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ORIGINAL ARTICLE

Erectile Dysfunction

Cavernous nerve reconstruction with autologous vein graft and platelet-derived growth factors

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In this study, we investigated the feasibility of using autologous vein graft and platelet-derived growth factors to bridge transected cavernous nerve in a rat model. A short defect in the bilateral cavernous nerve was created and repaired with vein graft from the right jugular vein or vein graft plus platelet-derived growth factors. The 32 rats were divided into four groups, namely Group 1 - no repair as a negative control, Group 2 - vein graft alone, Group 3 - vein graft plus platelet-derived growth factors, and Group 4 - sham operation as a positive control. We evaluated nerve regeneration and functional recovery using retrograde tracing study with FluoroGold, Toluidine blue staining of cavernous nerve, and the intracavernous pressure at 3 months. Three months after surgery, rich FluoroGold-positive cells were observed in the sham and vein graft plus platelet-derived growth factors group, but very few were found in the no repair group. The number of myelinated axons of regenerated cavernous nerve and intracavernous pressure were increased obviously in the two vein graft groups, especially in the vein graft plus platelet-derived growth factors group. These findings confirm the feasibility of using autologous vein as guides for cavernous nerve regeneration, and the regeneration can be further enhanced when the vein is filled with platelet-derived growth factors.

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INTRODUCTION

With the development of the anatomic radical prostatectomy (RP) described by Walsh and Donker,[1] impotence rates following RP have decreased evidently. However, in some patients, they require wide resection of the prostate involving one or both cavernous nerves, which usually results in erectile dysfunction (ED). Therefore, methods of restoring cavernous nerve (CN) function in these patients are required.

While the concept of autologous nerve transplantation is well-established, clinical studies using autologous nerve to bridge transected cavernous nerves following RP have revealed only modest success,[2,3] some artificial materials and tubulization techniques have been studied in the past in attempts to facilitate regrowth of the excised CN, such as silastic tube, poly L-lactic acid and E-caprolactone copolymer conduit, or adipose tissue-derived acellular matrix thread.[4–7] Except for these materials, accumulating evidence indicates that many growth factors such as insulin growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) play a very important role in neural regeneration and up-regulation of neuronal nitric oxide synthase (nNOS), as well as in the recovery of erectile function after CN injury.[8–13]

Platelet-rich plasma (PRP) is a biologic product of concentrated platelets that is collected from autologous blood.[14] Platelets contain various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), IGF-1, bFGF, and VEGF. When platelets are activated, they release these factors, which play important biological roles in various conditions.[15] Over the past decade, PRP has been used to improve clinical outcomes in cardiac, plastic, periodontal surgical therapy, and intrabony defects. In our previous report, we showed that the application of PRP to the site of CN-crush injury facilitated nerve regeneration and recovery of erectile function.[16]

Autogenous vein grafts have been experimentally and clinically to be supportive conduits for nerve fibers to regeneration and mature.[17–19] The advantages of no donor nerve morbidity and the ease of harvesting veins make vein grafting an attractive alternative to standard nerve grafting.[20]

In this study, we investigated the feasibility of using autologous vein graft combined with platelet-derived growth factors to bridge transected CN in a rat model.

MATERIALS AND METHODS

Animal studies

A total of 36 male Sprague–Dawley rats weighting 250–300 g were used in this study. All rats were obtained from the Experimental Animal Centre of Wuhan University and Wuhan University's Animal Care and Use Committee approved all procedures in the current study.

Experimental groups

Thirty-two rats were divided into four equal groups. In Groups 1, 2, and 3, the main and ancillary CN were exposed and a 2 mm segment on each side was excised. In Group 1 (negative control), there was no attempt to repair the defect. In Group 2, the main CN defect was repaired by using autologous vein graft. In Group 3, the defect was repaired by using autologous vein graft combined with platelet-derived growth factors. Group 4 (sham control) received a sham operation

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and identification of the cavernous nerves bilaterally, with no further surgical manipulation.

Preparation of platelet-derived growth factors

Four rats were used for the preparation of platelet-derived growth factors according to the procedure from Tajima *et al*.[21] The whole blood of four rats was drawn preoperatively via cardiac puncture into sterile syringes containing the anticoagulant, citrate phosphate dextrose (CPD). The blood was subjected to centrifugation for 10 min at 312 ×*g* and the supernatant was transferred to another tube. The supernatant was subjected to centrifugation for 10 min at 1248 ×*g* to yield platelet poor plasma (PPP) and PRP. The top layer, which consisted of the PPP, was discarded. The remaining were PRP. To activate the PRP, 334 μl of 2% calcium chloride were added to activate platelets and to convert fibrinogen to fibrin gel. The tubes were incubated for 4 h at room temperature to completely release the growth factors from the platelets, and then centrifuged at 1500 ×*g* for 10 min to precipitate fibrin gels. The supernatants, which included platelet-derived growth factors, were filtered with a 0.2 μm pore filter and then stored at −80°C until use during the operation.

Surgical procedures

For the surgical procedure, the rats were anesthetized by an intraperitoneal injection with sodium pentobarbital (40 mg kg^{-1}). In the excision group, the major pelvic ganglia (MPG) and the CN were exposed through a low abdominal incision. The main and ancillary cavernous nerves were then bilaterally excised 2 mm below the MPG. In the autologous vein graft groups, the right external jugular vein with a length of 1 cm was first harvested and divided into two equal parts. Then, the cut segment of the external jugular vein was carefully washed in sterile saline solution and used in two experimental paradigms. In the autologous vein graft group, 5 mm vein segment was interposed between the proximal and distal CN stumps. The nerve stumps were secured within 1 mm of the ends of the conduit with 10-0 nylon stay suture. The conduit ends were then tightened around the nerve stumps with circumferential 6-0 nylon suture ties. In the autologous vein graft combined with platelet-derived growth factors group, the vein was filled with 5 μl of platelet-derived growth factors to bridge transected CN. In the sham operation group, the MPG and cavernous nerves were exposed only.

Retrograde tracing study

Three months after operation, the animals were anesthetized, and 5 μl FluoroGold (FG) (4.0%) (Biotium Inc., Hayward, CA, USA) were injected into each bilateral penile crus 7 days before dissection of the MPG. After dissection, the MPG was fixed in ice-cold 4% paraformaldehyde for about 48 h and then immersed in 0.1 mmol l^{-1} phosphate buffer containing 25% sucrose. Transverse MGP sections of 10 μm thickness were serially prepared on a cryostat at intervals of 60 μm. FG-positive cells were identified through a wide-band ultraviolet filter and the entire field of each section was examined. FG-positive cells in each MPG were counted using 20 serial sections. The images were captured directly from the fluorescence microscope using a digital camera.

Measurement of erectile responses

The erectile response was assessed in all rats after 3 months by measuring intracavernous pressure (ICP), following electrostimulation of the MPG. Through a repeat midline abdominal incision, the MPG was exposed and isolated. The skin overlying the penis was incised and the crura of the penis were identified. A 23-gauge scalp-vein needle filled with 250 U ml^{-1} of heparin solution was connected to polyethylene-50 tubing and inserted into the right crus body to measure the ICP. Systemic mean arterial pressure (MAP) was monitored by inserting a 22-gauge cannula into the carotid artery on the left side of the incised neck. A bipolar stainless steel electrode was used to directly stimulate the MPG (probes 2 mm in diameter and separated by 1 mm). Monophasic rectangular pulses were generated by a computer with a custom-built constant current amplifier. The stimulus parameters were 1.5 mA, 20 Hz, pulse width 0.2 ms, and duration 50 s. The ICPs and MAP were recorded in all rats using a bioinformation acquisition system (BL-420F, Chengdu TME Technology Co., Ltd., Chengdu, China). Then, the maximal ICP/MAP ratios in four groups were calculated.

Toluidine blue staining

After functional testing, samples of the normal, ablated CN, and grafted autologous vein segments were collected for toluidine blue staining. All harvested samples were fixed in 3% (weight/volume) cold glutaraldehyde. The samples were dehydrated with ethanol and postfixed with 1% osmium tetroxide before being infiltrated with a graded araldite-propylene oxide mixture and embedded in EPON 812 (Heidelberg, New York, USA). An LKB III Ultramicrotome (LKB Produkter A.B., Bromma, Sweden) was used to obtain 1 μm cross-section of the embedded sample, which was then stained with 1% toluidine blue and examined under microscopy. Images were captured using an Olympus-DP12 camera (100 × oil immersion objective) and then processed using Image-Pro Plus 3.0 (Media Cybernetics, Bethesda, MD, USA). The nerves were analyzed for the number of myelinated axons.

Statistical analysis

Data were expressed as mean ± s.d. The results were first analyzed by one-way ANOVA with significance indicated at $P < 0.05$. If the difference was significant, Student–Newman–Keuls test was performed.

RESULTS

Retrograde neural tracing of FG

Three months after surgery, in the retrograde tracing study, FG-positive cells were hardly seen in the MPG of the excision group (**Figure 1a**). In the two vein graft groups, the number of FG-positive cells showed a remarkable increase at 3 months (**Figure 1b** and **1c**), especially in the group of vein graft combined with platelet-derived growth factors, but they were still less than the number of FG-positive cells in the sham group (**Figures 1d** and **2**).

Evaluation of the erectile function

To evaluate the recovery of erectile function, the maximal ICP was measured. **Figure 3** shows representative ICP responses. There was no ICP response in the excision rats. On the other hand, ICP responses in the two vein graft groups with or without platelet-derived growth factors were facilitated. The ICP-to-MAP ratio increased in vein graft groups when compared to the negative control group ($P < 0.05$), especially in the group of vein graft combined with platelet-derived growth factors (**Figure 4**).

Toluidine blue staining

At the time of harvest, Group 1 animals demonstrated significant bilateral neuroma formation in the area of nerve ablation (**Figure 5a**). The regeneration of CN was detected in the two vein graft groups with or without platelet-derived growth factors (**Figure 5b** and **5c**). Group 4 animals had normal histology of CN (**Figure 5d**). Group 3 showed a statistically significant increase in the number of myelinated axons of

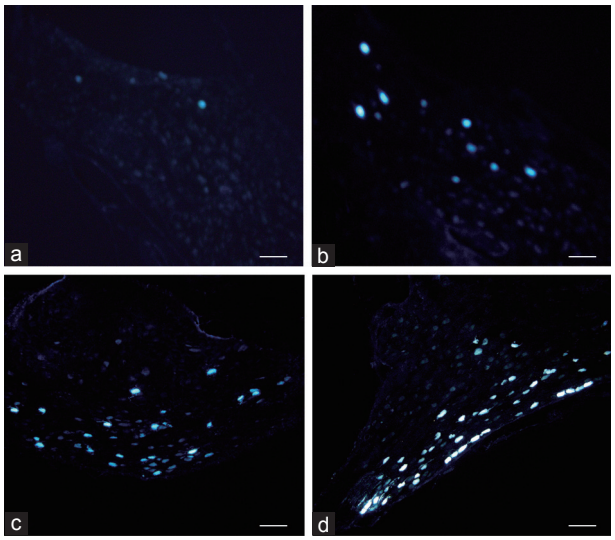

Figure 1: Representative retrograde FluoroGold transport into major pelvic ganglia in four groups at 3 months. (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, (**d**) Group 4. All ×100 Scale bars: 600 μm.

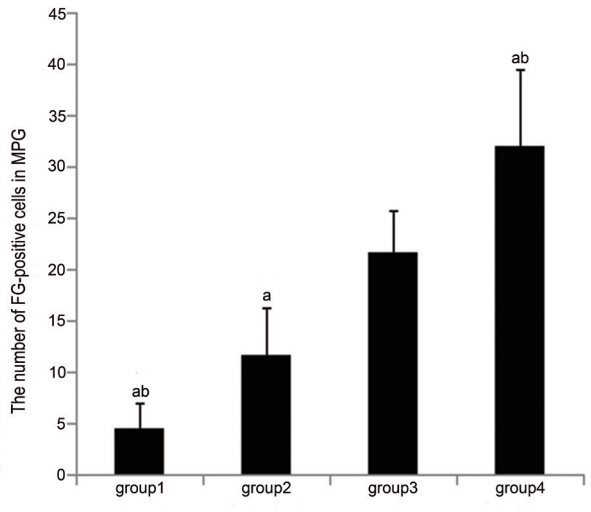

Figure 2: Number of FluoroGold positive cells in major pelvic ganglia. [a]$P < 0.01$, versus Group 3; [b]$P < 0.05$, versus Group 2.

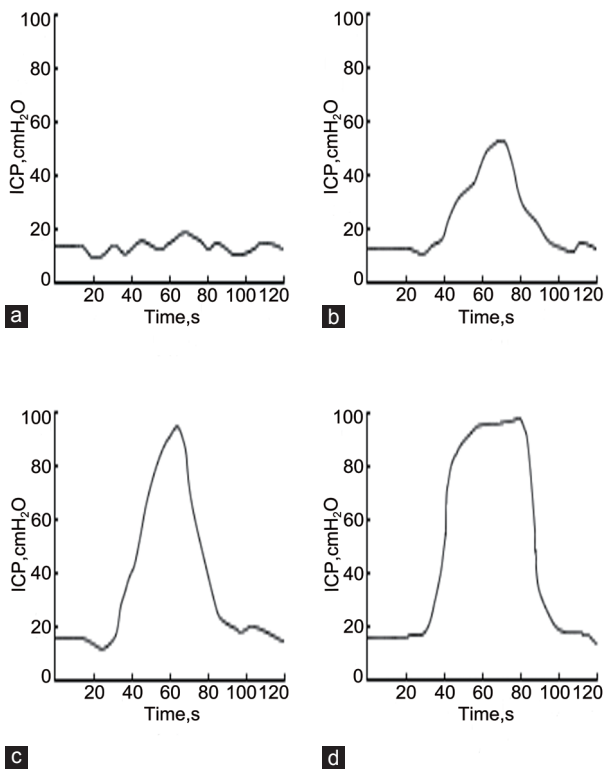

Figure 3: Example of maximal intracavernous pressure (ICP) changes after electrostimulation of major pelvic ganglia at 3 months. (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, (**d**) Group 4.

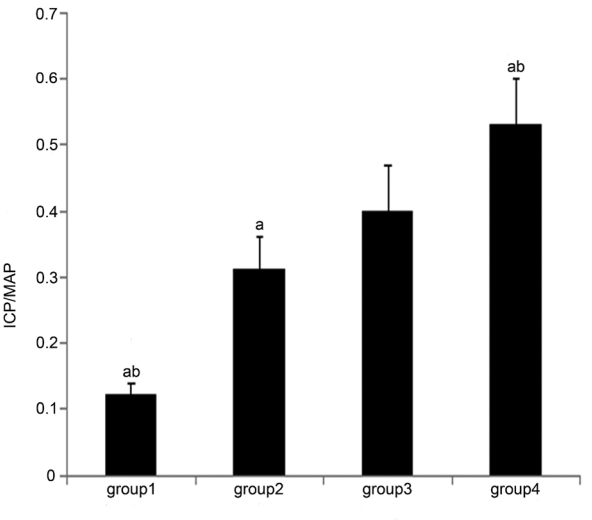

Figure 4: Maximal intracavernous pressure/mean arterial pressure (ICP/MAP) ratio with ICP responses by electrical stimulation in four groups. [a]$P < 0.01$, versus Group 3; [b]$P < 0.05$, versus Group 2.

regenerated CN compared with Group 2, but it was still less than that in the Group 4 (**Figure 6**).

DISCUSSION

Our study confirms the feasibility of using autologous vein as guides for CN regeneration, and the regeneration of CN can be further enhanced when the vein is filled with platelet-derived growth factors.

For histological confirmation of regenerated CN, we used toluidine blue staining of CN, which is more direct than the nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining of intracavernosal erectile tissue. The identification and quantification of myelinated axon of regenerated CN is an ideal process by which to confirm the regeneration of CN. To clarify the evidence on regeneration of the CN and re-projection to its target, we performed a retrograde tracing study using FG. FG injected into a target organ is taken from the nerve terminals if the nerve re-projects into the injected area and then it is transported retrograde and accumulated in the original neurons of the MPG.[22] This study clearly demonstrated that there was no re-projection of unreconstructed nerves into the target organ 3 months after excision. Reconstruction with vein graft plus platelet-derived growth factors achieved significant re-projection into the corpus cavernosum

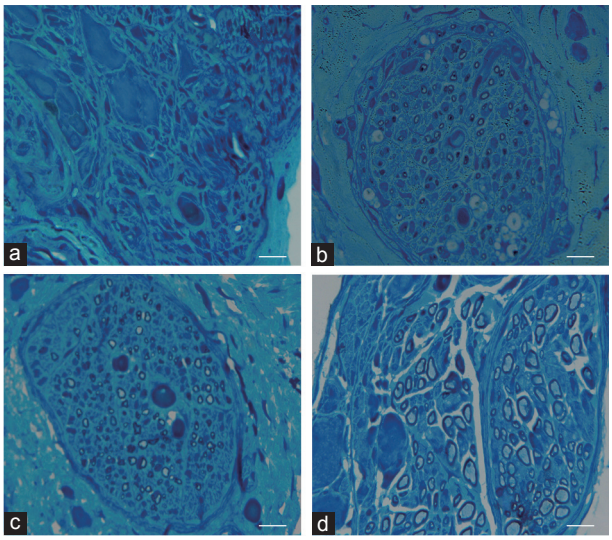

Figure 5: Toluidine blue staining of cavernous nerves: (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, (**d**) Group 4. In (**a**), there was neuroma formation. In (**b**), there was a paucity of myelinated axon of regenerated cavernous nerve into and through the vein cavity. In (**c**), there was a significant increase in the number of myelinated axon of regenerated cavernous nerve relative to (**b**). In (**d**), there was abundant of myelinated axons of cavernous nerves that had a normal morphological appearance. All ×1000 Scale bars: 600 μm.

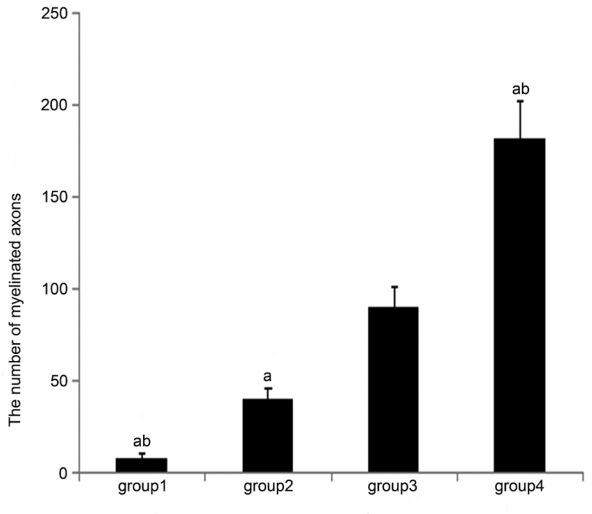

Figure 6: Number of myelinated axons of cavernous nerves in four groups. [a]$P < 0.01$, versus Group 3; [b]$P < 0.01$, versus Group 2.

compared with the vein alone. The result was accordance with the ICP changes among four groups.

The rationale for the autologous vein graft for axonal regeneration is supported by several advantages. Veins are nonimmunogenic, cause less inflammatory reaction, are easy to collect, have a longer life than bioabsorbable tubes, and are available in a wide variety of sizes. Removal of the vein after nerve regeneration is not necessary because the vein is autologous tissue. The walls of veins are thin, but are resilient enough to act as a barrier against scar ingrowth and are permeable enough to provide a favorable internal milieu for nerve regrowth, allowing diffusion of the proper nutrients.[23]

Numerous studies in the CN have showed the potential role of growth factors in facilitating CN regeneration and function recovery. It has been demonstrated that intracavernosal injection with VEGF and brain-derived neurotrophic factor (BDNF) or adeno-associated virus-mediated BDNF (AAV-BDNF) enhanced nerve regeneration and erectile recovery after bilateral cavernosal nerve injury.[24,25] Jung *et al*.[8] created a rat model of CN neurotomy to study changes in nNOS-containing nerve fibers and the RNA expression of IGF-1, nerve growth factor (NGF), TGF-α, TGF-β_1, β_2, β_3, and NOS on the penis, and their findings suggested that IGF-1 and TGF-β_2 may contribute to the regeneration of nNOS-containing fibers. In addition to *in vivo* studies in rats undergoing injury to CN, Lin *et al*.[26] group established a ganglia culture system to screen various growth factors as potential therapeutic agents for pelvic nerve injuries. They found that both BDNF and VEGF facilitated the outgrowth of neurite from the major pelvic ganglia that stained positive for nNOS. Further study indicated that BDNF promoted neurite growth in main pelvic ganglia primarily by activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.[27,28] Both the *in vivo* and *in vitro* studies mentioned above demonstrated the beneficial role of growth factors in neural regeneration and up-regulation of nNOS, as well as in the recovery of erectile function. Some studies also showed that augmented PDGF-β expression after nerve injury might contribute to peripheral nerve regeneration because PDGF-β is a Schwann cell mitogen and survival factor with trophic activity on neurons.[29,30] Allamargot *et al*.[31] found that PDGF accelerated the rate of re-myelination and improved regeneration of the nervous system. In the current study, it was observed that platelet-derived growth factors enhanced the regeneration and maturation of the CN obviously, when it was incorporated into the vein. On account of the prevention of neurodegeneration and promotion of survival of axons at an early stage after nerve injury are critical. A cocktail of neurotrophic factors administered at critical time points may be required to prevent neurodegeneration.[32] Hence, we believe that platelet-derived growth factors in PRP are responsive for the positive effect on cavernous nerve regeneration and functional recovery. When platelets are activated, they release platelet-derived growth factors. The platelet-derived growth factors can not only react individually, but also have compound effect to enhance the regeneration of CN.

In summary, this study shows that autologous vein graft combined with platelet-derived growth factors can bridge transected cavernous nerves well in a rat model. Given the limitations of the study, future work will include finding the optimal dose of platelet-derived growth factors and identifying the primary molecular signaling pathway in the process of nerve regeneration. Although much is yet to be determined, our study indicates that autologous vein graft with platelet-derived growth factors may provide a good alternative to fresh autologous nerve grafts for the regeneration of CN injuries.

AUTHOR CONTRIBUTIONS

XGD, SWL, and YL conceived and designed the experiments; XGD and YL performed the experiments; XGD and YL analyzed the data; XGD wrote the paper; XMZ, SWL, and XHW participated in supervision. All authors have read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing financial interests.

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