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Original Article

Pubertal Development of Penile Nitric Oxide Synthase (NOS)-Containing Nerve Fibers in the Rat

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ABSTRACT

Objectives: To evaluate the expression of nitric oxide synthase (NOS)-containing nerve fibers in penile tissue in different age groups in the rat and to measure serum testosterone levels during this developmental process.

Material and Methods: Fifteen male Sprague-Dawley (SD) rats divided into 3 equally numbered groups of different age (40 days (d), 54d and 65d) were used in this study. Penile erection was evaluated using cavernous nerve electrostimulation. Before sacrificing the rats, a penile mid-shaft specimen was taken for nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining and blood samples were collected to evaluate the testosterone levels.

Results: Electrostimulation of the cavernous nerve revealed no significant difference in the maximal intracavernous pressure in the different age groups (44.9 ± 6.4, 54.8 ± 24.8, 45.9 ± 16.8 cm H_2O, respectively; $p>0.05$), but the latency of the response to electrostimulation was significantly shorter in 54d and 65d than in 40d-old rats (5.4 ± 0.8 and 5.0 ± 0.7 vs. 9.0 ± 5.4 sec., respectively; $p<0.05$). NADPH diaphorase staining revealed a significantly greater number of fibers in the corpus cavernosum (CC) and dorsal nerves (DN) of 54d and 65d-old rats compared to 40d-old rats (CC: 192.3 ± 34.5 and 238.8 ± 32.7, respectively, vs. 126.0 ± 11.3 $p<0.0l$, DN: 941.5 ± 77.8 and 848.4 ± 264.3, respectively, vs. 560.0 ± 114.2 $p<0.05$), but the differences between 54d and 65d-old rats were not statistically significant. Serum testosterone concentrations were significantly different between the 40d and the 54d-old rats (total: 6.5 ± 4.6 nmol/L vs. 16.8 ± 14.2 nmol/L vs. 22.8 ± 16.2; $p<0.05$, free: 16.8 ± 14.2 nmol/L vs. 73.5 ± 56.6; $p<0.05$), but the differences between the 54d and 65d-old rats (total: 22.8 ± 16.2 vs. 19.5 ± 8.9 nmol/L, free: 73.5 ± 56.6 vs. 58.7 ± 34.3 nmol/L) were not statistically significant.

Conclusion: The number of NADPH-positive fibers increases with age; however similar tumescence was recorded following electrostimulation in all age groups. NOS-nerve growth correlates with serum testosterone level. The increase of NADPH-positive fibers was accompanied by a decrease in the delay of onset of penile erection following electrostimulation.

Key words: Growth process, nitric oxide nerve fibers, penis, erection, blood testosterone, rats

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INTRODUCTION

Many types of penile dysfunction in adolescents are associated with insufficient organ growth, with or without degeneration[1-3]. It has been recently proposed that nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining of cavernosal biopsy specimens may provide a new diagnostic tool for neurogenic impotence[4]. A better understanding of the mechanisms implicated in the functional development of the penis would help us further recognize the etiology of erectile dysfunction in adolescents and thus open the way to develop new diagnostic tools and treatment modalities.

In recent years, the physiology and pathophysiology of penile erection have been extensively studied clinically and experimentally both in vivo and in vitro[1,5]. Among these, the role of smooth muscle relaxation in the corpora cavernosa as well as different neurotransmitters have been mainly emphasized[3]. As a key factor in cavernous smooth muscle relaxation, nitric oxide (NO) and its releasing nerve fibers are being studied extensively[6,7]. The discovery of nitric oxide (NO) as an intercellular messenger or neurotransmitter has opened a new era for identifying the important mechanisms underlying physiological and pathophysiological events in autonomically innervated organs and tissues; it has also paved the way for the development of new therapies based on a novel concept of molecule and cell interaction[6]. Endothelium-derived relaxing factor (EDRF) has been proved to be NO, a labile gaseous molecule, that modulates vascular tone, platelet aggregation and adhesion, and vascular smooth muscle proliferation[6,7]. NO was also determined to act as a non-adrenergic, non-cholinergic (NANC) neurotransmitter of postganglionic parasympathetic nerve fibers, innervating a variety of smooth muscles, including the penile corpus cavernosum (CC)[8]. The nerve is called "nitrergic" or "nitroxidergic". Although CC sinusoidal endothelial cells also produce and liberate NO in response to chemical and possibly physical stimuli, roles of neurogenic NO in penile erection appear to be more attractive and convincing[8]. NO is formed from L-arginine via catalysis by NO synthase (NOS) isoforms, neuronal (nNOS), endothelial (eNOS), and inducible NOS[8]. NO from nerves and possibly endothelia plays a crucial role in initiating and maintaining intracavernous pressure increase, penile vasodilatation, and penile erection that are dependent on cyclic GMP synthesized with activation of soluble guanylyl cyclase by NO in smooth muscle cells[8]. Despite all this, important physiological processes during development, implicating neurons, smooth muscles and hormones in penile growth and thus erection, are rarely studied. This project involves a correlation analysis of serum testosterone levels, erectile function and expression of NOS-containing nerve fibers in the penis during development in rats.

MATERIAL AND METHODS

Three groups of five male Sprague-Dawley (SD) rats 40, 54, and 65 days (d) old, were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). The 3 age groups were chosen in the adult life of the male SD rat before aging (90 days), with adequate periods between different age groups to assess any age-related differences in expression of NOS–containing nerve fibers and changes in serum testosterone levels. The SD rats were housed in controlled 12h light/12h dark cycle allowing free access to water and rat chow (Charles River). Under general anesthesia with phenobarbitol (Somnotol ®) 50mg/kg different tests were performed: 1) electrostimulation of the cavernous nerve, 2) penile tissue sampling and 3) measurement of serum testosterone levels. Ethical approval was obtained from the animal care and use committee at McGill University Health Care Centre prior to initiating this study.

After taking blood samples, PE-50 tubing was inserted into the left carotid artery exposed via a midline neck incision to record the blood pressure. Through a lower abdominal midline incision under operating microscope (Leica) the posterolateral areas of the prostate were explored on both sides so that the major pelvic ganglions, the pelvic nerves and the cavernous nerves were identified. Then the proximal ends of both cavernous nerves were dissected (about 2 mm) for stimulation. The skin overlying the penis was incised and both penile crura were exposed by removing part of the overlying ischiocavernous muscle. A 23G needle filled with 250 u/ml of heparin solution and connected to the PE-50 tubing was inserted in the left crus and fixed to the tunica. Intracavernous pressures were measured and recorded with a Hewlett-Packard 1270 Km pressure transducer (Hewlett Packard, Andover MA) connected to a Gould Slatham Recorder 2400 (Gould mc, Cleveland OH). Electrostimulation was performed with a delicate stainless-steel bipolar hood electrode attached to a multi-joined clamp (each pole was 0.2 mm in diameter; the two poles were

separated by 1 mm). Monophasic rectangular pulses were delivered by a signal generator (Hewlett-Packard 801213) with a custom-built constant current amplifier. Stimulus parameters were 1.5 mA, frequency 20Hz, pulse width 0.2 msec, duration 1 mm[9]. Cavernous nerves were stimulated on both sides. After electrostimulation was completed, a mid-shaft penile segment was taken for staining of the cavernous tissue and dorsal nerve. The distal end of each penile segment was marked with methylene blue to allow future identification of the right or left side.

Blood samples for testosterone measurement were taken at 9 am following anesthesia. One milliliter (mL) of blood was taken by intra-cardiac puncture. Total and free serum testosterone levels were evaluated with radioimmunoassay following the technique of Collins et al.[10] (DiaSorin Inc, Stillwater MN).

Mid-shaft sections of the penis were fixed for four hours in 4°C, freshly prepared solution of 2% formaldehyde, 0.002% picric acid in 0.1M phosphate buffer, pH 8.0. Tissues were cryoprotected for 24 hours in 4°C 15% sucrose in 0.1M phosphate buffer, pH 8.0. They were then embedded in O.C.T. compound (Tissue- Tek, Miles Laboratory) frozen in liquid nitrogen and stored at —70°C. Cryostat tissue sections were cut at 7 μm and adhered to changed slides, air-dried in convective oven for 5 min, and hydrated for 10 min with 0.1M $P0_4$ pH 8.0. Sections were incubated with 0.1mM NADPH, 0.2mM nitroblue tetrazolium, 0.2% Triton X-100 in 0.1 M $P0_4$ pH8.0 for 60 min at room temperature. The reaction was terminated by washing sections in buffer; slides were then cover-slipped with buffered glycerin as the mounting medium[11]. The presence of NADPH diaphorase-positive nerves was seen as highly localized, densely blue granules or fibers. Negative controls were included by omitting the primary antibody. The staining pattern was assessed by counting the number of NADPH-positive nerve fibers present in each CC and in the entire DN (magnification 400X; endothelium staining not included in this count). Counting was done with the evaluator blinded to the treatment group. An average of 4 different areas from the midpenile shaft were assessed for each specimen and the mean recorded.

Data were analyzed using the t-tests for paired or unpaired samples and logistic regression using the Statview 4.02 software. Values were considered significant at $p<0.05$. Data are expressed as mean ± SD, unless otherwise stated.

RESULTS

Both total and free serum testosterone le- vels were significantly lower in the 40d-old rats than in the 54d and 65d-old animals ($p<0.05$), but there were no statistically significant differences between the rats aged 54d and 65d (Table 1).

The erectile response following electrostimulation of the cavernous nerves failed to show significant differences in maximal intracavernous pressure among the three age groups (Table 2). The mean response latency following cavernous nerve stimulation showed a significant delay in onset of increased intracavernous pressure in the 40d-group rats compared to both the 54d and 65d-old rats ($p<0.05$). However, no significant difference was noted between the 54d and 65d-group rats (Table 3).

NADPH diaphorase staining revealed significantly increased numbers of NO-containing nerve fibers within the corpus cavernosum (CC) and dorsal nerve (DN) of the 54d-group rats compared to the 40d-group rats. The increase is from 4.7 to 27.2 fibers per day (8 fields, 400X) in CC and DN; respectively. There were no statistically significant differences between the 65d and 54d groups (Table 4).

Linear regression statistics revealed that the relationship between free serum testosterone concentrations and the number of NADPH-positive nerve fibers in DN and CC were significantly related ($r = 0.132$, $p<0.0l$; $r = 0.304$, $p<0.01$).

Table 1: Blood testosterone levels at different age groups of rats. (mean±SD).

Group	Testosterone	
	Total (nmol/L)	Free (nmol/L)
40d	6.5 ± 4.6	16.8 ± 14.2
54d	22.8 ± 16.2**	73.5 ± 56.6**
65d	19.5 ± 8.9**	58.7 ± 34.3**

**p<0.05 40d vs. 54d and 65d

Table 2: Mean maximal intracavernous pressure in response to cavernous nerve stimulation. (mean±SD).

Group	Intracavernous pressure (cmH_2O)
40d	44.9 ± 6.4*
54d	54.8 ± 24.8*
65d	45.9 ± 16.8*

*p>0.05

DISCUSSION

NO, a gaseous messenger molecule, has been established as an important mediator of arterial dilation and smooth muscle relaxation within the sinusoids that brings about tumescence of penile erectile tissue[12-13]. In vitro, many experiments have demonstrated a relaxant effect of NO or NO-related compound synthesized from L-arginine on strips of CC[14]. Furthermore, smooth muscle relaxation is elicited by NO donors and blocked by NO scavengers or NOS inhibitors. However, NO's labile nature has hampered its definitive identification and has made it necessary to indirectly study its distribution. The enzyme NADPH-diaphorase is a co-enzyme in the synthesis of NO and has been demonstrated to specially stain the same sites as a specific neuronal NOS antibody[15-20]. NADPH diaphorase-positive tissue is therefore considered NOS-containing tissue.

In this study, we found a significant increase in the number of NADPH-positive nerve fibers from 40d to 54d of age. Previous studies from our laboratory have shown that at birth NADPH-positive nerve fibers are not present in the CC and only a few fibers are present in the DN[21]. The number of NADPH-positive fibers steadily increases in the DN up to 65d of age. On the other hand, it is only at 30d that positive fibers appear in the CC and also increase steadily up to 65d[21]. In developmental studies, reflexive erections in male rats appear as early as 28d, flips at 31d and then cups at 40d[22]. Therefore, increased numbers of NADPH-positive fibers in the penis seem to be related to erections.

The increase in NADPH-positive nerve fibers over the 40-65d period is rapid when compared to younger rats[21]. This rapid growth period is important for the development of penile erectile mechanisms. Whether the nerve growth is sufficient or not will directly influence penile erectile quality. In clinical practice, we should pay attention to this stage. Factors such as insufficient serum testosterone, virus infection or drugs preventing sufficient nerve growth may result in erectile dysfunction.

Interestingly, our results showed that the increase in NADPH-positive nerve fibers is accompanied by increased serum testosterone concentrations. There are several publications demonstrating that testosterone may regulate the number of NOS-containing nerve fibers in tissues[23-25]. Baba et al. observed a decrease in the number of NOS-

Table 3: Latency period after cavernous nerve stimulation.

Group	Latency period (seconds)
40d	9.0 ± 5.4
54d	5.4 ± 0.8**
65d	5.0 ± 0.7**

**p<0.05 40d vs. 54d and 65d

Table 4: Number of NADPH diaphorase-positive nerve fibers in different age groups.

Group	Number of nerve fibers	
	Corpus cavernosum	Dorsal nerve
40d	126.0 ± 11.3	560.0 ± 114.2
54d	192.3 ± 34.5**	941.5 ± 77.8**
65d	238.8 ± 32.7**	848.4 ± 264.3**

**p<0.05 40d vs. 54d and 65d

positive nerve fibers following castration in rats with concurrent down-regulation of the maintenance and generation of NO[23]. Conversely, administration of a NOS-inhibitor led to increases in serum and testicular interstitial fluid levels of testosterone[23]. NO may therefore negatively regulate the secretion of testosterone[24]. In the present study, we found that growth of NADPH-positive nerve fibers and higher serum testosterone concentrations occur concurrently, suggesting that the growth of NO nerve fibers is modulated by testosterone, indirectly supporting testosterone as a modulator of NOS production. However, male rats have low plasma testosterone levels which begin to rise at about 40d of age[22,26]. Södersten et al. have shown that male changes in sexual behavior always precede changes in plasma testosterone[27]. Even when male rats were not allowed to copulate, the surge in plasma testosterone occurred at about 51d of age which is significantly later than the onset of mounts and intromission[22]. Therefore, testosterone may not be the only modulator of erectile function in rats.

Electrostimulation of cavernous nerves is a reliable method to measure erectile function as confirmed by several studies[9,28,29]. In this study, we evaluated the intracavernous pressure following electrostimulation of the cavernous nerves and found no significant difference between the three groups. We find this interesting, particularly because the number of NADPH-containing nerve fibers was lower at 40d compared to 54d and 65d of age. This raises the question on how the 40d rats are able to achieve similar maximal intracavernous pressure as the 54d and 65d rats. This can be explained by the presence of a minimal number of functional NOS nerve fibers at 40d capable of stimulating full erection. The excess NOS nerve fibers present after this age may act as reserves responsible for decreasing the delay of erection, as shown in this study. The more the NOS-nerve fibers, the stronger the compensatory power, and the shorter the delay.

Limitations of this study include the fact that we did not perform a more rigorous quantitative analysis of NOS, such as immunoblotting for neuronal NOS, and we did not elucidate precisely the expression of different constitutive NOS isoforms. However, we believe that we were able to establish a definite correlation between NOS-nerve fibers and serum testosterone that can be a trigger for further studies.

In conclusion, there exists a very important growth period for NADPH-positive nerve fibers in the rat penis from 40d to 65d of age. NADPH-positive nerve fiber growth coincides with higher serum testosterone levels. This provides further evidence that NOS-nerve fiber growth is modulated by testosterone.

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