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Original Article

The effect of platelet-rich plasma on cavernous nerve regeneration in a rat model

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Abstract

The aim of this study was to investigate the effect of platelet-rich plasma (PRP) on cavernous nerve (CN) regeneration and functional status in a nerve-crush rat model. Twenty-four Sprague–Dawley male rats were randomly divided into three equal groups: eight had a sham operation, eight underwent bilateral nerve crushing with no further intervention and eight underwent bilateral nerve crushing with an immediate application of PRP on the site of injury. Erectile function was assessed by CN electrostimulation at 3 months and nerve regeneration was assessed by toluidine blue staining of CN and nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining of penile tissue. Three months after surgery, in the group that underwent bilateral nerve crushing with no further intervention, the functional evaluation showed a lower mean maximal intracavernous pressure (ICP) and maximal ICP per mean arterial pressure (MAP) with CN stimulation than those in the sham group. In the group with an immediate application of PRP, the mean maximal ICP and maximal ICP/MAP were significantly higher than those in the injured control group. Histologically, the group with the application of PRP had more myelinated axons of CNs and more NADPH-diaphorase-positive nerve fibres than the injured control group but fewer than the sham group. These results show that the application of PRP to the site of CN-crush injury facilitates nerve regeneration and recovery of erectile function. Our research indicates that clinical application of PRP has potential repairing effect on CN and peripheral nerves.

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Keywords: platelet rich plasma, impotence, erectile dysfunction, nerve regeneration

1 Introduction

With the development of the anatomic radical prostatectomy (RP) described by Walsh and Donker [1], impotence rates following RP have decreased significantly. However, even now, a significant proportion of the patients undergoing anatomic nerve-sparing RP often experience incomplete or delayed recovery of erectile function.

In many instances, the cavernous nerves (CNs) may have been inadvertently damaged by manipulation during nerve-sparing prostatectomy. The recovery of erectile function may depend on re-growth of nerves from the remaining neural tissue [2]. Accumulating evidence indicates that neuroimmunophilin ligand and many growth factors, such as insulin growth factor-1 (IGF-1), brain-derived growth factor (BDNF) and vascular en-

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dothelial growth factor (VEGF) play a significant 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 [3–10].

Platelet-rich plasma (PRP) is prepared by centrifugation of the patient's own blood [11]. Platelets contain various growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, IGF-I and VEGF. When platelets are activated, they release those factors, which play important biological roles in various conditions [12]. These findings suggest that the application of PRP to the injured CN could facilitate nerve repair.

The aim of this study was to evaluate whether the application of PRP to the site of CN injury had neuromodulatory effects on the regeneration of CNs and also to evaluate the recovery of erectile function after bilateral CN-crush injury in a rat model.

2 Materials and methods

2.1 Experimental animals

Twenty-four healthy male Sprague-Dawley rats (3 months old, 250–300 g) were randomly divided into three equal groups: group 1 received a sham operation comprising a midline incision and identification of the CNs bilaterally, with no further surgical manipulation; group 2 underwent CN dissection and a subsequent intentional 2-min crush injury with a hemostatic clamp; and group 3 was treated as group 2, but followed by an immediate application of activated PRP to the site of CN injury. All rats were obtained from the Experimental Animal Centre of Wuhan University (Wuhan, China), and the Wuhan University Animal Care and Use Committee approved all the procedures followed in this study.

2.2 PRP preparation

The PRP preparation was obtained from six age-matched male Sprague-Dawley rats. The whole blood of six rats was drawn preoperatively through cardiac puncture into ethylene diamine tetraacetic acid-coated tubes. 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 1 248 × *g* to yield platelet-poor plasma (PPP) and PRP. The top layer, which consisted of the PPP, was aspirated and put into a new tube. The remaining layer was the PRP. Approximately 400 μL of PRP was activated with 40 μL of 10% calcium chloride solution and 1 000 U mL^{-1} bovine thrombin (Sigma-Aldrich, St. Louis, MO, USA) to form a gel for application to every rat in group 3 during the operation.

2.3 Surgical procedures

For the surgical procedure, rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital (40 mg kg^{-1}). The rats were kept isothermic by placing them on a heating pad at 37ºC. A lower midline abdominal incision was made after the abdomen was shaved and prepared with an iodine-based solution. With the aid of an operating microscope (SXP-1C, Medical Optical Instruments Factory of Shanghai Medical Instruments Co. Ltd, Shanghai, China), the prostate gland was exposed and the posterolateral CNs and major pelvic ganglion were identified bilaterally. There was no further surgical manipulation in group 1. In group 2, the CNs were isolated and a crush injury was applied using a hemostat clamp (HC-X020, Cheng-He Microsurgical Instruments Factory, Ningbo, China) for 2 min, and then the abdomen was closed. In group 3, after bilateral nerve crushing, 200 μL of activated PRP was applied immediately to each site of the CN injury. In all rats, the abdomen was closed in two layers.

2.4 Measurement of erectile responses

The erectile response was assessed in all rats after 3 months by electrostimulation of the CNs and by measuring intracavernous pressure (ICP). Through a repeat midline abdominal incision, the CNs were exposed and isolated. The skin overlying the penis was incised and the crura of the penis was identified. A 23-G 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-G cannula into the carotid artery on the left side of the incised neck. A bipolar stainless steel electrode was used to directly stimulate the CNs (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 as follows: amplitude, 1.5 mA; frequency, 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). The mean of the maximum right and

left ICPs was then determined for each rat. Finally, the maximal ICP/MAP ratios in the three groups were calculated.

After functional testing, samples of CNs and the mid-shaft penis were collected for toluidine blue staining and nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining.

2.5 Toluidine blue staining

Two to three mm of CN was obtained from the main trunk of both sides at equivalent points, which were distal to the site of injury. All harvested nerves were fixed in 3% (weight/volume) cold glutaraldehyde. The nerve segments 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, NY, USA). An LKB III Ultramicrotome (LKB Produkter A.B., Broma, Sweden) was used to obtain 1 μm cross-sections of the embedded nerve, which were then stained with 1% toluidine blue and examined with light microscopy. Images were captured using an Olympus-DP12 camera (× 100 oil immersion objective) and processed using Image Pro-Plus 3.0 (MediaCybernetics, Bethesda, MD, USA). The nerves were analyzed to determine the number of myelinated axons.

2.6 Nicotinamide adenine dinucleotide phosphate-diaphorase staining

Samples of penile tissue were fixed for 4 h in phosphate buffer containing 0.002% picric acid and 2% formaldehyde, and then transferred to 30% sucrose before freezing. Serial cryosections (10 μm) were adhered to the charged slides. After being air dried for 5 min, the sections were incubated with 0.1 mmol L^{-1} NADPH, 0.2 mmol L^{-1} nitroblue tetrazolium and 0.2% Triton X-100 (Sigma-Aldrich) in a buffer, with constant microscopic monitoring for color development. When the medium was deep blue for NADPH-diaphorase-positive nerves, rinsing in buffer terminated the reaction. The staining pattern was assessed by counting the number of NADPH-diaphorase-positive nerves in the dorsal nerves at an original magnification of × 400 with light microscopy. The endothelium staining was excluded from the count.

2.7 Statistical analysis

Data were expressed as mean ± SD. The results were first analyzed using one-way analysis of variance (ANOVA) with significance indicated at $P < 0.05$. If the difference was significant, a Student–Newman–Keuls test was carried out.

3 Results

3.1 Evaluation of erectile function

To evaluate recovery of erectile function, the maximal ICP was measured (Figure 1). No erectile dysfunction was identified in the sham group, with a pressure of 108.9 ± 12.8 cmH_2O. The injured control group showed a significant reduction in maximal ICP

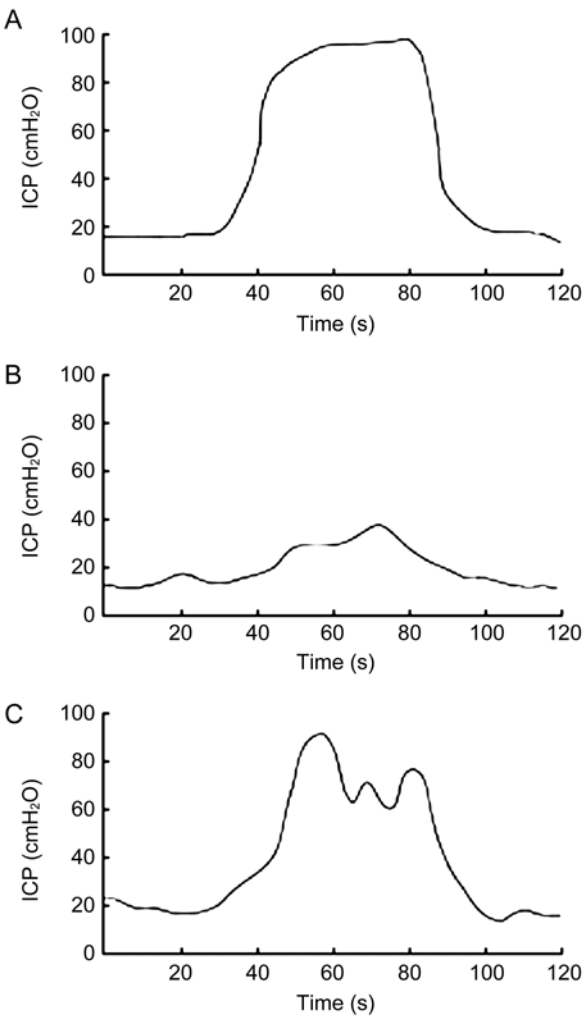

Figure 1. Example of Maximal intracavernous pressure (ICP) changes after electrostimulation of the cavernous nerves at 3 months. (A): Group 1; the pressure reached 100 cmH_2O. (B): Group 2; the pressure reached 38 cmH_2O. (C): Group 3; the pressure reached 92 cmH_2O.

of 32.7 ± 12.2 cmH_2O, consistent with a state of erectile dysfunction. In the PRP-treated group, the mean maximal ICP was 83.1 ± 12.9 cmH_2O, which was significantly higher than that in the injured control group ($P < 0.05$). The maximal ICP/MAP ratio was increased significantly in the PRP treated group compared with the injured control group, but it was still less than that of the sham group (Figure 2).

3.2 Toluidine blue staining of cavernous nerve

The myelinated axons of CNs were assessed by toluidine blue staining of histological specimens. In concordance with the functional result, the number of myelinated axons in the injured control group was significantly less than that in the sham group ($P < 0.05$) (Table 1 and Figure 3). The group treated with PRP showed a statistically significant increase in the number of myelinated axons compared with the injured control group ($P < 0.05$), but it was still less than that in the sham group ($P < 0.05$).

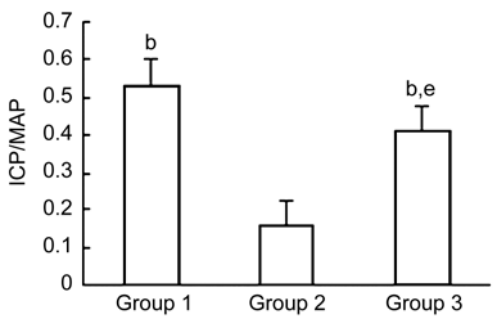

Figure 2. Maximal intracavernous pressure (ICP)/mean arterial pressure (MAP) ratio with ICP responses by electrical stimulation in three groups. $^{b}P < 0.05$, compared with group 2; $^{c}P < 0.05$, compared with group 1.

Table 1. Changes in the number of myelinated axons of the CN and NADPH-diaphorase-positive nerve fibres in the dorsal nerve in the three groups of rats (mean ± SD).

Group	Myelinated axons	NADPH-diaphorase-positive nerve fibres
Sham	180.9 ± 20.7[a]	285.6 ± 57.3[a]
Injured	60.3 ± 14.5	77.5 ± 16.6
Treated with PRP	114.9 ± 23.9[a,b]	151.8 ± 20.3[a,b]

Abbreviations: CN, cavernous nerve; NADPH, nicotinamide adenine dinucleotide phosphate; PRP, platelet-rich plasma.
[a]$P < 0.05$, compared with group 2.
[b]$P < 0.05$, compared with group 1.

3.3 Nicotinamide adenine dinucleotide phosphate-diaphorase staining

There were significantly fewer NADPH-diaphorase-positive nerve fibres in the dorsal nerves of the injured control group than in the sham group ($P < 0.05$) (Table 1 and Figure 4). In the PRP-treated group, the number of NADPH-diaphorase-positive nerve fibres was increased significantly compared with the injured control group ($P < 0.05$).

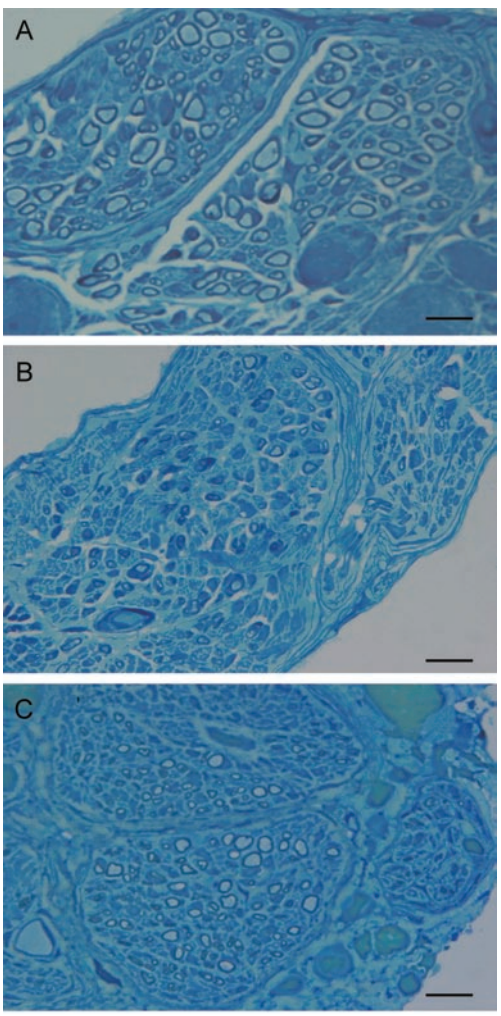

Figure 3. Toluidine blue staining of cavernous nerves. (A): Group 1; (B): Group 2; (C): Group 3. In group 1, there were abundant myelinated axons of cavernous nerves that had a normal morphological appearance. In group 2, the number of myelinated axons decreased substantially and displayed the phenomenon of atrophy. In group 3, there was a significant increase in the regeneration of well-orientated myelinated axons relative to group 2. Bars = 600 μm.

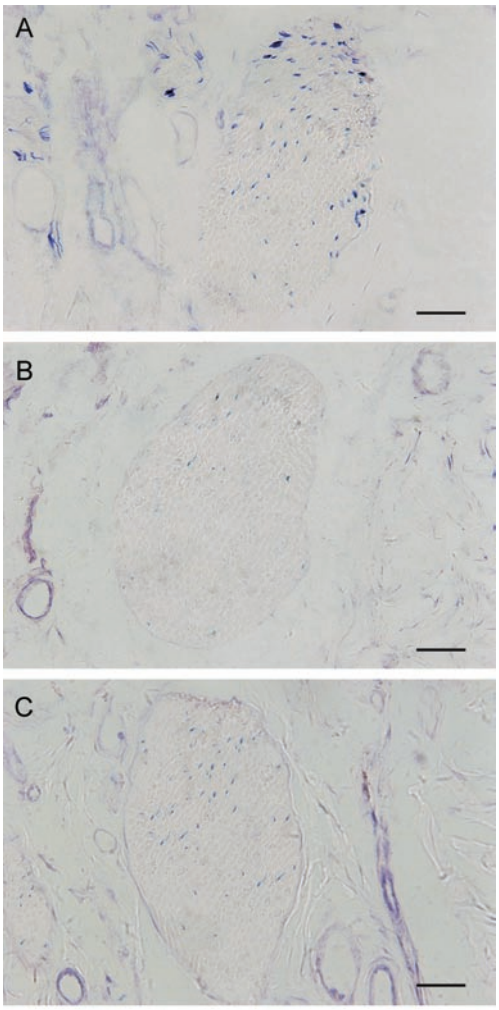

Figure 4. NADPH-diaphorase staining of dorsal nerves. (A): Group 1; (B): Group 2; (C): Group 3. In group 1, there was predominant blue staining of nerve fibres. In group 2, there was a paucity of blue-stained fibres. In group 3, there was a significant increase in the number of blue-stained nerve fibres relative to group 2. Bars = 600 μm.

4 Discussion

Our results show a measurable neurotrophic effect of PRP on CN regeneration after crush injury in a rat model. This is on the basis of the following statistically significant results found when the injured CN was treated with PRP: (1) during CN electrostimulation, the measurement of the maximum ICP and ICP/MAP in this group showed good functional recovery after treatment; (2) histologic evaluation of this group showed a significant increase in the number of myelinated axons, which indicated the recovery of CNs directly; and (3) an increase of NADPH-diaphorase-positive nerve fibres in the dorsal nerves also showed the recovery of the CNs indirectly after application of PRP. These findings are in agreement with an earlier report of the neurotrophic and neuroprotective effects of PRP on facial nerve regeneration in a rat model [13].

The specific mechanism for regeneration of damaged CNs is still unknown, but growth factors are likely to play an important role in this process. Jung *et al*. [14] created a rat model of CN neurotomy to study changes in nNOS-containing nerve fibres and the RNA expression of IGF-I, nerve growth factor, TGF-α, TGF-β_1, β_2 and β_3 and NOS on the penis. Their results suggested that IGF-I and TGF-β_2 may contribute to the regeneration of nNOS-containing fibres. In a more recent study, Hu *et al*. [15] found that autologous vein grafts combined with local injection of IGF-I facilitated repair of ablated CNs. Researchers also found that intracavernous injection with VEGF and BDNF or adeno-associated virus-mediated BDNF enhanced nerve regeneration and erectile recovery after bilateral CN injury [8–10]. In addition to *in vivo* studies in rats undergoing injury to CNs, Lin *et al*. [16] established an *in vitro* model of pelvic ganglion culture. Among several growth factors tested, they found that both BDNF and VEGF facilitated the outgrowth of axons that stained positive for nNOS. Other studies indicated that BDNF promoted neurite growth in the main pelvic ganglion, primarily by activating the Janus kinase–signal transducer and activator of the transcription (JAK–STAT) pathway [17, 18]. Both the *in vivo* and the *in vitro* studies mentioned above showed the beneficial role of growth factors in neural regeneration and up-regulation of nNOS, as well as in the recovery of erectile function.

In this study, the application of PRP to the site of CN injury shows the positive effect on CN regeneration and functional recovery. We believe that the growth factors and platelet gel in PRP are all responsible for this property. The platelets in the PRP are activated by the addition of thrombin and excess calcium, which promotes both platelet activation and the formation of a thrombus-like gelatinous substance (platelet gel). The activated platelets are trapped on the platelet gel, where they continue to excrete growth factors and slowly diffuse into the injury site. Such a platelet gel could be a good delivery vehicle for the growth factors PDGF, TGF-β, IGF-I and VEGF, which are present in PRP. The above-mentioned researchers have found that

TGF-β_2, IGF-I and VEGF contribute to the neural regeneration and up-regulation of nNOS. As for PDGF, it has been shown that neurons express PDGF receptors, and PDGF-β has been proven to be a mitogen and survival factor for Schwann cells with trophic activity on neurons [19]. Also, an augmented PDGF-β expression in peripheral neurons has been found after peripheral nerve injury, suggesting a role for PDGF-β in peripheral nerve regeneration [20]. Another study found that PDGF improved regeneration and remyelination of the nervous system [21]. In our study, we also found that the group treated with PRP showed a statistically significant increase in the number of myelinated axons compared with the injured control group. Maybe it is partly contributed to the PDGF in PRP. The growth factors in PRP not only react individually but may also have a compound effect. Therefore, we believe that the neuromodulatory effect of PRP may be associated with local releasing growth factors at the injured site and promotion of the myelinated axon's regeneration.

In summary, this study shows that the application of PRP to the site of CN injury has neuromodulatory effects on the regeneration of CNs and the recovery of erectile function. Given the limitations of the study, future work will include finding the optimal dose of PRP and identifying the primary molecular signaling pathway in the process of nerve repair. Although much is yet to be determined, our study indicates the potential of a new clinical application for PRP in CN or other peripheral nerve regeneration.

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