saRNA Guided iNOS Up-Regulation Improves Erectile Function of Diabetic Rats

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Abbreviations and Acronyms

cGMP = cyclic guanosine monophosphate
CSMC = cavernous smooth muscle cell
ds = double stranded
ED = erectile dysfunction
eNOS = endothelial NOS
ICP = intracavernous pressure
iNOS = inducible NOS
MOI = multiplicity of infection
nNOS = neuronal NOS
NO = nitric oxide
NOS = NO synthase
p- = phosphorylated
PCR = polymerase chain reaction
PDE5 = phosphodiesterase type 5
RNAa = RNA activation
RT-PCR = reverse transcriptase-PCR
saRNA = small activating RNA
shRNA = short hairpin RNA
STZ = streptozocin
TSS = transcription start site

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Purpose: Promoter targeted saRNAs mediate sequence specific up-regulation of gene expression. We explored the therapeutic effect of RNA activation mediated iNOS gene activation on improving erectile function in a rat model of diabetes mellitus.
Materials and Methods: An optimal saRNA sequence specific for iNOS promoter was cloned into an adenoviral vector, resulting in AdU6/shiNOS and AdU6/shControl. The corresponding viruses were used to transduce cultured rat cavernous smooth muscle cells. Streptozotocin induced diabetes models were established in rats and used to test the effects of intracavernous delivery of iNOS saRNA viruses on erectile function. iNOS expression in the cavernous smooth muscle cells or penile tissue of treated rats was assessed by reverse transcriptase-polymerase chain reaction and Western blot. Cyclic guanosine monophosphate was analyzed by enzyme-linked immunosorbent assay. Intracavernous pressure in response to cavernous nerve stimulation was measured using a data acquisition system on post-injection days 1, 3, 5, 7, 10 and 14.
Results: Adenovirus mediated expression of iNOS saRNA caused sustained up-regulation of iNOS in cavernous smooth muscle cells. Intracavernous injection of AdU6/shiNOS activated iNOS expression in vivo and significantly increased peak intracavernous pressure in streptozotocin induced diabetic rats via nitric oxide/intracellular cyclic guanosine monophosphate activation.
Conclusions: Results show that saRNA mediated iNOS over expression in the penis can restore erectile function in streptozocin diabetic rats via the nitric oxide-cyclic guanosine monophosphate pathway.

Key Words: penis; erectile dysfunction; RNA; diabetes mellitus, experimental; nitric oxide synthase type II

Erectile dysfunction is a common problem affecting about 50% of men 40 to 70 years old.[1] It often develops as a complication of diabetes with a 27% to 75% prevalence in men with diabetes.[2–4] Current pharmacological treatments for ED include oral, intracavernous and intraurethral administration of erectogenic drugs.[5]

The development of PDE5 inhibitors revolutionized ED management.[5,6] Orally administered PDE5 inhibitors are effective in most ED cases and less invasive than other treatment modes.[5] Local intracavernous and intraurethral pharmacotherapy is a second line treatment in cases of failure of or contraindication to oral pharmacotherapy.[5] Men with diabetes who have more severe forms of ED are less responsive to current pharmacological therapy.[4] Moreover, the

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drugs require on demand access and have limited effectiveness for diabetic ED that develops after prostatectomy or due to severe veno-occlusive disease with only 50% to 60% of these cases benefiting from PDE5 inhibitor therapy.[7] Therefore, better alternatives are needed to treat ED.

NO, which is synthesized by NOS, is a postganglionic nonadrenergic neurotransmitter and an essential mediator of penile erection. After release into the corpus cavernosum, NO diffuses into the vasculature and adjacent trabecular smooth musculature of the penis, where it stimulates cGMP activity and leads to decreased intracellular Ca^{2+} and to smooth muscle cell relaxation with subsequent penile erection.[8] The 3 NOS isoforms are eNOS, nNOS and iNOS.[9,10] All 3 isoforms have been investigated as potential gene therapy targets to modulate the erectile response.[10] Of the 3 isoforms iNOS does not directly intervene with physiological penile erection but it is Ca^{2+} independent and can produce NO in a sustained manner at a high concentration.[11] Thus, iNOS over expression in the corpus cavernosum of the penis would potentially reverse ED.

Recently, some researchers reported that dsRNA targeting promoter sequences could induce a phenomenon termed RNAa, which is sequence specific transcriptional gene activation, and such dsRNA molecules are termed saRNAs.[12,13] Several studies suggest that dsRNA could activate tumor suppressor genes and inhibit tumor progress, including prostate,[12] bladder and lung cancer,[14] and hepatocellular carcinoma.[15] Activating commonly dysregulated genes with saRNAs offer a practical, cost-effective approach to control gene expression. Thus, RNAa may hold great promise as a therapeutic option for treating disease.

To test the hypothesis that iNOS RNAa in the penis could improve erectile function, we used an adenovirus to mediate the expression of an iNOS saRNA in cultured CSMCs and in the penis of diabetic rats. iNOS saRNA activated iNOS expression in vitro and in vivo, and restored erectile function in diabetic rats.

MATERIALS AND METHODS

CSMC Isolation and Culture

Rat CSMCs were isolated from 3-month-old rats and cultured as previously described.[16] Cells reached confluence in approximately 5 days and were passaged every 5 to 6 days. Cells at passages 3 and 4 were used in this study.

Construction of iNOS Specific saRNA Adenovirus Vector

The iNOS promoter sequence was obtained from Ensembl genome databases (http://useast.ensembl.org/index.html) and analyzed against several genome and sequence databases, including dbTSS (http://dbtss.hgc.jp/), AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=mouse&q=Ngfb) and the UCSC Genome Browser (http://genome.ucsc.edu/), to determine the TSS. The program assigned weighted scores to all possible targets and returned a list of targets ranked by sum scores. A candidate target sequence was ACTATGCTGCCCAAACTAA at a location between −612 and −594 bp relative to the TSS of the iNOS gene. Recombinant adenovirus (AdU6/shiNOS) generation, amplification and titer were performed according to the method of Li et al.[17] A control shRNA sequence was also cloned to generate control adenovirus (AdU6/shControl).

Adenovirus Infection

Cells were incubated with the corresponding virus (AdU6/shiNOS or AdU6/shControl) at an MOI of 25, 50 or 75 at 37C. After adsorption for 2 hours, 2 ml fresh growth medium were added and cells were incubated for 72 hours before harvest. Experiments were performed using virus at an MOI of 75 except as otherwise indicated.

Diabetes Induction in Rat

Procedures were approved by the animal care and use committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. A total of 117 male Sprague-Dawley® rats at age 8 weeks were randomly divided into 2 groups. The nonSTZ group of 13 rats received intraperitoneal injection of citrate buffer (50 mM sodium citrate, pH 4.5). The STZ group of 104 rats received intraperitoneal injection of 60 mg/kg STZ (Sigma®) in citrate buffer (50 mM sodium citrate, pH 4.5).[18] At 72 hours after STZ injection rats with a blood glucose of greater than 16.7 mmol/L were considered diabetic. Six rats were excluded from study because blood glucose was below 16.7 mmol/L. Two rats died of unknown reasons in the nonSTZ group and 19 in the STZ group died of complications associated with diabetes mellitus (tumor and/or infection).

At 2 months the remaining 79 rats in the STZ group were randomly divided into 8 subgroups of 9 to 11 per group, including the STZ sham treated, STZ shControl, and STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups. The STZ sham group received sham treatment. The STZ shControl group received 1 intracavernous injection of AdU6/shControl virus and were analyzed on day 1 after injection. The STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups received 1 intracavernous injection of AdU6/shiNOS virus and were analyzed on post-injection day 1, 3, 5, 7, 10 and 14, respectively. Body weight and blood glucose were determined before intraperitoneal injection of STZ and before sacrifice.

In Vivo Gene Delivery to Corpus Cavernosum

The penis was exposed after the rat was anesthetized with sodium pentobarbital (35 mg/kg intraperitoneally). A dose of 15 μl AdU6/shControl (7.94×10^9 IU/ml), AdU6/shiNOS (7.94×10^9 IU/ml) or saline was injected into the right side of the corpora with a 30 gauge needle.

Erectile Response Measurement

Erectile function was assessed by cavernous nerve stimulation, as described previously.[19] Each penis was cut transversely into 3 segments and each segment was sub-

Real-time quantitative PCR primer sequences

Primer	Sequence	Amplicon Size (bp)
nNOS:		132
Forward	5′-CCTATGCCAAGACCCTGTGTGA-3′	
Reverse	5′-CATTGCCAAAGGTGCTGGTG-3′	
eNOS:		80
Forward	5′-GATCCTAACTTGCCTTGCATCCT-3′	
Reverse	5′-TGTAATCGGTCTTGCCAGAATCC-3′	
iNOS:		101
Forward	5′-CTCACTGTGGCTGTGGTCACCTA-3′	
Reverse	5′-GGGTCTTCGGGCTTCAGGTTA-3′	
β-actin:		160
Forward	5′-AAGAGCTATGAGCTGCCTGA-3′	
Reverse	5′-TACGGATGTCAACGTCACAC-3′	

divided into 3 slices. One slice was randomly selected from each segment to form a new group. The 3 groups were randomly assigned for RNA, protein isolation and enzyme-linked immunosorbent assay, respectively.[20]

Real-Time Quantitative RT-PCR

Total RNA was isolated from penile tissue and CSMCs using TRIzol® reagent according to manufacturer instructions. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix. The final PCR reaction volume was 25 μl. The table lists the sequences of the RT-PCR primers used.

Western Blot

For protein detection cells and penile tissue lysates were harvested. Equal amounts of total protein were separated and incubated with primary antibodies, including anti-p-eNOS (Ser-1177, 1:1,000), anti-eNOS (1:1,000), anti-nNOS (1:1,000), anti-iNOS (1:1,000) (Abcam®) and anti-β-actin (1:50,000) (Sigma-Aldrich®). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:5,000 or 1:10,000) (ProMab Biotechnologies, Richmond, California) were used. Immunoreactive protein was detected using the enhanced chemiluminescence method (Amersham, Aylesbury, United Kingdom), as described by the manufacturer.

Cavernous cGMP Measurement

Penile tissue was homogenized, centrifuged and extracted with H_2O saturated diethyl ether. Samples were assayed for cGMP using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan).

Statistical Analysis

All experiments were repeated at least 3 times. Results are presented as the mean ± SD. Statistical analysis was performed with 1-way ANOVA, followed by the Bonferroni post test for multiple comparisons and the LSD t test. The paired t test was used to compare parameters of the same group at different time points. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Design and Cloning of iNOS Specific saRNA Expressing shRNA

To deliver RNAa to corpus cavernosum tissue in vivo, we used an adenovirus to express saRNA as

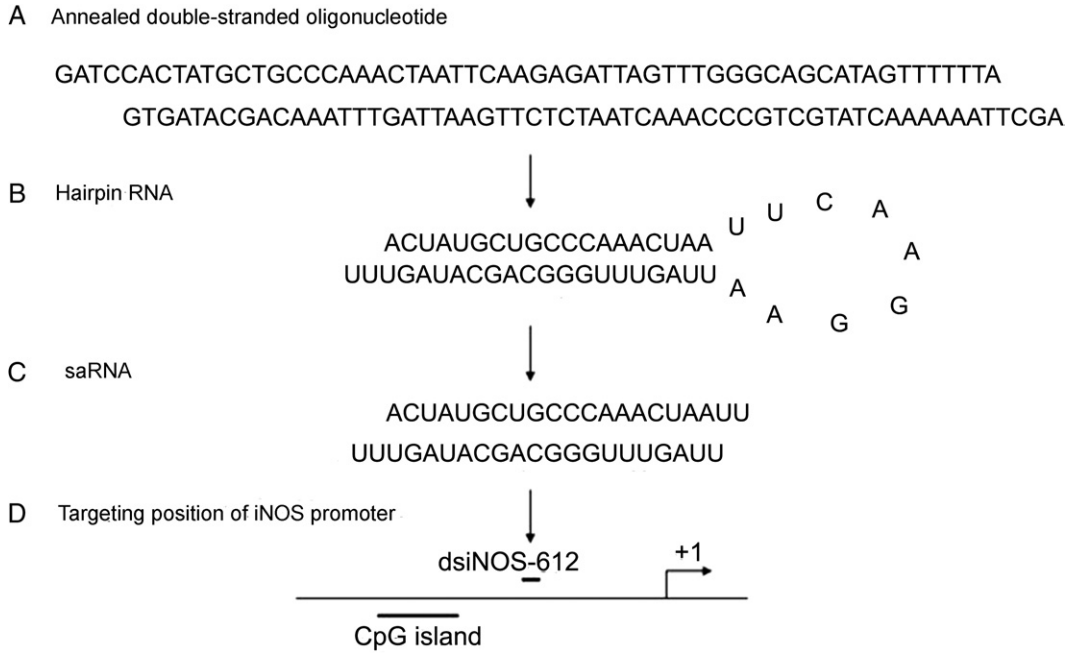

Figure 1. Oligonucleotide sequence and predicted RNA structure of specific iNOS sequences. *A*, annealed ds oligonucleotides were inserted in pDC316-EGFP-U6 between BamHI and HindIII sites. *B*, after packaging into adenovirus and transduction into CSMCs, oligonucleotides were transcribed into hairpin RNA. *C*, hairpin RNA was processed into functional saRNA. *D*, saRNA targeted position of iNOS promoter at position −612 relative to transcription start site.

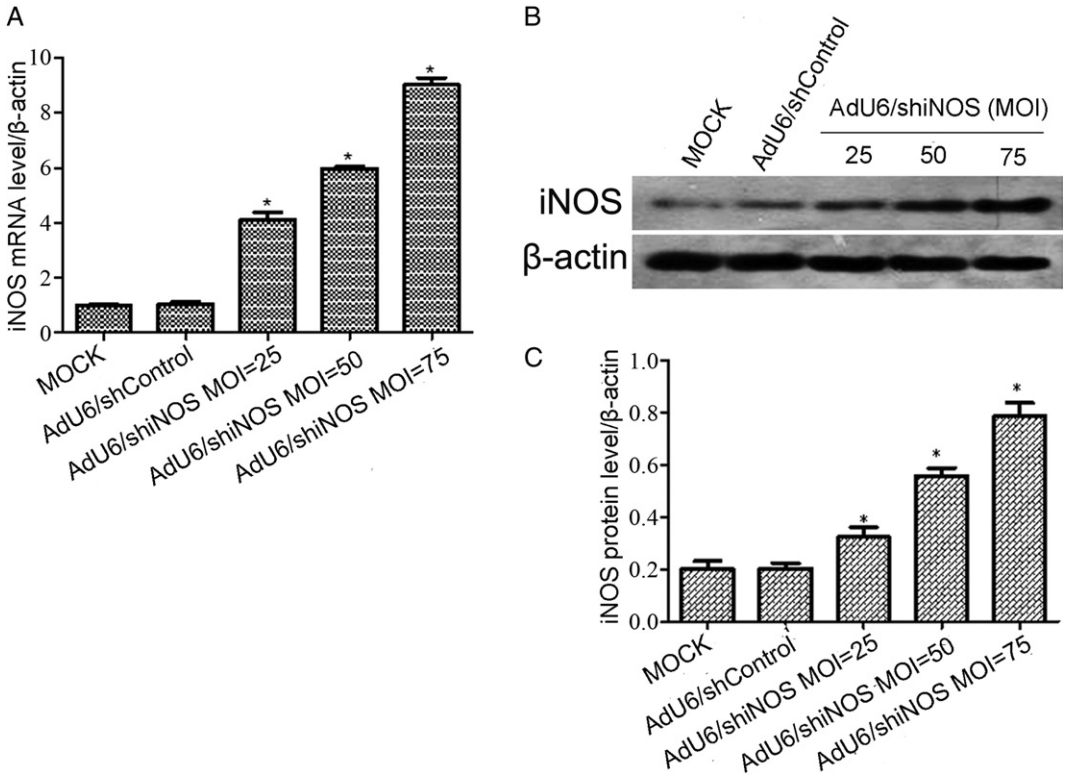

Figure 2. iNOS saRNA activated iNOS expression at mRNA and protein levels in CSMCs. Cultured CSMCs were mock transduced (*MOCK*) or transduced with virus at indicated MOI for 72 hours and harvested for RNA and protein isolation. *A,* iNOS mRNA was detected by real-time RT-PCR. Results are shown as mean ± SD of 3 independent experiments. *B,* proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. iNOS protein was detected by Western blot using iNOS antibody. β-actin protein was also detected as protein loading control using β-actin antibody. *C,* relative iNOS protein was determined by quantifying Western blot membrane band intensity (*B*). Results are shown as mean ± SD of 3 independent experiments. Asterisk indicates $p < 0.05$ vs mock transduction and AdU6/shControl groups.

shRNA. Based on published saRNA design rules,[13] we selected a target on the iNOS promoter at a location between −612 and −594 bp relative to the TSS of the iNOS gene (fig. 1).

iNOS Up-Regulation by iNOS Promoter Targeted saRNA in Cultured CSMCs

We infected cultured rat CSMCs with 25, 50 and 75 MOI of AdU6/shiNOS with a mean transduction efficiency of 82.2% ± 2.7%, 82.5% ± 3.3% and 83.3% ± 3.5%, respectively. Cells infected with mock or 75 MOI AdU6/shControl served as controls. No viral toxicity in cells was observed at any MOI during transduction. Compared to AdU6/shControl infected cells, cells infected with AdU6/shiNOS showed significant higher iNOS mRNA levels ($p < 0.05$) and protein expression in a dose dependent manner (fig. 2). A 9.2-fold increase in iNOS mRNA expression was detected when AdU6/shiNOS was transduced at 75 MOI (fig. 2, *A*). Since AdU6/shiNOS at a MOI of 75 resulted in the strongest iNOS activation in CSMCs, all subsequent experiments were done at a MOI of 75.

iNOS saRNA Intracavernous Delivery

Improved erectile function. The final mean blood glucose concentration in STZ rats was significantly increased compared to that in nonSTZ controls and the final mean body weight of STZ rats decreased significantly (each $p < 0.05$). Increased iNOS slightly reduced blood pressure but the effect was not statistically significant ($p > 0.05$). The total ICP area on cavernous nerve stimulation in AdU6/shiNOS treated rats was significantly higher at all time points than in the STZ shControl and STZ sham groups ($p < 0.05$, fig. 3). The maximal response to intracavernous injection of AdU6/shiNOS was observed when ICP area was measured on day 5 after virus injection with ICP restored to levels close to those of the normal control nonSTZ group (fig. 3). It was particularly significant that the increased ICP area in the STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups remained detectable 14 days after virus injection (fig. 3). Results suggest that intracavernous delivery of iNOS saRNA leads to sustained improvement of erectile function in diabetic rats.

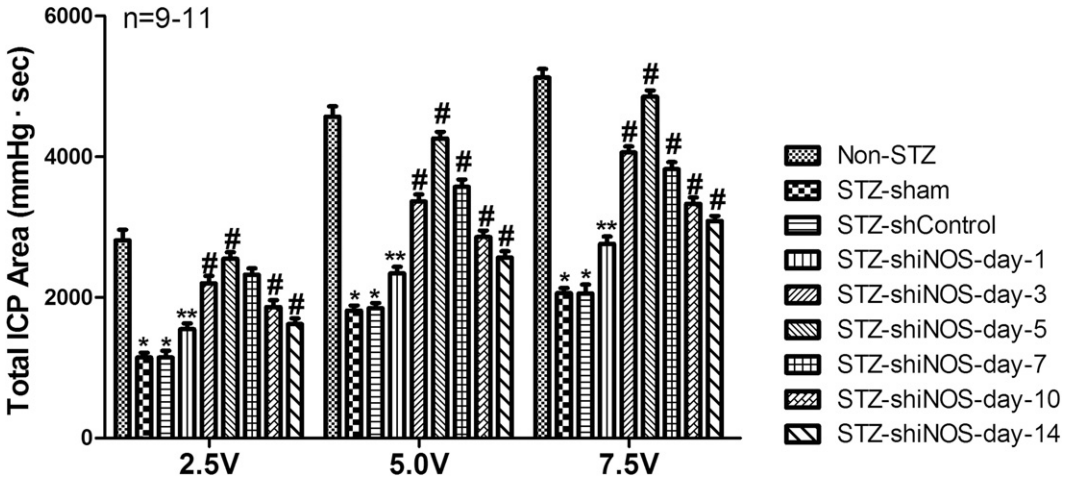

Figure 3. Intracavernous iNOS saRNA injection improved erectile function in diabetic rats. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Cavernous nerve was stimulated at different 2.5, 5 or 7.5 V at 15 Hz frequency and 1.2-millisecond pulse width for 1 minute at 3-minute intervals. Erectile response to cavernous nerve stimulation was measured as total ICP AUC in mm Hg per second on day 1, 3, 5, 7, 10 and 14 after injection. Results are shown as mean ± SD. Single asterisk indicates $p < 0.05$ vs nonSTZ group. Double asterisks indicate $p < 0.05$ vs STZ sham and STZ shControl groups. Pound sign indicates $p < 0.05$ vs STZ sham group.

Significantly higher induction of iNOS mRNA and protein levels was observed in diabetic rats that received intracavernous injection of AdU6/shiNOS virus, as analyzed at all time points (figs. 4, *A* and 5, *B*, $p < 0.05$). iNOS mRNA and protein expression followed a trend similar to that of ICP area (fig. 3), suggesting that improved erectile function was due to iNOS induction mediated by its saRNA.

To further assess whether iNOS induction by its saRNA was target specific, we evaluated the expression of eNOS and nNOS. Diabetic rats had significantly lower eNOS and nNOS expression than nondiabetic rats (figs. 4, *B* and 5, *B*). Injection of AdU6/shiNOS virus did not alter eNOS or nNOS mRNA or protein expression compared to that in diabetic rats that received AdU6/shControl virus injection. This suggests that iNOS saRNA caused specific iNOS activation in penile tissue.

Diabetic rats transfected with AdU6/shControl and AdU6/shiNOS showed no significant change in p-eNOS (Ser-1177) expression compared with diabetic rats without treatment (fig. 5, *A* and *C*).

Increased penile cGMP. A significant increase in cGMP in the penile tissue of STZ rats treated with AdU6/shiNOS virus was observed compared to AdU6/shControl rats and diabetic rats without treatment ($p < 0.05$, fig. 6, *A*). Peak cGMP occurred at day 5 after treatment. This suggests that iNOS induction by saRNA leads to activation of the intracellular cyclic GMP pathway.

DISCUSSION

We constructed an iNOS specific saRNA expression adenovirus and used it to transduce rat CSMCs and the corpus cavernosum of diabetic rats. Results revealed that iNOS levels in CSMCs and the corpus cavernosum of STZ rats were up-regulated by saRNA, leading to restored erectile function in STZ diabetic rats via the NO-cGMP pathway.

RNA interference is a promising therapy for many diseases due to the high efficacy and high specificity of gene knockdown, and the low toxicity of small RNA molecules.[21] However, many conditions are caused by aberrant inactivation of genes and there is a lack of safe methods to restore expression. Using the same therapeutic advantages of small RNA molecules, RNAa may provide expanded drug targets for small RNA based therapy for various conditions, including ED.

A previous study showed that lentiviral based delivery of saRNAs could activate vascular endothelial growth factor expression by targeting promoter sequences in an ischemic mouse model.[22] To our knowledge we established a new saRNA delivery system using adenovirus as the saRNA delivery vector. Since adenovirus provides high transduction efficiency and does not integrate into the host genome, it is safer.[23] Our results show that the AdU6/shiNOS adenovirus is highly infective to various cell lines and host types, and it can effectively activate iNOS expression in CSMCs in vitro and in penile tissue in vivo.

NO synthesis is catalyzed by NOS and, thus, it is an attractive target for ED gene therapy. Of the 3

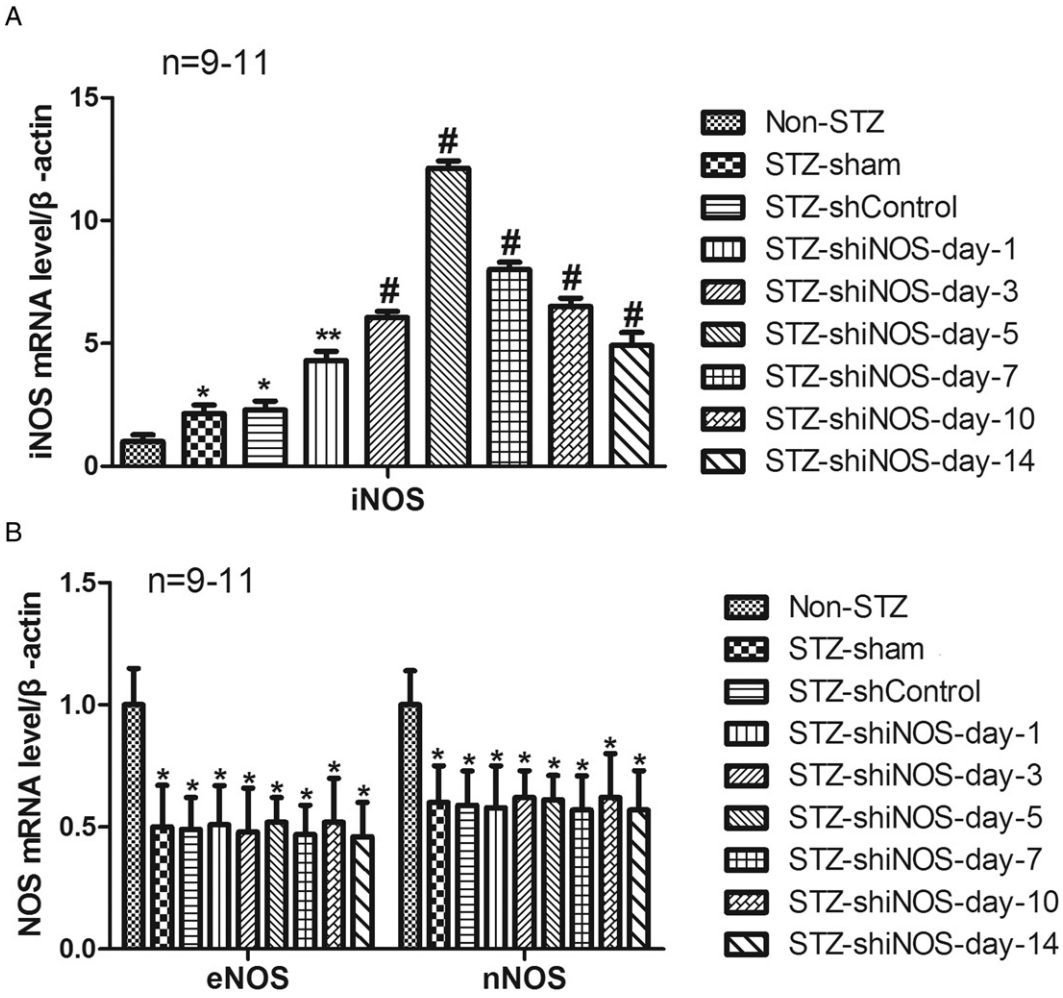

Figure 4. iNOS saRNA activated iNOS expression in vivo. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Penile tissue was harvested for RNA isolation after measuring erectile function. cDNA derived from isolated RNA by reverse transcription reaction was amplified by real-time RT-PCR. Result are shown as mean ± SD of mRNA expression normalized to β-actin in all rats in each treatment group and plotted as fold change vs nonSTZ group. *A,* iNOS mRNA. *B,* NOS mRNA. Single asterisk indicates $p < 0.05$ vs nonSTZ group. Double asterisks indicate $p < 0.05$ vs STZ sham and STZ shControl groups. Pound sign indicates $p < 0.05$ vs STZ sham group.

NOS isozymes iNOS synthesizes much more NO in a sustained manner and functions independently of intracellular calcium flux.[24] Therefore, even if iNOS is expressed in relatively few cells, a wide area of cells could be exposed to NO.[8] A recent study showed that iNOS has an important role in protecting the penile corpus cavernosum from the profibrotic effects of hyperglycemia.[25] In recent years researchers have used different methods to deliver iNOS into the corpus cavernosum and found that gene therapy targeting iNOS is feasible for treating ED.[8,26–28] For example, iNOS cDNA could be used as a potential antifibrotic agent to reverse fibrotic changes that impair cavernous function.[29] Another group used plasmid and adenovirus to deliver an exogenous iNOS gene into the corpus cavernosum.[27] They achieved a significant increase in erectile response by enhancing NO production.

However, the unbalanced production of NO and its derivatives, collectively termed reactive nitrogen species, leads to pathological changes.[30] Excessive NO production by iNOS may generate nitrosative stress and significant amounts of NO can lead to surrounding tissue damage due to peroxynitrite formation, inducing cell apoptosis.[30]

In our study enhanced NO production did not decrease eNOS or nNOS expression, or damage endothelial cells or neurons. Thus, at certain levels NO may have profibrotic effects and may not injure tissue, while excess NO production would damage tissue due to reactive nitrogen species. We used RNAa to restore endogenous iNOS gene expression. Adenovirus mediated RNAa successfully enhanced iNOS gene expression in CSMCs and in the penis of diabetic rats, resulting in prolonged improvement in erectile function in the rats. RNAa may provide unmatched advantages over conventional ectopic

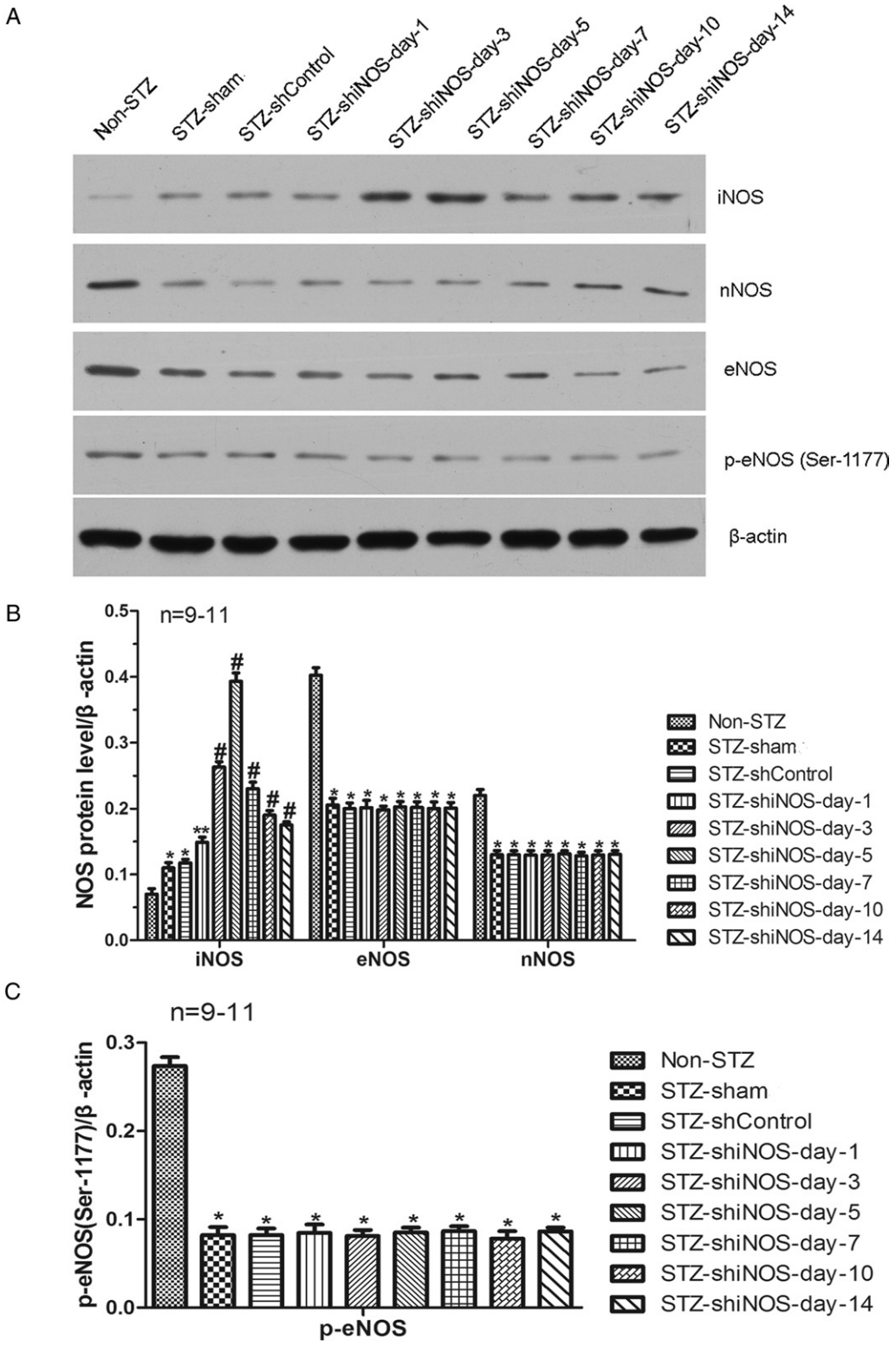

Figure 5. iNOS saRNA activated iNOS protein and impacted nNOS, eNOS and p-eNOS proteins in diabetic rat penile tissue. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Penile tissue was harvested for protein isolation after measuring erectile function. *A*, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. iNOS, eNOS, nNOS and p-eNOS proteins were detected by Western blot using iNOS, eNOS, nNOS and p-eNOS antibodies, respectively. β-actin protein was also detected as protein loading control using β-actin antibody. *B* and *C*, mean $\pm$ SD protein was determined by quantifying Western blot membrane band intensity (*A*) in all rats in each group. *B*, relative iNOS, eNOS and nNOS. *C*, relative P-eNOS and proteins. Single asterisk indicates $p < 0.05$ vs nonSTZ group. Double asterisks indicate $p < 0.05$ vs STZ sham and STZ shControl groups. Pound sign indicates $p < 0.05$ vs STZ sham group.

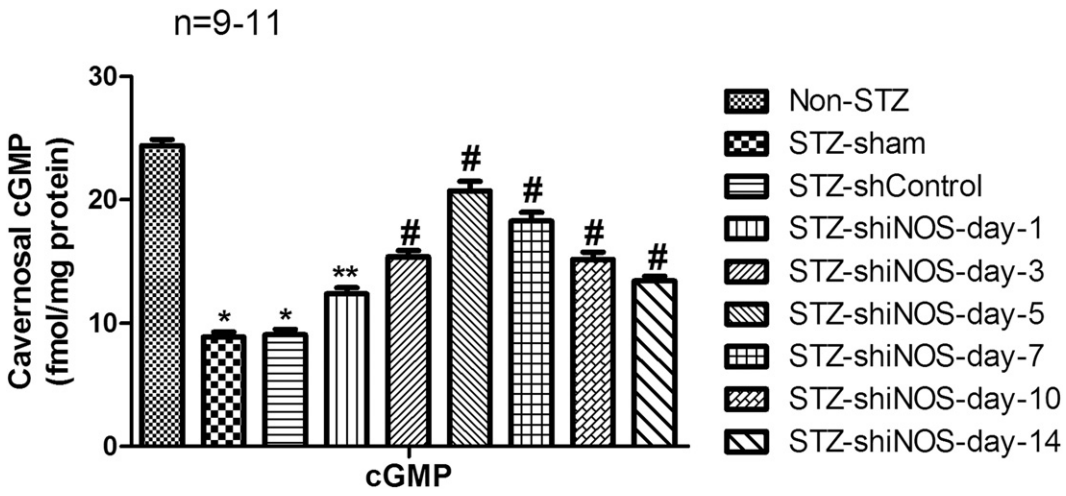

Figure 6. saRNA mediated iNOS activation increased cGMP. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Penile tissue was harvested for cGMP isolation after measuring erectile function. cGMP was measured by enzyme-linked immunosorbent assay. Results are shown as mean ± SD of all rats in each group. Single asterisk indicates $p < 0.05$ vs nonSTZ group. Double asterisks indicate $p < 0.05$ vs STZ sham and STZ shControl groups. Pound sign indicates $p < 0.05$ vs STZ sham group.

expression of exogenous genes due to its unique ability to restore endogenous gene expression.

CONCLUSIONS

Taken together, our results reveal adenovirus mediated RNAa activated iNOS gene expression in vitro and in vivo, and improved erectile function in diabetic rats for a prolonged period via activation of the NO-intracellular cGMP pathway. These findings provide proof of concept that RNAa mediated over expression of iNOS can be used to restore erectile function in diabetic rats.

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