

BASIC SCIENCE

Off-Target Effect of Sildenafil on Postsurgical Erectile Dysfunction: Alternate Pathways and Localized Delivery System



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ABSTRACT

Introduction: There is no consensus on the best oral phosphodiesterase type 5 inhibitor (PDE5I) for patients undergoing penile rehabilitation after surgical nerve injury.

Aim: To determine the mechanism of PDE5I on cultured neuronal cells and the effectiveness of local drug delivery using nanospheres (NSPs) to sites of nerve injury in a rat model of bilateral cavernous nerve injury (BCNI).

Methods: The effects of sildenafil, tadalafil, and vardenafil on cyclic adenosine monophosphate, cyclic guanosine monophosphate, and cell survival after exposure to hypoxia and H₂O₂ were measured in PC12, SH-SY5Y, and NTERA-2 (NT2) cell cultures. The effects of phosphodiesterase type 4 inhibitor (PDE4I) and PDE5I on neuronal cell survival were evaluated. Male rats underwent BCNI and were untreated (BCNI), immediately treated with application of empty NSPs (BCNI + NSP), NSPs containing sildenafil (Sild + NSP), or NSPs containing rolipram (Rol + NSP).

Main Outcome Measures: Viability of neuronal cells was measured. Intracavernous pressure changes after cavernous nerve electrostimulation and expression of neurofilament, nitric oxide synthase, and actin in mid-shaft of penis were analyzed 14 days after injury.

Results: Sildenafil and rolipram significantly decreased cell death after exposure to H₂O₂ and hypoxia in PC12, SH-SY5Y, and NT2 cells. PC12 cells did not express PDE5 and knockdown of PDE4 significantly increased cell viability in PC12, SH-SY5Y, and NT2 cells exposed to hypoxia. The ratio of intracavernous pressure to mean arterial pressure and expression of penile neurofilament, nitric oxide synthase, and actin were significantly higher in the Sild + NSP and Rol + NSP groups than in the BCNI and BCNI + NSP groups. Limitations included analysis in only two PDE families using only a single dose.

Conclusion: Sildenafil showed the most profound neuroprotective effect compared with tadalafil and vardenafil. Sildenafil- or rolipram-loaded NSP delivery to the site of nerve injury prevented erectile dysfunction and led to increased neurofilament, nitric oxide synthase, smooth muscle content in rat penile tissue after BCNI.

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Key Words: Phosphodiesterase Inhibitors; Cavernous Nerve Injury; Iatrogenic Erectile Dysfunction; Penile Rehabilitation; Radical Prostatectomy

INTRODUCTION

The risk of erectile dysfunction (ED) after radical prostatectomy (RP) is 20% to 80%.^{1,2} The etiology of post-RP ED is

believed to be multifactorial and involves a combination of ischemic, mechanical, thermal, and inflammatory injuries to the neurovascular bundles.^{3,4} Various treatment modalities for post-

Received March 16, 2016. Accepted October 12, 2016.

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<http://dx.doi.org/10.1016/j.jsxm.2016.10.003>

prostatectomy ED, including pharmacologic agents and devices, have been studied, but no standard treatment algorithm or guideline for effective penile rehabilitation has been developed. A promising and widely evaluated intervention is the use of phosphodiesterase type 5 inhibitors (PDE5Is).^{4,5}

Animal studies have shown that PDE5I alleviates ED and reverses adverse structural changes seen after bilateral cavernous nerve injury (BCNI).^{6–9} These previous studies reported that the main mechanisms of PDE5I include prevention of oxidative-associated tissue damage and preservation of penile smooth muscle and endothelial integrity. In addition, much attention has been given to the direct effect of PDE5I on damaged nerves. Hlaing et al¹⁰ reported that oral sildenafil after cavernous nerve resection in rats increases neurotrophic factors and has a neuro-regenerative effect on major pelvic ganglia. Furthermore, sildenafil has been shown to protect the central nervous system against stroke or chemical hypoxia in animal models by increasing blood flow and minimizing hypoxia.^{11–14} Although the precise mechanism underlying the neuroprotective effect of PDE5I remains unclear, cyclic guanosine monophosphate (cGMP) and nitric oxide (NO) have been implicated as key mediators.^{11,15}

Orally administered PDE5I treatments for post-RP ED often require long-term daily use and can cause dose-related side effects, such as headache, dizziness, flushing, dyspepsia, myalgia, and nasal congestion. Local delivery of these drugs might be more favorable than systemic (oral) delivery for the following reasons: relative ease of laparoscopic administration; high bioavailability by localized direct delivery of the therapy to the wound site; and decreased occurrence of unwanted systemic side effects. The use of nanospheres (NSPs) represents an innovative approach to increase the bioavailability, solubility, circulation time, and resistance to metabolic degradation of hydrophobic drugs. These NSPs are synthesized from a family of fully degradable, ABA-type triblock copolymers made of poly(ethylene glycol), oligomers of desamino-tyrosyl-tyrosine esters, and suberic acid¹⁶ and spontaneously self-assemble in aqueous media. Several preclinical studies have reported on the non-toxicity of these NSPs in vitro¹⁶ and in vivo¹⁷ and on their ability to deliver hydrophobic drugs.^{16–18}

In the present study, we investigated the mechanism underlying the neuroprotective effect and the impact of local delivery of PDEIs, using NSPs on erectile function (EF) after BCNI, in an effort to further understand the role of PDE5Is in the framework of post-RP ED.

METHODS

Chemicals and Reagents

Sildenafil citrate, tadalafil, vardenafil, and rolipram (Selleck Chemicals, Houston, TX, USA) were dissolved in dimethyl sulfoxide and diluted with complete media. H₂O₂ was diluted in complete media.

Cell Culture and Treatment

The human neuroblastoma cell line, SH-SY5Y (ATCC, Manassas, VA, USA), was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). We evaluated differentiated and undifferentiated SH-SY5Y cell lines in this study. To induce differentiation, we treated SH-SY5Y cells with DMEM containing 3% FBS and all-trans retinoic acid 10 μ mol/L for 72 hours. PC12 (ATCC), a rat pheochromocytoma cell line, was cultured in DMEM containing 10% horse serum and 5% FBS. Human pluripotent NTERA-2 (NT2) cell culture (ATCC) was cultured in 10% FBS and 1% penicillin and streptomycin with DMEM under an atmosphere of 5% CO₂ at 37°C. Generation of NT2 neurons was performed using a differentiation protocol in free-floating aggregates. Briefly, NT2 cells were seeded in bacteriological-grade Petri dishes at a density of 5×10^6 cells. On the first day, culture medium 10 mL was added to each Petri dish. On the next days, all-trans retinoic acid 10 μ mol/L was added. The media were changed every 2 to 3 days. After 8 days, the cells from Petri dish were transferred and seeded into 100-mm cell culture dishes and cultured for another 8 days in retinoic acid medium. The BD GasPak EZ Anaerobe Pouch System (Becton Dickinson, Franklin Lakes, NJ, USA) was used for the hypoxia assay. This system produces an anaerobic atmosphere (oxygen concentration < 0.1%) within 2.5 hours with greater than or equal to 10% carbon dioxide within 24 hours. Cells were cultured under hypoxia for 24 hours. For the H₂O₂ toxicity assay, NT2 cells, SH-SY5Y, and PC12 cells were incubated with the indicated concentration of H₂O₂ for 24 hours. Cells were incubated with different concentrations of sildenafil, tadalafil, vardenafil, and rolipram for 30 minutes before H₂O₂ administration. Cell density and viability were determined using the Vi-Cell Viability Analyzer (Beckman Coulter, Fullerton, CA, USA).

Cyclic Adenosine Monophosphate and cGMP Assay

Cells were seeded in 24-well plates and exposed to 24 hours of hypoxia after incubation with the indicated concentrations of sildenafil, tadalafil, or vardenafil. Extracellular levels of cyclic adenosine monophosphate (cAMP) and cGMP were measured with immunoassay kits (Abcam, Cambridge, MA, USA).

RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction

Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA). Isolated RNA was reverse transcribed (ImProm-II Reverse Transcription System; Promega, Madison WI, USA) and cDNA 1 μ g was used for polymerase chain reaction (PCR) over 35 cycles. Rat and human PDE primers were used (Table 1). β -Actin mRNA was used as a control. We also purified RNA from paraffin-embedded tissue using a Paraffin Embedded Tissue RNA Isolation Kit from Thermo Fisher (Waltham, MA, USA).

Table 1. List of primers for PCR and real-time PCR

	Forward	Reverse
Real-time PCR: rat		
NOS	GCTCCAGATCTGACCCAAGG	GGTCGATCGGCTGGACTTAG
NF	GCTGCAGTCCAAGAGCATTG	CTGGATGGTGTCTGGTAGC
Actin	CGTGACTACTGCTGAGCGTG	GTGATCACCTGCCCATCAGG
GAPDH	TCTCTGCTCCTCCCTGTTCTA	TACGGCCAAATCCGTTTACA
PCR: human		
hPDE1A	CACTGGCTCACTGAACTGGA	AAAGTGGGGAAAATGGAAGC
hPDE1B	CAAGGCCCTGTCTCTACTGC	CGTCAATGGACATCTGGTTG
hPDE1C	CCTTCTGAGGTCCGAGACTG	AAAGATCTCCAGCTCCGTCA
hPDE2A	CCCATTCTGCTACCTGCTC	CCGCGATCTTTCTCGTAGTC
hPDE3A	AGAATGGGACCACAAACGAG	TGAGGGTCATCATCACTGGA
hPDE3B	GACCGTCGTTGCCTTGATT	ATGCCAAATCTTGGTGAGG
hPDE4A	TGACGTGGCTACCATAACA	GGATGCGGTGGAGTAGTTA
hPDE4B	ATCACCTTGCTGTGGGTTTC	CTGAGCATCAGGCTGTACCA
hPDE4C	GTCATAACACGCCAATGTG	TTCGGTCGGAATAGTTGTCC
hPDE4D	CATCCTGGTGTGTCCAATCA	ATTTTTCCACGGAAGCATTG
hPDE5A	TTCAACCGAAATGACGAACA	CAGCAATCAGCAATGCAAGT
hPDE6A	CTCCTGACCATTGGGCTTTA	CCAGCTCCTCTTCTCACAC
hPDE6B	AGCAAAGGGTACCGGAGAAT	TCCAGGGACAGGTACTCCAC
hPDE6C	CATTGCTCAGGAAATGCTCA	TGGTGCCTCTGTGGTCAATA
hPDE7A	GCCATTAGAAAAGCAGGCAAC	CTCTCTGCAGTCCCTTCCAG
hPDE7B	TATACCAACAAGCCCTCTGC	ATCGCCAGTGATGATTCTCC
hPDE8A	GCGGCAAGAAGGTAGCAGTA	AGTTGTGATCGCACCTCTCC
hPDE8B	CCTGCGACAGAGCTGGTTAT	CACGTGGTCATCGCTTGTTA
hPDE9A	GCTCTCTCCAGAGACCATCG	ATCTGCTTGAACCATCAGG
hPDE10A	TCAGGTGCAGGTATGCAGAG	GTTGACCATCTGCACCACAC
hPDE11A	TTTTGTGGACTTGGCATCAA	CTTGGAAGGCATTGTTGGTT
hPDE12	TGTTTCGAATCAAGCAGCAC	CATCTTTCCTCCTCCCAT
h β -actin	GGCATCGTGATGGACTCC	GCTGGAAGGTGGACAGCG
PCR: human		
rPDE1a	TCAACCGCTTCAAGATTCTT	CCGTGGAGTATCAGGGACAT
rPDE1b	AACCAACAGCTTCCACATCC	GGCTGGACACTCTTTTCTGC
rPDE1c	TTGAAAAGCCGAAAGCCTTA	AGCTAGGCGAGCCTTCTCTT
rPDE2a	AGGTGGTGGAGGACAAACAG	GCATCTGGGATGTTCAAGAT
rPDE3a	TTCCGTTCTGGAGAACCATC	CTTCCGGGTCATCAGTGTCT
rPDE3b	TAGTCTGCCAGGTGTGCATC	ATCCACCTGCAGTTGTGTTCC
rPDE4a	CTCCTGGCTGACCTGAAGAC	CCTCCAAGGAATCCTCTTCC
rPDE4b	CATCTCACGCTTTGGAGTCA	AGTGTCTGGCGTTGCTTCTT
rPDE4c	CCCACCTCCCAAAGTACAGA	CAGCCTCTGACTCACACTGC
rPDE4d	AGCTCACCCACCTCTCTGAA	GTGAAAACAGCCTCCAAAGC
rPDE5a	GGGAAGAGGTGCTGGTGTGA	CTTCTGATGCAGTGCGTGTT
rPDE6a	GTCTGGAAGCAAGGTCTTCG	CTCCATAAGGGTCTCGTCCA
rPDE6b	ACGAGCGAAGACGAAGATGT	CTTGATCACCCACCTGACT
rPDE6c	GGCTTCTACAGGCTGAATGC	CCTTGTGAAACTGTGCTCA
rPDE7a	TGGACAAGCCAAGTGTATGC	AGCACCTATCTGAGCCTCCA
rPDE7b	CCAAAGGCACTTCCATGAAT	GTAGACCGCCAGTGGTGATT
rPDE8a	AGTTGGCATTGGAGAGGTG	ACTTCGCTTGTCTGGAGGA
rPDE8b	GCCAAGGAGGAAAATTAGG	GCATCGAGAGGTGACTGTGA
rPDE9a	ATGACCTTGGTCTGGTCAGG	TCAGATGCTCCTCATTGCTG
rPDE10a	AAGGCCTATCTTTCCCTCCA	GCAGTGACAATAGGCAAGCA
rPDE11a	CTCGATGGGAAACCTTTTGA	CTTGGAAGGCATTGTTGGTT
rPDE12	GTGTGTCCCAAACCTCGGTCT	TGCAAACAGATGAGGTCTGC
β -actin	GCTACAGCTTACCACCACA	CAGTGAGGCCAGGATAGAGC

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; hPDE = human phosphodiesterase; NF = neurofilament; NOS = nitric oxide synthase; PCR = polymerase chain reaction; rPDE = rat phosphodiesterase.

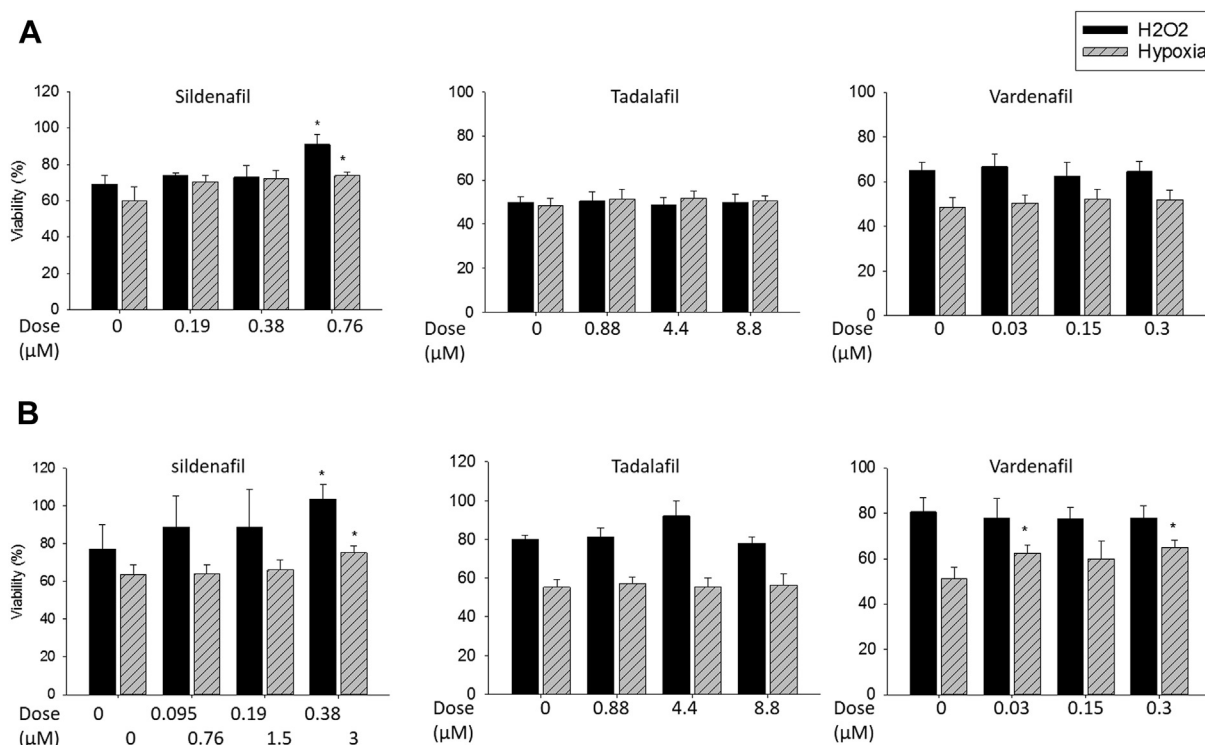


Figure 1. Panels A and B show the effect of phosphodiesterase type 5 inhibitors sildenafil, tadalafil, and vardenafil against H₂O₂ toxicity and hypoxia in the PC12 and SH-SY5Y cell lines, respectively. Sildenafil significantly increased cell survival in the two neuronal cell types exposed to hypoxia and H₂O₂. *Statistically significant ($P < .05$) vs group without phosphodiesterase type 5 inhibitor treatment.

Transient Transfection

For transfection, 50,000 cells were plated onto six-well plates. PDE4a, PDE4b, PDE4d, or PDE5a small interfering RNA (Qiagen, Valencia, CA, USA) was transfected using a PC12 transfection reagent (Altogen Biosystem, Las Vegas, NV, USA) or Lipofectamine-2000 (Invitrogen). PDE expression studies and toxicity assays were performed 72 hours after transfection.

Western Blot Analysis

Cells were lysed with the lysis buffer (phenyl methyl sulphonyl fluoride 1 mmol/L, NaCl 150 mmol/L, ethylenediaminetetraacetic acid 1 mmol/L, ethylene glycol tetraacetic acid 1 mmol/L, 1% Triton, sodium pyrophosphate 2.5 mmol/L, b-glycerolphosphate 1 mmol/L, and Na₂VO₄ 1 mmol/L in Tris 20 mmol/L; pH 7.4). Cell lysates were incubated on ice for 20 minutes and centrifuged at 10,000g for 15 minutes at 4°C. Supernatant containing 20 to 50 μg of protein was loaded onto a sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane and probed with appropriate primary and secondary antibodies.

Animal Model

This study was approved by institutional animal care and use committee (I13-007-1). Fifty 12-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were divided into five groups ($n = 10$ per group). The groups included control (no surgery), BCNI (no treatment after BCNI), BCNI +

NSP (BCNI with application of empty NSPs 20 mg), Sild + NSP (BCNI with application of sildenafil 50.7 ng in NSPs 20 mg), and Rol + NSP (BCNI with application of rolipram 50.7 ng in NSPs 20 mg). Freeze-dried powdered formulations of sildenafil- or rolipram-loaded NSPs were used. These doses were calculated to deliver sildenafil at approximately 0.38 μmol/L (254 ng/mL) or rolipram at approximately 1 μmol/L (275 ng/mL) to the cavernous nerve area in 10 days. The volume around the cavernous nerve was estimated to be 20 μL. The Supplemental Appendix presents details on the preparation of NSPs loaded with sildenafil and rolipram. The crushed nerve injury rat model has been previously described. Briefly, exploratory laparotomy was performed, and the cavernous nerve was found bilaterally at the posterolateral aspect of the prostate and then crushed with a hemostat. It was applied for 30 seconds, removed for 30 seconds, and then reapplied for 30 seconds for each side.^{19,20}

Functional Study

Fourteen days after nerve injury, rats were evaluated for EF using neurostimulation of the cavernous nerve with a square pulse stimulator at a frequency of 20 Hz and 5-ms pulse width at 4, 6, and 8 V (ADInstruments, Inc, Colorado Springs, CO, USA). Stimulation duration was 1 minute with a 3- to 5-minute rest period between stimulations. Mean arterial pressure (MAP) and intracavernosal pressure (ICP) were measured by cannulation of the carotid artery and corpus cavernosum, respectively. The ICP/MAP ratio was calculated as an index for EF at each of the three instantaneous voltages.

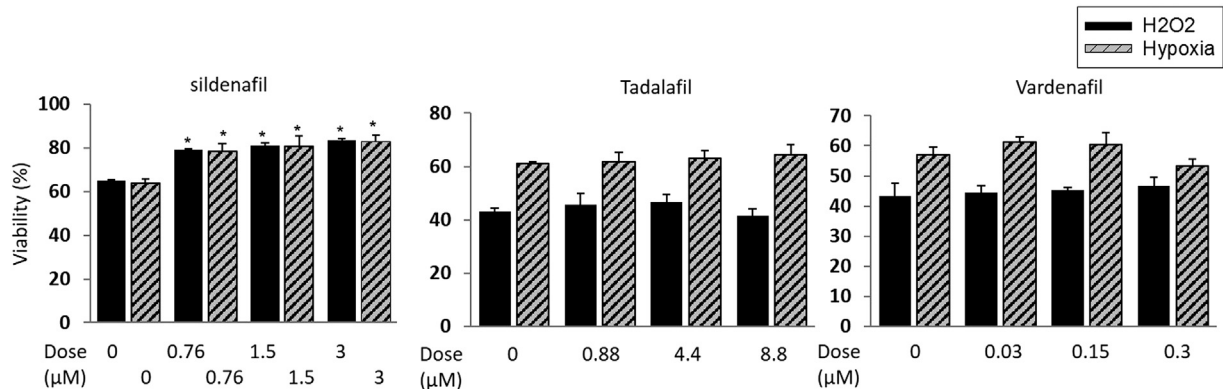


Figure 2. Effect of phosphodiesterase type 5 inhibitors sildenafil, tadalafil, and vardenafil against H₂O₂ toxicity and hypoxia in NTERA2 cells. Sildenafil significantly increased cell survival in NTERA2 cells exposed to hypoxia and H₂O₂. *Statistically significant (*P* < .05) vs group without phosphodiesterase type 5 inhibitor treatment.

Real-Time PCR

Real-time PCR amplification was performed with StepOnePlus (Thermo Fisher) to quantify the expression of neurofilament (NF), NO synthase (NOS), and actin from paraffin-embedded samples of penile mid-shaft. Real-time PCR assays were performed in micro-reaction tubes (Corbett Research, San Francisco, CA, USA) using the RT² qPCR SYBR Green mix (Qiagen). All samples were

run in triplicate. Glyceraldehyde-3-phosphate dehydrogenase served as the endogenous RNA reference gene (Table 1 lists rat primers for quantitative PCR).

Histology and Immunohistochemistry

Penile mid-shafts were dissected and fixed with 4% formaldehyde in phosphate buffered saline. Penile tissues were embedded in

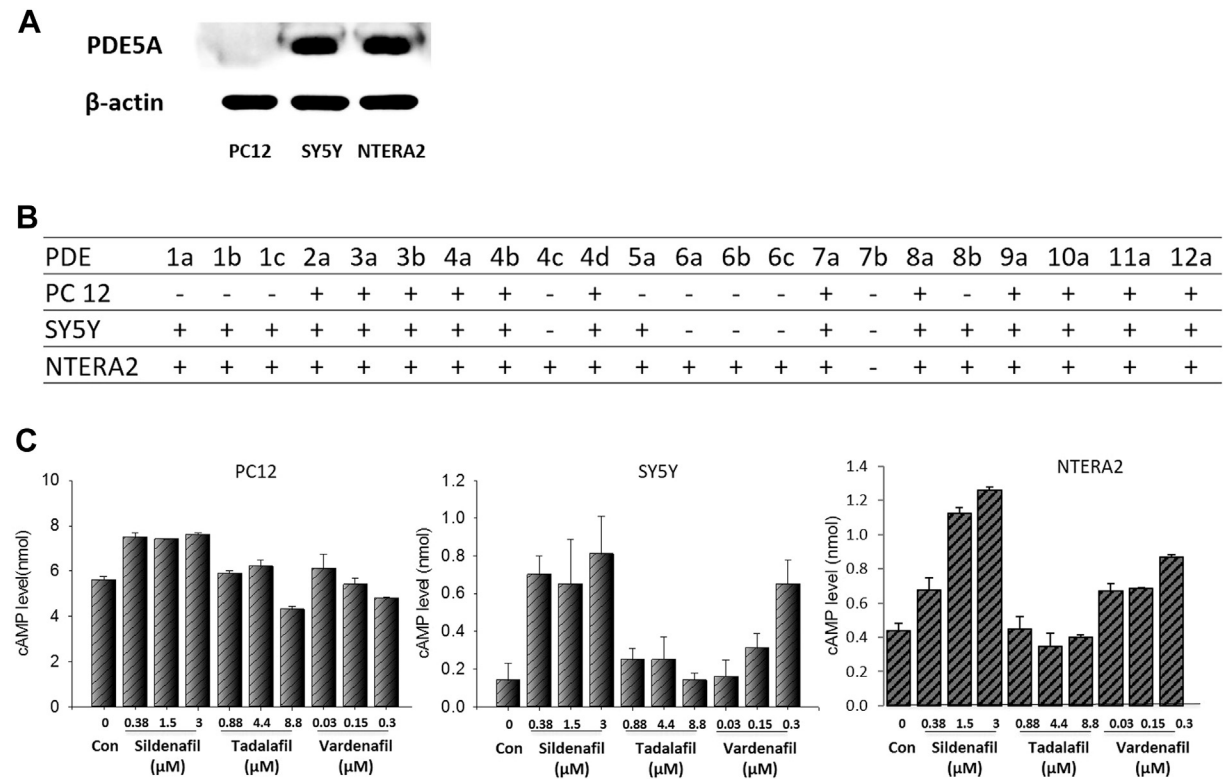


Figure 3. Cyclic adenosine monophosphate levels in media of PC12, SH-SY5Y, and NTERA2 cells after hypoxia and pretreatment with a phosphodiesterase (PDE) type 5 inhibitor. Panel A shows western blot analysis of PDE expression in PC12, SH-SY5Y, and NTERA2 cell lines. PC12 cells did not express PDE type 5a (PDE5A). Panel B shows PDE expression in PC12, SH-SY5Y, and NTERA2 cell lines with reverse transcription polymerase chain reaction. Panel C shows cyclic adenosine monophosphate levels in media of PC12, SH-SY5Y, and NTERA2 cells after hypoxia and pretreatment with a PDE type 5 inhibitor. It shows significantly higher cyclic adenosine monophosphate levels in the sildenafil-treated groups compared with the tadalafil- and vardenafil-treated groups after hypoxia. The cell counts did not differ in these groups. Con = control.

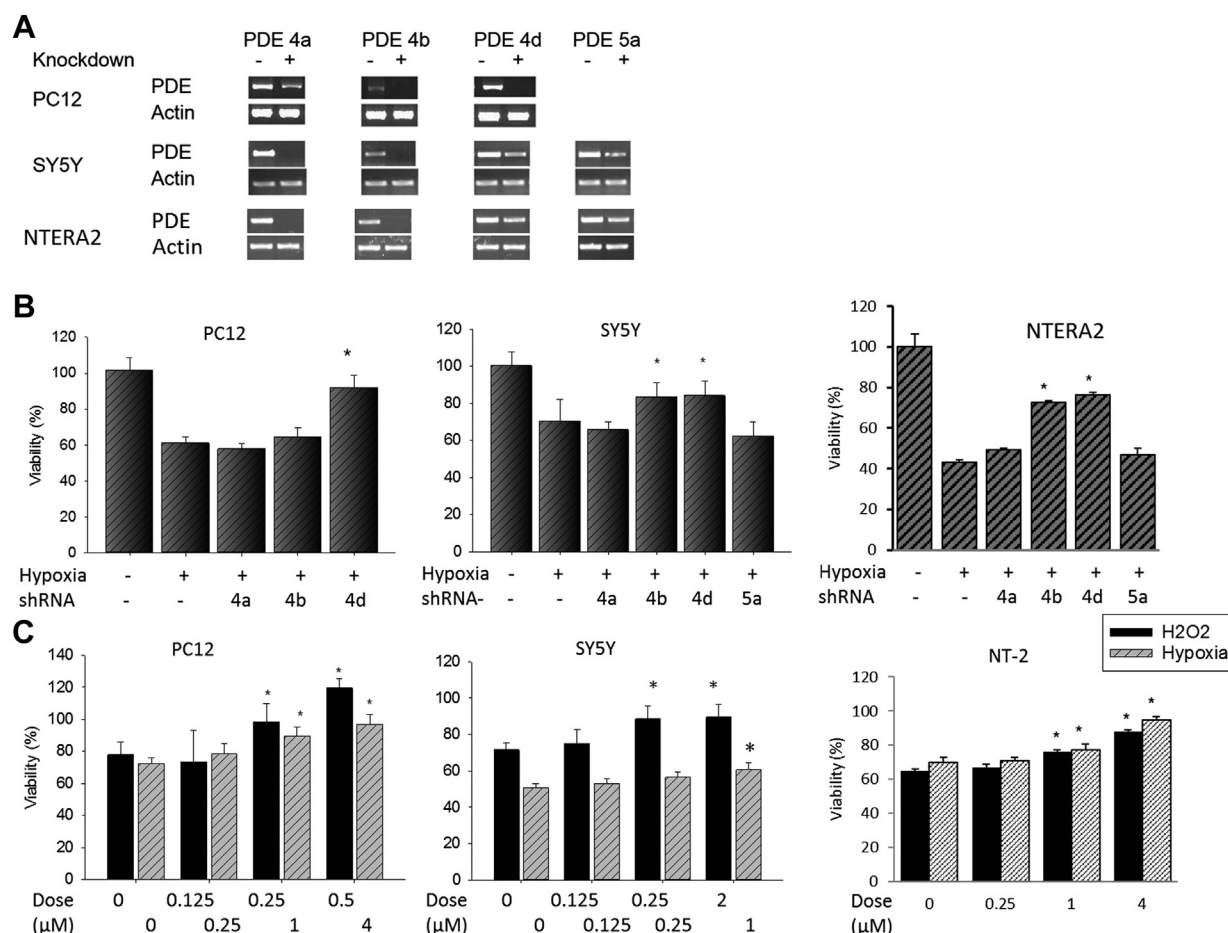


Figure 4. Panel A shows phosphodiesterase (PDE) 4a, 4b, 4d, and 5a expressions in knockdown PC12, SH-SY5Y, and NTERA2 cell lines by small interfering RNA. Reverse transcription polymerase chain reaction shows that these PDEs were successfully suppressed in these cells. Panel B shows the effect of hypoxia on PC12, SH-SY5Y, and NTERA2 cell lines with knockdown PDE expression (for PC12 and NTERA2 cells, $*P < .05$ vs PC12 cells [normal cells] under hypoxia; for SH-SY5Y cells, $*P < .05$ vs PDE4a or PDE5a knockdown cells). Inhibition of PDE4b and PDE4d showed a protective effect against hypoxia. Panel C shows the effect of rolipram against H₂O₂ toxicity and hypoxia in PC12, SH-SY5Y, and NT2 cell lines ($*P < .5$ vs group without rolipram treatment). shRNA = short hairpin RNA.

paraffin and cut into 5-μm sections. This sectioned tissue was used for histologic analysis. Sections were deparaffinized and blocked for 1 hour with 10% normal goat serum in phosphate buffered saline and 0.1% Triton X-100. Primary antibodies were incubated overnight: rabbit anti-neuronal NOS (1:100; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and mouse anti-NF (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). Fluorescence-conjugated secondary antibodies Alexa-488 (Life Technologies, Carlsbad, CA, USA) and Alexa-594 (Life Technologies) were incubated for 1 hour at $25 \pm 2^\circ\text{C}$. Nuclei were stained with 4',6-diamidino-2-phenylindole.

Image Analysis

Image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The threshold level of each eight-bit image was set to calculate only the neuronal NOS- or NF-positive area, and the total dorsal nerve area was calculated for each section.

Data Analysis

Data analysis was performed using SPSS 21 (SPSS, Inc, Chicago, IL, USA) and PowerLab software (ADInstruments). Comparisons between the experimental and control groups were performed using analysis of variance. Multiple groups were compared using the Tukey-Kramer test. The Mann-Whitney test was used for non-normally distributed analysis. Statistical significance was set at a P value less than 0.05.

RESULTS

Sildenafil Inhibits Neuronal Cell Death in Tissue Culture

After exposure to hypoxia and H₂O₂, sildenafil significantly increased cell viability in PC12, SH-SY5Y, and NT2 cells (Figures 1A, B and 2). Vardenafil had a more modest effect on protecting against hypoxia, whereas tadalafil had no effect.

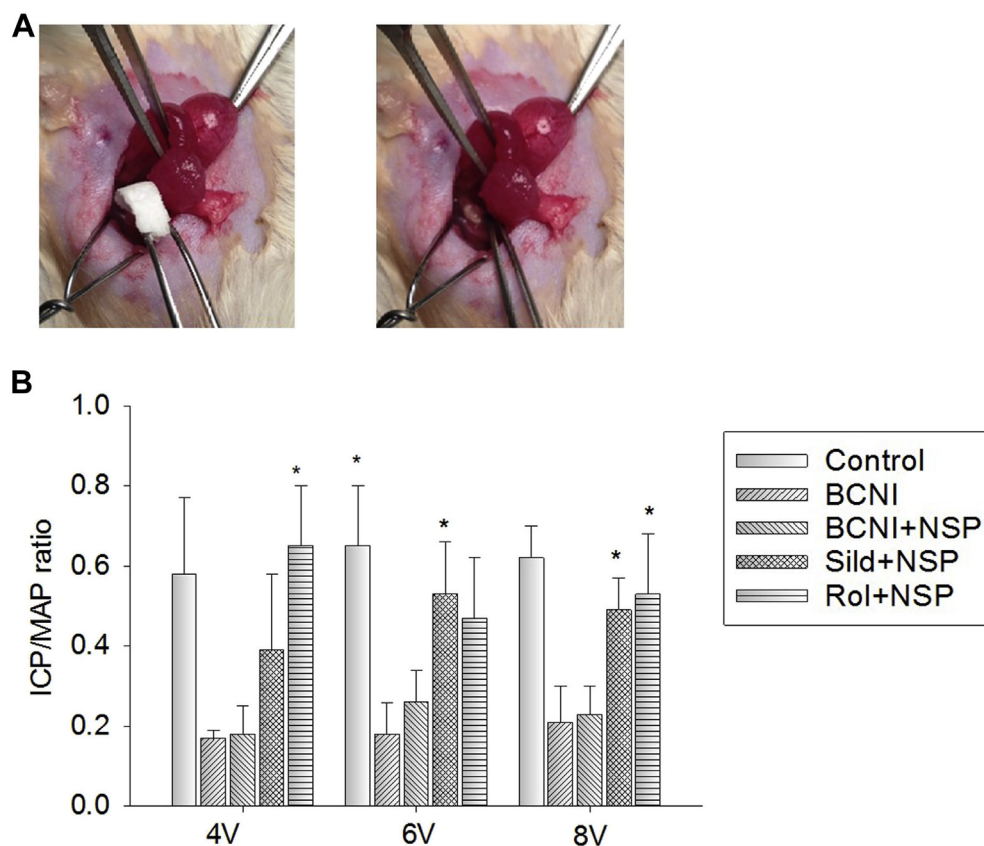


Figure 5. Panel A shows the application of nanospheres to the site of nerve injury. The nanosphere was used in a dry powder formulation, which dissolved immediately after placement. Panel B shows the ratio of intracavernosal pressure (ICP) to mean arterial pressure (MAP) in each group (* $P < .05$ vs group with bilateral cavernous nerve injury). The intracavernosal pressure response to electrostimulation of the cavernous nerve was improved in groups treated with sildenafil-loaded and with rolipram-loaded nanospheres compared with groups treated with bilateral cavernous nerve injury only and bilateral cavernous nerve injury and empty nanospheres. [Figure 5 is available in color at www.jsm.jsexmed.org.](http://www.jsm.jsexmed.org)

PDE4 and Neuronal Cell Viability

Semiquantitative reverse transcription PCR and Western blot demonstrated that PC12 did not express PDE5 (Figure 2A). This absence of PDE5 in PC12 suggested that sildenafil's protective effect against cell death is likely due to an off-target effect. Therefore, the expression of all known PDE isoforms was measured using reverse transcription PCR (Figure 2B).

To further investigate the underlying mechanism of sildenafil's protective effect against cell death in neuronal cell culture, we evaluated the downstream effectors of PDEs, cAMP, and cGMP after administration of PDE5I. In the sildenafil group, cAMP levels were significantly higher than those in the tadalafil- and vardenafil-treated groups (Figure 2C). However, all three PDE5Is had similar effects on cGMP (data not shown). Next, knockdown studies of PDE4a, PDE4b, PDE4d, and PDE5 were carried out (Figure 3A). The results showed that blocking PDE4 subtypes significantly inhibited cell death induced by hypoxia and H_2O_2 in PC12, SH-SY5Y, and NT2 cell lines (Figure 3B). More importantly, knocking down PDE5 in SH-SY5Y and NT2 cells had no effect on survival. We next studied the effect of rolipram, a PDE4I, on cell death. When PC12, SH-SY5Y, and NT2 cells were exposed to H_2O_2 or hypoxia, the decrease in the

viable cell count was significantly reversed by the administration of rolipram (Figure 3C).

In Vivo Studies

To assess the effect of sildenafil and rolipram in vivo, the BCNI rat model was used (Figure 4A). The ICP/MAP ratio was decreased in the BCNI group. Local delivery of sildenafil and rolipram using NSPs increased the ICP/MAP ratio significantly compared with the BCNI group (Figure 4B).

Penile tissue staining showed that the BCNI and BCNI + NSP groups had significantly lower NF and neuronal NOS content compared with the control group. The Sild + NSP and Rol + NSP groups showed a significantly higher NF content compared with the BCNI group (Figures 5 and 6). Real-time PCR data showed higher expression of NF, NOS, and actin in the Sil + NSP and Rol + NSP groups compared with the BCNI and BCNI + NSP groups (Figure 6A–C).

DISCUSSION

This study showed that sildenafil was more effective than tadalafil and vardenafil at protecting cells against cytotoxic nerve

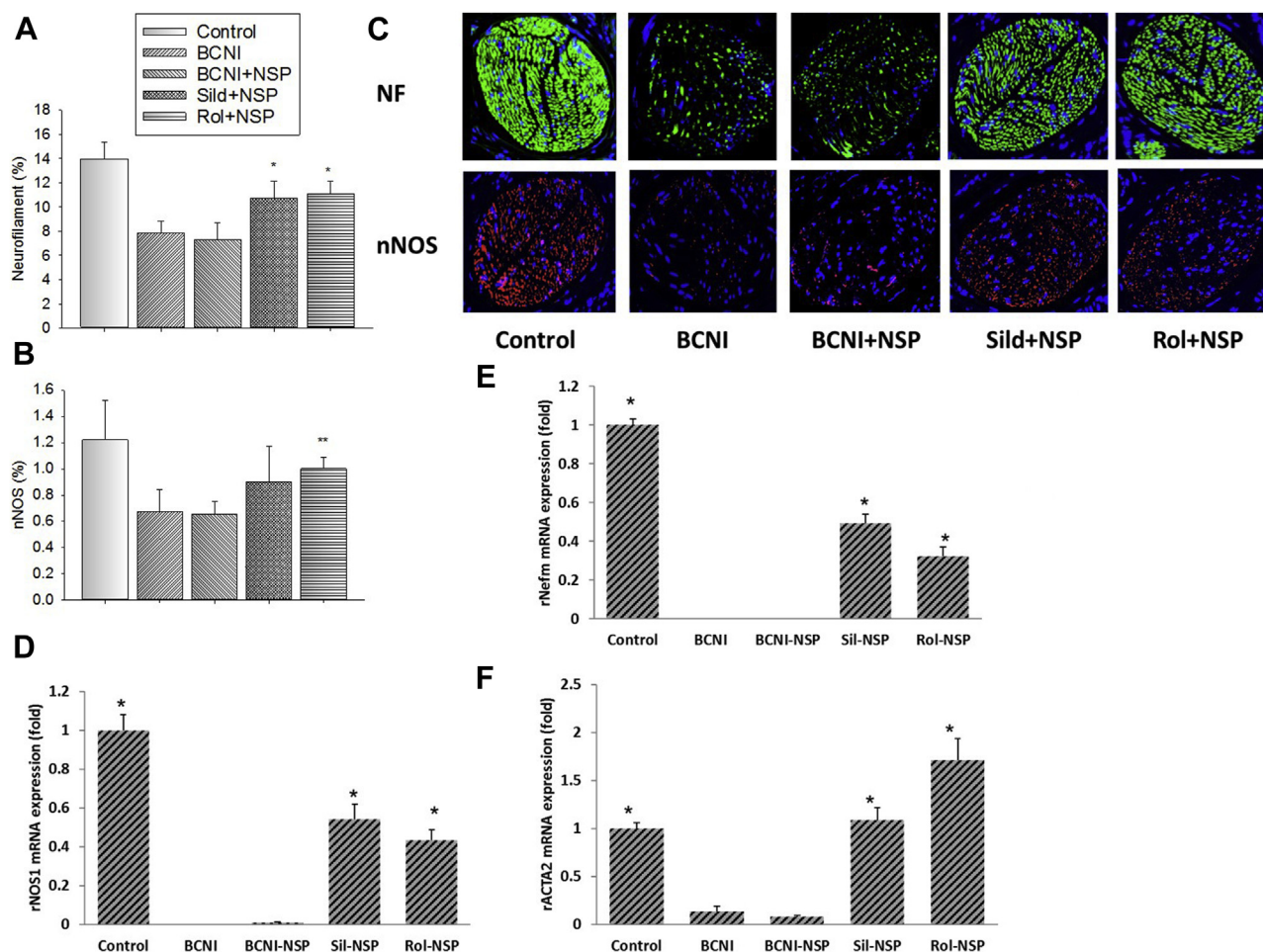


Figure 6. Panel A shows the percentage area of neurofilament (NF) expression in the penile dorsal nerve. Panel B shows the percentage area of neuronal nitric oxide synthase (nNOS) expression in the penile dorsal nerve. Panel C shows representative NF and nNOS staining for each group (original magnification $\times 400$). Nuclei were stained with 4',6-diamidino-2-phenylindole. Expressions of NF (D), NOS (E), and actin (F) in the mid-shaft of the penis were measured by real-time polymerase chain reaction (* $P < .05$ vs BCNI and BCNI + NSP groups; ** $P < .05$ vs BCNI + NSP group). BCNI = bilateral cavernous nerve injury; BCNI + NSP = bilateral cavernous nerve injury and immediate treatment with empty nanospheres; Rol + NSP = rolipram-loaded nanospheres; Sild + NSP = sildenafil-loaded nanospheres. Figure 6 is available in color at www.jsm.jsexmed.org.

damage in a concentration-dependent manner. This neuroprotective property of sildenafil is likely attributed to the off-target effect on other PDEs such as PDE4, as suggested from the selective expression of PDE4 isoform assays and the absence of PDE5 expression in these neuronal cell lines. These findings were further corroborated by the finding of increased cAMP levels, which has a known association with PDE4,²¹ in the sildenafil-treated group. PDE4 subgroup gene knockdown and treatment with rolipram, a PDE4 selective inhibitor, resulted in similar patterns of cell death, suggesting that PDE4 blockage might be responsible for the sildenafil-mediated prevention of neuronal cell death from hypoxia and reactive oxygen species.

When these conclusions were tested in an in vivo study, rats treated with sildenafil-loaded NSPs had improved histologic parameters compared with the BCNI group for NF, NOS, and smooth muscle content. This could reflect an improved efficacy of dorsal nerve preservation and vasorelaxation, two major factors

in improved EF. In the setting of post-RP ED, these results might be clinically applicable.

Even in the era of robotic surgery with various nerve-sparing techniques, a significant number of patients complain of ED after surgery.² Unfortunately, there is no consensus on the best agent or treatment regimen for penile rehabilitation.²² Among the three commonly used PDE5Is, vardenafil is the most potent.²³ However, consistent with pharmacokinetic data showing that sildenafil has the highest relative selectivity for PDE4 in its half-maximal inhibitory concentration for PDE5,²⁴ the present study found that sildenafil is most effective in neuronal cell protection, likely attributable to the inhibition of other PDEs.

Although used as a treatment modality in respiratory disease,²⁵ PDE4Is have not been extensively investigated in sexual medicine.²⁶ However, emerging evidence from a series of in vivo studies strongly suggests a neuroprotective role of PDE4Is by the cAMP pathway. For example, Lau et al²⁷ in a study of late larval stage

lampreys reported that administration of a cAMP analogue at the time of spinal cord transection increased the number of axons, prevented abnormal axonal growth patterns, increased levels of synaptotagmin within axon tips, and increased the survival of neurons. Similarly, rolipram has been found to have a neuroprotective effect after spinal cord injury in mice.^{28,29} Schaal et al²⁹ reported that rolipram reverses neuronal injury-induced PDE4B1 and PDE4A5 production, PDE4A5 phosphorylation, monocyte chemoattractant protein-1 expression, and immune cell infiltration. Taken together, these results strongly suggest that blocking PDE4 could be effective in preventing neuronal cell death. The results of the present study are consistent with these data, because cell viability in PDE4b and PDE4d knockdown cells was significantly higher than that in cells with PDE5a knockdown cells.

Because intraoperative cavernous nerve damage is believed to account for a large proportion of post-RP ED, the use of PDE4Is in conjunction with traditional PDE5Is should be explored in these patients. This combination has been studied in the pulmonology literature. Treatment using low-dose roflumilast, a PDE4I, in combination with a PDE5I has shown a favorable response in a model that tested allergen-induced airway hypersensitivity.²⁵ Active research on pharmacologic intervention targeting PDE4 and PDE5 is underway, so adequately mixed inhibitors could benefit patients with neurogenic ED after RP.

In addition to the introduction of a potential therapeutic target (PDE4) in ED, this rat model study demonstrates a novel route of PDEI administration. We used intraoperative administration of NSP-loaded PDEI to the site of nerve injury. This resulted in increased smooth muscle content and reversal of adverse histologic changes after nerve injury.

In this NSP delivery system, the total local release of sildenafil and rolipram from the NSP was 81% and 85%, respectively, at 10 days (Supplemental Appendix). Liquid chromatographic-mass spectrometric analysis of hourly rat blood samples did not show traces of sildenafil at any time point. Thus, the localized drug administration with extended release could be superior to systemic oral intake in treating patients with cavernous nerve injury because of direct delivery and fewer systemic side effects. Therefore, this nanotechnology might become a convenient alternative or adjunct to traditional oral dosing. Timing of penile rehabilitation after RP is controversial, and in most trials it starts 10 to 14 days after surgery. Based on this study, we believe that starting penile rehabilitation the same day as surgery might have a better outcome.

This study has some relevant limitations. First, it is essential to keep in mind that post-RP ED is a multifactorial disease process that cannot be sufficiently explained by the neurogenic model alone. Second, the studied PDE gene families were limited to PDE4 and PDE5, and possible modulating effects from other PDE families on cAMP were not evaluated in this study. Third, only one dose of sildenafil and of rolipram was used in the in vivo study to show the safety and efficacy of the NSP delivery system. However, the use of a single dose might be justified, because the goal of this study was to establish a proof of concept. Additional studies are required to

determine clinically appropriate doses when given locally by NSP. Fourth, we did not compare the efficacy of local NSP delivery of rolipram or sildenafil with systemic administration of these drugs. Therefore, we cannot conclude that local delivery is superior to oral administration in this animal model. Fifth, because direct evaluation of the major pelvic ganglion and the cavernous nerve was not performed,³⁰ it is difficult to definitively interpret the favorable outcome of local therapy as the direct effect of neurogenesis and reinnervation of the cavernous nerve. The combination of sildenafil and rolipram was not tested in this study, and therefore we cannot report on any synergistic effect that might exist.

CONCLUSION

This study demonstrated a superior neuroprotective effect for sildenafil compared with vardenafil and tadalafil. The therapeutic targeting of PDE4 should be considered for men with ED after RP. Furthermore, application of NSP-loaded sildenafil or rolipram to the site of nerve injury resulted in improved EF and fewer adverse histologic changes.

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Conflicts of Interest: The authors report no conflicts of interest.

Funding: None.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jsxm.2016.10.003>.