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Intracavernosal vascular endothelial growth factor (VEGF) injection and adeno-associated virus-mediated VEGF gene therapy prevent and reverse venogenic erectile dysfunction in rats

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Penile veno-occlusive dysfunction (venogenic erectile dysfunction) is a common cause of erectile dysfunction (ED). We investigated whether vascular endothelial growth factor (VEGF) can be used to prevent and reverse venogenic ED in a rat model. Pharmacological cavernosometry was developed and validated using adult male rats with either arteriogenic or venogenic ED. Castrated animals were treated with intracavernous VEGF as either a recombinant protein (C+VEGF) or adeno-associated virus (AAV)-mediated VEGF gene therapy (C+VEGF gene) in an attempt to prevent the development of venogenic ED. Other animal groups received testosterone replacement (C+testosterone) or intracavernous AAV-LacZ gene (C+LacZ). Animals with documented venogenic ED were treated with intracavernous VEGF in an attempt to reverse their ED. Functional analysis (pharmacological infusion cavernosometry) was performed following treatment. Penile specimens were harvested for immunohistochemistry and electron microscopic evaluation. Castrated rats showed a decrease in papaverine-induced intracavernous pressure and an increase in maintenance and drop rates during pharmacological cavernosometry. These changes were prevented by systemic testosterone and intracavernous VEGF or AAV-VEGF therapy. Moreover, intracavernous VEGF was able to reverse the venogenic ED produced by castration. The quantity of penile smooth muscle detected by alpha actin staining decreased after castration but not in the C+T, C+VEGF, or C+VEGF gene groups. Transmission electron microscopy revealed atrophy of penile smooth muscle cells and nerves in the castrated rats. In VEGF-treated rats, regeneration of smooth muscle and nerves as well as endothelial cell hypertrophy and hyperplasia were the prominent features. In our animal model, systemic testosterone replacement or intracavernous VEGF (protein and VEGF gene) prevented the veno-occlusive dysfunction in castrated animals. In rats with established venous leakage, VEGF treatment reversed the cavernosometric findings of leakage. Intracavernous injection of either VEGF protein or VEGF gene may be a preferred therapy to preserve erectile function in patients in whom testosterone therapy is contraindicated.
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Introduction

Following sexual arousal and release of nitric oxide to the erectile tissue, three events must take place to ensure an erection: trabecular smooth muscle relaxation, arterial dilation and venous compression.[1] During erection, blood filling of sinusoidal spaces compresses subtunical venules, thereby reducing venous outflow. With normal erectile function, a high intracavernous pressure (ICP) is maintained with a low inflow rate.[2] Patients with veno-occlusive dysfunction (venous leakage) exhibit a poor response to intracavernous injection with vasoactive agents (papavarine, prostaglandin E1, phentolamine, or combinations, for example), despite good arterial flow demonstrated by duplex ultrasound.

Following radical prostatectomy, approximately 30% of patients have vasculogenic erectile dysfunc-

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tion (ED) in addition to neurogenic ED and at least half of these men have venous leakage.[3] Regardless of the etiology of organic ED (neurogenic, traumatic, hormonal, and vascular, etc), venous leakage is a common final condition resulting from smooth muscle atrophy.[4] Furthermore, veno-occlusive dysfunction is the most common etiology of ED among nonresponders to medical management of ED.[5]

We propose that vascular endothelial growth factor (VEGF) may hold promise as a therapeutic agent for veno-occlusive dysfunction because of its known angiogenic effects and possible role as an androgen mediator. Following castration, rats do not display copulatory behavior or erectile function.[6] They have depressed expression of nitric oxide synthase in the penis,[7] undergo vascular involution in the ventral prostate[8] and demonstrate decreased expression of VEGF in the prostate.[9] Additionally, these animals display venogenic ED as assessed by ICP monitoring and blood flow studies.[10,11] With testosterone repletion, VEGF synthesis is induced and followed by vascular regrowth in the ventral prostate of castrated rats,[12,13] suggesting that VEGF may be the mediator of androgenic effects in the prostate. For this reason, we hypothesized that VEGF may also mediate androgenic effects in the penis and that VEGF administration may prevent ED after castration in rats.

The objectives of this study were (1) to establish normal values for pharmacologic cavernosometry in the rat and validate a rat model of venogenic ED (Experiment 1: Model validation), (2) to *prevent* venogenic ED (Experiment 2: Prevention trial), and (3) to *reverse* venogenic ED (Experiment 3: Treatment trial). Since the cavernosal sinusoids are lined with numerous endothelial cells, the target cell type of VEGF, the penis is an ideal organ for VEGF therapy. Our previous study also revealed that penile smooth muscle cells possessed VEGF receptors and VEGF treatment enhanced penile smooth cell proliferation and migration in culture.[14] Additionally, VEGF has also been reported to be neuroprotective as well as neurotrophic.[15,16] Since impairment of erectile nerves, endothelial cells, and the cavernous smooth musculature is the final common pathway of various types of organic ED, we conducted this study to examine whether VEGF can restore the integrity of the above-mentioned tissues and thus prevent or cure ED.

Experimental animals and methods

Animal groups

Male Sprague–Dawley rats aged 3–6 months (wt 350–450 g) were used in this study. They were housed in our animal care facility with rat chow and water available *ad libitum* on a 12 h light/dark cycle. All animal care, treatments, and procedures were approved by the Committee on Animal Research at our institution. Rats were randomly divided for the animal model of vasculogenic ED (Experiment 1) and the VEGF prevention trial (Experiment 2). For the VEGF treatment trial (Experiment 3), the animals underwent castration and then were treated with VEGF after venous leak was demonstrated, to evaluate the efficacy of VEGF treatment in reversing established venogenic ED.

Experiment 1: To determine normal values for rodent pharmacologic cavernosometry and validate the model of venogenic ED in the rat, vasculogenic ED was induced. Arterial insufficiency was produced after performing a bilateral ligation of the internal iliac arteries. The acute and chronic effects of arterial insufficiency were evaluated 7 days and 30 days after bilateral iliac artery ligation. Venogenic ED was induced by castration, and pharmacologic cavernosometry was performed 6 weeks after surgery. Control animals underwent a sham laparotomy and studied 6 weeks later.

Experiment 2: For the *prevention* trial of VEGF in rats with venogenic erectile ED, adult males were castrated and immediately treated with hormone, intracavernosal VEGF. Hormone replacement was accomplished using a subcutaneously placed testosterone-filled silastic implant, as previously described.[17] A therapeutic testosterone serum titer was confirmed in this animal group by testosterone radioimunoassay[18] performed by the biomedical core lab at our institution. Intracavernous treatment with VEGF was administered using either recombinant VEGF protein or an adeno-associated virus vector expressing the VEGF gene (AAV-VEGF). Control animals received a silastic implant containing saline, an intracavernous injection of normal saline, or an adenovirus transfection vector expressing lacZ reporter gene without the VEGF gene (AAV-LacZ).

Experiment 3: The trial of VEGF *treatment* was performed in castrated animals that were shown, 6–8 weeks following castration, to develop venogenic erectile ED by pharmacologic cavernosometry. These animals were treated with intracavernous VEGF protein and then after 1 month cavernosometry repeated to measure the effect of VEGF treatment.

Animal treatments

Surgical preparation: Prior to all surgical procedures, animals received anesthesia consisting of isoflurane inhalation as preanesthetic followed by an intraperitoneal injection of sodium pentobarbital (40 mg/kg). After the animal was asleep, electric clippers were used to trim the ventral abdominal

hair and the skin was prepped with clorhexidine scrub. Antiseptic technique was maintained for all procedures. Following surgery, the anterior abdominal fascia and skin were approximated with 4-0 silk suture, analgesic buprenorphine (0.5 mg/kg s.c.) was administered and the animal allowed to awaken covered with a heating pad. Euthanasia was accomplished by an intraperitoneal injection of sodium pentobarbital (200 mg/kg), followed by bilateral thoracotomy when the animal was fully asleep.

Arterial ligation surgery: A 2 cm midline longitudinal low abdominal incision was made and a wheatlander retractor placed so that the plane between the prostate and sigmoid colon could be bluntly opened. Under a dissecting microscope with $\times 2.5$–10 objectives, using sterile cotton-tipped swabs, the iliac artery bifurcation was identified and the common iliac exposed to the external iliac take-off. The internal iliac arteries were identified as those medial branches off the common iliac between the iliac bifurcation and the take-off of the external iliac. These were doubly ligated using 7-0 nylon sutures. After this was performed on both the right and left side, the incision was closed and the animal recovered as noted above.

Castration: A 2 cm midline longitudinal low abdominal incision was made and each testicle was grasped using forceps and brought into the incision. Each gubernaculum was divided using electrocautery and then the spermatic cords ligated with 4-0 silk suture and divided. After confirming hemostasis, the abdomen was closed and the rat recovered as above.

Intracorporal injections: A 1.5 cm oblique incision was made in the lower abdominal skin extending from the midline just above the penile hilum to below the level of the glans about 1 cm lateral to the midline. The skin was sharply dissected from the anterior surface of the penis and then the penis was retracted anteriorly using a towel clamp placed around it atraumatically with the foreskin left intact. Using blunt dissection the penile base and crura were exposed. The ischiocavernosus muscles were sharply dissected off the anterior surface of the crus until the white of the tunica albuginea of the corpora cavernosa was identified. The crus was then gently cannulated using a 23 gauge butterfly needle, and saline flush with a visual erectile response was used to confirm that the needle tip was truly intracavernosal. The intracavernosal injections were then administered with either VEGF protein (Calbiochem, Inc. La Jolla, CA, USA), AAV-VEGF, or AAV-LacZ. VEGF protein was administered at a dosage of 4 μg/injection in 0.1 ml phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA). AAV-VEGF and AAV-LacZ constructs have been described previously[19] and were administered at 10^{10} viral particles/injection in 0.1 ml PBS. Following injection, the needle was left in place for 5 min and then removed to allow the medication to diffuse throughout the cavernosal space. Immediately thereafter, pinpoint electrocautery was applied to the needle hole for hemostasis and then the wound was closed and the animal recovered as above.

Testosterone replacement: Following castration, testosterone- or saline-filled silastic implants were placed in the subcutaneous tissue of the anterior abdominal wall, as previously described.[17] Implants were prepared using sterile silastic tubing (Dow Corning, Midland, MI #602-265, inner diameter 0.062″) that was filled with testosterone propionate powder (Sigma Chemical, St Louis, MO, USA) with the aid of wall suction. By radioimmunoassay,[18] serum testosterone titer was found to be undetectable in the castrated animals and in the normal range for animals given testosterone implants.

Pharmacologic cavenosometry in the rat: To perform pharmacologic cavernosometry, both the right and left crura were separately cannulated using 23 gauge butterfly needles as described above. One cannula was flushed with sterile heparinized saline (100 U heparin/ml) and attached to a pressure detector for continuous intracorporal pressure (ICP) monitoring as previously described.[11] The contralateral cannula was attached to an infusion pump (Harvard Pump, Southwick, MA #55-2222), filled with sterile dilute heparinized saline (20 U heparin/ml). The baseline ICP was recorded (flaccid ICP) and then a dose of papavarine (1 mg in 0.1 ml) was administered through the infusion cannula. Overall 5 min was allowed for the papavarine to diffuse throughout the corpora and then the infusion cannula was flushed with heparinized saline and the pressure monitor cannula vented to normalize ICP after flushing. After another 5 min, the ICP was again recorded (ICP after papavarine) and the infusion started. An infusion rate of 0.05 ml/min was started and increased (by 0.05 ml/min every 10 s) until the ICP started to rise. Subsequent increases in inflow rate were made only after the ICP reached a plateau pressure. By slowly adjusting the inflow rate, an ICP of 100 cm H_2O (erectile pressure) was reached and the infusion rate required to maintain this pressure recorded (the maintenance rate). After this pressure was steady for 20 s, the infusion was terminated and the change in ICP over the subsequent 60 s was recorded (the drop rate).

Tissue preparation: After pharmacologic cavernosometry was performed, the penis was amputated at the crural bony attachments and immediately placed in ice-cold saline. The Y-shaped crura was sharply cut from the penile base and then a 1 mm thick slice cut for electron microscopy and placed in Karnofsky's solution (3% gluteraldehyde, 1% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.4). A 3 mm thick section of the distal penile shaft was then cut and placed in 10% normal buffered formalin for paraffin sections, and the balance of the penile shaft was flash frozen using dry ice in OCT

compound (Sakura Finetek USA, Torrance, CA, USA) for frozen sectioning and immunohistochemistry.

Immunohistochemistry: Frozen sections were cut at 10 μm, adhered to charged slides, air-dried for 15 min, and then rehydrated with 0.05 M PBS for 5 min. Sections were treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. After rinsing, sections were washed twice in PBS for 5 min, then incubated with 3% horse serum and 0.3% Triton X-100 at room temperature for 30 min. The serum solution was drained and then sections were incubated for 60 min with mouse monoclonal anti-alpha-smooth muscle actin (Sigma, St Louis, MO, USA) at a dilution of 1:4000 in PBS. After washing, sections were immunostained using the avidin–biotin–peroxidase method (Elite ABC, Vector Labs, Burlingame, CA, USA), with diaminobenzidine as the chromogen, followed by counterstaining with hematoxylin. Immunochemistry was performed in penile tissues from four rats randomly chosen from each subgroup.

Enzyme-linked immunosorbent assay: Serum samples from both systemic and penile blood were collected after whole-blood centrifugation. Solid-phase enzyme-linked immunosorbent assay for VEGF was performed using the Quantikine M mouse VEGF Immunoassay Kit (R&D Systems, Minneapolis, MN, USA) as previously described.[14] Briefly, samples were diluted and added to microplate strip wells that were then treated with the enzyme-labeled immunoreactant VEGF conjugate. After incubation for 2 h and washing, the substrate solution was added and incubated for 30 min.The stop solution was added and then the optical density in each well determined using a microplate reader set to 450 nm. The results were plotted on a curve generated by the optical density of VEGF standards ranging from 0 to 500 pg/ml.

Transmission electron microscopy: The penis was dissected, thinly sliced (~1 mm thick), placed in Karnovsky's fixative (1% para-formaldehyde/3% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4) at room temperature for 30 min, and then stored at 4°C. The fixed tissue was then rinsed in buffer, postfixed in 2% aqueous OsO_4, and stained *en bloc* with uranyl acetate before being dehydrated in ethanol, cleared in propylene oxide, and embedded in eponate12 (Ted Pella Co., Redding, CA, USA). Thick sections were cut and stained with toludine blue, examined under light microscope to select the area to be thin sectioned. Thin sections were cut by Leica ultracut E microtome (Bannockburn, IL, USA), stained with uranyl acetate and Reynold's lead to enhance contrast and examined under Philips Tecnai 10 electron microscope (Eidhoven, the Netherlands).

Statistical analysis: We used computer software from Primer of Biostatistics, 3rd edn (Glantz SA, McGraw-Hill, Inc., New York, 1992) for statistical analysis. The data were first analyzed by one-way analysis of variance. If the difference was significant, Student–Neuman–Keuls test was used to perform the pairwise comparisons. A paired *t*-test was used where values represent findings before and after treatment in the same animals (Experiment 3, Table 3).

Results

Experiment 1: Model validation. The first goal of this study was to obtain normal values for pharmacologic cavernosometry in a rat model of vasculogenic ED. As shown in Table 1, flaccid ICP were comparable, in the range of 30 cm H_2O, among different groups of rats. However, after papavarine injection, control animals had a steep rise in ICP to >100 cm H_2O, while the castrated and ligated animals had lower responses. Only a minimal increase in ICP was noted in the animals following either castration or chronic internal iliac ligation, characteristic of vasculogenic ED. Acute ligation animals had a better response to papavarine than the chronic ligation group. After the infusion was started, both the control and acute ligation group promptly achieved erectile pressure with minimal inflow required. When the infusion was stopped, these animals had a minimal pressure drop, evidencing their intact veno-occlusive mechanism. The castration and chronic ligation groups, on the other hand, required a significantly higher infusion rate to maintain erectile pressure and experienced a steep pressure

Table 1 (Experiment 1) Cavernosometric findings in rat model of vasculogenic ED

	Flaccid ICP (cm H_2O)	*ICP after papavarine (cm H_2O)**	*Maintenance rate (ml/min)***	*Drop rate in 1 min (cm H_2O)****
Control (*n*=5)	35.4(±9.3)	104(±59)	0.024(±0.3)	9(±13)
Castration (*n*=7)	22.0(±5.2)	35.0(±5.0)	1.14(±0.5)	75(±5.4)
Acute ligation (*n*=6)	28.7(±7.6)	73.3(±10)	0.06(±0.12)	13.2(±13.8)
Chronic ligation (*n*=4)	29.3(±7.6)	38.3(±19)	1.9(±1.8)	45.8(±19)

* $P \leq 0.05$, control *vs* castration; control *vs* chronic ligation.
** $P \leq 0.05$, control *vs* chronic ligation; chronic ligation *vs* acute ligation.
*** $P \leq 0.05$, between all pair-wise comparisons except control *vs* acute ligation.

Table 2 (Experiment 2) Cavernosometric findings in castrated animals after prevention trial of testosterone replacement (C+testosterone), VEGF protein treatment (C+VEGF), AAV-VEGF gene therapy (C+VEGF gene), or LacZ control (C+LacZ control)

	Flaccid ICP (cm H_2O)	*ICP after papavarine (cm H_2O)* *	*Maintenance rate (ml/min)* **	*Drop rate in 1 min (cm H_2O)* ***
C+saline (*n*=5)	23.4(±5.3)	29.4(±15)	0.51(±0.26)	56.1(±15)
C+testosterone (*n*=7)	28.9(±7.5)	87.7(±26)	0.09(±0.1)	11.7(±16)
C+VEGF (*n*=5)	27.8(±5.2)	85.0(±28)	0.04(±0.09)	9.0(±20)
C+VEGF gene (*n*=11)	23.4(±7.1)	61.4(±36)	0.04(±0.03)	27.8(±18)
C+LacZ (*n*=5)	27.0(±6.7)	36.0(±12.8)	0.25(±0.31)	58.6(±8.1)

*$P \leq 0.05$, C+saline *vs* C+testosterone, C+VEGF; C+testosterone *vs* C+LacZ; C+VEGF *vs* C+LacZ.
**$P \leq 0.05$, C+saline *vs* C+testosterone, C+VEGF, and C+VEGF gene.
***$P \leq 0.05$, C+saline *vs* C+testosteone, C+VEGF *vs* C+VEGF gene; C+LacZ *vs* C+VEGF, C+testosterone, and C+VEGF gene.

Table 3 (Experiment 3) Cavernosometric findings in VEGF treatment trial

	Flaccid ICP (cm H_2O)	*ICP after Papavarine (cm H_2O)*	*Maintenance rate (ml/min)*	*Drop rate in 1 min (cm H_2O)*
Before VEGF treatment (6 weeks after castration)	22.4(±6.9)	33.0(±12.3)	0.19(±0.18)	45.1(±18)
1 month following VEGF treatment	25.3(±8.5)	83.9(±31)*	0.08(±0.15)*	17.4(±24)*

Animals (*n*=8) were castrated and then shown to have venous leak (after approximately 6 weeks) by pharmacologic cavernosometry. They were then treated with intracavernosal VEGF and 1 month later underwent repeat cavernosometry (*$P \leq 0.05$ for values before *vs* after VEGF treatment).

drop when the infusion was terminated. These findings are characteristic of venous leakage in the chronic ligation and castration groups.

Experiment 2: Prevention trial. Our second goal was to perform a prevention trial using intracavernosal VEGF either in the form of recombinant protein or AAV-mediated gene expression. As shown in Table 2, flaccid ICP was again in the range of 30 cm H_2O in each of the animal groups. After papavarine administration, however, both control groups (castration only and castration with LacZ injection) exhibited only weak rises in ICP, required a significant infusion rate to sustain an erectile ICP of 100 cm H_2O, and had a steep pressure drop after the inflow was terminated. In contrast, the three treatment groups exhibited nearly normal erectile function with high ICP in response to papavarine, a very low maintenance rate to sustain erectile ICP, and minimal pressure drop when the infusion was stopped. Of note, the VEGF gene-treated animals showed a lesser response to papavarine and a higher drop rate than the animals treated with either testosterone replacement or intracavernosal VEGF protein.

Experiment 3: *Treatment trial*. Our third goal was to perform a treatment trial using intracavernosal VEGF in animals with venous leak. Animals were castrated and, 4–6 weeks later, underwent cavernosometry. As shown in Table 3, before VEGF treatment, this animal group displayed a weak response to papavarine with ICP reaching 33 cm H_2O compared to normal animals that attained nearly 100 cm H_2O (see Table 1). Also, these castrates required a relatively high maintenance rate (0.19 ml/min) to achieve erectile pressure and a steep drop rate when the infusion was terminated (45.1 cm H_2O in 60 s), evidencing venous leak. After these animals received intracorporal VEGF treatment, however, nearly normal erectile function returned with a prompt rise in intracorporal pressure after papavarine (to 84 cm H_2O), a low maintenance rate (0.08 ml/min) to achieve erectile pressure, and a minimal drop in ICP (17.4 cm H_2O in 60 s) after the infusion was terminated.

Immunohistochemistry: Quantitative analysis of the smooth muscle content (by alpha actin staining), as measured by computerized image analysis, produced the following pixel numbers: sham (43 518±21 677), castrated (37 214±18 814), castrated+testosterone treated (60 518±18 733), castration+AAV-VEGF treated (51 690±7 109), castrated+VEGF protein treated (52 990±9 512). No significance between the groups was noted by one-way analysis of variance.

Transmission electron microscopy

Dorsal nerve. In sham-operated rats (Figure 1a), the dorsal nerve was filled with both myelinated and nonmyelinated nerve bundles. The mean diameter

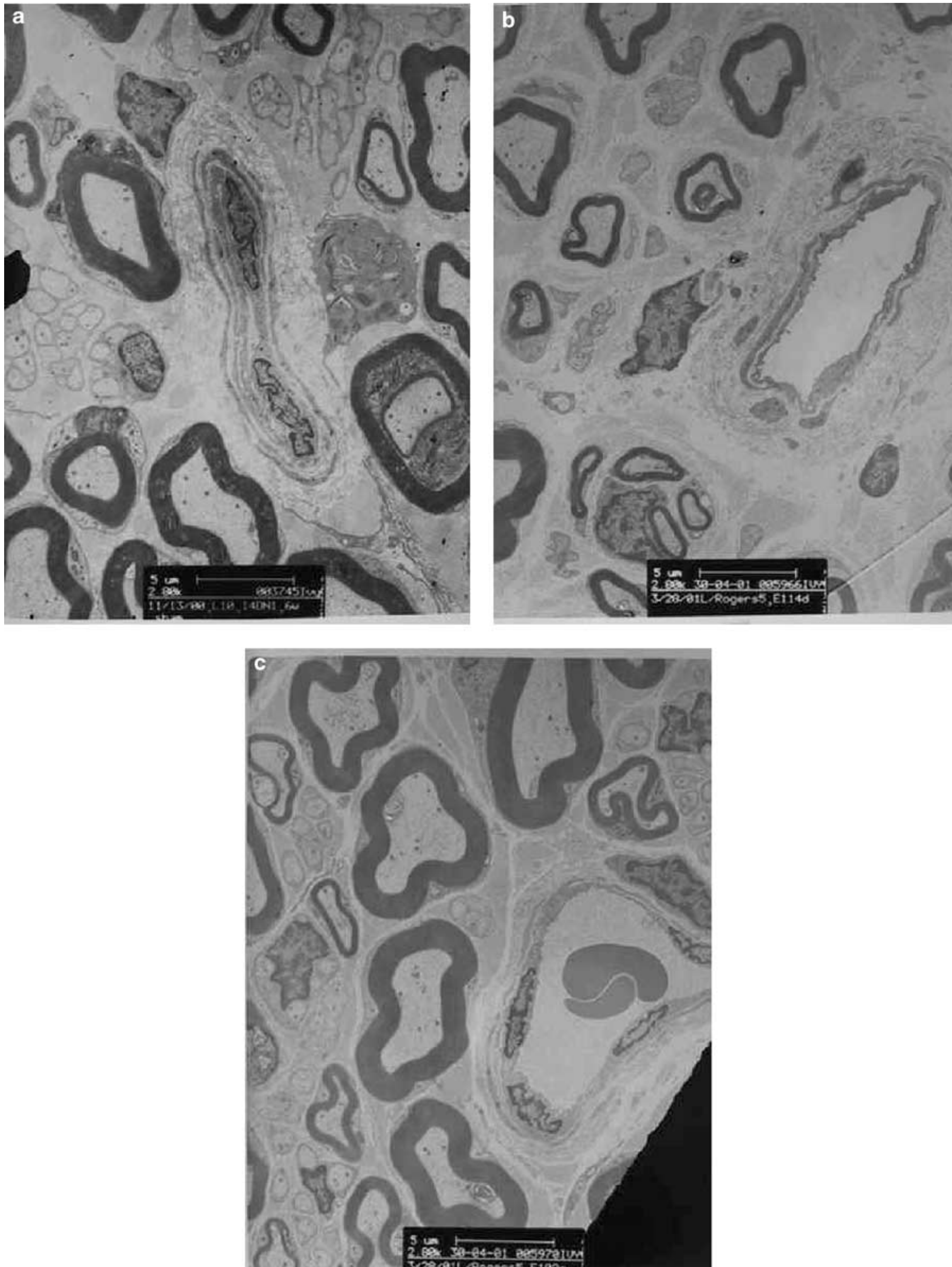


Figure 1 Representative electron micrographs of cross-sections of penile dorsal nerves ($\times 6500$). (a) Sham-operated rat. The dorsal nerve is filled with both myelinated (arrows) and nonmyelinated nerve fibers (arrow heads). The nuclei of Schwann cells are seen occasionally near the nerve fibers (curved arrow). (b) Castrated rat treated with LacZ. The diameter of the myelinated and nonmyelinated nerve fibers appear smaller than those of the sham-operated rats. Many nonmyelinated nerve fibers become indistinct and smaller. (c) Castrated rat treated with VEGF protein. Although many small myelinated nerve fibers are still present, larger fibers with thick myeline sheaths are also noted. The size of both myelinated and nonmyelinated nerve fibers appears larger than that of the castrated + Lac Z group.

of the individual myelinated axon (excluding myelin sheath) was $2.54 \pm 1.04\,\mu m$. The mean thickness of the myelin sheath was $0.74 \pm 0.21\,\mu m$. The mean diameter of the nonmyelinated axon was $0.97 \pm 0.35\,\mu m$. The nuclei of Schwann cells were seen occasionally.

In castrated rats, with or without LacZ injection (Figure 1b), the diameter of both the myelinated and nonmyelinated axons appeared smaller than those of the sham-operated rats. Mean diameters were the following: myelinated axon $1.64 \pm 1.0\,\mu m$; myelin sheath $0.49 \pm 0.13\,\mu m$; nonmyelinated axon $0.64 \pm .32\,\mu m$. Comparing the castrated rats to the sham-operated rats, the P values were 0.06, 0.004, and 0.001, respectively. Many nonmyelinated nerve fibers became indistinct and smaller. There was also an increase in the number of nucleated Schwann cells.

Although many small myelinated nerve fibers were still present in castrated rats treated with VEGF or AAV-VEGF (Figure 1c), larger fibers with thick myelin sheaths were also noted. The mean diameters of the myelinated nerve and myelin sheath were 2.36 ± 0.92 and $0.93 \pm 0.44\,\mu m$, respectively. The nonmyelinated nerve fibers were more clearly defined but were not as abundant as the sham group. The mean diameter of nonmyelinated axons was $0.96 \pm 0.33\,\mu m$. Comparing the VEGF-treated group to the castrated + Lac Z group, the P values of myelinated axon, meylin sheath, and nonmyelinated axon were 0.113, 0.05, and 0.000 respectively. The nerve fibers and myelin sheath in the testosterone replacement group appeared similar to the sham group.

Intracavernosal tissues

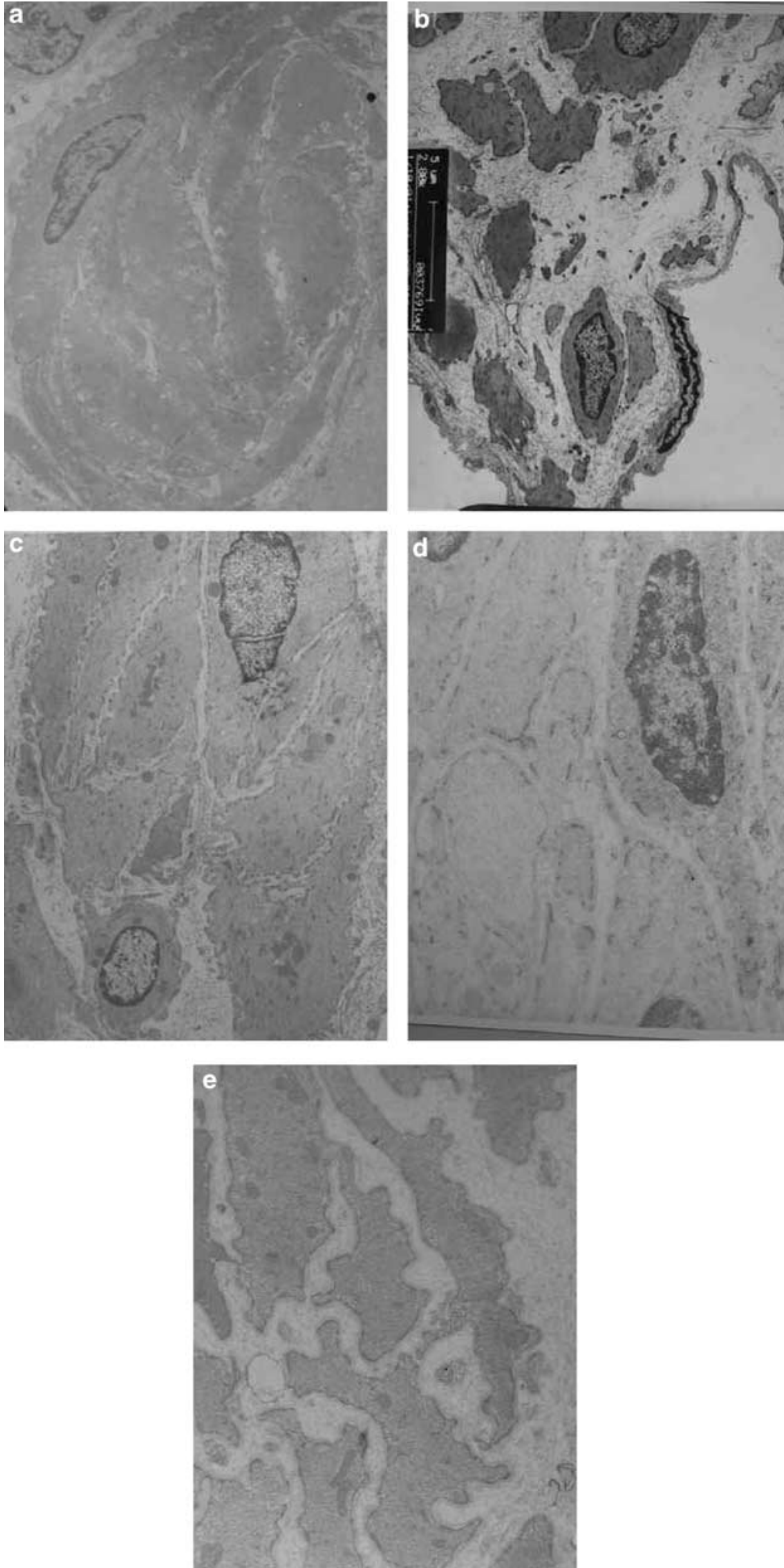
Intracavernous smooth muscle cells. In sham-operated rats, the smooth muscle cells (myocytes) were usually arranged in clusters and were separated by fine strands of fibroconnective tissue (Figure 2a). The cytoplasm of these myocytes contained abundant contractile myofilaments and dense bodies. Occasionally, small aggregates of organelles, including mitochondria, rough endoplasmic reticulum, and Golgi apparatus, were found adjacent to the nucleus. The cell membrane (sarcolemma) consisted typically of alternating dense bands and light bands. The light bands contain numerous pinocytotic vesicles (caveolae). The intercellular spaces among myocytes were usually quite narrow with many gap junctions connecting individual cells. Nerve terminal varicosities were frequently seen located near clusters of smooth muscle cells. In low-power micrographs ($\times 6500$) of castrated rats with or without LacZ, the smooth muscle cells appeared scattered in a field of connective tissues (Figure 2b). The major differences between the castrated and castrated + testosterone rats were the increase in cytoplasmic myofilaments and the decrease in intercellular spaces in the latter group of rats. The myocytes in testosterone-treated rats appeared packed in clusters rather than scattered.

Striking differences were noted when comparing the AAV-VEGF and VEGF protein-treated rats to the castrated + Lac Z rats. The smooth muscles were arranged in clusters with minimal intercellular spaces (Figure 2c). Under high power ($\times 9400$), we noted the following: an increase in myofilaments and dense bodies, a decrease in dense bands, and an increase in the number of caveolae within the light bands of the sarcolemma (Figure 2d, e).

Endothelial cells. In sham-operated rats (Figure 3a), the cavernous sinusoids were lined by intact endothelium, the cytoplasm of which contained numerous pinocytotic vesicles (caveolae), mitochondria, rough endoplasmic reticulum, and Golgi apparatus. The nuclei of the endothelial cells were occasionally seen and appeared oval shaped or elongated. In castrated rats with or without LacZ, the appearance of the capillaries and cavernous sinusoidal endothelium was similar to the sham-operated group. In AAV-VEGF and VEGF-protein-treated rats, the nuclei of the endothelial cells lining most of the capillaries and sinusoids were plump and more numerous, indicative of endothelial hypertrophy and hyperplasia (Figure 3b, c).

Enzyme-linked immunosorbent assay: To examine whether the AAV-VEGF-treated animals had increased VEGF expression in the penis, blood samples were taken from the penis (penile bleed following glans amputation) and from the abdominal aorta for animals treated with AAV-VEGF ($n = 7$) or AAV-LacZ ($n = 5$). While the mean VEGF titer in the systemic serum of animals that did not receive the VEGF gene (AAV-LacZ group) was 9.5 ± 5.3 pg/

Figure 2 Representative electron micrographs of cross-sections of intracavernous erectile tissues. (a) Sham-operated rat ($\times 6500$). The smooth muscle cells (myocytes) are arranged in clusters and are separated by fine strands of fibroconnective tissue. The cytoplasm of these myocytes contained abundant contractile myofilaments. The cell membrane (sarcolemma) consists of alternating dense bands and light bands. The intercellular spaces among myocytes are narrow. (b) Castrated + AAV LacZ-treated rat ($\times 6500$). The smooth muscle cells (myocytes) appear scattered and separated by larger amounts of connective tissue. (c) Castrated + VEGF-protein-treated rat ($\times 6500$). The smooth muscles were arranged in clusters with minimal intercellular spaces. (d) Higher power microphotography of the intracavernous erectile tissue of a castrated + AAV LacZ-treated rat ($\times 21\,620$). The smooth muscle cells (myocytes) are separated by large amounts of connective tissue. The sarcolemma is lined mostly by dark bands. (e) Higher power microphotography of the intracavernous erectile tissue of a castrated + VEGF-protein-treated rat ($\times 21\,620$). The intercellular spaces are narrower than in (d). There is an increase in myofilaments and dense bodies in the cytoplasm. The alternating light and dark bands on the sarcolemma are more distinctive (arrow).



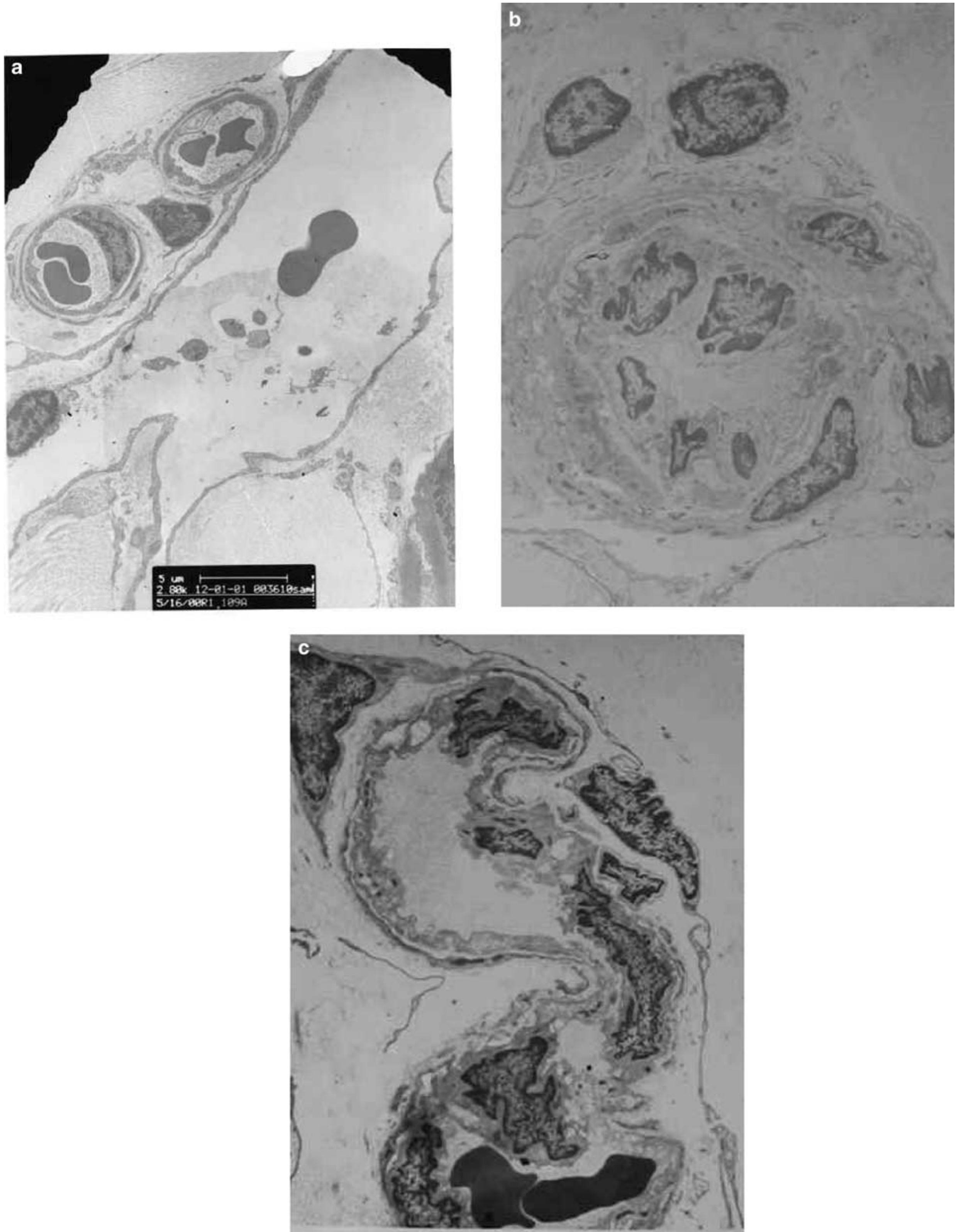


Figure 3 Representative electron micrographs of cross-sections of intacavernous penile tissues ($\times 6500$). (a) Castration + LacZ rats. Both the capillaries and cavernous sinusoids are lined by intact endothelium. The nuclei of the endothelial cells were occasionally seen (arrow) and appeared oval-shaped or elongated. In VEGF-protein-treated rats, the nuclei of the endothelial cells (arrows) lining many of the capillaries (b) and sinusoids (c) were large and more numerous, indicative of endothelial hypertrophy and hyperplasia.

ml, the AAV-VEGF-treated animals demonstrated a marked increase in VEGF titer at 23.3 ± 4.5 pg/ml ($P = 0.04$). Similarly, serum from the penile blood in the AAV-LacZ group had a VEGF titer of 13.6 ± 4.7 pg/ml compared to a mean of 29.7 ± 6.8 pg/ml in the group receiving the intracavernosal VEGF gene ($P = 0.039$). These differences are statistically significant, suggesting an increase of VEGF expression in the penile tissue after treatment with VEGF gene.

Discussion

Erectile function is a hemodynamic process of blood inflow and pressure maintenance in the cavernosal spaces.[1] As such, the penis is a predominantly vascular organ, and vascular insufficiency is the most common etiology of ED. Sinusoidal smooth muscle atrophy and collagen deposition are commonly found in men with long-standing ED of various etiologies, whether hormonal, neurological, or vascular.[20] Such degradation in smooth muscle quantity and quality leads to veno-occlusive dysfunction. This represents an end-stage muscular degeneration akin to myocardial changes with congestive heart failure or dilated cardiomyopathy for which no treatment currently exists.

Advancement in molecular biology has brought improved understanding of pathophysiology at the molecular level and offers treatment possibilities aimed at specific pathologic mechanisms. Treatment with VEGF in either protein or gene form has increased neovascularity in animal models of vasculopathies such as limb claudication[21] and coronary artery disease.[22] It has also been shown to improve symptomatic angina and wound healing in humans with inoperable heart disease and critical limb ischemia, respectively. The penis represents a convenient tissue target for gene therapy because of its external location, ubiquity of endothelial-lined spaces and low-level blood flow in the flaccid state. In addition, the penis is filled with billions of endothelial and smooth muscle cells, both of which are rich in VEGF receptors.[14] In fact, three recent reports have shown beneficial effects of intracavernous VEGF therapy in animals with arteriogenic ED.[23–25] The goal of the current study was to evaluate the efficacy of VEGF treatment in preventing and reversing the development of venogenic ED.

To this end, we first developed an animal model of venogenic ED by castration, as Mills *et al*[10,11,26] have previously shown that castration leads to venogenic ED in rats within 6–8 days and testosterone repletion had a preventive effect. In these earlier studies ganglionic electrostimulation was used to generate an erection and the penile response was gauged with cavernosometric monitoring of ICP[11] or Doppler measurement of penile arterial inflow.[10] Our goal was to devise a technique for evaluating venous leak in animals similar to the technique used in humans. For this reason, erection was generated using pharmacologic agents (papavarine) instead of ganglionic electrostimulation. The physiologic parameters (maintenance inflow rate and ICP drop rate) used to diagnose venous leak in humans[27,28] were reproduced in a rat model. Using this technique, pharmacocavernosometric findings were determined in normal animals and animals with venogenic and arteriogenic ED. This method was found to be a sensitive and reproducible technique to evaluate penile arterial insufficiency and venous leak in a rat model.

This model was then used to evaluate the efficacy of VEGF, administered intracorporally as recombinant protein or AAV gene vector, to prevent the development of venogenic erectile ED (Experiment 2: Prevention trial). It has been previously shown that castration induces an involution of the prostate gland and its vasculature.[12] Furthermore, after testosterone replacement, endothelial cell proliferation is stimulated and both blood flow and vascular volumes are normalized. After castration, prostatic VEGF synthesis is downregulated, as determined by RT-PCR, Western blot, and immunohistochemical analysis.[13] Also, testosterone induces VEGF synthesis, suggesting that VEGF may be a tissue mediator of androgenic effects on the prostate. The goal of Experiment 2 was to determine if VEGF could prevent the development of venous leak in the rat model. Both testosterone replacement and VEGF treatment maintained erectile function when administered immediately after castration. Animal groups receiving no testosterone replacement or intracorporal AAV-LacZ showed persistent venogenic erectile ED after castration. Histological examination of smooth muscle content and morphology revealed deterioration in both the quality and quantity of penile smooth muscle after castration. Electron microscopic examination also revealed alteration of cell membrane and widening of intercellular spaces. Smooth muscle content as measured by alpha actin staining was normalized in animals receiving either testosterone or VEGF, evidence of preserved smooth muscle integrity with such preventative treatment.

The final phase was a treatment trial (Experiment 3) in which animals were first documented to have venous leak, 6 weeks after castration, and then treated with intracorporal VEGF protein. Cavernosometry was repeated and restoration of near normal erectile function was found 1 month after such treatment. We believe that this is the first experimental evidence of any medical therapy improving venogenic erectile ED. The exact mechanism by which VEGF improves erectile function is unknown. Nevertheless, we observed clear evidence of restoration of neural and smooth muscle integrity as well as hyperplasia and hypertrophy of endothelial cells after VEGF treatment. Conceivably, in-

creased cavernosal neovascularity may lead to functional or structural changes in the nerve and smooth muscles. Alternatively, the direct effect of VEGF on the nerve and smooth muscle may also play a role since VEGF has been reported to have a direct trophic effect on the penile smooth muscle cells[14] and spinal neurons in culture.[15,16] Further studies are under way to study the mechanism of VEGF action in the penis.

Conclusions

The technique presented here for pharmacologic cavernosometry is a simple and reproducible method to evaluate vasculogenic ED in a rat model. Normal erectile function and ED because of arterial insufficiency or venous leak may be diagnosed by characteristic cavernosometric findings. Using this technique, the presence of veno-occlusive disease may be diagnosed in animals 6 weeks after either castration or ligation of the internal iliac arteries.

Animals treated with testosterone replacement at the time of castration retain normal erectile function, while those without testosterone replacement develop venous leak. If these animals are treated with intracavernosal recombinant VEGF protein or AAV-VEGF at the time of castration, their erectile function is maintained and venous leak is prevented. The mechanism for this is not known at present, but we find a decrease in penile smooth muscle content in the castrated group compared with either the testosterone replacement group or the group receiving intracavernosal VEGF or AAV-VEGF. Penile smooth muscle morphology is uniformly degenerated after castration. Animals treated with the intracorporal AAV-VEGF transfection vector demonstrate significantly more VEGF protein in their penile serum compared to the systemic serum, and markedly more than control animals, indicating an increased expression of penile VEGF in these animals.

When rats with established venogenic ED are treated with one dose of intracavernosal recombinant VEGF protein, their erectile function returns to nearly normal, with reversal of the veno-occlusive defect. Electronmicroscopy revealed endothelial cell hyperplasia and hypertrophy as well as restoration of smooth muscle and neural integrity in the penile tissue after VEGF treatment. Since impairment of erectile nerve, endothelial cell, and the cavernous smooth musculature is the final common pathway of various types of organic ED, VEGF therapy may hold the key to prevention and cure of many forms of ED.

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References

1 Lue TF. *Contemporary Diagnosis and Management of Male Erectile Dysfunction*. Handbooks in Health Care Co.: Newtown, Penn., 1999.
2 Karadeniz T *et al*. Judgment of color Doppler ultrasound with respect to cavernous artery occlusion pressure in dynamic infusion cavernosometry when evaluating arteriogenic impotence. *Urol Int* 1996; **57**: 85–88.
3 Mulhall JP, Graydon RJ. The hemodynamics of erectile dysfunction following nerve-sparing radical retropubic prostatectomy. *Int J Impot Res* 1996; **8**: 91–94.
4 Mersdorf A *et al*. Ultrastructural changes in impotent penile tissue: a comparison of 65 patients. *J Urol* 1991; **145**: 749–758.
5 Rajfer J, Rosciszewski A, Mehringer M. Prevalence of corporeal venous leakage in impotent men. *J Urol* 1988; **140**: 69–71.
6 Meisel RL, O'Hanlon JK, Sachs BD. Differential maintenance of penile responses and copulatory behavior by gonadal hormones in castrated male rats. *Horm Behav* 1984; **18**: 56–64.
7 Zvara P *et al*. Nitric oxide mediated erectile activity is a testosterone dependent event: a rat erection model. *Int J Impot Res* 1995; **7**: 209–219.
8 Lekås E *et al*. Decrement of blood flow precedes the involution of the ventral prostate in the rat after castration. *Urol Res* 1997; **25**: 309–314.
9 Häggström S, Wikström P, Bergh A, Damber JE. Expression of vascular endothelial growth factor and its receptors in the rat ventral prostate and Dunning R3327 PAP adenocarcinoma before and after castration. *Prostate* 1998; **36**: 71–79.
10 Mills TM, Lewis RW, Stopper VS. Androgenic maintenance of inflow and veno-occlusion during erection in the rat. *Biol Reprod* 1998; **59**: 1413–1418.
11 Mills TM, Stopper VS, Wiedmeier VT. Effects of castration and androgen replacement on the hemodynamics of penile erection in the rat. *Biol Reprod* 1994; **51**: 234–238.
12 Franck-Lissbrant I, Häggström S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats [see comments]. *Endocrinology* 1998; **139**: 451–456.
13 Häggström S, Lissbrant IF, Bergh A, Damber JE. Testosterone induces vascular endothelial growth factor synthesis in the ventral prostate in castrated rats. *J Urol* 1999; **161**: 1620–1625.
14 Liu X *et al*. Vascular endothelial growth factor promotes proliferation and migration of cavernous smooth muscle cells. *J Urol* 2001; **166**: 354–360.
15 Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor: direct neuroprotective effect in *in vitro* ischemia. *Proc Natl Acad Sci USA* 2000; **97**: 10242–10247.
16 Sondell M, Sundler F, Kanje M. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci* 2000; **12**: 4243–4254.
17 Verdonck A *et al*. Effect of testosterone replacement after neonatal castration on craniofacial growth in rats. *Arch Oral Biol* 1998; **43**: 551–557.
18 Parker CR Jr, Ellegood JO, Mahesh VB. Methods for multiple steroid radioimmunoassay. *J Steroid Biochem* 1975; **6**: 1–8.
19 Su H, Lu R, Kan YW. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. *Proc Natl Acad Sci USA* 2000; **97**: 13801–13806.
20 Karadeniz T *et al*. Correlation of ultrastructural alterations in cavernous tissue with the clinical diagnosis vasculogenic impotence. *Urol Int* 1996; **57**: 58–61.
21 Baumgartner I *et al*. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia [see comments]. *Circulation* 1998; **97**: 1114–1123.

22 Symes JF *et al*. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg* 1999; **68**: 830–836; discussion 836–837.
23 Henry GD *et al*. Intracavernosal injections of vascular endothelial growth factor protects endothelial dependent corpora cavernosal smooth muscle relaxation in the hypercholesterolemic rabbit: a preliminary study. *Int J Impot Res* 2000; **12**: 334–339.
24 Byrne RR *et al*. Vascular endothelial growth factor restores corporeal smooth muscle function *in vitro*. *J Urol* 2001; **165**: 1310–1315.
25 Lee MC *et al*. The effect of vascular endothelial growth factor on a rat model of traumatic arteriogenic erectile dysfunction. *J Urol* 2002; **167**: 761–767.
26 Mills TM, Dai Y, Stopper VS, Lewis RW. Androgenic maintenance of the erectile response in the rat. *Steroids* 1999; **64**: 605–609.
27 Hatzichristou DG *et al*. *In vivo* Assessment of trabecular smooth muscle tone, its application in pharmaco-cavernosometry and analysis of intracavernous pressure determinants [see comments]. *J Urol* 1995; **153**: 1126–1135.
28 Karadeniz T *et al*. Value of color Doppler sonography in the diagnosis of venous impotence. *Urol Int* 1995; **55**: 143–146.