimens, two volume supernatant was added to a solution consisting of eight volume of 0.3 M Na_2HPO_4 $2H_2O$ and one volume of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate). Absorbances were measured at 412 nm with a spectrophotometer and GSH levels were calculated with 13 600/M/cm coefficient.

Mann–Whitney *U*-test was used for statistical analysis.

Results

Routine blood tests and hormonal profiles of all patients were normal. In the diabetic group, all the patients were type II diabetes. The duration of diabetes was between 2 and 5 years and their serum glucose was under control with either insulin or oral antidiabetics.

Diabetic group

Following papaverine test there was no erection in five patients and partial erection was found in three patients. PPDU showed cavernosal artery pathology in all patients. NPT was diminished in one patient and negative in seven patients. More conservative treatments like oral (sildenafil) and intracavernosal pharmacotherapy or vacuum devices failed in these patients.

Non-diabetic group

In the pscyhogenic ED group, papaverine, pharmacopenile colour Doppler ultrasonography and nocturnal penile tumescence tests were found to be normal (NPT was accepted as normal when there was a change in circumference of at least 3 cm at the base of the penis and at least 2 cm at the tip with a rigidity of >70% measured with RigiScan device). Psychosexual counselling and other treatment modalities like pharmacotherapy failed in the patients. None of the eight patients with Peyronie's disease had normal erection. Also PPDU and NPT test results were pathological in these patients. Penil prostheses implantation was discussed after oral or intracavernosal pharmacotherapy and vacuum device were tried.

Nitrite, nitrate, MDA and GSH levels of diabetic and non-diabetic patients are listed in Table 1.

Table 1. Nitrite, nitrate, MDA and GSH levels of corpus cavernosum in diabetic and non-diabetic group

	Diabetic Group ($n = 8$) (mean ± SD) (nM/g tissue)	Non-diabetic group ($n = 14$) (mean ± SD) (nM/g tissue)	*p*-value
Nitrite	21.25 ± 5.05	35.59 ± 2.16	$p < 0.001$
Nitrate	31.01 ± 8.84	57.31 ± 2.23	$p < 0.01$
MDA	77.42 ± 1.97	55.08 ± 6.12	$p < 0.001$
GSH	4.97 ± 0.26	4.26 ± 0.60	$p < 0.01$

There was a statistically significant difference between the diabetic and non-diabetic groups according to the nitrite ($p < 0.001$), nitrate ($p < 0.01$), MDA ($p < 0.001$) and GSH ($p < 0.01$) levels.

Discussion

The presentation of ED in DM is usually gradual, insidious and progressive. ED is a common complication and an important cause of decreased quality of life in men with diabetes. These patients present a risk of ED threefold higher than the general population (Feldman *et al.*, 1994). The corpus cavernosum smooth muscle tissue of men with DM has impairment of both, NO-mediated smooth muscle relaxation and the autonomic nerve-mediated relaxation. Further, the degree of impairment seems to be associated with the duration of DM. The incidence of complications is correlated with hyperglycemia level. This correlation may explain the hypothesis that diabetic AGEs cause cavernosal tissue or non-adrenergic non-cholinergic nerve fibre damage via membrane lipid peroxidation (Seftel *et al.*, 1997). Lugg *et al.* (1996) found that NOS activity decreased in patients with DM, androgen deprivation, adrenalectomy and chronic smoking. Importantly, AGEs have also quench NO in vitro and in vivo (Burnett *et al.*, 1992; Monnier *et al.*, 1992). Thus, it is hypothesized that AGEs, which accumulate in the long term, may adversely affect erectile function by interacting with NO (Seftel *et al.*, 1997). Cartledge *et al.* (2001b)n stated that AGEs are more prevalent in the erectile tissue of diabetic animals, and aminoguanidine reversed the impairment in neuronal and endothelial NO-mediated penile smooth muscle relaxation seen in diabetes by reacting with intermediates of glycation products.

We found that cavernosal tissue nitrite and nitrate levels were significantly lower in the diabetic than in the non-diabetic group. A link between the pathogenesis of ED and decreased local NO activity has been suggested by De Tejada *et al.* (1989), as in isolated corpus cavernosal strips from diabetic patients with ED, both neurogenic and endothelium-dependent relaxations were impaired. Seftel *et al.* (1997) investigated the levels of pentosidine, a diabetic

AGE, in the serum and corpus cavernosum of men with DM who were undergoing penile prostheses implantation. A significant elevation of pentosidine was found in the penile tissue, but not in the serum, of diabetic patients in comparison with non-diabetic patients. Cavernosal tissue NOS enzyme activity was lower in the diabetic group, leading to the confusion that AGEs interact with NO directly or alters NOS activity. Our findings could be explained with this mechanism. Contrary to our findings, Moriel *et al.* (1993) could not find any difference between the cavernosal blood and serum nitrite and nitrate levels before and during erection in men with normal erectile function. The contradiction may, in part, be explained by measurement of NO metabolites in the serum.

The second possible explanation that could account for diabetic ED resides in autonomic nerve-mediated relaxation. Rendell *et al.* (1999) showed that sildenafil was successful in 56% of 121 insulin-dependent DM patients with ED. Similarly, Lincoln *et al.* (1987) demonstrated that vasoactive intestinal polypeptidergic, cholinergic and adrenergic nerves of the penis are affected in DM and proposed that cavernosal tissue damage is not the unique cause of the diabetic ED. Besides this, Cellek *et al.* (1999) showed that in DM nitrergic nerves undergo selective degeneration whereas the noradrenergic nerves which are anti-erectile remained intact, and conluded that this selective degeneration was NO-dependent and inhibition of NO synthase was neuroprotective in this condition. The present study does not exclude the possibility that non-adrenergic non-cholinergic nerve fibre damage may contribute to the development of ED in diabetic patients but a detailed discussion on the autonomic innervation of ED is beyond the scope of the present study.

We found that MDA, one of the end products of lipid peroxidation, was significantly higher in the diabetic than in the non-diabetic group. Basaga (1990) demonstrated that tissue MDA level is the most reliable indicator of tissue damage caused by membrane lipid peroxidation. In an experimental animal model, Kakkar *et al.* (1995) found that MDA levels were significantly higher in the cavernosal tissue following DM and concluded that high MDA levels were an indicator of cavernosal tissue damage. The results reported here would tend to support this view. In our study high levels of cavernosal tissue MDA might be an indicator of corpus cevernosum smooth muscle damage caused by membrane lipid peroxidation.

Tirmenstein & Reed (1988) suggested that GSH, either alone or in conjunction with added proteins, can protect microsomes against lipid peroxidation. Ursini *et al.* (1982) found that GSH peroxidase system is one of the most important defensive mechanisms against membrane lipid peroxidation-induced tissue damage. They thought that GSH increased in tissue as a reactive agent or as a substrate of GSH peroxidase enzyme system, preventing cells from oxidative stress. In the present study, GSH levels were higher in the diabetic group. This might be attributed to the deficiency of GSH peroxidase enzyme system leading to high GSH levels or independently of this enzyme system GSH has become higher as a reactive agent (Gibson *et al.*, 1985).

In conclusion, the present results demonstrate that cavernosal tissue nitrite and nitrate were lower, and MDA and GSH levels were higher in the diabetic group than in the non-diabetic group. Our data provide evidence that NO deficiency, possibly due to the membrane lipid peroxidation and defective antioxidant defence system, may contribute to the development of diabetic ED and thus is involved in the pathogenesis of ED in diabetic patients. For further evaluation, we need larger patient groups which also includes normal potent patients.

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