Gene Therapy Using Replication-Defective Herpes Simplex Virus Vectors Expressing Nerve Growth Factor in a Rat Model of Diabetic Cystopathy

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Diabetic cystopathy is one of the common complications of diabetes and current therapy is limited. In the present study, the effects of gene therapy, using replication-defective herpes simplex virus type 1 (HSV-1) vectors to deliver and express the nerve growth factor (NGF) gene (HSV-NGF) on tissue NGF levels and bladder function, were evaluated in streptozotocin (STZ)-induced diabetic rats. Diabetic rats exhibited a significant decrease in NGF levels in the bladder and lumbosacral dorsal root ganglia (DRG) detected by enzyme-linked immunosorbent assay and displayed marked bladder dysfunction 12 weeks after STZ injection. In contrast, rats with bladder wall injection of the NGF expression vector 8 weeks after STZ treatment exhibited a significant increase of NGF levels in the bladder and L6 DRG 4 weeks after HSV-NGF injection. Along with the restoration of tissue NGF expression, in metabolic cage studies and cystometry, HSV-NGF–injected rats also showed significantly reduced bladder capacity and postvoid residual volume than diabetic rats injected with the control vector (HSV-lacZ), indicating that voiding function was improved after HSV vector–mediated NGF gene delivery. Thus, HSV vector–mediated NGF gene therapy may prove useful to restore decreased NGF expression in the bladder and bladder afferent pathways, thereby improving hypoactive bladder function in diabetes. Diabetes 53:2723–2730, 2004

Diabetes is a major health concern that often induces urological complications. As many as 85% of patients with diabetes exhibit bladder dysfunction characterized by impaired sensation of bladder fullness, increased bladder capacity, reduced bladder contractility, and elevated residual urine volume (1,2). It has been documented that diabetic cystopathy is induced by polyneuropathy that predominantly affects sensory and autonomic nerve fibers (3). Because diabetic cystopathy is often irreversible, treatment options for diabetic cystopathy are limited, and it has proven to be difficult to restore bladder function in diabetic patients (4). The development of new therapeutic approaches for diabetic cystopathy is therefore needed.

Alteration in the tissue level of nerve growth factor (NGF), one member of the neurotrophic factor family, has been a major focus as a contributing factor inducing diabetic neuropathy. Previous studies have reported that a deficiency in retrograde axonal transport of NGF from target organs to sensory pathways may play an important role in inducing diabetic neuropathy (5). We have also reported that diabetic rats displaying measurable bladder dysfunction demonstrated a decrease in tissue NGF levels in the bladder and lumbosacral dorsal root ganglia (DRG) that correlated with the progression of diabetic cystopathy, suggesting that reduced availability of NGF is also attributable to diabetic cystopathy (4). It has been well documented that NGF is necessary for maintaining normal function of mature sensory and sympathetic neurons, in addition to its ability to promote the survival of the neurons during development (6). The neuroprotective effects of NGF on sympathetic and sensory neurons after various neurotoxic treatments have also been demonstrated (7). Therefore, NGF supplement therapy has been performed and shown to be effective for the treatment of diabetic neuropathy in animals (8,9).

Herpes simplex virus type 1 (HSV-1) represents a viral vector system with several biological features that make it attractive for gene delivery to the peripheral or visceral nervous system (10,11). HSV-1 is a large (152-kb) double-stranded DNA virus and has a large ample genome space capable of accommodating large or multiple cDNA inserts (10–15 kb) (12) and is readily cultured and can be propagated to high titers (10). Moreover, the virus is neurotropic, displaying an affinity for sensory nerves where the viral genome persists as an episomal molecule within these sensory nerve cell bodies as part of the normal life cycle of the virus (13). Replication-defective virus recombinant vectors have been recently created, with a loss of certain immediate-early genes that allow an extra margin of safety for clinical applications and reported reduced cellular toxicity (14,15). The potential utility of HSV gene therapy has been shown by expression of a number of genes or proteins (7,16–20). In addition, HSV possesses a natural promoter system, the latency active promoters

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LAP1 and LAP2, which may be used to drive long-term transgenic expression in neurons of the peripheral nervous system (16).

Based on these observations, it is tempting to hypothesize that gene therapy using replication-defective HSV vectors expressing the NGF gene can restore the tissue NGF levels not only in the bladder but also in the bladder afferent pathways and can improve bladder dysfunction induced by peripheral neuropathy in diabetics. We have previously reported the proof of concept for in vivo gene transfer in normal rats that demonstrated an increase of tissue NGF levels in the bladder and lumbosacral DRG after bladder wall injection of HSV vectors encoding the NGF gene (16). In the present study, we investigated whether NGF gene therapy using replication-defective HSV-1 vectors can restore the tissue NGF level in the bladder and L6 DRG, which contain bladder afferent neurons, and thereby improve bladder function in streptozotocin (STZ)-induced diabetic rats.

**RESEARCH DESIGN AND METHODS**

Vectors. Replication-defective HSV-1 vectors with (SLN vectors) and without (SHZ vectors) the β-NGF gene, the detailed construction of which was previously described (7), were used. In brief, the murine β-NGF cDNA fused to the SV40 late polyA signal was juxtaposed downstream of the HSV-1 LAP2 promoter (21) in a plasmid that contains the lacZ gene cassette under the control of the HSV-1 glycoprotein C late gene promoter. The expression cassettes were introduced into the ICP4-deleted vector backbone by Cre-lox recombination to yield the SLN expression vector (7,16). The expression control virus SHZ was engineered in a similar manner, except these recombinants lack the NGF cDNA expression cassette and fail to express NGF in culture or in vivo (7). Replication-defective vectors were propagated on the E5 cell line (22) and purified on Optiprep (Life Technologies, Gaithersburg, MD) gradients (23). Gradient-purified viruses were aliquoted and stored at −80°C and titered on the E5 complementing cell line. All preparations of replication-defective vectors were examined on Vero cells for the presence of replication-competent recombinants.

**Induction of diabetes.** Adult female Sprague-Dawley rats (weighing 250–300 g) obtained from Hilltop Laboratory (Pittsburgh, PA) were used in all of the studies. Diabetes was induced by a single intraperitoneal injection (65 mg/kg body wt) of STZ (Sigma, St. Louis, MO) freshly dissolved in 0.9% sterile saline before injection. The care and handling of animals were in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

**Viral injection.** HSV-1 vectors were injected 8 weeks after diabetes induction. Under halothane anesthesia, a low midline incision was made to expose the bladder, and vectors were injected into the bladder wall using a 30-gauge Hamilton syringe. A total of 30 × 10^8 plaque-forming units (PFU) of either SLN or SHZ was injected at four sites around the bladder base. After the abdomen was closed, rats were allowed to recover from halothane anesthesia for 1 h, five rats in each group (normal SHZ- or SLN-injected group) were anesthetized with urethane (0.84 g/kg s.c.), and single cystometry (CMG) analysis was performed under a urethane-anesthetized condition. After withdrawing intravesical fluid through the catheter, first by gravity and then by manually compressing the bladder, saline at room temperature (20–22°C) was infused at the same rate (0.08 ml/min) until bladder voiding contraction was observed, and bladder capacity was then calculated. Thereafter, the solution was switched to saline containing 0.25% acetic acid to elicit bladder nociceptive responses, which were evaluated by a reduction of intercontraction intervals of repetitive bladder contractions.

**Measurement of NGF by enzyme-linked immunosorbent assay.** Under urethane anesthesia, bladder and L5–L6 DRG tissues were removed from SHZ-injected diabetic rats (n = 6) and SLN-injected rats (n = 8), which were used for cystometry. Bladder and L5–L6 DRG tissues were also removed from a separate group of 12 normal rats and used as the control. The tissues were weighed, homogenized at −22°C and stored at −80°C until protein extraction. The tissue NGF and protein levels in the bladder and L6 DRG were measured as described previously (4). Briefly, the samples were homogenized in homogenization buffer, and the homogenate was centrifuged at 10,000 g for 4 min. The supernatant was diluted with four volumes of Dulbecco’s PBS. The samples were acidified by the addition of 10 N HCl to pH 2.0–3.0 for 15 min at room temperature (20–22°C) and then neutralized by the addition of 10 N NaOH to pH 7.5–8.0. After acid treatment, the samples were stored at −80°C until assayed. The samples were assayed in triplicate in an antigen-capture enzyme-linked immunosorbent assay (Promega, Madison, WI) according to the manufacturer’s instructions. Plates were read at 450 nm on an Eks800 microplate reader (Bio-Tek Instruments, Winooski, VT). Total protein concentration for the same samples was also determined with a protein assay kit (Pierce, Rockford, IL). All tissue NGF values were then standardized to tissue protein levels and expressed as picograms per microgram protein.

**Histochemical staining.** Four weeks after SHZ bladder wall injection in diabetic rats, the bladder and L6 DRG were harvested under urethane anesthesia. Tissues were fixed lightly in acetone for 2 min, treated in 10–30% sucrose solutions for 3 h, and then kept at −80°C until sectioning. Cryostat sections (14 μm) of the tissues were mounted on gelatin-coated slides. The slides were fixed for 2 min in 1% glutaraldehyde (Sigma, St. Louis, MO), rinsed twice in PBS, and incubated overnight at 37°C in X-gal substrate ([0.4 mg/ml 5-bromo-chloro-3-indolyl-β-D-galactosidase (Boehringer Mannheim, Indianapolis, IN), 1 mmol/l MgCl2, 5 mmol/l KFe(CN)6, and 5 mmol/l KFe(CN)6] in PBS). Sections were then counterstained with eosin. Tissues from the same animals were also incubated overnight at 37°C in X-gal–free substrate [1 mmol/l MgCl2, 5 mmol/l KFe(CN)6, and 5 mmol/l KFe(CN)6] in PBS] as a negative control. After X-gal staining, the samples were photographed using a digital-imaging camera connected to the microscope (Olympus, Tokyo). The density of X-gal-stained regions of the sections were measured using Image-Pro Express software (Media Cybernetics, Silver Spring, MD).

**Statistical analysis.** All data were expressed as means ± SE. Nonparametric tests (unpaired t test) were used to test differences in the ICI, voided volume per micturition, bladder capacity, postvoid residual volume, and tissue NGF levels. For all statistical tests, P < 0.05 was considered significant.

**RESULTS**

**Blood glucose level.** When compared with untreated rats, diabetic rats showed 8–18% weight loss from the initial body weight as well as increased fluid intake during a period of 12 weeks after STZ injection. In addition, similar reductions in body weight were found in diabetic rats injected with SHZ control vectors and SLN vectors. Blood glucose levels in diabetic rats were elevated (331.5 ± 43.9 mg/dl) when cystometry was performed, and there was no significant difference in blood glucose levels in diabetic rats injected with SHZ control vectors and SLN.
vectors. Blood glucose levels of >300 mg/dl were used as inclusion criteria to place animals in the diabetic group for all further studies involving HSV vector injection.

**Metabolic cage study.** Measurements of micturition patterns on animals placed in metabolic cages 4 weeks after viral injection (12 weeks after diabetes induction) revealed that voided volume per micturition in diabetic rats injected with the SHZ control vector was significantly greater than that observed in normal rats (3.31 ± 0.25 vs. 0.65 ± 0.08 ml, P < 0.01) (Fig. 1). Voided urine volume for 24 h was also significantly greater in diabetic rats than in normal rats (97.6 ± 5.3 vs. 13.2 ± 0.9 ml, P < 0.01). However, voided urine volume for 24 h was not different in diabetic rats treated with SLN and SHZ injection (76.4 ± 9.4 vs. 97.6 ± 5.3 ml).

**Cystometry.** Cystometry (Fig. 2) performed in conscious diabetic rats revealed that bladder capacity for inducing voiding significantly increased (1.75 ± 0.19 ml, P < 0.01) in diabetic rats with SHZ injection (Fig. 2) when compared with normal rats (0.56 ± 0.07 ml). Bladder capacity in diabetic rats with SLN injection (0.84 ± 0.07 ml, P < 0.05) was significantly smaller (P < 0.01) than that of diabetic rats with SHZ injection, although it was still significantly larger compared with normal rats (Fig. 2). Postvoid residual volume in diabetic rats with both SHZ and SLN injection (0.22 ± 0.02 and 0.08 ± 0.02 ml, respectively; P < 0.01) was greater than that in normal animals (0.01 ± 0.01 ml). However, the postvoid residual volume of diabetic rats with SLN injection was significantly smaller (P < 0.01) than that of diabetic rats with SHZ injection rats (Fig. 2B). These reductions in bladder capacity and postvoid residual volume were observed in all SLN-injected rats tested when compared with SHZ-injected rats. Other parameters, such as pressure threshold for voiding, voiding efficiency, and maximal voiding pressure, were not significantly different in diabetic rats with SLN or SHZ injection.

Cystometry under urethane anesthesia also demonstrated that diabetic rats with SLN injection showed significantly smaller bladder capacity (1.78 ± 0.09 vs. 3.19 ± 0.38 ml, P < 0.01) than diabetic rats injected with SHZ. When acetic acid was infused into the bladder of these animals, the reduction in the ICI in both groups of diabetic rats with SHZ and SLN injection was significantly smaller than that in normal rats (31.0 ± 9.0 and 22.5 ± 7.8 vs. 79.7 ± 8.7% reduction, respectively; P < 0.01) (Fig. 3). However, there was no significant difference in the average reduction of ICI after intravesical acetic acid infusion between diabetic rats with SLN and SHZ injection (Fig. 3), suggesting that *NGF* gene delivery did not enhance blad-

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**FIG. 1.** Micturition patterns in a metabolic cage study to evaluate the efficacy of HSV vector–mediated NGF delivery to the bladder. \( A: \) Representative traces of voided urine volume plotted against time in normal rats (upper trace) and diabetic rats (middle trace: diabetic rats with SHZ control vector injection; lower trace: diabetic rats injected with SLN). \( B: \) Averaged voided volume per micturition (normal rats: \( n = 9; \) SHZ: \( n = 10; \) SLN: \( n = 11) \). **\( P < 0.01, *P < 0.05. \)
der nociceptive responses induced by acetic acid infusion into the bladder.

**Tissue NGF levels.** There were no significant differences in total protein levels per tissue weight in the bladder (78.34 ± 4.14 vs. 85.66 ± 12.50 mg/g wet tissue, n = 4) and L6 DRG (270.30 ± 2.01 vs. 225.70 ± 2.49 mg/g wet tissue, n = 4) obtained from normal and diabetic rats. As we reported previously (4), tissue NGF levels in the bladder (46.26 ± 7.82 vs. 90.87 ± 11.33 pg/μg protein, P < 0.02) and L6 DRG (24.07 ± 1.31 vs. 51.35 ± 3.70 pg/μg protein, P < 0.01) were significantly decreased in 12-week diabetic rats with SHZ injection compared with normal rats (Fig. 4A). However, 4 weeks after SLN injection (12 weeks after diabetes induction), tissue NGF levels in the bladder (75.35 ± 5.96 pg/μg protein, P = 0.01) (Fig. 4A) and L6 DRG (34.61 ± 3.54 pg/μg protein, P = 0.03) (Fig. 4B) were significantly increased when compared with diabetic rats with SHZ infection, suggesting that HSV vector-mediated NGF delivery partially restored the NGF levels in the bladder and L6 DRG, which contains bladder afferent neurons.

**Immunohistochemical staining.** Positive histochemical staining for β-galactosidase was observed in both the bladder and L6 DRG 4 weeks after bladder wall injection of SHZ vectors in diabetic rats. Bladder sections in diabetic rats showed specific activity 4 weeks after SHZ injection (Fig. 5A). HSV vector-mediated β-gal expression was observed mainly in the bladder neck area, where virus vectors were injected, suggesting that HSV vectors were confined at the injection sites without significant diffusion in the bladder. DRG sections also demonstrated positive staining in cell bodies in L6 DRG (Fig. 5B), demonstrating that the vectors were able to infect the correct target tissue in diabetic animals, as previously shown in normal rats (16).

## DISCUSSION

Previous studies have demonstrated that 1) diabetic cystopathy is one of the common complications of diabetes, 2) bladder dysfunction in diabetes is attributable to diabetic neuropathy that induces sensory and autonomic nerve dysfunction, and 3) diabetic cystopathy can develop insidiously and may be found even in the early stages of diabetes (24). Although there are some conservative treatments available for diabetic cystopathy, none can currently restore bladder function (24).

It has been reported that one of the major pathological changes inducing diabetic neuropathy is an alteration in the availability of neurotrophic factors such as NGF produced in target organs (25). Previous studies have also revealed that diabetic rats exhibited decreased NGF pro-
duction in target organs, as well as reduced axonal transport of NGF in afferent pathways (5,26). We have also recently found that the time-dependent reduction in tissue NGF levels in the bladder and lumbosacral DRGs, which contain bladder afferent neurons, is well correlated with bladder dysfunction in diabetic rats up to 12 weeks after STZ injection (4), although early increases in NGF levels in the diabetic rat bladder have also been reported (27). In addition, it has also been shown that change in the levels of other neurotrophic factors, such as tissue neurotro-

FIG. 3. CMG analyses in urethane-anesthetized animals to evaluate the efficacy of HSV vector-mediated NGF delivery to the bladder. A: Representative traces of CMGs in normal rats (upper trace) and diabetic rats (middle trace: diabetic rats injected with SHZ; lower trace: diabetic rats injected with SLN) before and after intravesical application of acetic acid (0.25%). B: Averaged percent reduction in ICI after acetic acid infusion (normal rats: n = 5; diabetic rats injected with SHZ: n = 5; diabetic rats injected with SLN: n = 5). **P < 0.01.

FIG. 4. NGF levels measured by enzyme-linked immunosorbent assay in the bladder (A) and L6 DRG (B) of normal rats (n = 12), untreated diabetic rats 12 weeks after STZ injection (DM12W), diabetic rats with SHZ injection (SHZ, n = 6), and diabetic rats with SLN injection (SLN, n = 8). Virus vectors were injected 8 weeks after diabetes induction, and NGF levels were measured 4 weeks after virus injection. Note that reduced NGF levels in the bladder and L6 DRG were significantly elevated in diabetic rats with SLN injection compared with untreated diabetic rats and diabetic rats with SHZ injection (bladder: P < 0.01; L6 DRG: P < 0.05). *P < 0.05, **P < 0.01. prot., protein.
phin-3 levels, could play a role in the pathogenesis of diabetic neuropathy (28–31). Overall, it seems reasonable to assume that the reduced production of neurotrophic factors including NGF in target organs (e.g., urinary bladder), which leads to the reduced transport of those factors to their afferent pathways, contributes at least in part only to diabetic neuropathy, but also to bladder dysfunction in diabetics.

Therefore, it is likely that the NGF supplement therapy represents a reasonable approach for treating diabetic neuropathy and/or cystopathy. In this regard, new therapeutic approaches using NGF for diabetic neuropathy have been reported in both basic and clinical studies. For example, Apfel et al. (9) demonstrated that NGF administration restored responses to thermal stimuli in diabetic rats in vivo. Unger et al. (8) have also demonstrated that NGF treatment reversed distinct diabetes-related alterations in myelinated nerve fiber morphology. In clinical studies, phase I and II trials of systemic administration of human recombinant NGF revealed safety and potential efficacy as a treatment for diabetic polyneuropathy (32). However, the phase III trial did not show significantly beneficial effects (33), because the doses required to achieve efficacy in humans, compared with comparable doses used in the preclinical animal studies, result in a variety of unwanted side effects. It has been well documented that the contributing factors to the failure of the large-scale phase III NGF clinical trial included inadequate dosage of NGF that was limited by painful or burning sensation syndrome after systemic NGF injection (34). It might also be speculated that development of anti-NGF antibodies after systemic NGF injection by patients could be another factor involved in the failure of the NGF clinical trial. These complications may be avoided by administration of the trophic factor directly to the cells that require the factor. In contrast to the above approach, the present study showed that NGF gene therapy using replication-defective HSV-1 vectors restored tissue NGF levels decreased by diabetes in the bladder and L6 DRG (Fig. 4), which contains bladder afferent neurons, in STZ-induced diabetic rats 4 weeks after viral vector injection into the bladder wall. In addition, this restoration of NGF levels in the bladder and L6 DRG was accompanied by improved voiding function, evidenced by a reduction in bladder capacity inducing the micturition reflex (Figs. 1 and 2) without enhancing bladder nociceptive responses induced by intravesical chemical irritation (Fig. 3). Thus, it is assumed that NGF gene therapy using replication-defective HSV-1 vectors could be effective to treat diabetic cystopathy, which is characterized by impaired sensations of bladder fullness, increased bladder capacity, reduced bladder contractility, and elevated postvoid residual urine volume (2,35).

It is well known that sensory signals originating from the bladder are carried by two kinds of sensory fibers to the spinal cord (i.e., Aδ-fibers and C-fibers) (36). Conscious voiding depends on Aδ-fiber bladder afferents even though Aδ-fiber and C-fiber bladder afferents are mechanosensitive, whereas C-fibers are responsible for bladder nociceptive responses in normal rats (37,38). We have previously reported that diabetes induced the damage of both Aδ- and C-fiber populations in bladder afferent pathways, which was evidenced by increased bladder capacity as measured by conscious cystometry and reduced bladder hyperactivity induced by intravesical acetic acid instillation, respectively, in association with the reduction of tissue NGF levels in the bladder and lumbosacral DRG (4).

FIG. 5. HSV vector–mediated β-gal expression in bladder smooth muscles and L6 DRG from the diabetic rat. A: Bladder wall with large area of positive staining smooth muscle cells present along the wall of the bladder (40×). B: L6 DRG demonstrating positively stained DRG neurons (arrows) (100×) after bladder inoculation of HSV vector–mediated β-gal expression. Calibration bar: 250 μm (A) and 100 μm (B).
The present study further revealed that restored levels of NGF in the bladder and lumbosacral DRG induced by HSV vector–mediated NGF delivery improved Aδ-fiber–mediated voiding function, as evidenced by the reduction in enlarged bladder capacity and increased postvoid residual urine volume in conscious diabetic rats. Therefore, it is likely that HSV vector–mediated NGF gene therapy was effective in ameliorating damaged Aδ-fiber function, thereby improving the micturition reflex in diabetic rats.

In contrast to the improvement of Aδ-fiber–mediated voiding function after HSV vector–mediated NGF gene therapy, diminished C-fiber–mediated bladder nociceptive responses in 12-week diabetic rats were not reversed by the therapy. This result was unexpected because previous studies have demonstrated that NGF instillation into the bladder can induce nociceptive responses, such as bladder hyperactivity in normal rats (39,40), and that high-affinity NGF receptors (trkA) are abundantly found on C-fibers, although they are found in Aδ-fibers as well (25). It has been reported that small-caliber C-fiber afferents are often the earliest affected, although all fiber classes are affected in diabetic neuropathy (41). Thus, it might be possible to speculate that the damage of C-fiber bladder afferents is more pronounced than the damage of Aδ-fiber afferents in diabetes and that NGF gene therapy using HSV vectors in our study might not have rescued damaged C-fibers in bladder afferent pathways, although there is the possibility that higher vector dose or repeated vector injection may improve bladder dysfunction mediated by C-fiber neuropathy. It has also recently been reported that in diabetic neuropathy affecting unmyelinated C-fiber afferents, non-peptidergic unmyelinated sensory neurons are vulnerable to diabetes and that administration of glial cell line–derived neurotrophic factor, but not NGF, reversed the deficit caused by diabetes (41). Thus, it also seems likely that HSV-mediated NGF gene therapy may not be effective to reverse diabetes-induced C-fiber neuropathy, at least in the bladder afferent pathway. Further studies are needed to examine the effects of glial cell line–derived neurotrophic factor on C-fiber– and Aδ-fiber–mediated bladder activity in diabetic cystopathy. Nevertheless, our results that localized NGF gene therapy using replication-defective HSV vectors restored normal voiding function without inducing nociceptive responses in the bladder seems encouraging because previous clinical trials using systemic administration of NGF were hampered by the adverse effects of painful or burning sensation syndromes, which were dose limiting for NGF (32–34).

In conclusion, this study provides the first evidence of the efficacy of NGF gene therapy using replication-defective HSV-1 vectors for diabetic cystopathy. In STZ-induced diabetic rats, bladder wall injections of replication-defective HSV-1 vectors expressing β-NGF restored the decreased tissue NGF levels in the bladder and L6 DRG that contained bladder afferent neurons. Along with the restoration of tissue NGF levels, voiding function was significantly improved without affecting bladder nociceptive responses. Therefore, NGF supplementation using HSV vector–based NGF gene therapy could prove useful to treat diabetic bladder dysfunction. The ultimate goal of these studies is to use an HSV vector–based approach in human clinical trials.

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