

ORIGINAL ARTICLE

The effect of regular exercise on penile nitric oxide synthase expression in rats

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Summary

Erectile dysfunction (ED) is a major public health problem that seriously affects the quality of life of patients and their partners and its prevalence increases significantly with ageing. In this study, we tested the hypothesis that age-associated decrease in penile endothelial (eNOS) and neuronal nitric oxide synthase (nNOS) activity in aged rats may be increased by regular exercise. A total of 28 young (4 m) and aged (24 m) male rats were divided into four equal groups: group 1 – young control; group 2 – young trained; group 3 – old control and group 4 – old trained group. Groups 2 and 4 rats were trained to swim for 30 min a day and 5 days a week, which lasted 8 weeks. At the end of 8 weeks, rats were sacrificed and penile tissues evaluated for eNOS and nNOS activities. eNOS and nNOS activities were evaluated by immunohistochemistry in paraffinized penile tissues and results assessed semiquantitatively. Results also were compared with healthy age-matched and adult (4 m) controls. Serum level of testosterone (T) was determined using ELISA kits (Beckman Coulter, Fullerton, CA, USA). In penile tissues of aged control rats, eNOS and nNOS staining were weakly positive; however in trained groups, eNOS and nNOS immunoreactivity were increased. In young control group, eNOS and nNOS activities were more intense than aged control. eNOS and nNOS activities were higher in adult trained group than control. Serum T concentrations were significantly higher in young and aged trained group than in control groups. We can suggest that regular exercise upregulates eNOS and nNOS expressions in the aged and young rat penis. Regular exercise may improve penile erection by increasing penile neurotransmitter in both young and aged rats.

Introduction

Penile erection is evoked by elevated pressure from the corpus cavernosum as a result of the relaxation of cavernous smooth muscles and arterioles. The relaxant substance has been clarified as consisting mainly of nitric oxide (NO) that is liberated from neurons and endothelium in the corpus cavernosum (Burnett *et al.*, 1992). NO is derived from L-arginine and molecular oxygen, a reaction that is catalysed by NO synthase (NOS). NOS exists in Ca⁺²-dependent constitutive neuronal (nNOS) and endothelial (eNOS) forms, and in a Ca⁺²-independent inducible (iNOS) form (Bredt & Snyder, 1994).

Vascular risk factors, including hypercholesterolaemia, atherosclerosis, hypertension and diabetes mellitus, can interfere with the neurovascular mechanisms underlying normal erection (Krane *et al.*, 1995; Siroky & Azadzo, 2004; Wang *et al.*, 2004). These conditions are known to induce oxidative tissue injury because of accumulation of reactive oxygen species (ROS), such as superoxide, H₂O₂ and hydroxyl radicals (Slater, 1984). Oxidative injury occurs when the oxidative burden of the body exceeds its antioxidant capacity. The mechanism of oxidative injury is thought to involve lipid peroxidation, protein oxidation, DNA oxidation, decreased synthesis and bioavailability of eNO and nNO and the upregulation of

proinflammatory cytokine growth factors and tissue-specific receptors (Slater, 1984).

The theory of free radicals is one of the hypotheses associated with ageing (Harman, 1956). It is believed that the antioxidant defence is generally weakened in old age (De & Darak, 1991). Several studies have shown an ageing-associated reduction in endothelial function in conduit and resistance arteries, including arterioles isolated from skeletal muscle (Hongo *et al.*, 1988; Tominaga *et al.*, 1994; Delp *et al.*, 1995; Muller-Delp *et al.*, 2002). Ageing is also associated with reduction in serum testosterone (T) production in male rats and humans.

Aerobic exercise training has been reported to ameliorate age-associated reductions in both central and peripheral cardiovascular function. For example, cardiac output, stroke volume and maximal aerobic capacity are increased as a result of exercise training (Ogawa *et al.*, 1992). Notably, physical training reduces vascular oxidative stress at least in part via increased activity of eNOS and extracellular superoxide dismutase, which in turn could exert beneficial vascular effects (Kojda *et al.*, 2001; Fukai *et al.*, 2000).

Nitric oxide synthase activity was decreased in penile homogenates from very old rats and diabetic BB and BBZ rats (Garbán *et al.*, 1995; Vernet *et al.*, 1995). The nNOS is the main NOS isoform responsible for the synthesis of the physiological mediator of penile erection, NO (Burnett, 1997; Gonzalez-Cadavid & Rajfer, 1997). The number of NOS-containing nerve fibres in the penile shaft of aged rats with erectile dysfunction (ED) appears to be reduced based on NADPH diaphorase staining (Carrier *et al.*, 1997). nNOS in the nitreigic nerves and eNOS in the endothelium of lacunar spaces and penile vessels are responsible for NO generation in the penis. Thus, the aim of this study was to evaluate the effects of regular exercise on nNOS and eNOS expression in penile tissue in aged rats as well as serum T levels and compare the results with healthy adult control and trained rats.

Materials and methods

Animals

Fourteen 4-month-young and 24-month-aged male Sprague Dawley rats weighing 250–300 g were divided into four groups as young control (group 1, $n = 7$), young trained (group 2, $n = 7$), aged control (group 3, $n = 7$) and aged trained (group 4, $n = 7$) groups. All rats were given standard rat chow and tap water ad libitum and were housed at $23 \pm 2^\circ\text{C}$ on a 12 h dark and 12 h light cycle. All animal experiments were approved by the Animal Ethics Committee.

Exercise procedures and tissue preparation

The exercise-trained group swam for 30 min a day, 5 days a week during 8 weeks of housing (09.00–11.00 h on each training day). The old and young control rats were housed under the same conditions as the swimming rats and they were handled as often as the exercise group. Exercise was performed by swimming in two glass tanks (length 100 cm, width 50 cm, depth 50 cm) containing tap water maintained at $32\text{--}34^\circ\text{C}$. The duration of first swimming experience was limited to 10 min and increased by 10 min daily until it reached 30 min a day (Gündüz *et al.*, 2004). Twenty-four hours after the last session, rats were sacrificed and penises were excised, isolated from surrounding tissues, perfused with saline and then paraffinized. All procedures were performed under general anaesthesia with 50 mg/kg ketamine HCL administered intraperitoneally.

Immunohistochemistry

For the immunohistochemical evaluation, specimens were processed for light microscopy and sections incubated at 60°C overnight and then de-waxed in xylene for 30 min. After rehydrating in a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37°C for 15 min and washed with PBS. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. Then, sections were incubated with eNOS Ab-1 (RB-9279-R7, Neomarkers, Labvision, Fremont, CA, USA) and nNOS (sc-648, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies. The Ultra-vision (Labvision) horseradish peroxidase/3-amino-9-ethylcarbazole staining protocol was used at this stage.

Sections prepared for each case were examined by light microscopy. Positive and negative controls were conducted in parallel with NOS stained sections. Staining of sections with commercially available antibodies served as the positive control. Negative controls included staining tissue sections with omission of the primary antibody.

The sections were evaluated for diffuseness and staining. Penile eNOS and nNOS were evaluated according to the diffuseness and intensity of staining in penile cavernous smooth muscle. According to the diffuseness of the staining, sections were graded as 0 = no staining; 1 = staining $<25\%$; 2 = staining $25\text{--}50\%$; 3 = staining $50\text{--}75\%$; 4 = staining $>75\%$. According to staining intensity, sections were graded as follows: 0 = no staining; 1 = weak but detectable staining; 2 = distinct; 3 = intense

staining (Moochhala *et al.*, 1996). Immunohistochemical values were obtained by adding the diffuseness and intensity scores.

Serum testosterone levels

Serum level of T was determined using ELISA kits (Beckman Coulter).

Statistics

Statistical analyses of the T levels were performed using the Mann–Whitney *U*-test. Immunohistochemical values were obtained by adding the diffuseness and intensity scores and the results were compared using the chi-squared test. $p < 0.05$ was accepted as statistically significant.

Results

In young control group, staining of eNOS and nNOS activities were mild-to-moderate. However, after 8 weeks of regular swimming exercise, nNOS activities were more

evident than in young control group (Fig. 1a–d). In aged control rats, eNOS and nNOS staining was poor; however, in aged trained rats, NOS activities were more intense than in aged controls (Fig. 2a–d). Serum T levels were significantly higher in aged rats of the young and aged trained group when compared with the rats of control ($p < 0.05$, Table 1).

Discussion

Previous studies have shown similar relationships between impotence and ageing. Pearlman & Obashi (1972) found impotence to be an age-dependent disorder with a prevalence of 1.9% at age 40 years and 25% at age 65 years. Studies by Frank *et al.* (1978), Wabrek & Burchell (1980) and others in the 1970s have shown impotence-related age dependency. The association of impotence with vascular disease in the literature is strong. Impairments in the haemodynamics of erection have been demonstrated in patients with myocardial infarction (Gundle *et al.*, 1980), coronary bypass surgery (Agarwal & Jain, 1989), cerebrovascular accidents and peripheral vascular disease (Ruzbarský & Michal, 1977).

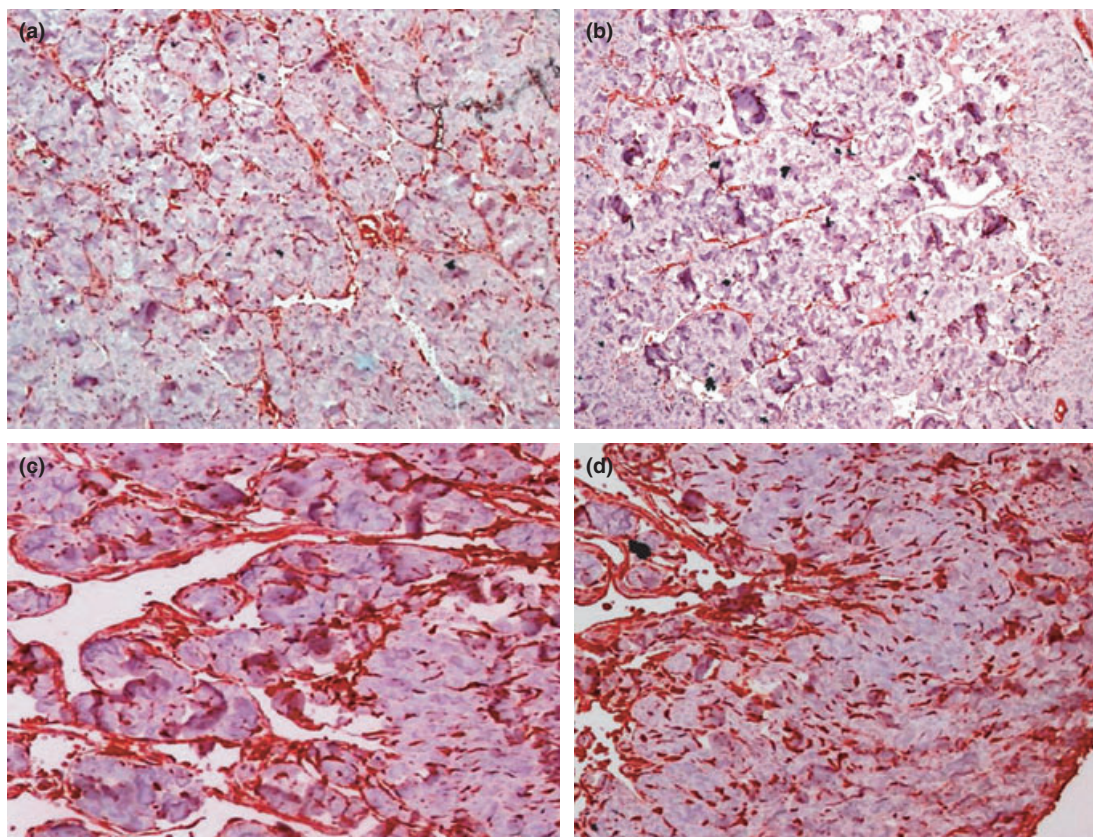


Figure 1 Mild eNOS (a) and nNOS (b) stainings in young rats. Moderate eNOS (c) and nNOS (d) staining in young trained rats (IHC 400 $\times$). eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

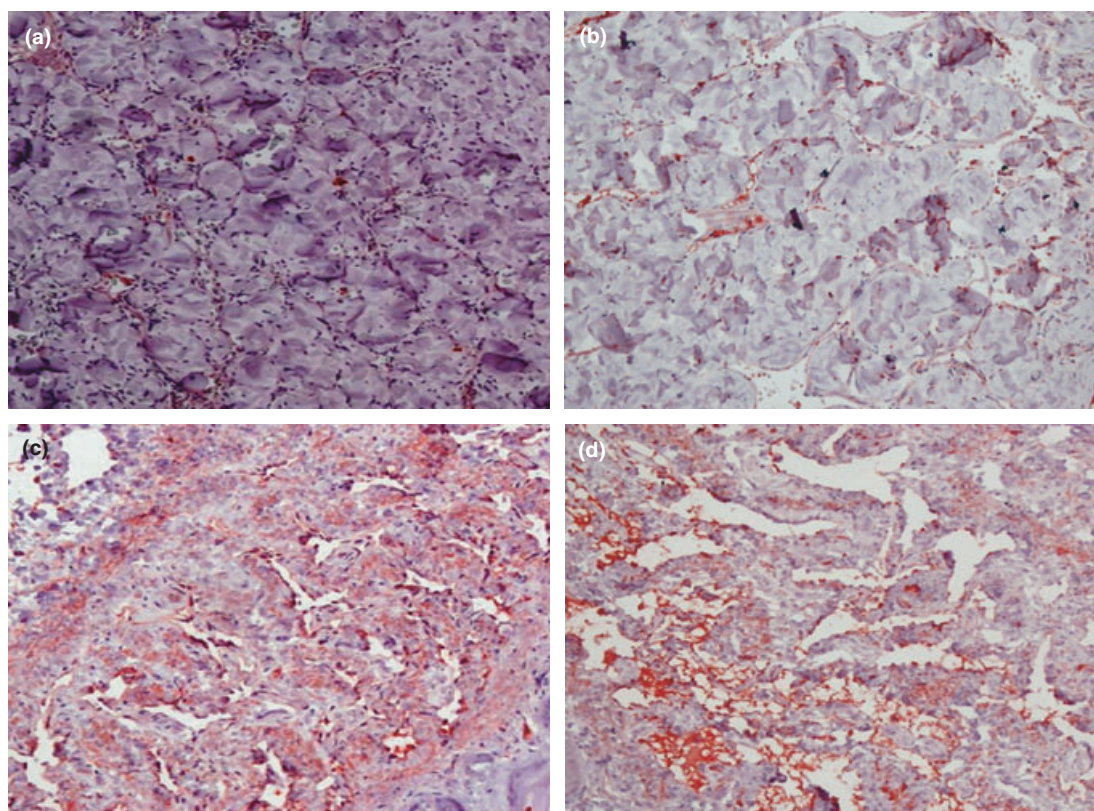


Figure 2 Poor eNOS (a) and nNOS (b) stainings in aged rats. Mild eNOS (c) and nNOS (d) staining in aged trained rats (IHC 400 $\times$).

Table 1 Serum testosterone concentrations of the groups

Variable	Young control	Young + trained	Aged control	Aged + trained
Serum testosterone (ng/dL)	320 $\pm$ 24.3	452.8 $\pm$ 34.6 ^{a*}	143.67 $\pm$ 12.14	296.01 $\pm$ 28.82 ^{b*}

Values are expressed as mean $\pm$ SD for seven rats in each group. The superscript letter 'a' compared with young control group and 'b' compared with aged control group.

* $p < 0.001$.

Immunohistochemical staining showed that chronic ischaemia led to disproportionate changes in NOS expression, favouring iNOS over nNOS and eNOS. It appears that as nNOS and eNOS expression decreases during the course of cavernosal ischaemia, iNOS expression increases (Azadzoi *et al.*, 2004). Nakatani *et al.* (2005) found that habitual exercise induces resistance to oxidative stress and suppresses the initiation step of carcinogenesis because of ROS. At the same time, habitual exercise (HE) suppresses increases in body weight.

Nitric oxide, formed in the vascular endothelium and derived from a biochemical reaction catalysed by eNOS, appears to play a role in exercise-induced dilation of blood vessels supplying to the cardiac and skeletal muscles. Endothelium-dependent, NO-mediated vasodila-

tation is augmented by exercise training (Bearden *et al.*, 2007). In coronary arterioles isolated distal to chronic occlusion, exercise training increases NO-mediated relaxation and eNOS mRNA expression, a response similar to that observed in coronary arterioles of normal animals (Griffin *et al.*, 2001).

Woodman *et al.* (2002) reported that a lower Ach-mediated vasodilatation of soleus muscle feed arteries was associated with lower levels of eNOS protein in old rats. Muller-Delp *et al.* (2002) found that the stimulus for the ageing-induced decrement in endothelial NO-dependent vasodilatation in skeletal muscle arterioles was because of physical inactivity and deconditioning. Miyauchi *et al.* (2003) found that the levels of eNOS mRNA and eNOS protein in the lungs were significantly higher in exercise rats than in control rats.

We found that in penile tissues of aged rats, eNOS staining was weakly positive; however, in trained group, eNOS immunoreactivity was increased. In young control group, eNOS was more intense than in aged control. nNOS is more interesting to researchers and may play a principal role in penile erection (Magee *et al.*, 1996; Burnett *et al.*, 1995). De Young *et al.* (2004) suggest that vitamin E was shown to protect penile tissue from injury and to preserve nerve and endothelial function, as demonstrated by nNOS and endothelial cell staining patterns and a measured rise in intracorporal pressure. NO released from nitrergic nerves in the penis plays a key role in trabecular smooth muscle relaxation and penile erection; reduced expression of nNOS in this tissue could be determinant for reduced NO production and the appearance of diminished erectile responses in the old rats sedentary.

In the ageing process, the augmentation of the antioxidant defence because of regular exercise will provide a significant advantage for various pathophysiological processes in old age. Miyauchi *et al.* (2003) found that the levels of nNOS mRNA and nNOS protein in the lungs were significantly higher in exercised rats than in control rats. In this study, immunohistochemical staining showed that nNOS immunoreactivity was more intense in aged trained group compared with that in aged control rats. We think that regular exercise improves ageing-associated vascular risk factor and oxidative injury in penile tissues and increases nNOS and eNOS. In this study, regular exercise increased penile nNOS and eNOS activity in aged rats. The increased penile NOS activities were also observed in young trained rats.

Many investigators believe that androgens act primarily to stimulate or maintain the activity of enzyme NOS. The role of androgens on the expression of NOS isoforms in the penile tissue has been demonstrated (Lugg *et al.*, 1995; Seo *et al.*, 1999; Traish *et al.*, 2007). In castrated animals, replacement with T restored erectile function as well as NOS expression in the corpora cavernosa (Schirar *et al.*, 1997; Traish *et al.*, 2007). The effects of exercise on serum T levels depend on the severity and duration of exercise. It is reported that moderate intensity exercise increases serum T levels (Grandys *et al.*, 2009); however, intensive exercise suppressed T production (Manna *et al.*, 2003; Jana *et al.*, 2008). Similarly, continuous exercise decreases T under normoxia and hypoxic conditions, intermittent exercise increases T during normoxia, but suppresses during hypobaric hypoxia (Hu *et al.*, 1998). In this study, serum T levels were significantly higher in rats of the young and aged trained group when compared with the rats of control.

In conclusion, the regular exercise upregulates eNOS and nNOS expressions in both aged and young rat penile

tissues. Regular exercise may improve penile erection by increasing penile neurotransmitter in both young and aged rats.

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