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Inhibition of Rho-Kinase Improves Erectile Function, Increases Nitric Oxide Signaling and Decreases Penile Apoptosis in a Rat Model of Cavernous Nerve Injury

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Abstract

Purpose—Bilateral cavernous nerve injury results in up-regulation of ROCK signaling in the penis. This is linked to erectile dysfunction in an animal model of post-prostatectomy erectile dysfunction. We evaluated whether daily treatment with the ROCK inhibitor Y-27632 (Tocris Bioscience, Ellisville, Missouri) would prevent erectile dysfunction in a rat model of bilateral cavernous nerve injury.

Materials and Methods—Sprague-Dawley® rats underwent surgery to create sham (14) or bilateral (27) cavernous nerve injury. In the injury group 13 rats received treatment with Y-27632 (5 mg/kg twice daily) and 14 received vehicle. At 14 days after injury, rats underwent cavernous nerve stimulation to determine erectile function. Penes were assessed for neuronal and nitric oxide synthase membrane-endothelial nitric oxide synthase. ROCK2 was assessed by Western blot. Cyclic guanosine monophosphate was determined by enzyme-linked immunosorbent assay. Cavernous homogenates were tested for ROCK and protein kinase G enzymatic activity. Penile apoptosis was evaluated using the Apostain technique (Alexis, San Diego, California). Data were analyzed on ROCK using ANOVA and the t test.

Results—While erectile function was decreased in rats with bilateral cavernous nerve injury, daily administration of Y-27632 improved erectile responses. Injury decreased neuronal and nitric oxide synthase membrane-endothelial nitric oxide synthase but ROCK2 was significantly increased. Y-27632 treatment restored neuronal nitric oxide synthase, nitric oxide synthase

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membrane-endothelial nitric oxide synthase and cyclic guanosine monophosphate levels, and protein kinase G activity. Treatment significantly decreased ROCK2 protein and ROCK activity. There were significantly fewer apoptotic cells after treatment than in injured controls.

Conclusions—These results provide evidence for up-regulation of the RhoA/ROCK signaling pathway with detrimental effects on erectile function after bilateral cavernous nerve injury. ROCK inhibition improved erectile dysfunction associated with bilateral cavernous nerve injury by preserving penile nitric oxide bioavailability and decreasing penile apoptosis.

Keywords

penis; apoptosis; prostatectomy; erectile dysfunction; rho-associated kinases

Erectile dysfunction is a highly prevalent complication of RP associated with significantly decreased quality of life.[1,2] The putative mechanisms responsible for post-RP ED are the focus of current research and remain incompletely defined. To preserve potency in patients who undergo RP, research efforts have focused on designing pharmacological and gene/stem cell based therapies to improve neuroregeneration after BCNI.[3] Other pharmacological approaches have aimed to prevent secondary collagen deposition/fibrosis of the penile vasculature, and associated endothelial and smooth muscle cell loss in the corpus cavernosum by targeting antifibrotic and anti-apoptotic pathways.[4] In the clinical setting available pharmacological options seek to improve corporeal blood flow and oxygenation, thus, preserving penile integrity. Unfortunately, the results of these efforts are disappointing and the need persists for potency preserving strategies after RP.

RhoA is a member of the Ras guanosine triphosphatase family and a key intracellular regulator of cellular responses, including migration and contraction of smooth muscle.[5] ROCK, the major downstream target of RhoA, consists of 2 isoforms (ROCK1 and ROCK2). After activation, RhoA/ROCK enhances calcium sensitivity through phosphorylation of MLC phosphatase targeting subunit and inhibits MLC phosphatase activity, resulting in increased MLC phosphorylation and smooth muscle contraction.[5] We previously investigated the role of RhoA/ROCK signaling in the penis after BCNI.[6] Nerve injury caused RhoA/ROCK up-regulation and activation in the penis, mainly the ROCK2 isoform, which was associated with neurogenic ED. Inhibition of RhoA/ROCK in pathophysiological disease states associated with vasculogenic ED previously resulted in improved erectile function by directly targeting the hypercontractile state of penile smooth muscle.[7–10] Additionally, ROCK inhibition regulates growth cone formation and axonal elongation after optic nerve injury.[11,12] However, to our knowledge the role of RhoA/ROCK in autonomic regulation of the penis has not been examined.

We evaluated the various mechanisms associated with the development of neurogenic ED in a rat model of post-RP ED, including up-regulated ROCK, corporeal apoptosis, and decreased NO bioavailability and the specific NOS isoforms responsible for NO loss. Therefore, we sought to identify the molecular mechanisms by which ROCK inhibition may suppress ED in a rat model of post-RP ED.

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MATERIALS AND METHODS

Experimental Design

We used 41 adult male Sprague-Dawley rats weighing 200 to 250 gm. All experiments were done in accordance with Johns Hopkins University School of Medicine guidelines for animal care and use. Rats were treated with sham surgery (14), BCNI (14) or BCNI plus twice daily intra-peritoneal injections with the nonselective ROCK1 and 2 inhibitor Y-27632 (5 mg/kg) (13).[13] Sham operated and BCNI rats were treated intraperitoneally with vehicle (saline). Treatment was stopped 72 hours (washout) before erectile function testing, which was performed 14 days after surgery.[13] Following erectile function testing, the rats were sacrificed and the penes were removed for further analysis.

Bilateral CN Injury

Using ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia, the prostate was exposed via a midline laparotomy and the CNs were identified.[6,14] BCNI was induced by applying a forceps for 3 × 15 seconds to the nerve 2 to 3 mm distal to the major pelvic ganglion.

Erectile Response Measurement

Using anesthesia, the CN was identified. The left cru was cannulated with a 25 gauge needle connected to a pressure transducer to measure ICP. The right carotid artery was cannulated for continuous measurement of MAP. The CN distal to the crush injury was stimulated with a square pulse stimulator (Grass Instruments, Quincy, Massachusetts) at a frequency of 20 Hz, with a 0.5-millisecond duration and a 30-second pulse width at increasing voltages (2, 4, 6 and 8 V) for 1 minute. The outcome parameters were ICP/MAP, peak ICP, total erectile response (AUC) and time for ICP to attain 80% of peak ICP.[14]

Western Blot Analysis

The penis was excised, the urethra was removed and the corpus cavernosum was homogenized in tris-HCl buffer (pH 7.5). Cytosolic and membrane fractions were isolated for cytosolic nNOS, ROCK2, and m-eNOS protein by Western blot. Protein (50 μg) was loaded on 4% to 20% tris-HCl gel (Bio-Rad®) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibodies (nNOS 1:500, m-eNOS 1:250, ROCK2 1:500 and GAPDH 1:10,000) (BD™ Bioscience and Amersham, Piscataway, New Jersey) overnight at 4C.[6,8] Membranes were incubated with horseradish peroxidase linked secondary antibody and visualized with an enhanced chemiluminescence kit (Amersham). Densitometry results were quantified using Image J (National Institutes of Health) and normalized to GAPDH.

ROCK and PKG Activity Assay

Corporeal tissue was homogenized in tris-HCl buffer (pH 7.5). Total ROCK activity was analyzed in the presence of 0.1 mM adenosine triphosphate by a ROCK activity assay kit (Cyclex, Nagano, Japan). PKG activity was determined by colorimetric analysis of protein (30 μg per sample) according to manufacturer instructions (Cyclex). ROCK and PKG

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activity is expressed as a percent of total sham operated ROCK and PKG activity, respectively.[6,15]

cGMP Measurement

Penile tissue was homogenized in ice-cold 6% trichloroacetic acid (pH 4.0) and centrifuged at 1,500 × gravity for 10 minutes at 4C. Trichloroacetic acid was extracted with H_2O saturated diethyl ether. Samples were assayed for cGMP levels in pmol/mg protein using an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, Michigan).[6,15]

Apoptosis Determination

At 14 days after BCNI the penes were paraffin embedded and stained using the Apostain technique. Sections were deparaffinized, rehydrated and blocked for nonspecific antibody binding in 0.1% bovine serum albumin and 1% nonfat dry milk. They were incubated overnight with F7–26 anti-single strand DNA specific mouse anti-rat antibody (Zymed, South San Francisco, California). The slides were washed and incubated for an hour with bio-tinylated rat anti-mouse antibody. Secondary antibody was visualized with avidin-biotin-peroxidase complex using an Elite® ABC Kit and with diaminobenzidine. Sections were counterstained with hematoxylin, viewed with a Nikon® microscope equipped with a camera. Images were captured using QCapture software (QImaging®). Apoptotic cells were counted in blinded fashion.[16] Four rats were examined in the sham operated and BCNI plus drug groups, and 5 were examined in the BCNI group. Six sections per penis were analyzed (3 controls and 3 stained samples) with approximately 40 high power fields per section.

Statistical Analysis

Data are shown as the mean ± SEM. Differences between multiple groups were compared by ANOVA, followed by the Tukey multiple comparisons test. Two-group analysis was performed by the unpaired t test and <0.05 was considered statistically significant.

RESULTS

ROCK Inhibition Effect on Erectile Response

There was a significant decrease in the erectile response in BCNI rats compared to sham operated rats in all parameters measured ($p < 0.05$, fig. 1). Y-27632 treatment in 7 rats significantly improved erectile function greater than twofold compared to that in 8 rats in the BCNI group ($p < 0.05$) and it did not differ from that in 8 sham operated rats. Peak ICP and ICP/MAP significantly increased greater than twofold in rats with BCNI plus Y-27632 compared to untreated BCNI rats. Erectile responses were not different from those of sham operated rats ($p < 0.05$, fig. 1, *A* and *B*). Total ICP in BCNI plus Y-27632 rats was increased 2 to 3 times higher than in BCNI rats ($p < 0.05$, fig. 1, *C*). Importantly, the time required to attain 80% of maximal ICP in Y-27632 treated rats did not differ from that in sham operated rats ($p < 0.05$, fig. 1, *D*).

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ROCK Protein and Activity Measurement

ROCK2 protein levels were 2.5-fold higher in BCNI than in sham operated penes in 6 rats per group ($p = 0.0029$, fig. 2, *A*). Daily treatment with Y-27632 decreased ROCK2 protein 62% compared to that in the penes of BCNI rats ($p = 0.0236$, fig. 2, *A*). Penile total ROCK activity was 23% higher in BCNI rat penes compared to that in sham operated penes in 5 rats per group ($p = 0.0463$, fig. 2, *B*). Treatment with ROCK inhibitor significantly decreased total ROCK activity in BCNI rats to 8% less than that in sham operated rats ($p = 0.0059$, fig. 2, *B*).

NOS Protein

Penile nNOS protein levels were decreased 70% in the BCNI group compared to the sham operated group in 6 rats per group ($p < 0.0001$, fig. 3, *A*). Similarly, m-eNOS protein was decreased by 50% in the 6 rats in the BCNI group ($p = 0.0346$, fig. 3, *B*). After Y-27632 treatment, nNOS protein in 6 BCNI rats doubled compared to that in 6 untreated BCNI rats ($p = 0.0346$, fig. 3, *A*). Also, Y-27632 treatment caused a profound twofold increase in m-eNOS protein, which that was greater than that in sham operated and untreated BCNI rats ($p = 0.0317$ and 0.0043, respectively, fig. 3, *B*).

cGMP Concentration and PKG Activity

cGMP levels and PKG activity were significantly decreased by 30% and 45%, respectively, in the 6 BCNI rat penes ($p = 0.0246$ and 0.0397, fig. 4). Y-27632 treatment resulted in a 7% increase in cGMP and a 50% increase in PKG activity. Each was significantly higher than in untreated BCNI penes in 6 rats per group ($p = 0.0038$ and 0.0017, respectively, fig. 4).

Apoptosis

BCNI led to increased apoptosis in endothelial and smooth muscle cells, while treatment decreased the number of apoptotic cells by about 90% in 5 preparations per group (fig. 5, *A* to *C*). The number of apoptotic positive endothelium and smooth muscle cells in BCNI rats was quantitatively higher than in sham operated rats, while Y-27632 significantly decreased the number of apoptotic cells in BCNI penes ($p = 0.0001$, fig. 5, *D*).

DISCUSSION

To our knowledge this report represents the first study to demonstrate that 2-week inhibition of RhoA/ROCK prevented ED onset in a post-RP BCNI rat model. Daily administration of Y-27632 resulted in significant improvement in CN mediated erectile responses after BCNI. The protein level of ROCK2 and total ROCK activity in cavernous tissue was decreased after Y-27632 treatment. Also, Y-27632 improved nNOS and m-eNOS protein levels, and increased cGMP and PKG activity. Finally, Y-27632 therapy suppressed the number of apoptotic cells after nerve injury.

Altered RhoA/ROCK activity in the penis is a pathogenetic factor contributing to ED development. We previously reported that BCNI increased RhoA/ROCK activity through increased ROCK2 protein in the corporeal vasculature and decreased nNOS in the dorsal penile nerve of the penis.[6] Furthermore, BCNI is associated with transforming growth

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factor-β1 induced corporeal fibrosis with loss of smooth muscle.[17] Others observed that increased ROCK activity contributes to penile smooth muscle hyperactivity in animals with neurodegenerative conditions, such as diabetes and aging.[8,18] Treating diabetic animals with atorvastatin successfully improved sildenafil induced penile erection by inhibiting RhoA/ROCK signaling in the penis.[9] Also, 2-week treatment with a ROCK inhibitor restored erectile function in diabetic rats and decreased corporeal apoptosis.[19] Thus, as shown in the current series and those of others, improvements in erectile function via ROCK inhibition provides further evidence that ROCK is a promising target for ED intervention.

RhoA/ROCK up-regulation suppresses eNOS activity and expression in the penis. We tested the effect of 2-week Y-27632 administration on the amount of m-eNOS, and the level and activity of the downstream effectors of NO, namely cGMP and PKG.[8,15,20] BCNI induced a 50% decrease in m-eNOS in the corpus cavernosum. This resulted in reduced second messenger cGMP, indicating decreased biosynthesis of NO, which translates into less PKG activity. PKG initiates the cellular events that decrease intracellular calcium and cause smooth muscle relaxation.[21] Interestingly, PKG also has a regulatory role in smooth muscle contraction by decreasing ROCK activity through phosphorylation of its upstream regulatory guanosine triphosphatase RhoA.[22] Therefore, it appears that the increase in ROCK activity after BCNI down-regulates the NO-cGMP pathway and results in greater ROCK activity, further down-regulating eNOS activity, and NO and cGMP bioavailability. Daily Y-27632 treatment prevented the decrease in m-eNOS and preserved cGMP concentration and PKG activity. Other groups used long-term phosphodiesterase type 5 inhibition after BCNI and noted improved erectile function with increases in corporeal cGMP and decreased apoptosis.[23] We hypothesize that this may have occurred through a ROCK dependent mechanism.

In addition to eNOS, the level of nNOS in the corpus cavernosum was decreased 60% after nerve injury. BCNI reduced nNOS protein levels in the penile dorsal nerve as a result of neurodegeneration after neuropraxia.[24,25] Y-27632 administration prevented a decrease in nNOS protein in BCNI rats, as measured by Western blot, but ROCK inhibition only partially prevented decreased nNOS. The preservation of nNOS protein expression and neurogenic mediated erectile responses suggest that the autonomic innervation of the corpus cavernosum is partially conserved in animals treated with ROCK inhibitor. It remains to be determined whether the preserved innervation of the corpus cavernosum is the result of preventing nerve degeneration or encouraging nerve regeneration and what the putative role of RhoA/ROCK is in the CN and major pelvic ganglion.

As previously noted, BCNI results in an increased apoptotic cell count in the corpus cavernosum.[16,26] Y-27632 treatment suppressed apoptosis about 90% after BCNI. This may be the result of preserving cGMP and PKG at physiological levels due to a maintained NO supply.[27] Additionally, ROCK has an important role in membrane blebbing during the process of apoptosis and ROCK inhibition may directly decrease apoptosis in the penis.[28] The suppression of apoptosis and preservation of corpus cavernosum architecture may contribute to the improvement in erectile function.

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Penile hemodynamics and nNOS levels were preserved after daily Y-27632 treatment. However, a limitation is that it remains undetermined whether this was an effect of nerve preservation or regeneration. Further studies of the expression and role of the RhoA/ROCK pathway in the major pelvic ganglion and penile innervation are ongoing at our laboratory to elucidate these mechanisms. Another limitation is the short time from BCNI to functional evaluation. More studies are required to determine whether the beneficial effects of ROCK inhibition persist. Nevertheless, we believe that ROCK may be an interesting target in clinical penile rehabilitation studies after RP.

CONCLUSIONS

The results of our study provide further evidence that the combined effect of up-regulation of the RhoA/ROCK signaling pathway and decreased eNOS/nNOS levels contribute to end organ dysfunction after BCNI. We noted beneficial effects of 2-week Y-27632 administration on erectile function after BCNI, which appeared to result from the preservation of penile NO bioavailability via eNOS and nNOS, and decreased corporeal apoptosis. Inhibition of the RhoA/ROCK pathway may represent a suitable therapeutic approach to post-RP ED.

Abbreviations and Acronyms

BCNI	bilateral CN injury
cGMP	cyclic guanosine monophosphate
CN	cavernous nerve
ED	erectile dysfunction
eNOS	endothelial NOS
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ICP	intracavernous pressure
MAP	mean arterial pressure
m-eNOS	membranous eNOS
MLC	myosin light chain
nNOS	neuronal NOS
NO	nitric oxide
NOS	NO synthase
PKG	protein kinase G
ROCK	Rho-associated protein kinase
RP	radical prostatectomy

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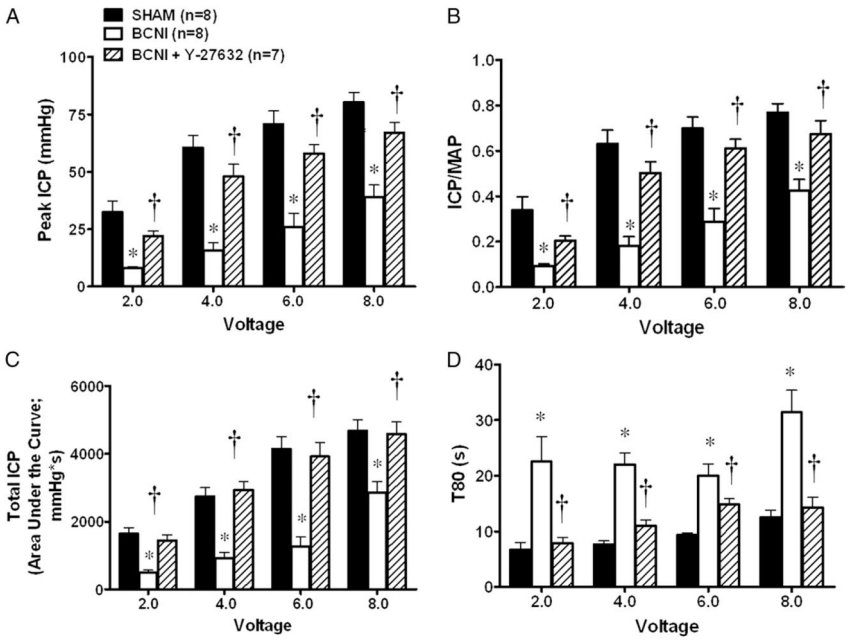


Figure 1.

In vivo erection experiments were done 14 days after CN injury via CN electrostimulation. Bar graphs shows voltage dependent erectile responses measured by peak ICP in mm Hg (*A*), ICP/MAP (*B*), total ICP or erectile curve AUC (*C*) and time in seconds (*s*) to attain 80% of peak ICP (*D*) after 1-minute CN stimulation in all groups. Asterisk indicates significant difference vs sham operated rats ($p < 0.05$). Dagger indicates significant difference vs BCNI rats without treatment ($p < 0.05$).

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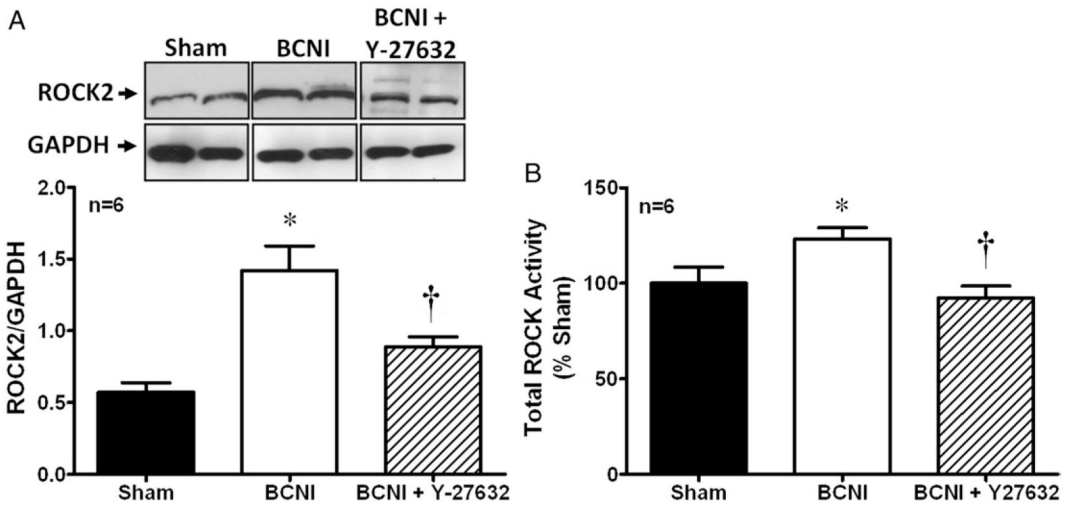


Figure 2.

Western blot reveals ROCK2 proteins in penile tissue of all groups (*A*). Total ROCK activity was decreased in penes treated with Y-27632, as determined by activity assays in 6 tissue samples per group (*B*). Asterisk indicates significant difference vs sham operated rats ($p < 0.01$). Dagger indicates significant difference vs BCNI rats without treatment ($p < 0.05$).

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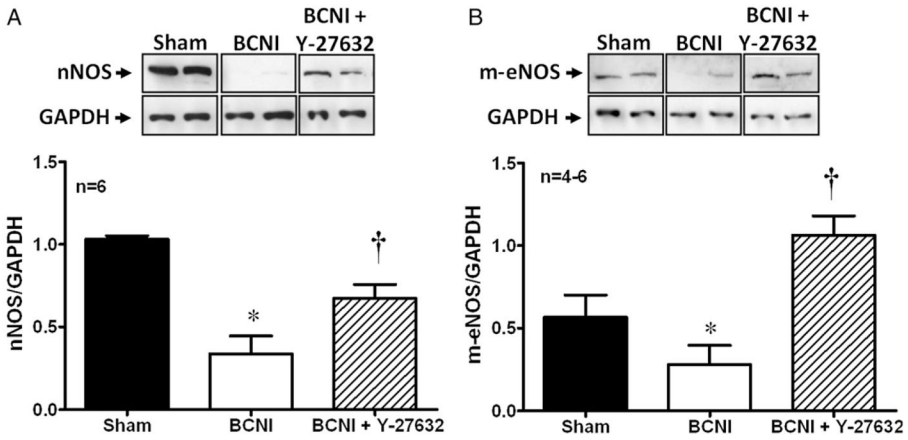


Figure 3.

Representative Western blots of nNOS and GAPDH protein (*A*), and m-eNOS and GAPDH protein (*B*) in penile tissue samples of all groups. Densitometry analysis shows nNOS-to-GAPDH (*A*) and m-eNOS-to-GAPDH (*B*) ratios in penile tissue samples of all groups. Asterisk indicates significant difference vs sham operated rats. Dagger indicates significant difference vs BCNI rats without treatment $p < 0.05$).

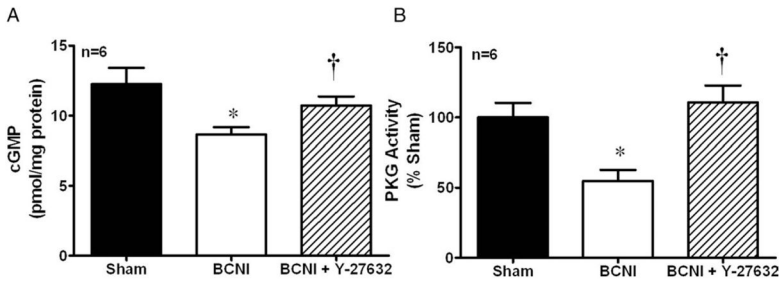


Figure 4.

cGMP levels (*A*) and cGMP dependent PKG activity (*B*) in rat penile tissue samples of all groups. Asterisk indicates significant differenced vs sham operated rats ($p < 0.05$). Dagger indicates significant difference vs BCNI rats without treatment ($p < 0.05$).

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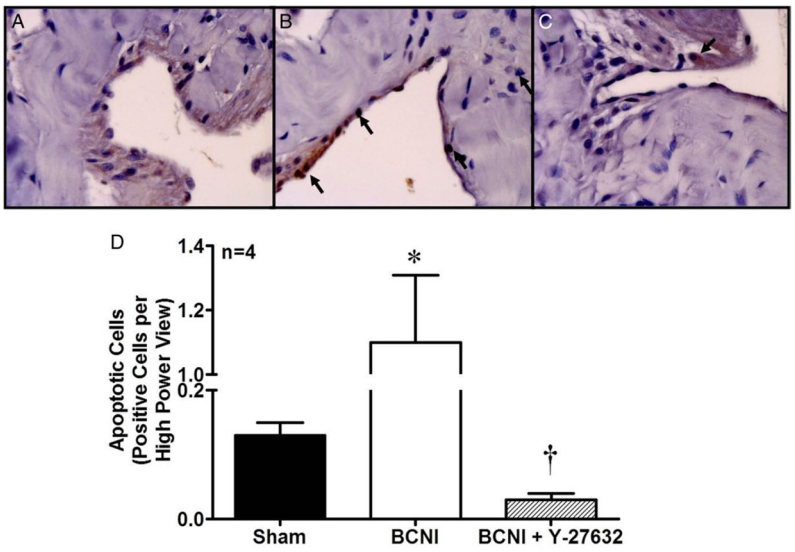


Figure 5.

Representative images reveal corpus cavernosum stained with Apostain (brown areas) and counterstained with hematoxylin in sham operated (*A*), BCNI (*B*) and Y-27632 treated (*C*) rats. Arrows indicate apoptotic cells with brown stained nuclei on sections of penis with BCNI (*B*). Reduced from ×40 (*A* to *C*). Bar graph shows that ROCK inhibition significantly decreased apoptotic cell death in 4 tissue samples per group (*D*). Asterisk indicates significant difference vs sham operated rats ($p < 0.0001$). Dagger indicates significant difference vs BCNI rats without treatment ($p < 0.0001$).