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Gene and stem cell therapy for erectile dysfunction

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Erectile dysfunction (ED) is defined as the inability to attain and/or maintain penile erection sufficient for satisfactory sexual performance. ED is a highly prevalent health problem with considerable impact on the quality of life of men and their partners. Although the treatment of ED with oral phosphodiesterase type V (PDE5) inhibitors is effective in a wide range of individuals, it is not efficacious in all patients. The failure of PDE5 inhibitors happens mainly in men with diabetes, non-nerve sparing radical prostatectomy, and high disease severity. Therefore, improved therapies based on a better understanding of the fundamental issues in erectile physiology and pathophysiology have recently been proposed. Here, we summarize studies on ED treatment using gene and stem cell therapies. Adenoviral-mediated intracavernosal transfer of therapeutic genes, such as endothelial nitric oxide synthase (eNOS), calcitonin gene-related peptide (CGRP), superoxide dismutase (SOD), and RhoA/Rho kinase and mesenchymal stem cell-based cell and gene therapy strategy for the treatment of age- and diabetes-related ED are the focus of this review.
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Penile erection, referred to as engorgement of the penis with blood, is the result of the penile arterial dilation which increases blood inflow, the relaxation of smooth muscle of the corpus cavernosum which increases blood storage, and penile venous occlusion which decreases blood outflow. The physiology of penile erection is complicated and this neurovascular event requires the functional integrity of nerves, endothelium, and smooth muscle.[1] Nitric oxide (NO), produced by both neuronal nitric oxide synthase (nNOS) in the nonadrenergic noncholinergic (NANC) nerves and endothelial nitric oxide synthase (eNOS) in the endothelium of penile arteries and cavernosal sinusoids is the principal mediator of penile erection.[2–5] NO formed in nerves or endothelial cells then diffuses to neighboring smooth muscle cells and activates soluble guanylyl cyclase. This activation results in increased cGMP formation, which mediates smooth muscle relaxation.[5] Besides NO, other molecules such as vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), substance P, and pituitary adenylate cyclase-activating polypeptide (PACAP) are also important for penile erection.[6] Causes contributing to erectile dysfunction (ED) can be broadly classified into two categories: organic and psychological. Aging, vascular diseases, neurological injuries, and diabetes mellitus are the common causes of organic ED.[7] Depressive stress is the common cause of psychological ED.[8,9]

Although the treatment of ED with oral phosphodiesterase type V (PDE5) inhibitors (sildenafil, tadalafil, and vardenafil) is effective in a wide range of individuals with ED, it is not efficacious in all patients. For example, the response rate to sildenafil decreased from 72% in men 18–49 y of age to 53% in men 50 y or older.[10] The failure of sildenafil therapy happened mainly in men with diabetes, non-nerve sparing radical prostatectomy, and high disease severity.[11–13] Therefore, improved therapies based on a better understanding of the fundamental issues in erectile physiology and pathophysiology are needed. Here we summarize recent studies on ED treatment using gene and stem cell therapy strategies.

eNOS gene therapy for ED

NO, the major mediator of penile erection, is synthesized by nNOS and eNOS.[5,14] It has been shown that NOS activity decreases with age and

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decreased NOS activity is associated with age-related ED.[15,16] To determine whether adenoviral-mediated overexpression of eNOS could enhance erectile responses, we injected recombinant adenovirus containing the eNOS gene or the β-galactosidase reporter gene lacZ driven by the cytomegalovirus (CMV) promoter (AdCMVeNOS or AdCMVlacZ) into the corpus cavernosum of the aged rat. The expression of β-galactosidase was observed in cavernosal tissue 1 day after intracavernosal injection of AdCMVlacZ. At 1 day after administration of AdCMVeNOS, eNOS transgene expression in cavernosal tissue was confirmed by Western blot analysis, and cGMP level in cavernosal tissue was increased. The increase in intracavernosal pressure in response to cavernosal nerve stimulation was enhanced in rats treated with AdCMVeNOS, and erectile responses to acetylcholine and zaprinast were enhanced at a time when erectile responses to the NO donor sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate were not altered. These results suggest that *in vivo* gene transfer of eNOS via adenoviral vector, alone or in combination with a type V phosphodiesterase inhibitor such as zaprinast, may constitute a new therapeutic intervention for the treatment of ED.[17] In order to find a better promoter for longer eNOS transgene expression in the corpus cavernosum, the second set of experiments using recombinant adenovirus containing eNOS gene or lacZ gene driven by the Rous sarcoma virus (RSV) promoter (AdRSVeNOS or AdRSVlacZ) was conducted. In this study, two groups of animals were treated with adenovirus containing the transgene driven by RSV promotor: (1) aged rats (60 weeks) with AdRSVlacZ; and (2) aged rats (60 weeks) with AdRSVeNOS. At 5 days after intracavernosal injection of adenovirus, these animals underwent cavernosal nerve stimulation to assess erectile function and responses were compared with young control rats (20 weeks). Cross-sections of rat penis injected with AdRSVeNOS were examined after trichrome staining. The transduction efficiency of adenoviral-mediated lacZ gene transfer was measured by the galacto-light chemiluminescent reporter gene assay in the cavernosal tissue of rats intracavernosally injected with AdRSVlacZ. The eNOS transgene expression was examined by RT-PCR in rats intracavernosally injected with AdRSVeNOS. The eNOS and iNOS protein levels were measured by Western blot analysis and cGMP level were assessed in cavernosal tissue by enzyme immunoassay. Adenoviral expression of the β-galactosidase reporter gene lacZ was observed in cavernosal tissue for up to 30 days, with peak expression level at 5 days after intracavernosal injection of AdRSVlacZ. Cross-sections of rat penis injected with AdRSVeNOS revealed no pathological (morphological or histological) changes. At 5 days after administration of AdRSVeNOS, eNOS protein, eNOS mRNA, and cGMP level in the corpus cavernosum were significantly increased ($P<0.05$), while iNOS protein level remained unchanged ($P>0.05$). At 5 days after administration of adenovirus, erectile responses to cavernosal nerve stimulation in the aged rat treated with AdRSVeNOS were significantly increased, similar to the responses observed in young rats. There was no increase in erectile responses to cavernosal nerve stimulation in the aged rat intracavernosally injected with AdRSVlacZ. These data suggest that *in vivo* adenoviral gene transfer of eNOS driven by the RSV promoter can physiologically improve erectile function in the aged rat for a relatively longer period of time.[18]

The major contributing factor to diabetes-related ED is the reduced production of NO caused by the significant decrease in both nNOS and eNOS protein expression and activity in the penis.[19–21] To determine whether adenoviral gene transfer of eNOS to the penis of streptozotocin (STZ)-induced diabetic rats with ED[22] could improve the impaired erectile function, rats were intracavernosally injected with adenovirus. The following experimental groups of animals were treated with adenovirus (1) STZ-induced diabetic rats with AdCMVlacZ; and (2) STZ-induced diabetic rats with AdCMVeNOS. STZ-induced diabetic rats had a significant decrease in erectile function, as determined by peak and total intracavernosal pressure (ICP) after cavernosal nerve stimulation, compared to age-matched healthy control rats. At 1–2 days after intracavernosal injection of AdCMVlacZ, β-galactosidase staining was localized to the endothelium and smooth muscle cells of the rat penis. At 1–2 days after AdCMVeNOS administration, the rats underwent cavernosal nerve stimulation to assess erectile function and their responses were compared with those of age-matched healthy control rats. STZ-induced diabetic rats treated with AdCMVeNOS had peak and total ICP similar to those in control animals. There was no increase in peak or total ICP in STZ-induced diabetic rats treated with AdCMVlacZ. This change in erectile function was a result of eNOS over expression with an increase in eNOS protein expression and constitutive NOS activity as well as an increase in nitric oxide biosynthesis, as reflected by an increase in cavernous nitrate plus nitrite formation. There was no change in nNOS protein expression or iNOS activity after adenoviral administration. Therefore, these data demonstrate that intracavernosal administration of adenoviral vector containing eNOS can improve erectile function in the STZ-induced diabetic rat with ED.[23] To determine whether a combination of eNOS gene transfer and sildenafil could have better therapeutic effects for ED related to diabetes mellitus, another set of experiments was carried out. In this study, five groups of animals were used: (1) age-matched healthy control rats, (2) STZ-induced diabetic rats, (3) STZ-induced diabetic rats + sildenafil (2 mg/kg

i.v.), (4) STZ-induced diabetic rats intracavernosally injected with AdCMVlacZ or AdCMVeNOS, and (5) STZ-induced diabetic rats intracavernosally injected with AdCMVeNOS + sildenafil (2 mg/kg i.v.). At 2 months after i.p. injection of STZ, groups 4 and 5 were intracavernosally injected with adenoviruses and 1–2 days later all animals underwent cavernosal nerve stimulation to assess erectile function. STZ-induced diabetic rats had a significant decrease in erectile function as determined by the peak and total ICP after cavernosal nerve stimulation when compared to control rats. STZ-induced diabetic rats intracavenosally injected with AdCMVeNOS had the peak and total ICP similar to control animals. STZ-induced diabetic rats treated with sildenafil showed a significant increase in peak ICP at the 5 and 7.5 V settings, while the total ICP was significantly increased at all voltage (V) settings. The increase in both peak and total ICP at all V settings of STZ-induced diabetic rats intracavernosally injected with AdCMVeNOS was greater than STZ-induced diabetic rats intracavernosally injected with AdCMVlacZ. STZ-induced diabetic rats intracavernosally injected with AdCMVeNOS and administered with sildenafil had a significant increase in total ICP that was greater than eNOS gene transfer alone. Further, cGMP level in cavernosal tissue was significantly decreased in STZ-induced diabetic rats, but was increased after intracavernosal injection with AdCMVeNOS to values greater than control animals. In conclusion, overexpression of eNOS in combination with sildenafil significantly increased both the peak and total ICP to cavernosal nerve stimulation in the STZ-induced diabetic rat, which were similar to the responses observed in healthy control rats. Moreover, the total erectile response in STZ-induced diabetic rats receiving eNOS gene transfer plus sildenafil was greater than STZ-induced diabetic rats receiving eNOS gene transfer or sildenafil alone.[24]

CGRP gene therapy for ED

Calcitonin gene-related peptide (CGRP), a 37-amino-acid neuropeptide, is a potent vasodilator.[25–29] CGRP is extensively localized in perivascular or periadventitia nerves throughout the body and exerts vasodilator effect through interaction with its receptors on endothelial and vascular smooth muscle cells via endothelium-dependent or independent mechanisms, depending on vessel type and species.[30–34] Localized in corpora cavernosa, CGRP also contributes to smooth muscle relaxation in the corpus cavernosum and is downregulated in the aging penis.[35,36] CGRP relaxes the smooth muscle of corpora cavernosa by hyperpolarization via K^+-channel opening and activation of adenylate cyclase, with subsequent increases in intracellular cAMP leading to erection.[37] When CGRP is administered intracavernosally in patients suffering from age-related ED, a dose-related increase occurs in penile arterial inflow and erection.[35,38] Therefore, we sought to determine whether adenoviral-mediated CGRP gene transfer into corpora cavernosa could enhance erectile responses in aged rats with ED. In this study, a significant decrease in CGRP concentration and cAMP and cGMP levels in cavernosal tissue was found in aged rats, compared to young rats. Our data also showed that aged rats had significantly lower erectile responses to cavernosal nerve stimulation, compared to young rats. At 5 days after intracavernosal injection with AdRSV-prepro-CGRP (an adenoviral vector containing prepro-CGRP gene under the control of RSV promoter), these aged rats had a three-fold increase in cavernosal CGRP level, compared to animals intracavernosally injected with AdRSVntlacZ (an adenoviral vector containing nuclear-targeted β-galactosidase reporter gene ntlacZ). The AdRSVprepro-CGRP-treated rats also showed an increase in CGRP mRNA and immunohistochemical localization of CGRP protein in the smooth muscle of corpora cavernosa. In addition, cAMP level in corpora cavernosa was significantly increased, whereas cGMP level remained unchanged. Adenoviral transduction efficiency of ntlacZ was measured by chemiluminescence and was observed in cavernosal tissue 5 days after treatment with AdRSVntlacZ. More importantly, 5 days after administration of AdRSVprepro-CGRP, a significant increase was observed in erectile responses to cavernosal nerve stimulation in the aged rat, similar to responses observed in young rats. These data demonstrate that intracavernosal adenoviral gene transfer of CGRP can physiologically improve erectile function in the aged rat with ED.[39]

SOD gene therapy for ED

The reduced NO bioavailability contributing to age-related ED can also be caused by the enhanced inactivation of NO due to the oxidative stress in the aging penis.[40–43] Although NO is rapidly diffusible from nerves or endothelial cells to the neighboring smooth muscle cells for the induction of smooth muscle relaxation, it can be scavenged by its interaction with superoxide anion (O_2^-) within vessel walls or corporal sinusoids to form the toxic molecule peroxynitrite ($ONOO^-$).[44,45] O_2^- is involved in oxidative stress and increased O_2^- production in aging may contribute to vascular or smooth muscle dysfunction observed in the normal aging process through its destruction of NO.[46] Superoxide dismutase (SOD), an antioxidant enzyme catalyzing the conversion of O_2^- to H_2O_2 and O_2, plays an important role in the protection of cells against O_2^- radicals and the prevention of the formation of

peroxynitrite, which is extremely cytotoxic and contributes to tissue injury, vascular tone alteration, and organ dysfunction.[47] There are three known isoforms of SOD and the extracellular superoxide dismutase (EC-SOD) is thought to play a critical role in modulating the redox state of the vascular interstitium and thereby prevents the pathophysiological effects of O_2^- in the vasculature.[45,48] EC-SOD is released from cells into extracellular space such as blood, lymph, synovial fluids, and cerebrospinal fluid, highly expressed in blood vessels, and is the primary extracellular antioxidant enzyme for O_2^-.[48–50] The increased levels of O_2^- in the endothelium and smooth muscle of aging corpora cavernosa may cause the decrease in NO bioavailability observed in the aging penis. Thus reducing local O_2^- level by intracavernosal EC-SOD gene transfer may be an effective method to preserve NO bioavailability or bioactivity in the penile vasculature. Therefore, the following experiments were carried out to: (1) investigate the expression level of O_2^- in the penis of young and aged rats; (2) examine the effect of adenoviral gene transfer of EC-SOD to the penis to determine the consequence of overexpression of EC-SOD on O_2^- level and erectile function in the aged rat. In the cavernosal tissue of aged rats, lucigenin-enhanced chemiluminescence assay showed a three-fold increase in superoxide formation, and the oxidative fluorescent probe hydroethidine analysis indicated higher superoxide levels throughout the aged penis. This increase in superoxide level was associated with the impaired cavernosal nerve-mediated and agonist-induced erectile responses, increased nitrotyrosine staining, and decreased cGMP level. However, there was no compensatory change in cavernosal EC-SOD mRNA or protein in the aged rat. Intracavernosal injection with AdCMVEC-SOD (an adenoviral vector containing the EC-SOD under the control of CMV promoter) into aged rats resulted in a significant increase in erectile responses to cavernosal nerve stimulation, acetylcholine, and zaprinast to a magnitude similar to young rats. *In vivo* adenoviral gene transfer of EC-SOD to the penis resulted in higher expression of EC-SOD mRNA and protein, higher SOD activity and cGMP level, and lower nitrotyrosine staining. These data provide evidence in support of the hypothesis that ED associated with aging is related in part to an increase in cavernosal superoxide formation. Intracavernosal EC-SOD gene transfer reduces superoxide formation, restores age-associated erectile function and may represent a novel therapeutic strategy for the treatment of ED.[42]

RhoA/Rho kinase gene therapy for ED

Contraction and relaxation of corporal smooth muscle is essential for normal erectile function.[2,4] Smooth muscle contraction is mediated by phosphorylation of myosin light chain (MLC) via the Ca^{2+}/calmodulin-dependent activation of MLC kinase and actin/myosin cross-bridge formation.[51,52] Smooth muscle relaxation is mediated by dephosphorylation of MLC via MLC phosphatase.[53] A principle regulator of MLC phosphatase is Rho-kinase, which belongs to the serine/threonine kinase family. RhoA, a GTP-binding protein, mediates agonist-induced activation of Rho-kinase.[54] The exchange of GDP for GTP on RhoA and translocation of RhoA from the cytosol to the membrane are markers of activation, and enable the downstream stimulation of Rho-kinase.[55,56] It was previously found that the Rho-kinase protein expression was increased in diabetic rabbit corporal tissue, suggesting that RhoA/Rho-kinase signaling pathway may contribute to diabetes-related ED.[57] To test the hypothesis that RhoA/Rho-kinase contributes to diabetes-related ED and downregulates eNOS in the penis, we conducted the following experiments in the STZ-induced diabetic rats with ED.[22] First, we demonstrate that colocalization of Rho-kinase and eNOS protein was present in the endothelium of the corpus cavernosum by immunostaining assay. It was then observed that the levels of RhoA/Rho-kinase protein and MYPT-1 phosphorylation at Thr-696 were elevated in the STZ-induced diabetic rat penis. In addition, eNOS protein expression, cavernosal constitutive NOS activity, and cGMP level were reduced in the corpus cavernosum of the STZ-induced diabetic rats. To determine the functional role of RhoA/Rho-kinase in the penis, we evaluated the effects of intracavernosal injection of AAVTCMV19NRhoA (an adeno-associated virus containing the dominant-negative RhoA mutant driven by the CMV promoter) on RhoA/Rho-kinase, eNOS and erectile function in the STZ-induced diabetic rat with ED. There was a significant decrease in erectile responses to cavernosal nerve stimulation in the STZ-induced diabetic rat. Intracavernosal administration of AAVTCMV-19NRhoA improved erectile function in the STZ-induced diabetic rats to values similar to control rats. Further, STZ-induced diabetic rats intracavernosally injected with the RhoA inhibitor AAVCMVT19NRhoA had a reduction in both RhoA/Rho-kinase and MYPT-1 phosphorylation at a time when cavernosal eNOS protein, constitutive NOS activity, and cGMP level were restored to levels found in the age-matched healthy control rats. These data demonstrate a new mechanism for the downregulation of penile eNOS in diabetes mediated by activation of the RhoA/Rho-kinase pathway. More importantly, these data suggest that inhibition of RhoA/Rho-kinase can increase eNOS protein content and NOS activity in corpora cavernosa, thus restoring erectile function in diabetes.[58]

Mesenchymal stem cell-based cell and gene therapy for ED

Although intracavernosal injection of adenovirus or adeno-associated virus containing eNOS, CGRP, SOD, or dominant-negative RhoA mutant can augment erectile responses in aged or diabetic animals with ED as described above, the disadvantage of this therapeutic strategy such as a local inflammatory response and random transgene expression in almost all cell types could limit its application to human patients.[59,60] Therefore, improved therapies for ED are needed. Mesenchymal stem cells (MSCs), also known as marrow stromal cells, are multipotent adult stem cells from bone marrow that can differentiate into osteoblasts, chondrocytes, adipocytes, and other cell types. Therefore, MSCs can be used for tissue repair and tissue regeneration in adult stem cell-based cell therapy.[61–65] MSCs can be easily isolated by their adherence to cell culture plastic, readily *ex vivo* expanded, and efficiently gene engineered. MSCs do not elicit immune rejection and can survive for a long period of time *in vivo* after autologous transplantation. These properties make MSCs attractive vehicles for *ex vivo* gene therapy of various diseases.[66,67]

In order to develop improved therapies for ED using mesenchymal stem cell-based cell and gene therapy strategy, we conducted the following studies. In the first set of experiments, we demonstrate for the first time the successful adenoviral gene transfer of eNOS into *ex vivo* expanded MSCs. In this study, rat mesenchymal stem cells (rMSCs) were isolated, *ex vivo* expanded, and transduced with AdRSVeNOS, an adenoviral vector containing the eNOS gene under the control of RSV promoter. The production of eNOS protein in AdRSVeNOS-transduced rMSCs was confirmed by immunohistochemical and Western blot analysis. Transduction efficiency was dose dependent, and eNOS transgene expression in rMSCs persisted for greater than 21 days in culture. The rMSCs retained multipotential differentiation capability after adenoviral-mediated eNOS gene transfer. The eNOS protein expressed in AdRSVeNOS-transduced rMSCs was demonstrated to be biologically active by calcium-dependent conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline assay.[68]

In the second set of experiments, we demonstrate for the first time the successful adenoviral gene transfer of CGRP into *ex vivo* expanded MSCs. In this study, rMSCs were isolated, *ex vivo* expanded, and transduced with adenovirus. AdRSVprepro-CGRP and AdRSVntlacZ, adenoviral vectors containing prepro-CGRP or nuclear-targeted β-galactosidase reporter gene ntlacZ under the control of RSV promoter, were used. We found that transduction efficiency of adenoviral-mediated gene transfer into *ex vivo* expanded rMSCs is dose dependent, transgene expression persists for more than 21 days in culture, and adenoviral transduction does not alter the proliferation or viability of rMSCs. Transduced rMSCs retained multipotentiality and transgene expression after cell differentiation. The expression and secretion of CGRP by AdRSVprepro-CGRP-transduced rMSCs was confirmed by Western blot analysis and enzyme immunoassay. The secretion of CGRP by AdRSVprepro-CGRP-transduced rMSCs was dose dependent, and the transduced cells released as much as 9.5 pmol CGRP/1×10^6 cells/48 h into culture medium at a multiplicity of infection (MOI) of 300. Furthermore, culture supernatant from AdRSVprepro-CGRP-transduced rMSCs increased intracellular cAMP level in pulmonary artery smooth muscle cells in culture, confirming the biological activity of the CGRP released by AdRSVprepro-CGRP-transduced rMSCs.[69]

To test our hypothesis that therapeutic gene-modified MSCs can be used in stem cell-based cell and gene therapy of ED, we conducted the following study. rMSCs were transduced with AdRSVeNOS or AdRSVntlacZ at MOI 300 for 48 h. The cells were then washed with PBS three times and 5×10^5 cells were intracavernosally injected into the aged rat with ED. After 7 days, both eNOS protein expression level and the constitutive NOS activity in cavernosal tissue were increased in rats treated with AdRSVeNOS-rMSCs by Western blot analysis and calcium-dependent conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline assay. These AdRSVeNOS-rMSCs treated rats also underwent cavernsal nerve stimulation to assess erectile function and both peak and total ICP were significantly increased, compared to rats treated with AdRSVntlacZ-rMSCs or PBS. According to X-gal staining for lacZ and immunostaining for eNOS, at 7 days after intracavernosal injection, significant numbers of the transplanted β-galactosidase-positive AdRSVntlacZ-rMSCs and eNOS-positive AdRSVeNOS-rMSCs were identified within the proximal, mid, and distal corporal sinusoids. Hematoxylin & Eosin staining for inflammation assay suggested that there were no inflammatory cells in rat corpora cavernosa 7 days after intracavernosal injection of adenoviral-transduced rMSCs. Therefore, intracavernosal injection of AdRSVeNOS-rMSCs increased eNOS expression in the corpus cavernosum, improved erectile function in aged rats with ED, and avoided the inflammation caused by intracavernosal injection of adenovirus.[68,70]

More recently, we found that at 21 days after intracavernal injection of wild-type rMSCs, AdRSVntlacZ-rMSCs, or AdRSVeNOS-rMSCs in the aged rat with ED, both eNOS protein expression level and the constitutive NOS activity in cavernosal tissue were increased in all the three groups of animals, compared to PBS-treated group. Both peak and total ICP in response to cavernous nerve stimulation were significantly increased in all the

three groups of rats, compared to PBS-treated rats. According to X-gal staining for lacZ, at 21 days after intracavernosal injection, some transplanted β-galactosidase-positive AdRSVntlacZ-rMSCs still can be identified within the corporal sinusoids and the stem cell might have integrated into the cavernosal structure. Hematoxylin & Eosin staining for inflammation assay showed that there were no inflammatory cells in rat corpora cavernosa 21 days after intracavernosal injection of wild-type rMSCs, AdRSVntlacZ-rMSCs, or AdRSVeNOS-rMSCs (Kadowtiz *et al*, unpublished data). These data suggest that intracavernosal injection of wild type rMSCs alone can increase eNOS expression in the corpus cavernosum and improve erectile function in the aged rat with ED. However, the mechanism of MSC-based cell therapy for age-related ED is not clear, and it will be one of the objectives of our future investigation.

In conclusion, we have used a series of effective gene and stem cell therapies in age and diabetes related ED in experimental animals and our results suggest that there is great clinical potential for using stem cell-based cell and gene therapies as an effective and safe treatment for human patients with ED.

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