Nitric Oxide-Releasing Polymeric Microspheres Improve Diabetes-Related Erectile Dysfunction

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ABSTRACT

Introduction. We have used a long-acting nitric oxide (NO)-releasing polymer to develop injectable biodegradable microspheres capable of localized NO release over prolonged periods of time.
Aim. The aim of this study was to evaluate the therapeutic potential of these microspheres for diabetes-related erectile dysfunction (ED) in the rat model.
Methods. NO-releasing microspheres were incubated in physiologic buffer, and *in vitro* NO release was measured using a Griess assay. To ensure no migration, microspheres were fluorescently tagged and injected into the corpus cavernosum of adult rats, and fluorescent imaging was performed weekly for 4 weeks, at which point rats were sacrificed. To assess physiologic efficacy, diabetes was induced in 40 rats using streptozotocin (STZ), whereas 10 rats were kept as age-matched controls. Diabetic rats were divided into four groups: no treatment, sildenafil, NO-releasing microspheres, and combination therapy. For each rat, the cavernosal nerve (CN) was stimulated at various voltages, and intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured via corpus cavernosum and carotid artery catheterization, respectively. Long-term efficacy was determined by injecting diabetic rats with microspheres and measuring erectile response at predetermined intervals for up to 5 weeks.
Main Outcome Measures. Erectile response was determined via calculation of mean peak ICP/MAP and area under curve (AUC) for each experimental group.
Results. Under physiologic conditions *in vitro*, microspheres continued NO release for up to 4 weeks. Fluorescent imaging revealed no detectable signal in tissues besides cavernosal tissue at 4 weeks postinjection. Upon CN stimulation, peak ICP/MAP ratio and AUC of diabetic rats improved significantly ($P < 0.05$) in microsphere and combination therapy groups compared with no treatment and sildenafil groups. In long-term efficacy studies, microspheres augmented the effect of sildenafil for 3 weeks following injection ($P < 0.05$).
Conclusions. NO-releasing microspheres significantly improved erectile response in diabetic rats for 3 weeks and hence offer a promising approach to ED therapy, either as monotherapy or combination therapy. **Soni SD, Song W, West JL, and Khera M. Nitric oxide-releasing polymeric microspheres improve diabetes-related erectile dysfunction. J Sex Med **;**:**–**.**

Key Words. Nitric Oxide; Diabetes; Erectile Dysfunction; Therapy; Corporal Smooth Muscle Relaxation; Rat

Introduction

Today, there are over 25.8 million people, or 8.3% of the population, living with diabetes in the United States. Even more worrisome is that 13 million men aged 20 years or older have diabetes, which accounts for 11.8% of this population [1]. It has been estimated that approximately 50–75% of diabetic men suffer from erectile dysfunction (ED), making it a major morbidity associated with diabetes [2].

The factors affecting erectile function are multiple and not completely understood, but nitric oxide (NO) is known to be a principal mediator in erectile physiology. NO released by the endothelial cells lining the corpus cavernosum and penile arteries acts by stimulating soluble guanylate cyclase to produce cyclic 3,5-guanosine

monophosphate (cGMP), which ultimately results in corporal smooth muscle relaxation and vascular inflow. However, it has been well established that release of endothelium-derived NO is diminished in diabetes, and the resultant NO deficiency has been implicated in the pathophysiology of diabetes-associated ED [2–6].

Therefore, investigators have previously sought to improve NO bioavailability via NO donors such as sodium nitroprusside (SNP) and gene transfer of endothelial NO synthase [7–9]. Although SNP produced good erections in animal models, doses required to achieve tumescence in human trials led to systemic toxicity making SNP an inadequate therapy. Also, while gene transfer showed promising results in animal models, given the possible risks and costs associated with gene therapy, it is currently not viewed as suitable therapy for ED.

Another class of NO donors called diazeniumdiolates enables controlled pharmacological delivery of NO over prolonged periods of time. These agents are formed by the reaction of two NO molecules with an amine group under basic conditions, and they release two NO molecules via dissociation in aqueous media. Champion et al. investigated the therapeutic efficacy of five different agents in this class with half-lives ranging from 2 seconds to 20 hours [10]. When injected intracavernosally, these agents produced dose-dependent increases in intracavernosal pressure. However, the duration of erectile response was significantly shorter than intracavernosal trimix therapy (phentolamine, papaverine, and prostaglandin E1), making these agents less attractive than trimix as monotherapy for on-demand dosing.

Of note, diazeniumdiolates can be covalently bound to poly(ethylene glycol) (PEG) polymers and then cross-linked into hydrogels and microspheres. This process can improve biocompatibility, prolong NO release, and help reduce migration into the bloodstream. Such NO-releasing polymers have previously been explored as a direct blood vessel coating for prevention of thrombosis following vascular injury and in vascular stents for prevention of stent restenosis. The polymers were found to be capable of slow, sustained NO release for over 60 days *in vitro* under physiologic conditions [11,12].

When incorporated into microspheres and administered intracavernosally, such NO-releasing polymers could theoretically improve NO bioavailability in cavernosal tissue and improve erectile function. In this study, we used the streptozotocin-induced type I diabetic rat model to investigate whether intracavernosal NO-releasing polymer microspheres, intravenous (IV) sildenafil, or combination therapy could improve diabetes-related ED.

Methods

Polymer Synthesis and Microsphere Fabrication

Unless otherwise specified, all products for microsphere synthesis were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acrylate-poly(ethylene glycol)-succinimidyl valerate (MW 3400, Laysan Bio, Arab, AL, USA) was first reacted with poly-L-lysine (degree of polymerization = 4) at an equimolar ratio in N-(2-Hydroxyethyl)piperazine-N′-(4-butanesulfonic acid) (pH 8.6) buffer for 4 hours to form ACRL-PEG-Lys$_4$. The copolymer was then dialyzed against Milli-Q water (EMD Millipore, Billerica, MA, USA) to remove unreacted product and lyophilized. The product was analyzed using gel permeation chromatography with an evaporative light scattering detector (Polymer Laboratories, Amherst, MA, USA), and successful formation of ACRL-PEG-Lys$_4$ was confirmed by a shift in position of the peak from the evaporative light-scattering detector. Next, ACRL-PEG-Lys$_4$ was dissolved in water and reacted with NO gas under pressure in a previously evacuated vessel to form diazeniumdiolates on the free amines of the lysine residues. A ninhydrin assay was used to confirm >90% conversion of free amine groups to diazeniumdiolates, and the resultant ACRL-PEG-Lys$_4$-NO polymer was lyophilized.

For fabrication of microspheres, PEG-diacrylate (PEGDA, MW 3400) was first synthesized by reacting 10 kDa PEG with 3 M excess acryloyl chloride and 1 M excess triethylamine in anhydrous dichloromethane under argon overnight. The reacted mixture was transferred to a separatory funnel, where 2 M K_2CO_3 was added at a 5 M excess to the PEG. After mixing well, the solution was allowed to separate, and the organic phase was collected, dried with magnesium sulfate and filtered. The final, clear liquid was precipitated in cold diethylether, filtered, and dried under vacuum.

ACRL-PEG-Lys$_4$-NO was then mixed with PEGDA in a 1:10 molar ratio in Hanks' Balanced Salt solution (HBS) containing 3.4 µl/mL N-Vinylpyrrolidone (NVP), 1.5% triethanolamine (TEOA), 0.1% pluronic F68, and 10 µM eosin Y photoinitiator. This precursor solution was then added to mineral oil containing 1,500 ppm 2,2-dimethoxy-2-phenyl acetophenone in a glass
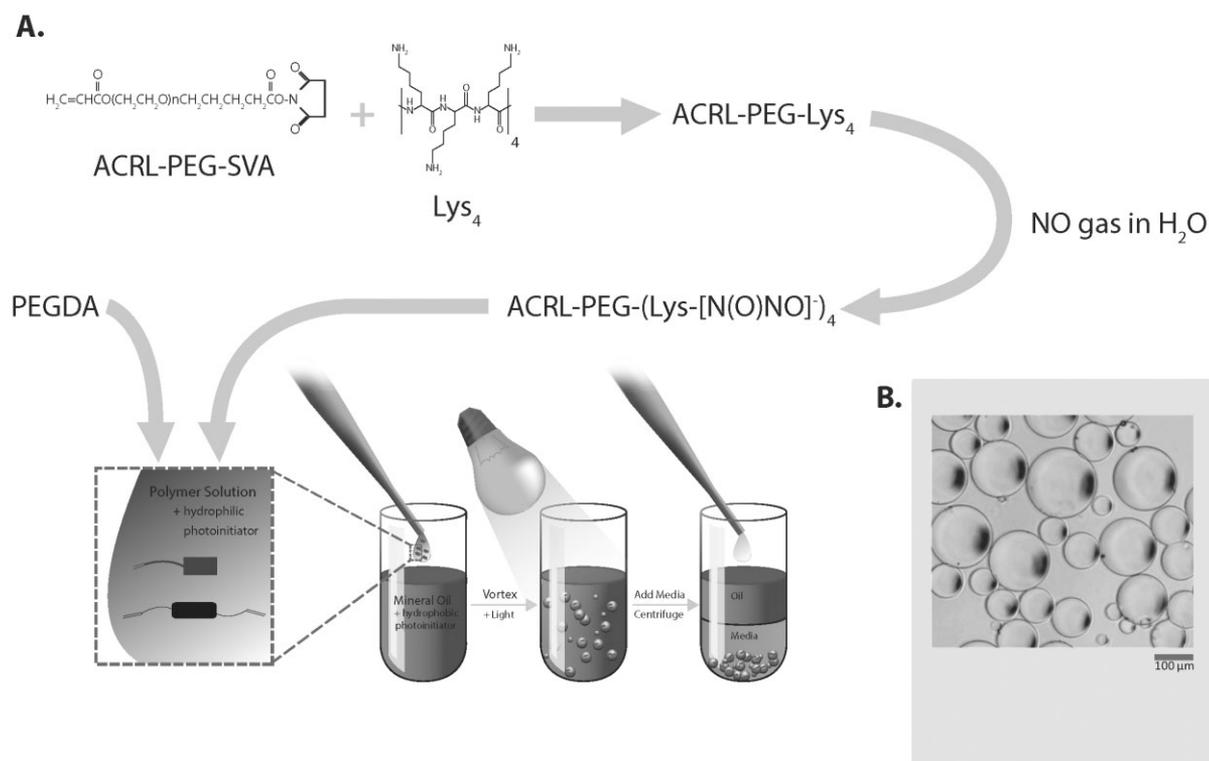
Figure 1 Protocol for PEG-Lys$_4$-NO microsphere synthesis and representative image of resultant NO-releasing microspheres.

test tube, vortexed at full speed for 5 seconds, and then exposed to light from a metal halide lamp (Dolan-Jenner, Boxborough, MA, USA) for 45 seconds. The lamp was modified with heat-absorbing and UV-blocking optical filters (Edmund Optics, Barrington, NJ, USA) for an excitation range of 365–700 nm. Cross-linked microspheres were separated from the oil phase by addition of phosphate buffered saline (PBS) and centrifugation. The oil layer was removed by aspiration, and the microspheres were washed twice more with PBS. The overall polymeric microsphere synthesis protocol is illustrated in Figure 1.

In addition, blank PEG-Lys$_4$ microspheres were fabricated by omitting the NO gas reaction step and were utilized for fluorescent imaging studies and as blank control microspheres.

In vitro NO Release Study

Following synthesis, PEG-Lys$_4$-NO microspheres were filtered for sizes 100–190 μm and 200 μL of the polymer microsphere suspension (0.74 umol polymer) was transferred to each of six different 2-mL vials. Buffer solution (HBS/1.5% TEOA, pH 7.4) was added to all vials for total volume of 2 mL. Microspheres were incubated in buffer solution under physiologic conditions (pH 7.4, 37°C) for 4 weeks, and a Griess assay was performed at weekly intervals to determine nitrite concentration in solution as a measure of NO release. Because the majority of NO is converted to nitrite rather than nitrate under *in vitro* conditions, measurement of nitrite provides a reasonable determination of NO release [13]. At the time of each measurement, all vials were centrifuged, and supernatant buffer was removed and replaced with fresh buffer solution.

Fluorescent Imaging

The Baylor College of Medicine Animal Care and Use Committee approved all animal protocols used in the current study. PEG-Lys$_4$ microspheres

Table 1 Weight and blood glucose levels of rats before and after 8-week diabetic period

	Control	Untreated diabetic	Sildenafil	Nitric oxide-releasing microspheres	Combination therapy
Initial weight (g)	306 ± 2	305 ± 2	307 ± 2	306 ± 2	307 ± 2
Final weight (g)	407 ± 5	214 ± 7	213 ± 7	218 ± 7	215 ± 10
Initial glucose (mg/dL)	84 ± 2	78 ± 2	83 ± 2	85 ± 3	81 ± 3
Final glucose (mg/dL)	70 ± 2	495 ± 4	487 ± 11	487 ± 9	489 ± 7

filtered for sizes 100–190 μm were reacted with an amine-reactive Alexa Fluor 750 fluorescent dye (Life Technologies, Grand Island, NY, USA) in 50 mM NaHCO$_3$ solution for 3 hours at 25°C. Microspheres were then washed twice with PBS to remove unreacted dye prior to injection. Adult rats were anesthetized via inhaled isoflurane and then injected with the fluorescently tagged microspheres (n = 5). For the injection procedure, sterile technique was used to make a 1 cm incision over the penis and remove overlying fascia to expose the left corpus cavernosum at the mid-shaft. Then 60 μL of microsphere suspension was injected into the corpus cavernosum under direct vision, and the needle hole in the corpus was sealed using Histoacryl tissue sealant (TissueSeal, Ann Arbor, MI, USA) to prevent extravasation of microspheres. The skin incision was then closed using a nonabsorbable suture.

All rats underwent whole animal fluorescence imaging using a Kodak *In Vivo* Imaging Station (Kodak, Rochester, NY, USA) with 755 nm emission and 830 nm excitation filters immediately following microsphere injection, and then at 7, 14, 21, and 28 days postinjection. Rats were then sacrificed, and organs were harvested and fluorescently imaged to evaluate for evidence of either local or distant migration of microspheres outside the corpus cavernosum. Net intensity of fluorescent signal was determined for all organs imaged using Kodak Molecular Imaging software.

Induction of Diabetes

Adult male Sprague-Dawley rats (Harlan, San Diego, CA, USA) were injected intraperitoneally with 60 mg/kg streptozotocin (STZ, Sigma-Aldrich) in citrate buffer (100 mM citric acid, 200 mM disodium phosphate, pH 7.0). For the erectile function studies, 10 rats were also kept as age-matched controls and administered intraperitoneal injections of citrate buffer alone. All rats were housed for 8 weeks, and glucose was monitored on all rats at 3 days, 4 weeks, and 8 weeks using an Accu-check blood glucose monitor (Roche Diagnostics, Indianapolis, IN, USA). Rats

were considered diabetic if blood glucose levels were greater than 250 mg/dL. If glucose was below 250 mg/dL at day 3, rats were again injected with 60 mg/kg STZ and glucose levels rechecked 3 days later. Rat body weights and blood glucose levels were recorded at the beginning and end of the 8-week period and are summarized in Table 1. As expected, a significant difference was observed in final weight and blood glucose levels between control and diabetic rats ($P < 0.05$). However, no significant difference was noted between diabetic rats within the various experimental treatment arms.

Erectile Function Studies

After the 8-week induction period, diabetic rats were randomly divided into four experimental groups: (i) no treatment, (ii) IV sildenafil alone, (iii) NO-releasing microspheres alone, and (iv) IV sildenafil and NO-releasing microspheres (n = 9–10 per group), as shown in Figure 2. An additional four diabetic rats were assigned to undergo injection with blank microspheres. Erectile function studies were then carried out on all control and diabetic rats.

For erectile response testing, rats were anesthetized with intraperitoneal injection of 38 mg ketamine and 2 mg xylazine per kilogram and placed in a supine position.

A carotid artery was catheterized using PE-50 (polyethylene) tubing and connected to a pressure transducer. The transducer was then connected to a data acquisition system (Dataq, Akron, OH, USA) to enable continuous measurement and recording of mean systemic arterial pressure (MAP). Next, a low midline abdominal incision was made, and the penis was exposed and freed of overlying skin and fascia. The ischiocavernosus muscle overlying the right corpus cavernosum was then divided to expose the crus. A 25-gauge needle filled with 200 U/mL heparin and linked to PE-50 tubing was inserted into the right penile crus and connected to a second pressure transducer to allow continuous measurement and recording of intracavernosal pressure (ICP). Next, the bladder and
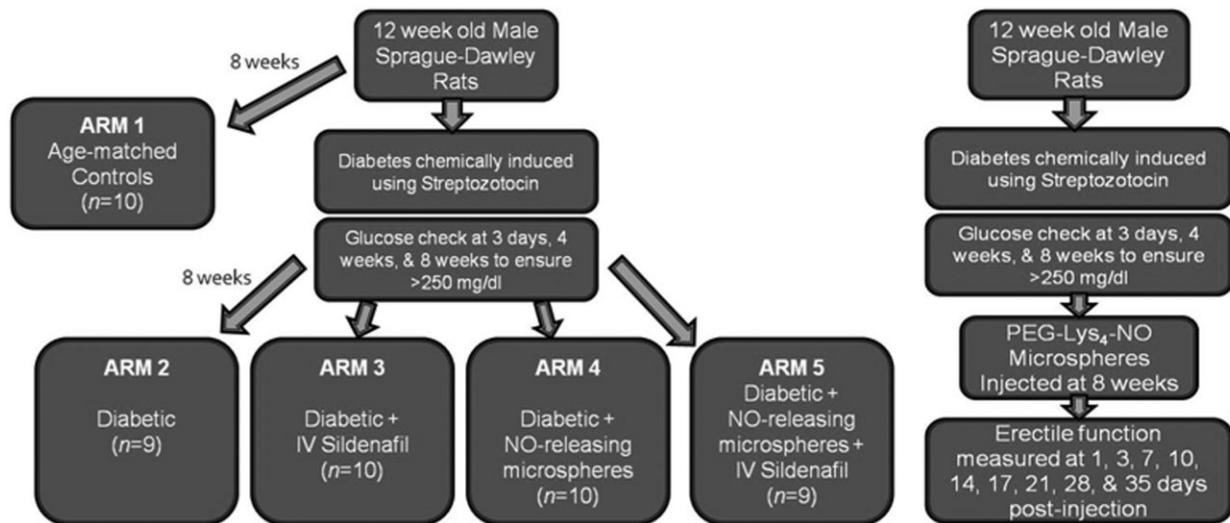
Figure 2 Experimental design for immediate (left) and long-term NO-releasing microsphere efficacy (right) studies.

prostate were exposed, and the right major pelvic ganglion and cavernous nerve were identified posterolateral to the prostate. A stainless-steel bipolar hook electrode connected to a nerve stimulator (Grass Instruments, Quincy, MA, USA) was then placed around the cavernous nerve.

For sildenafil treatment groups, sildenafil citrate (Sigma-Aldrich) was dissolved in normal saline at a concentration of 1 mg/mL, and 2 mg/kg sildenafil was administered as an IV bolus 10 minutes prior to erectile response testing. Likewise, rats undergoing NO-releasing microsphere treatment were injected using a 27-gauge needle with 40 μL (0.21 μmol PEG-Lys$_4$-NO polymer) of microspheres into the left crus 20 minutes prior to erectile function testing. For both sildenafil and microsphere treatment, the ICP did rise by 5–20 mm Hg upon administration but was allowed to return to baseline levels prior to initiating nerve stimulation.

The cavernous nerve was then stimulated (20 Hz, 5 msec) in 60-second intervals at 2, 4, 6, and 8 V while measuring ICP and MAP. A rest period of 3–4 minutes was included between each successive stimulation period. The maximal ICP-to-MAP ratio (ICP/MAP) was calculated for each experiment to normalize for variations in systemic blood pressure. Also, total area under the curve (AUC, mm Hg-sec) was calculated for each experiment from the start of nerve stimulation until the ICP returned to baseline values. Upon completion of each experiment, the rat was sacrificed, and the cavernosal tissue was harvested.

Long-Term Efficacy Studies
After 8 weeks in a diabetic state, additional rats were injected with NO-releasing microspheres using an identical sterile technique to that described for injection of fluorescently tagged microspheres. Next, rats were randomly assigned to various time points for erectile response testing: 1, 3, 7, 10, 14, 17, 21, 28, and 35 days postinjection (n = 3–4 per group), as shown in Figure 2. At the designated time point, rats were administered 2 mg/kg IV sildenafil, and erectile response experiments were carried out as previously described. Average ICP/MAP was determined for each time point at 4, 6, and 8 V of stimulation. After each experiment, the rat was sacrificed, and the cavernosal tissue was harvested.

Statistics
All hemodynamic data are expressed as mean ± standard error of the mean and were analyzed using one-way analysis of variance with repeated measures, followed by Newman–Keuls post hoc analysis for multiple group comparisons using Graphpad Prism version 6 (Graphpad Software, San Diego, CA, USA). A *P* value of less than 0.05 was used as the criterion for statistical significance. Additionally, equivalency testing using a confidence interval approach was used to demonstrate equivalence among groups using a 90% confidence interval and assuming an equivalence interval of ±0.03 for ICP/MAP.
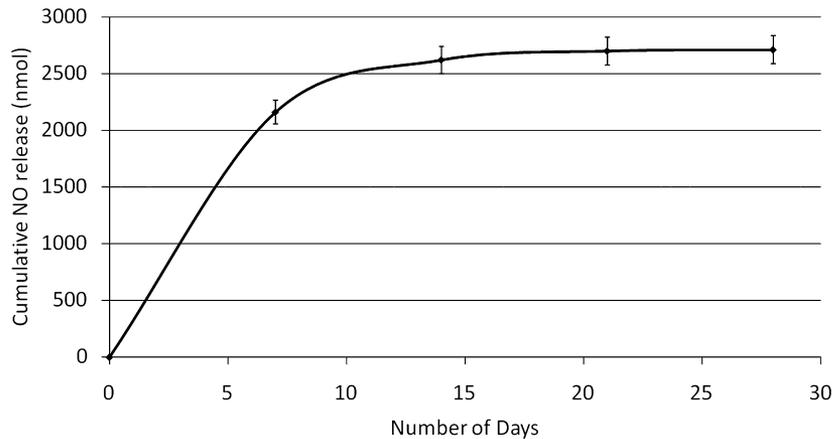
Figure 3 NO release profile for PEG-Lys$_4$-NO microspheres depicting sustained NO release for over 3 weeks in vitro under physiologic conditions.

Results

In vitro NO Release Study

The Griess assay demonstrated that there was continued release of NO from PEG-Lys$_4$-NO microspheres for over 3 weeks under physiologic conditions (pH 7.4, 37°C), as depicted in Figure 3. The highest rate of NO release from the microspheres was observed during the first 7 days of each study, and release appeared to taper off to undetectable levels after 4 weeks. Although the total number of nanomoles of NO released during each study was dependent on the concentration of PEG-Lys$_4$-NO microspheres incubated in solution, the overall NO release profile remained relatively constant for various microsphere concentrations tested. After no further NO release was detected, microspheres were incubated in pH 3 buffer solution to promote release of any NO trapped within microspheres. There was no detectable amount of unreleased NO remaining within the polymer microspheres after 4 weeks.

Fluorescent Imaging

Whole-animal fluorescent imaging performed at weekly intervals following intracavernosal injection of fluorescently labeled polymer microspheres demonstrated that microspheres did in fact remain within the corpus cavernosum for at least 4 weeks. Representative fluorescent overlay images from day 0 and day 28 postinjection are shown in Figure 4A. At 4 weeks postinjection, all animals were sacrificed, and major organs were harvested for further fluorescent imaging (Figure 4B). The net intensity of fluorescent signal emitted by each organ was determined for comparison with the intensity in the penis. As shown in Figure 4C, the net intensity of the fluo-

rescent signal was minimal from all organs imaged other than the corpus cavernosum.

In vivo Erectile Response Studies
Effect of Sildenafil on Erectile Response

At 8 weeks following induction of diabetes, all diabetic and control rats were administered the designated medication based on their treatment group and then underwent erectile response testing (n = 9–10 per group). As compared with age-matched control rats, diabetic rats had a significantly blunted erectile response ($P < 0.05$) for all levels of cavernous nerve stimulation (CNS). The maximal ICP/MAP was determined at four different voltage settings (2–8V) and found to be significantly lower in the untreated diabetic rats compared with control rats ($P < 0.05$). The AUC was also calculated from the beginning of stimulation until ICP returned to baseline levels and again demonstrated significantly lower values in diabetic rats ($P < 0.05$).

In order to assess the efficacy of sildenafil in improving diabetes-related ED, diabetic rats were administered 2 mg/kg IV sildenafil and then underwent erectile response testing 10 minutes later. Although a significant increase in both ICP/MAP and AUC was noted with sildenafil treatment at 2V and 6V of CNS ($P < 0.05$), the improvement at all other voltages tested did not reach significance, as shown in Figure 6. Overall, IV sildenafil treatment at the dose tested was not able to appreciably improve erectile function of the diabetic rats.

Effect of NO-Releasing Microspheres and Combination Therapy on Erectile Response

Likewise, diabetic rats assigned to the microsphere or combination therapy groups also underwent
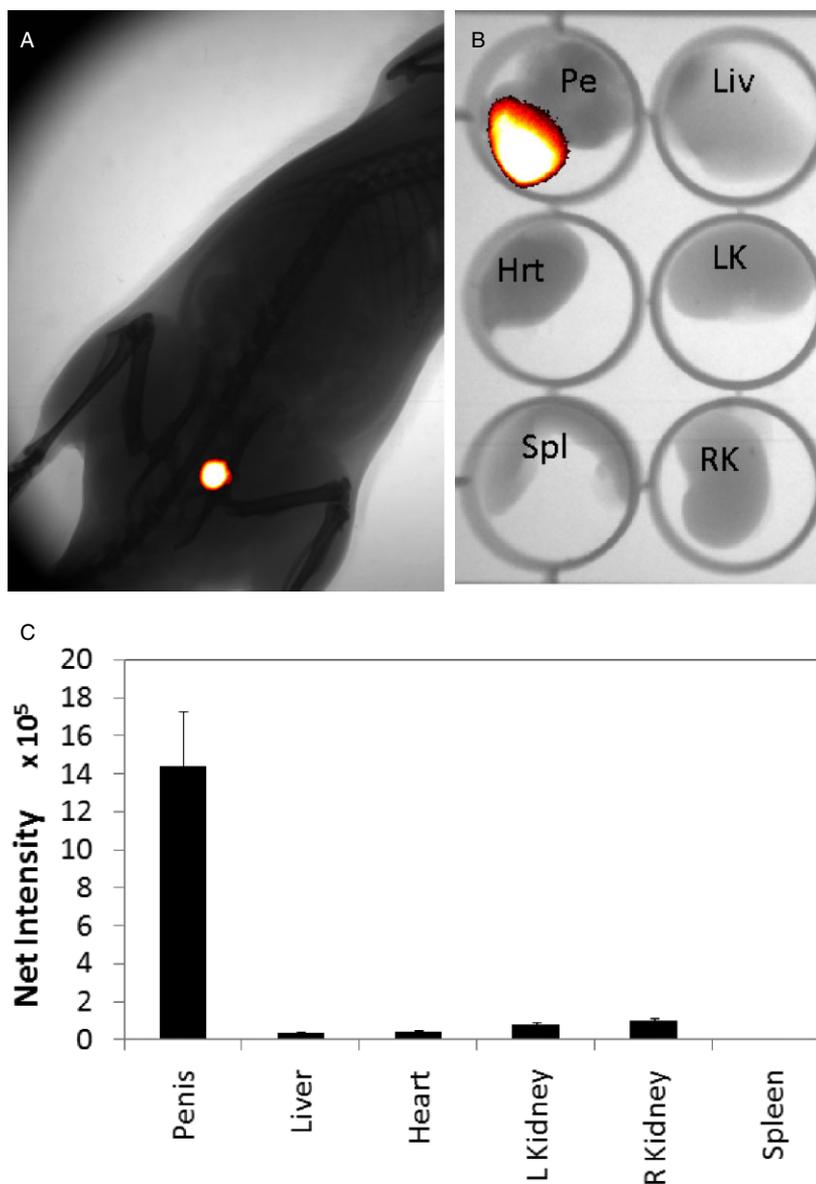
Figure 4 (A) Representative whole animal fluorescent imaging on days 0 and 28 following microsphere injection. (B) Representative fluorescent imaging of harvested organs (Pe = penis, Liv = liver, Hrt = heart, LK = left kidney, Spl = spleen, RK = right kidney). (C) Average net fluorescent intensity of harvested organs at 4 weeks following injection.

erectile response testing at 8 weeks following induction of diabetes. First, the impact of blank PEG-Lys$_4$ microspheres on erectile response was determined and showed no significant change from untreated diabetic rats, as shown in Figure 5. Next, NO-releasing PEG-Lys$_4$-NO microspheres were tested (Figure 6) and shown to significantly improve the ICP/MAP ratio and AUC as compared with untreated diabetic rats at all voltages tested ($P < 0.05$). Importantly, NO-releasing microspheres alone achieved a significantly higher ICP/MAP ratio at 4, 6, and 8 V as compared with sildenafil treatment alone ($P < 0.05$).

Combination therapy with IV sildenafil and intracavernosal NO-releasing microspheres achieved remarkable improvement in erectile function. At 2 V of CNS, rats given combination therapy achieved significantly higher ICP/MAP and AUC compared with untreated diabetic rats ($P < 0.05$). Moreover, CNS at higher voltages (4 V, 6 V, and 8 V) yielded statistically significant improvement in both maximal ICP/MAP ratio and AUC, even when compared with sildenafil therapy alone ($P < 0.05$). These results indicate that the addition of NO-releasing microspheres dramatically augments erectile response of diabetic rats compared to sildenafil treatment alone. In fact, diabetic rats administered combination therapy achieved ICP/MAP ratios at least equivalent to age-matched control rats in this study.
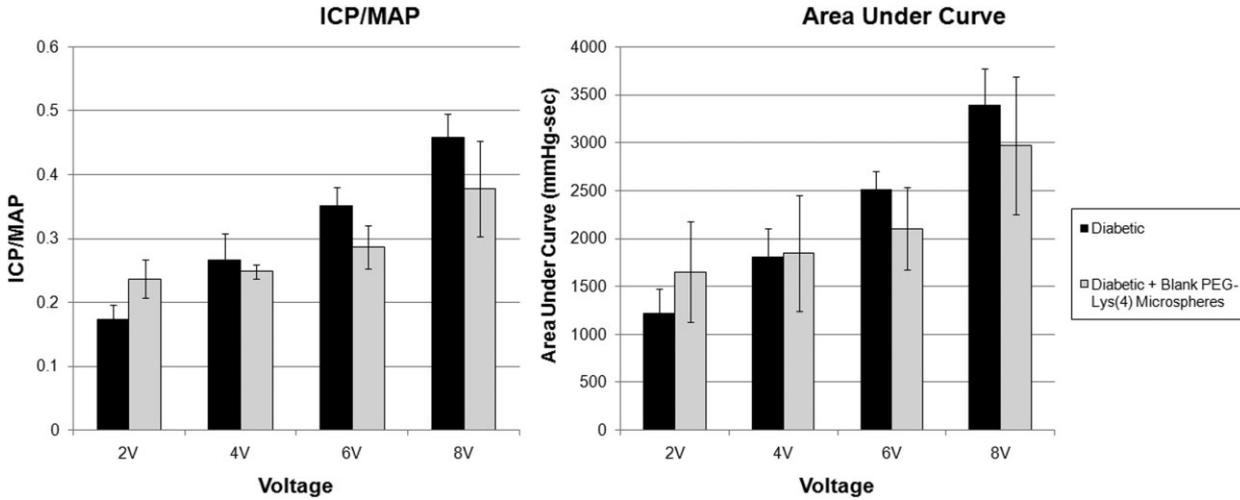
Figure 5 Effect of blank PEG-Lys$_4$ microspheres on erectile response in diabetic rats. No significant difference in erectile response was noted upon injection of blank microspheres.

Long-Term Efficacy Studies

To assess the long-term durability of the NO-releasing microspheres, additional rats were injected with microspheres at 8 weeks following diabetes induction. Rats then underwent erectile response studies on days 1, 3, 7, 10, 14, 17, 21, 28, and 35 with concomitant IV sildenafil (n = 3–4).

Maximal ICP/MAP ratio was then determined for each time point, and these data are summarized in Figure 7. Maximal ICP/MAP remained greater than 0.8 during the first 3 days but then steadily decreased until it reached a level equal to sildenafil alone. As compared with sildenafil alone, the addition of NO-releasing microspheres significantly
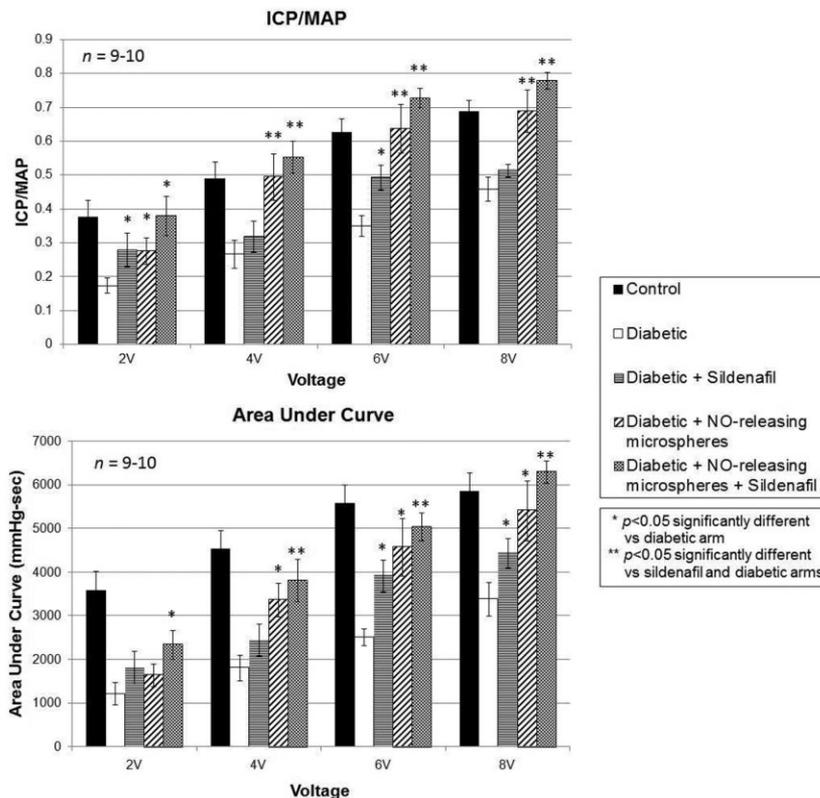

Figure 6 Results of erectile function studies depicting the average ICP/MAP (top) and AUC (bottom) achieved after CNS for 1 minute at 2–8 V in control rats, untreated diabetic rats, and diabetic rats treated with IV sildenafil, NO-releasing microspheres, or combination therapy. n indicates the number of rats tested per group.

Figure 7 Long-term NO-releasing microsphere efficacy at designated time points following microsphere injection. All rats administered concomitant 2 mg/kg IV sildenafil prior to erectile response testing. ICP/MAP achieved with sildenafil treatment alone (n = 10) included for comparison. n indicates the number of rats tested at each time point; *$P < 0.05$ response significantly different compared to sildenafil alone at 8 V only; **$P < 0.05$ response significantly different compared with sildenafil alone at all voltages tested.
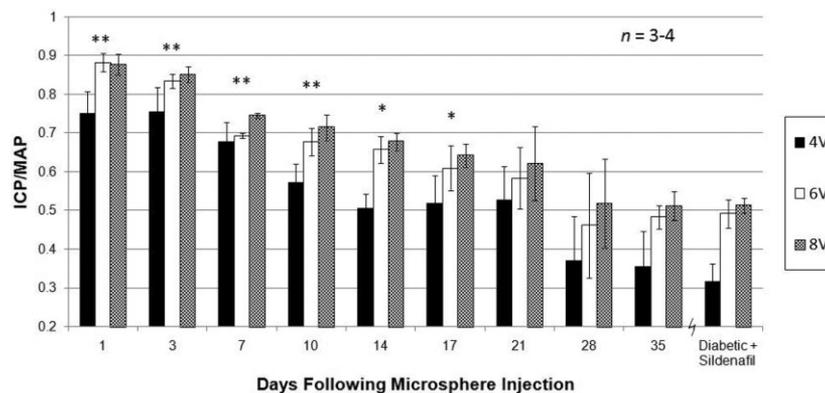
augmented the erectile response of diabetic rats for approximately 3 weeks following injection at 8 V of CNS ($P < 0.05$).

Discussion

Currently available medical therapies for ED include oral phosphodiesterase type 5 inhibitors (PDE5), intraurethral alprostadil, and intracavernosal trimix injections. Oral PDE5 inhibitors are able to potentiate the effects of NO by preventing intracellular cGMP breakdown, but they rely upon intact cavernous nerve function. However, release of neuronal and endothelial NO is often compromised in diabetic men due to angiopathic vascular changes and neuropathic changes and in postprostatectomy patients due to nerve injury [4,5,14,15]. Therefore, although these oral medications are widely utilized for ED therapy due to their ease of use, they suffer from unsatisfactory efficacy and high discontinuation rates in the hard-to-treat ED population consisting of diabetic and postprostatectomy patients [16–20].

Meanwhile, intraurethral alprostadil works independently of the cGMP pathway and does not rely on intact nerve function though it can suffer from poor compliance due to side effects [21]. Finally, intracavernosal trimix injection therapy is highly effective for triggering direct vasodilation and improving erectile quality, irrespective of nerve function [22–24]. However, many men are very reluctant to self-inject each time they want to obtain an erection [25]. As a result, many of these diabetic and postprostatectomy patients do not pursue treatment beyond oral PDE5 inhibitors or instead resort to surgical therapy with penile prostheses.

It is conceivable that many of these men would opt for an intermediate therapeutic option in which the patient or urologist injects a long-acting medication every few weeks, rather than the patient having to self-inject each time prior to intercourse. In men who achieve insufficient benefit from a PDE5 inhibitor, such a medication could be utilized as an adjunct therapy to augment the erectile response. Thus, such a long-acting medication could potentially bridge the therapeutic gap that currently exists between PDE5 inhibitors and on-demand intracavernosal injections by only requiring injection every few weeks.

Although NO-releasing polymers have previously been investigated in cardiovascular applications, the present study represents the first time they have been explored as a potential therapy for ED. In this study, we have incorporated diazeniumdiolates into PEG polymers, and then utilized these NO-releasing polymers to generate biocompatible microspheres. Under physiologic conditions *in vitro*, the microspheres are capable of sustained NO release for over 3 weeks. When injected intracavernosally, these polymeric microspheres do not migrate outside the corpus cavernosum of the rat either locally or distally. There was some apparent loss of signal intensity between day 0 and day 28 postinjection likely due to photobleaching and dissociation of the fluorescent dye over time.

In addition, while IV sildenafil was able to provide a modest improvement in erectile response in the diabetic rats, NO-releasing microspheres generated a substantial improvement that was significantly higher than sildenafil alone. Also of note, when diabetic rats were administered combination therapy with sildenafil and microspheres, they were able to achieve erectile responses equivalent to age-matched control rats. Finally, long-term efficacy studies demonstrated that the addition of NO-releasing microspheres provided significant improvement in erectile response for approximately 3 weeks as compared

with sildenafil alone at higher levels of nerve stimulation. However, it is possible that continuous exposure of the cavernosal tissues to NO donors could lead to tolerance. Additionally, various studies have shown that oxidative and nitrosative stress may play significant roles in development of ED [26–31]. At this point, it is unclear the exact impact that NO-releasing microspheres will have on oxidative and nitrosative stress in the penis. Further studies are certainly needed to evaluate the long-term impact of these microspheres.

Both the *in vitro* NO release studies and *in vivo* long-term durability studies demonstrated that the PEG-Lys$_4$-NO microspheres used in this study can maintain NO release and efficacy for about 3 weeks. However, it has previously been shown that longer polyamines tend to produce NO donor species with substantially longer half-lives due to formation of stabilizing hydrogen bonds [32]. Therefore, it is possible that slight alterations to the polymer structure or microsphere fabrication process could provide even further prolongation of NO release and therapeutic effect.

Conclusion

In the present study, we have developed biocompatible injectable polymeric microspheres that exhibit sustained NO release for over 3 weeks under physiologic conditions. In addition, we have shown that these microspheres do not migrate outside the penile tissues when injected into the corpus cavernosum. Furthermore, we have demonstrated therapeutic potential for improving erectile responses in the diabetic rat ED model with these materials. Although the NO-releasing microspheres alone produced significant improvement over sildenafil therapy, the maximal ICP/MAP and AUC achieved by combination therapy essentially matched control levels. Moreover, the microspheres demonstrated the ability to augment the erectile response of diabetic rats for up to 3 weeks following injection. Thus, these NO-releasing polymeric microspheres offer a novel approach to therapy for diabetes-related ED.

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Category 2
(a) Drafting the Article
Samit Soni
(b) Revising It for Intellectual Content
Samit Soni; Mohit Khera; Jennifer West

Category 3
(a) Final Approval of the Completed Article
Samit Soni; Weitao Song; Mohit Khera; Jennifer West

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