Expression of Caveolin-1 in Penile Cavernosal Tissue in a Denervated Animal Model after Treatment with Sildenafil Citrate

Edgardo F. Becher, MD, PhD,* Jorge E. Toblli, MD, PhD,† Cynthia Castronuovo, PhD,* Carlos Nolazco, MD,* Claudio Rosenfeld, MD,* Halina Grosman, PharmD,‡ Elba Vazquez, PhD,§ and Osvaldo N. Mazza, MD, PhD*

*Division of Urology, Hospital de Clínicas "José de San Martín," University of Buenos Aires, Buenos Aires, Argentina; †Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina; ‡Department of Clinical Biochemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina; §Department of Biologic Chemistry, School of Sciences, University of Buenos Aires, Buenos Aires, Argentina-CONICET

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ABSTRACT

Introduction. Radical pelvic surgery is a major cause of erectile dysfunction due to iatrogenic cavernous nerve damage. Endothelial nitric oxide synthase, which generates nitric oxide (NO) in the cavernosal tissues, localizes to specialized plasma membrane invaginations known as caveolae. Growing evidence suggests that caveolae are major components of signal trafficking and that stimuli that affect the concentration of the main structural protein of caveolae, caveolin-1 influence NO signaling.

Aim. To evaluate caveolin-1 expression as a marker of cavernous tissue damage and determine the impact of early sildenafil administration on caveolin-1 expression in animal models of partial and total surgical penile denervation.

Methods. Thirty-six rats were divided into six groups (N = 6 per group) that received bilateral or unilateral penile denervation or sham surgery, with and without sildenafil 10 mg daily for 7 weeks.

Main Outcome Measures. Sections were taken from the proximal middle portion of the penis of all animals. Cavernous tissue was delineated by the tunica albuginea, then the extent of immunostaining for the following parameters was quantitated to determine (i) cavernous smooth muscle layer in the cavernous space expressed as the percentage of α-smooth muscle actin (α-SMA) positive immunostaining per area and (ii) caveolin-1 expressed as a percentage of area.

Results. A marked decrease in both caveolin-1 and α-SMA expression in cavernous smooth muscle tissue and in the endothelium of rats was noted after a bilateral and unilateral neurotomy. Specimens from animals receiving sildenafil exhibited higher mean immunostaining values for both proteins in cavernous tissue. The differences were statistically significant compared with groups receiving the same surgical treatment without sildenafil.

Conclusion. Caveolin-1 and α-SMA expression in cavernous tissue is significantly reduced by pelvic nerve injury, and the loss is related to the extent of the neural damage. Early administration of sildenafil elicits caveolin-1 expression, which appears to preserve cavernous tissue. **Becher EF, Toblli JE, Castronuovo C, Nolazco C, Rosenfeld C, Grosman H, Vazquez E, and Mazza ON. Expression of caveolin-1 in penile cavernosal tissue in a denervated animal model after treatment with sildenafil citrate. J Sex Med **;**:**–**.**

Key Words. Alpha Smooth Muscle Actin; Endothelium; Immunohistochemistry; Neurotomy

Introduction

Radical pelvic surgery is a major cause of erectile dysfunction (ED) due to iatrogenic cavernous nerve damage and the hypoxia and fibrosis that may follow [1–3]. Postoperative ED is primarily related to the extent of nerve injury and might be influenced by early pharmacologic intervention, although the methodology is still controversial [4]. Other than the patient's preoperative erectile performance and the surgical technique, no markers are available to predict the extent of ED after radical pelvic surgery.

A histologic analysis of penile biopsy samples from men after prostatectomy showed increased collagen fibers and decreased elastin fibers and smooth muscle cells (SMC), reflecting a change in the "microstructure" of the penile tissues after prostatectomy [5]. Apoptosis of SMC occurs after neurotomy in rat models [6–8], which exhibit many of the same features observed in men with ED after prostatectomy. This SMC apoptosis was abrogated by daily treatment with sildenafil [6,8].

Nitric oxide (NO), through its activation of soluble guanyl cyclase, is the principal mediator of penile smooth muscle relaxation leading to an erection [9]. Endothelial nitric oxide synthase (eNOS), which generates NO in the cavernosal tissues, localizes to plasma membrane invaginations known as caveolae in cell culture models and in endothelial cells of conduit vessels and microvessels in vivo [10–12]. Caveolae are specialized organelles present at the plasma membrane of most cells and are involved in endocytosis. Growing evidence suggests that caveolae are major components of signal trafficking of certain cells and in cellular systems such as smooth muscle and endothelium [13,14].

eNOS is believed to be regulated in part through an inhibitory association with caveolin-1, the main structural protein present in caveolae [15]. eNOS also contains a binding domain for the calcium-dependent protein phosphatase, calmodulin. The calcium-dependent binding of calmodulin to eNOS is important in the homodimerization and activation of eNOS. Caveolin-1 appears to inhibit eNOS dimerization and activation by interfering with the binding of calmodulin to eNOS when intracellular calcium levels are low. The NO produced by eNOS activates guanyl cyclase, which in turn produces the second messenger, cyclic guanosine monophosphate (cGMP) [16]. The cGMP produced by guanyl cyclase activates a signaling cascade and is later degraded to inactive guanine monophosphate (GMP) by selective phosphodiesterases.

Haas et al. demonstrated an age-related decrease in relaxation of the corpora cavernosa in rabbit tissue strips after application of acetylcholine [17]. However, equivalent relaxation of penile tissue from old and young animals was achieved with the NO donor, sodium nitroprusside suggesting that the older rabbits' tissue was responsive to NO. Haas et al. also reported that eNOS expression was upregulated in the penile endothelium and smooth muscle in aging rabbits compared with young rabbits and suggested that the upregulation of eNOS might represent a compensatory mechanism. However, the immunohistochemical finding of eNOS in smooth muscle by Haas et al has been called into question. Burnett et al. found that eNOS was present at approximately 20% greater levels in the endothelial layer of the sinusoids of the corpora cavernosa using two different eNOS-specific antisera in neuronal NOS knockout mice [18]. These researchers also attempted to detect inducible NOS by Western blot analysis or immunohistochemistry of penile tissue but could not detect this isoform. Bakircioglu et al. found that caveolin-1, the isoform present in penile smooth muscle and endothelial caveolae, is reduced in penile tissues from aging rats compared with young rats [19]. This result suggested that, despite an observed increase in eNOS expression, the absence of caveolae for eNOS to associate with could result in decreased NO synthesis by abrogating the colocalization of eNOS with another resident protein of caveolae, arginine transporter protein [20].

Animal studies show that aging and hypertension decrease the ratio of trabecular smooth muscle to collagen and the concentration of caveolae, suggesting reduced expression of caveolin-1 [19,21]. Stimuli that affect the concentration of caveolins and caveolae influence signaling and the presence of eNOS [22–24], and the association of eNOS with caveolae and eNOS activity is dependent on the action of calmodulin [15]. Ultimately, decreased concentrations of caveolae are expected to affect endothelium-dependent smooth muscle relaxation.

A decline in eNOS activity would result in decreased signaling through the cGMP pathway [16]. The selective inhibitor of phosphodiesterase type 5, sildenafil inhibits cGMP hydrolysis in penile corpus cavernosum [25], prolonging signaling through this pathway in the absence of NO.

Linder et al. showed that soluble guanyl cyclase (sGC) and caveolin-1 colocalize on the corpora cavernosal endothelium, and that disruption of caveolae with methyl-β-cyclodextrin inhibited penile smooth muscle relaxation by electrical stimulation [26]. However, when the tissues were treated with the nonhydrolyzable cGMP analog, 8-bromo-cGMP, the smooth muscle relaxation was no longer inhibited by disruption of caveolae. These studies suggest that loss of innervation to the penis results in loss of SMC and may disrupt the caveolae in the corpora cavernosal endothelium, which is important for erectile function. If intact caveolae are essential for erectile function,

then one can hypothesize that levels of caveolin-1 are also affected by denervation.

The objective of this study was to evaluate caveolin-1 expression as a marker of cavernous tissue damage and to determine the impact of early sildenafil administration on caveolin-1 expression in animal models of partial and total surgical penile denervation.

Methods

Thirty-six adult Sprague-Dawley rats [27] were divided into six groups (N = 6 in each group) that received bilateral or unilateral penile denervation or sham surgery, with and without daily postsurgical administration of approximately 10 mg sildenafil. The animals were divided into groups as follows: group 1, bilateral neurotomy; group 2, unilateral neurotomy; group 3, sham surgery; group 4, bilateral neurotomy with sildenafil; group 5, unilateral neurotomy with sildenafil; and group 6, sham surgery with sildenafil.

The animals were housed and surgery was performed at the Institute for Cardiovascular Research, University of Buenos Aires School of Medicine (Buenos Aires, Argentina). The animals were sacrificed 7 weeks after the surgical procedure. The penile corporal tissue was analyzed by immunostaining with antibodies against caveolin-1 and α-smooth muscle actin (α-SMA) as a standard marker at the Laboratory of Experimental Medicine, Hospital Alemán (Buenos Aires, Argentina).

Surgical Procedure and Postoperative Handling

The animals were anesthetized using an intraperitoneal injection of ketamine 50 mg/kg and xylazine 6 mg/kg. A midline abdominal incision was made and the bladder and prostate were exposed. Extensive neurotomy of the pre-erectile periprostatic plexus was performed using a cautery on the lateral aspect of the prostate under optical magnification. The neurotomy was performed bilaterally on groups 1 and 4 and unilaterally on groups 2 and 5; no neurotomy was performed on groups 3 and 6 (sham groups). The rats were treated with postoperative analgesia consisting of nalbuphine 1 mg/kg twice daily.

After surgery, animals on active treatment (groups 4–6) were administered 50 mg of sildenafil per 100 mL of drinking water. With an average consumption of 20 mL/day, each rat received an estimated dose of 10 mg sildenafil. Seven weeks after surgery, the animals were sacrificed by administration of a lethal dose of intraperitoneal sodium thiopental. A midline abdominal incision was made, and the penises were excised in bulk from the crus to the distal aspect.

Immunolabeling and Light Microscopy

Immunolabeling of specimens was performed through a modified avidin-biotin-peroxidase complex technique. Following deparaffinization and rehydration, the sections were washed in phosphate-buffered saline (PBS) for 5 minutes. Quenching of endogenous peroxidase activity was achieved by incubating the sections in 1% hydrogen peroxide in methanol for 30 minutes. After washing the sections in PBS, pH 7.2, for 20 minutes, the sections were incubated with blocking serum for 20 minutes. Thereafter, the sections were incubated with the primary antibody overnight, rinsed in PBS, and incubated with biotinylated universal antibody for 30 minutes. After washing in PBS, the sections were incubated with Vectastain® Elite ABC reagent (Vector Laboratories, Birlingame, CA, USA) for 40 minutes and exposed to 0.1% diaminobenzidine (Polyscience, Warrington, PA, USA) and 0.2% hydrogen peroxide in 50 mM Tris buffer, pH 8, for 5 minutes. We used α-SMA to evaluate cavernosal smooth muscle status because it has been widely studied and used for this purpose [28].

α-SMA was quantified using antimouse α-SMA (Sigma Chemical Co., St. Louis, MO, USA) monoclonal antibodies and rabbit polyclonal IgG anti-caveolin-1 (N-20 SC-896; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:100 dilution.

Morphometric Analysis

Between six and eight transverse histologic sections from the penis of each animal were studied by an image analyzer (Image-Pro® Plus, version 4, Media-Cybernetics, Inc., Silver Spring, MD, USA). In order to compare similar segments of the corpus of all rats, sections were taken from the proximal middle portion of the penis of each animal. Morphological analyses were performed with the observer blinded to the animal group, and the data were averaged. Cavernous tissue was delineated by the tunica albuginea, then a quantification of the extent of immunostaining for the following parameters was performed: (i) cavernous smooth muscle layer in the cavernous space expressed as the percentage of α-SMA–positive immunostaining per area and (ii) caveolin-1 expressed as a percentage of area. Control sections used for determination of antibody specificity

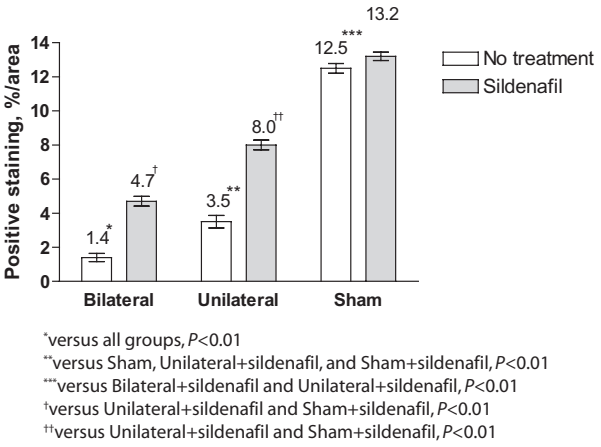


Figure 1 Expression levels of caveolin-1 in rat penises after surgery based on immunohistochemical staining. Data are expressed as the percentage of positive caveolin-1 staining per area.

included (i) positive controls of normal endothelium for caveolin-1 and (ii) negative controls (serial sections of each sample omitting the primary antibody). Caveolin-1 immunostaining was also qualitatively evaluated by the presence of positive or negative staining. All data were averaged in the final result.

Statistical Analysis

Values are expressed as the mean ± standard deviation. All statistical analyses were performed using absolute values and processed through GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA, USA). For parameters with non-Gaussian distribution, such as histologic data, comparisons were made by nonparametric analysis of variance and Dunn's multiple comparison test. A value of $P < 0.05$ was considered significant.

Results

There was a marked decrease in both caveolin-1 and α-SMA expression in cavernous smooth muscle tissue and in the endothelium of rats after bilateral and unilateral neurotomies (Figures 1–3).

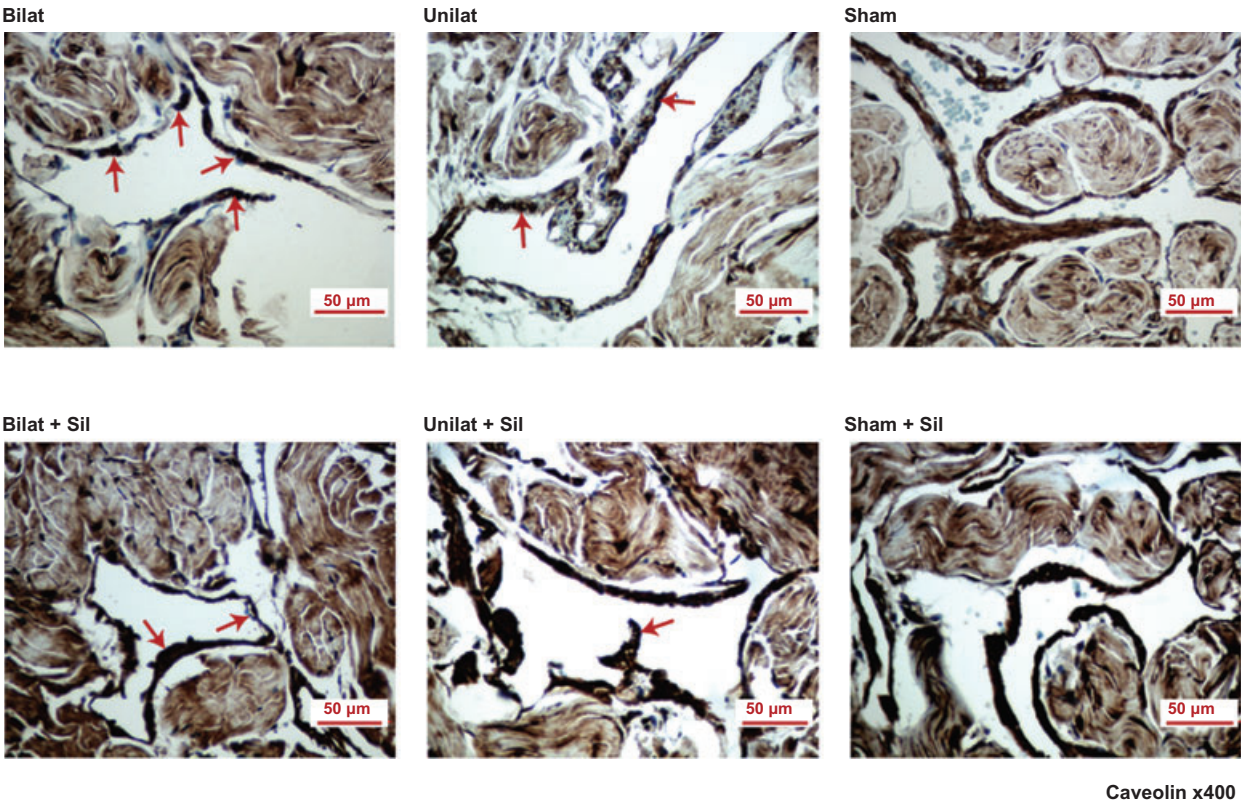
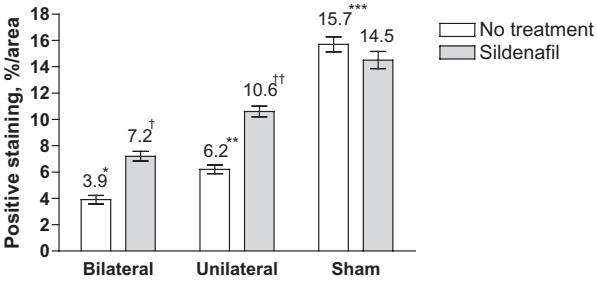


Figure 2 Immunostaining of caveolin-1 in cavernous smooth muscle in rats receiving bilateral (Bilat) or unilateral (Unilat) neurotomies, or no neurotomy (Sham), with or without subsequent sildenafil (Sil) treatment. Arrows point toward stained cells.



*versus all groups, $P<0.01$
**versus Sham, Unilateral+sildenafil, and Sham+sildenafil, $P<0.01$
***versus Bilateral+sildenafil and Unilateral+sildenafil, $P<0.01$
†versus Unilateral+sildenafil and Sham+sildenafil, $P<0.01$
††versus Unilateral+sildenafil and Sham+sildenafil, $P<0.01$

Figure 3 Expression levels of α-smooth muscle actin (α-SMA) in rat penises after surgery based on immunohistochemical staining. Data are expressed as the percentage of positive α-SMA staining per area.

The mean positive immunostaining areas indicating caveolin-1 and α-SMA expression were 1.4% ± 0.6% and 3.9% ± 0.8%, respectively, in animals in group 1, which received bilateral nerve injury but no sildenafil (Figures 1 and 3). This result was statistically significantly lower compared with all other groups ($P < 0.01$). Animals that received unilateral neurotomy but no sildenafil (group 2) had mean immunostaining values of 3.5% ± 0.9% for caveolin-1 and 6.2% ± 0.8% for α-SMA. In contrast, rats that received sham surgeries but no sildenafil (group 3) had mean values of 12.5% ± 0.7% for caveolin-1 and 15.7% ± 1.4% for α-SMA ($P < 0.01$ vs. groups 1 and 2).

Specimens from animals that received sildenafil treatment exhibited higher mean immunostaining values for both proteins in cavernous tissue. In the group 4 animals that received bilateral neurotomy and sildenafil, caveolin-1 expression was 4.7% ± 0.7% and α-SMA expression was 7.2% ± 0.9%; in the group 5 animals that received unilateral neurotomy and sildenafil, mean caveolin-1 was 8.0% ± 0.7% and mean α-SMA was 10.6% ± 1.0%. The differences were statistically significant compared with the groups that received the corresponding surgical treatment without sildenafil (Figures 1 and 3).

Discussion

There is growing evidence that caveolae and caveolins contribute to NO signaling and are necessary components for penile cavernosal smooth muscle function and endothelial health. Linder et al. showed that caveolae act as platforms for soluble guanylyl cyclase/cGMP signaling in cavernous smooth muscle, which is necessary for penile erection [26]. Carrier et al. reported a permanent decrease of NOS-positive nerve fibers after a bilateral neurotomy in rats but a significant regeneration after a unilateral surgery [29]. Also, Leungwattanakij et al. reported an increase in transforming growth factor-β_1, hypoxia inducible factor-1α, and collagen III synthesis responsible for cavernosal fibrosis in rats after a bilateral neurotomy [2].

Caveolin-1 has been shown to be significantly decreased in aging rats. Bakircioglu et al. found both a decrease in caveolin-1 expression in cavernosal endothelium and smooth muscle as well as a decreased concentration of caveolae upon electron microscopy in aging rats [19]. They also found a decrease in smooth muscle content and an increase in collagen, coincident with findings from human biopsy specimens.

Cavernosal caveolin-1 was also decreased in other morbid conditions such as hypertension [21]. We recently reported a decreased expression of caveolin-1 in corporal smooth muscle and endothelium in spontaneously hypertensive rats [21].

Regarding the method of cavernosal neural damage, Mullerad et al. reported the consequences of different cavernosal nerve damage using different surgical models and concluded that there were no significant differences in the hemodynamic alterations between neurotomy and various types of nerve crush injuries [30]. We elected to perform an ample cautery neurotomy to ensure full neural damage.

The expression of caveolin-1 changed markedly with the administration of sildenafil right after nerve damage in the treatment groups (groups 4 and 5 vs. groups 1 and 2). These results show that nerve damage causes decreased expression of caveolin-1 and that sildenafil plays a protective role in the corporal smooth muscle and endothelial function, presumably by increasing cGMP levels. The exact mechanism of cavernosal protection by sildenafil is not fully understood, but there is evidence supporting an antioxidative mechanism through an enhanced expression of glutathione peroxidase [31].

In the present study, the decreased corporal expression of caveolin-1 is similar to the expression of α-SMA, used as a standard marker. This finding shows that the method used to detect

caveolin-1 expression is reliable. Both proteins decreased after neural damage, with a more marked decrease in animals that received a bilateral neurotomy. One potential advantage of caveolin-1 is that this protein can be measured in serum. Tahir et al reported the feasibility of measuring caveolin-1 in human serum using a specifically developed immunoassay in patients with prostate cancer [32,33]. Although these findings are still in the early stages, the use of caveolin-1 as a potential biomarker for cavernosal health may be of interest in future investigations.

Conclusions

Caveolin-1 and α-SMA expression in cavernous tissue is significantly reduced by pelvic nerve injury and is related to the extent of the neural damage. Early administration of sildenafil elicits caveolin-1 expression, which appears to preserve cavernous tissue and suggests that an NO/cGMP mechanism is involved in this preservation. A greater impact occurred in animals with bilateral nerve injury.

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Corresponding Author: Edgardo F. Becher, MD, PhD, Division of Urology, Hospital de Clínicas "José de San Martín," University of Buenos Aires, Ave Cordoba 2424 Buenos Aires, C1120AAT Argentina. Tel: +54 (11) 4964-2424; Fax: +54 (11) 4964-0200; E-mail: ebecher@cdu.com.ar

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