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The Effect of Aging and Exogenous Testosterone Replacement on Nitric Oxide Concentration and Activity of Nitric Oxide Synthase in the Rat Corpus Cavernosum

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The effects of testosterone replacement on the nitric oxide (NO) concentration and activity of NO synthase (NOS) in the penis were investigated. Male Wistar rats (*n* = 39) were divided into 5 groups: 14-week-old males, 13-month-old males, 15-month-old males, 15-month-old males treated with low-dose testosterone replacement and 15-month-old males treated with high-dose testosterone replacement. The testosterone concentration in serum and the NO concentration in penile tissue were measured, and the endothelial NOS (eNOS) and neuronal NOS (nNOS) expressions were examined immunohistochemically. The testosterone concentration in serum tended to decrease with aging, but the 15-month-old testosterone-replaced rats maintained almost the same level as the 14-week-old rats. Nitrite and nitrite/nitrate concentrations in penile tissue tended to decrease with aging. Nitrite concentrations in the 15-month-old rats were significantly higher in the testosterone-replaced groups than in the non-replaced group, but no significant difference in nitrite/nitrate concentration was recognized between the 15-month-old rats not treated and treated with testosterone replacement. Immunohistochemical staining for eNOS and nNOS demonstrated a decreasing expression of the 2 NOSs with aging and recovering of the NOSs by testosterone replacement. The results of this study suggest that NO plays a major role in the mediation of penile erection, and testosterone replacement may favorably alter age-related erectile dysfunction.

Key words: aging; immunohistochemical staining; nitric oxide; testosterone replacement

Recently, several studies have revealed that nitric oxide (NO) is an important neural messenger which mediates penile erection (Ignarro et al., 1990; Holmquist et al., 1991; Kim et al., 1991; Burnett et al., 1992; Rajifer et al., 1992). Erection is mediated by the release of NO from non-adrenergic non-cholinergic nerve terminals, the endothelium of penile blood vessels, and corporal smooth muscle, producing smooth muscle relaxation and vasodilation (Burnett, 1997). NO stimulates the formation of guanylate cyclase in smooth muscle cells, converting GTP to 3'5'-cyclic GMP (cGMP) (Burnett, 1997). A cascade of cGMP-dependent intracellular events then leads to a decrease in intracellular calcium, ultimately causing smooth muscle relaxation, in part through changes in potassium conductance (Seftel et al., 1996; Burnett, 1997).

The production of NO is mediated by a family of NO synthase (NOS) that all represent distinct gene products. These enzymes produce NO through a complex set of redox reactions that result in the conversion of L-arginine to L-citrulline. The isoforms of NOS have been categorized as being either inducible or constitutive (Forstermann et al., 1995). The inducible isoform of NOS (iNOS) is associated primarily with macrophages and is activated by specific cytokines as part of the immune response. The endothelial (eNOS) and neuronal (nNOS) isoforms of NOS are constitutive and are activated,

Abbreviations: eNOS, endothelial NOS; iNOS, inducible NOS; mRNA, messenger RNA; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; PBS, phosphate-buffered saline

in part, by an increased concentration of intracellular calcium and calmodulin binding to the enzyme. Experimental evidence suggests that the constitutive isoforms of NOS may be responsible for NO production in penile erection (Bush et al., 1992; Burnett et al., 1993). Recent evidence suggests that eNOS and its subsequent production of NO may be a significant route by which NO-mediated cavernosal relaxation is brought about because transgenic mice lacking nNOS are still capable of erectile activity with pelvic nerve stimulation (Burnett et al., 1996).

A decrease in the serum testosterone level with aging may contribute to a reduction of sexual potency (Meisel and Sachs, 1994; Garban et al., 1995), although this reduction with aging is a multi-factorial phenomenon.

A recent study also hypothesized that androgens maintained and facilitated male sexual potency through enhancement or maintenance of NOS activity in the corpus cavernous tissue in the penis (Mills et al., 1996). In addition, several studies have shown that androgen replacement facilitated neural activities in some areas of the brain which mediate sexual function (Okamura et al., 1994a, 1994b; Pu et al., 1996).

In this article, the changes in the serum tes tosterone concentration, NO concentration and NOS protein expression in penile tissue were examined by using the rat as our model of aging, and the changes brought about by testosterone replacement, as well. The goal in this study was to evaluate whether testosterone replacement helps to resolve age-related erectile dysfunction.

Materials and Methods

Subjects used were 14-week-old (G1, n = 10), 13-month-old (G2, n = 5) and 15-month-old male Wistar rats (n = 24) (SLC, Shizuoka, Japan). All rats were housed in a room with controlled lighting and allowed access to food and water ad libitum. The 15-month-old rats were divided into 3 groups as follows: no testosterone replacement group (G3, n = 8), low-dose testosterone replacement group (G4, n = 8) and high-dose testosterone replacement group (G5, n = 8). In the replacement groups, Silastic tubes (Dow Corning, Midland, MI; outer diameter, 3.17 mm; inner diameter, 1.57 mm) which were 3 cm in length, and contained 40 mg of testosterone powder (Sigma, St. Louis, MO) were subcutaneously implanted in the backs of the rats when they were anesthetized with pentobarbital (30 mg/kg) at the age of 13 months, and the replacement was continued for 2 months. In G4, 4 tubes were implanted and in G5, 8 tubes were implanted. As each group reached the age desired for the experiment as mentioned above, blood (7–8 mL) was collected through the inferior vena cava of each rat under pentobarbital (30 mg/kg) anesthesia, and the serum was separated by centrifugation and stored at –80˚C until it was assayed. Total testosterone and free testosterone concentrations in serum were determined by radioimmunoassay (Total Testosterone Kit and Free Testosterone Kit, DPC Corp., Tokyo, Japan). Rats were killed in succession by means of additional pentobarbital (30 mg/kg), and the penis was removed and weighed. The penis was divided into 2 pieces, and one was used for measurement of NO concentration and the other was used for immunohistochemistry.

Measurement of NO concentration in penile tissue

The final products of NO in vivo are nitrite and nitrate. The relative proportion of nitrite and nitrate is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both nitrite and nitrate.

NO was measured by means of the Griess method. The penile tissue was weighed and homogenized in phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 10,000 × g for 20 min. The supernatant was ultracentrifuged at 100,000 × g for 15 min, and then the supernatant was ultrafiltered using a 30 kDa molecular weight cut-off filter. The sample was assayed by means of a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI). The values were estimated per tissue weight and

Table 1. Body and penile weight, and testosterone concentration in serum

	Young adult rats	Middle-aged rats			
	14-week-old	13-month-old	15-month-old		
	G1	G2	G3	Testosterone-replaced	
	[10]	[5]	[8]	Low-dose G4 [8]	High-dose G5 [8]
Body weight (g)	367 ± 14	596 ± 59	562 ± 39*	554 ± 19	587 ± 47
Penis weight (mg)	394 ± 40	659 ± 73	596 ± 42*	621 ± 45	621 ± 38
Total testosterone (ng/mL)	2.0 ± 0.4	1.0 ± 0.2	0.8 ± 0.1*	3.7 ± 0.4**	5.0 ± 1.1**
Free testosterone (pg/mL)	8.3 ± 1.9	5.2 ± 0.8	4.2 ± 1.2*	14.5 ± 3.6**	20.0 ± 6.7**

Values represent mean ± SEM.
[ ], number of animals.
Significant difference ($P < 0.05$): *G3 versus G1; **G4 or G5 versus G3.

per amount of protein in the tissue. Protein was determined using a commercial kit (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL).

eNOS and nNOS immunohistochemistry in penile tissue

The penis was immediately fixed with neutral buffered 15% formaldehyde-saline. The tissues were embedded in paraffin after the fixation. Sections (3 μm) were subjected to immunohistochemical stains for eNOS and nNOS. The sections were retrieved by micro-wave and treated with 3% methanol/hydrogen peroxide for 15 min at room temperature to reduce background staining. They were then reacted with 200 μg/mL of rabbit anti-eNOS or nNOS polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:5000 dilution in Dako antibody diluent (Dako, Carpenteria, CA) overnight at 4˚C. Sections were washed with PBS and the specifically bound first antibodies were visualized by means of biotinylated anti-rabbit secondary antibody and with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Again, sections were washed with PBS and incubated for 30 min with horseradish peroxidase-labeled streptavidin at room temperature. After the incubation period, tissue sections were washed and a diaminobenzidine peroxidase substrate solution (Vector) was applied for 5 min. The reaction was stopped by washing the sections in water. Mayer's hematoxylin was used for counterstaining.

Statistical analysis

Experimental values were expressed as mean ± SEM for the number of separate determinations indicated in each case. The non-parametric *t*-test was used for calculating probabilities when comparing 2 groups independent from the others, and those with *P* values less than 5% ($P < 0.05$) were considered significant.

Results

Body weight and penile weight

Among the group of 15-month-old rats, body weight and penile weight of rats treated without testosterone replacement (G3) were not significantly different from those treated with testosterone replacement groups (G4 and G5) (Table 1).

Serum testosterone concentration

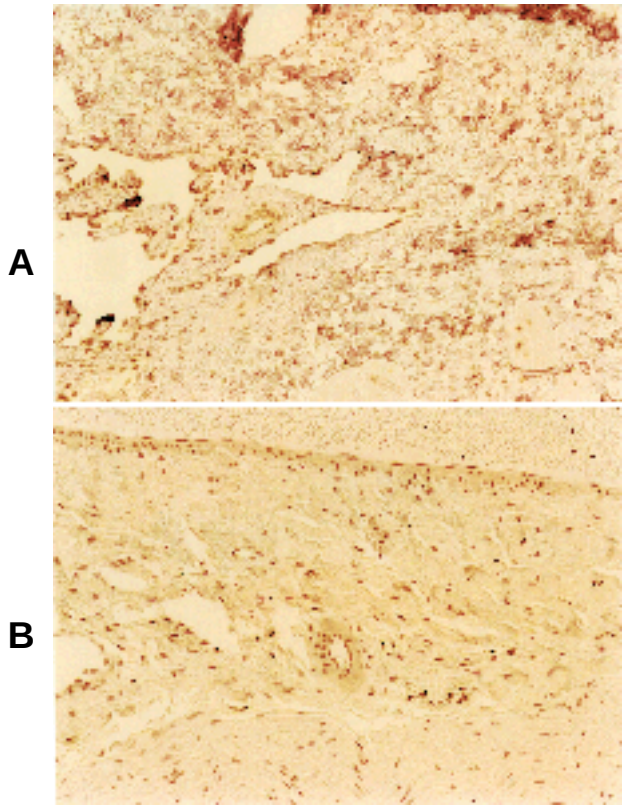
The total testosterone level tended to decrease with aging, but the level in the 15-month-old rats without testosterone replacement (G3) was significantly lower than that in 14-week-old rats (G1). Among 15-month-old rats, the rats with

Table 2. Nitrite and nitrite/nitrate concentration in penile tissue

	Young adult rats	Middle-aged rats			
	14-week-old	13-month-old	15-month-old		
	G1	G2	G3	Testosterone-replaced	
	[10]	[5]	[8]	Low-dose G4 [8]	High-dose G5 [8]
Nitrite† (μmol/g wt)	17.3 ± 3.4	16.2 ± 3.4	11.0 ± 1.9	31.5 ± 3.6*	27.0 ± 6.6*
Nitrite‡ (nmol/mg pro)	1.11 ± 0.24	1.05 ± 0.22	0.54 ± 0.05	2.59 ± 0.73*	1.61 ± 0.50*
Nitrite/nitrate† (μmol/g wt)	48.4 ± 7.1	42.1 ± 5.2	40.6 ± 4.3	43.4 ± 6.7	49.8 ± 10.4
Nitrite/nitrate‡ (nmol/mg pro)	3.28 ± 0.53	2.47 ± 0.33	2.32 ± 0.23	3.27 ± 0.54	3.10 ± 0.66

†Per tissue weight (μmol/g weight).
‡Per amount of protein in the tissue (nmol/mg protein).
Values represent mean ± SEM.
[ ], number of animals.
pro, protein; wt, weight.
Significant difference ($P < 0.05$): *G4 or G5 versus G3.

low- and high-dose testosterone replacement (G4 and G5) showed significantly higher levels of total testosterone than the rats without replacement (G3), but they did not have significantly higher levels than 14-week-old rats (Table 1). The free testosterone level in 15-month-old rats without testosterone replacement was significantly lower than that in 14-week-old rats. Among 15-month-old rats, the free testosterone level in the low- and high-dose testosterone replacement groups was significantly higher than that in the non-replacement group (Table 1).



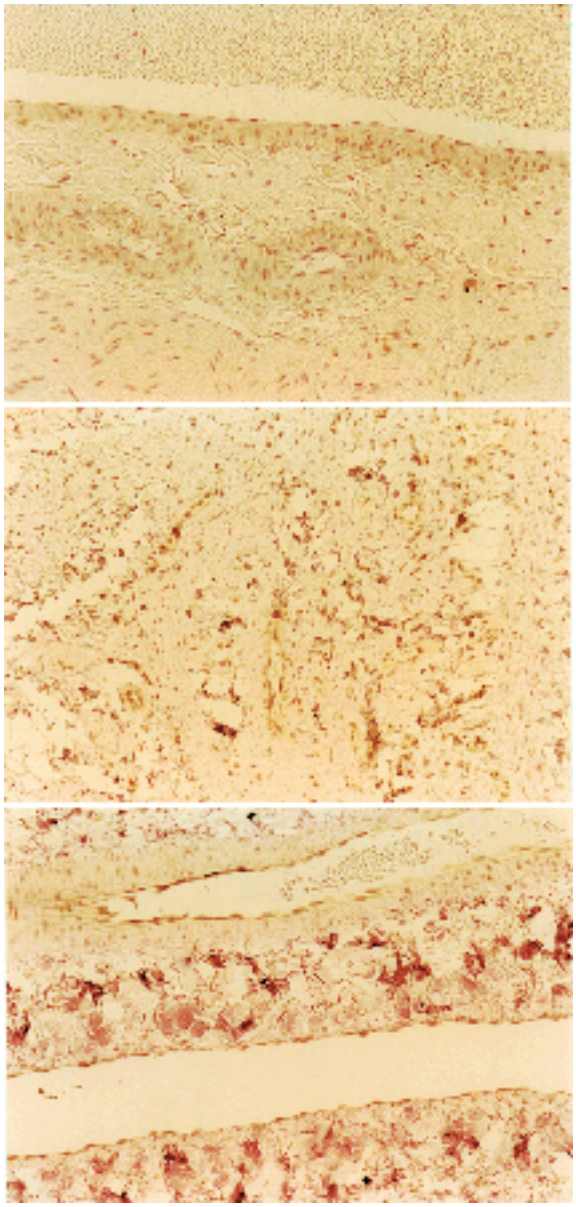
A: G1, young adult rat *(14-week-old)*
B: G2, middle-aged rat *(13-month-old)*

Fig. 1. Sections of rat corpus cavernosum tissue immunostained by ant-endothelial nitric oxide synthase (eNOS) antibodies (original magnification × 200). Sections are counterstained with hematoxylin.
A: Immunohistochemical localization of eNOS shows strong staining of the vascular endothelium.
C: Aged rats without replacement have weak staining.
D and E: Testosterone replacement restored the same level of staining as in the young adult group.
[Figs. 1A and B on p. 48 and Figs. 1C–E on p. 49]

Nitrite and nitrite/nitrate concentration in penile tissue

Nitrite level

This level tended to decrease with aging, but there was no significant difference between 14-week-old rats (G1) and 15-month-old rats (G3). Among 15-month-old rats, the low- and high-dose testosterone replacement rats showed a significantly higher nitrite level per amount of protein in the tissue and per tissue weight than rats without replacement. There was no significant difference in nitrite levels between the high- and the low-dose replacement rats (Table 2).



C: G3, middle-aged rat
D: G4, low-dose testosterone replaced middle-aged rat
E: G5, high-dose testosterone replaced middle-aged rat
(G3–G5: 15-month-old)

Figs. 1C–E. *Continued from the previous page.*

Nitrite/nitrate level

This level tended to decrease with aging, but there was no significant difference between 14-week-old rats and 15-month-old rats without testosterone replacement. The nitrite/nitrate level increased with testosterone replacement; however, the difference was not significant between 15-month-old rats treated with and without testosterone replacement (Table 2).

eNOS and nNOS immunohistochemistry in penile tissue

Figures 1 and 2 show light microscopic photographs of the corpus cavernosum from G1 to G5. Immunohistochemical localization of eNOS showed strong staining of the vascular endothelium in 14-week-old rats (G1) (Fig. 1A).

Thirteen-month-old (G2) and 15-month-old rats without testosterone replacement (G3) had weak staining (Figs. 1B and C), and testosterone replacement restored the level of staining (Figs. 1D and E). Immunohistochemical stainings in 14-week-old rats, 15-month-old rats with low- and high-dose testosterone replacement rats were almost at the same level. Immunohistochemical staining with anti-nNOS