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Neurotrophic and neuroprotective actions of *Achyranthes bidentata* polypeptides on cultured dorsal root ganglia of rats and on crushed common peroneal nerve of rabbits

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HIGHLIGHTS

- Naturally derived *Achyranthes bidentata* polypeptides (ABPP) have some neuroactivities.
- ABPP encouraged neurite outgrowth of rat dorsal root ganglia through ERK1/2 activation.
- ABPP accelerated peripheral nerve regeneration after common peroneal nerve crush.

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ABSTRACT

We have isolated *Achyranthes bidentata* Blume polypeptides (ABPP) from the aqueous extract of *A. bidentata* Blume, a traditional Chinese medicine with multiple therapeutic applications. In this study, we aimed to investigate neurotrophic effects of ABPP on cultured dorsal root ganglia (DRGs) of rats and neuroprotective effects on crushed common peroneal nerve of rabbits. Immunochemistry and Western blot analysis indicated that ABPP (0.01, 0.1, and 1.0 μg/ml) encouraged neurite outgrowth from cultured DRG explants/neurons in a concentration-dependent manner through activation of ERK1/2. After crush injury to rabbit common peroneal nerve, animals received daily administration of ABPP for 5 weeks. Electrophysiological assessments and histomorphological evaluation showed that 6.0 mg/kg ABPP significantly enhanced nerve regeneration and function restoration. Our findings suggest that ABPP could be used as a neurotrophic and neuroprotective agent to treat peripheral nerve crush injury.

1. Introduction

Although the peripheral nervous system is able to regenerate spontaneously, clinical intervention is necessary to enhance nerve regeneration and functional recovery for patients with peripheral nerve injury. Surgical therapy is applicable to nerve transection injury, while administration of appropriate drugs or agents (*e.g.* neurotrophins and vitamins) is useful for treating nerve crush injury [3,4]. Therefore, the search for new agents of natural origin attracts some attention.

Achyranthes bidentata Blume (Amaranthaceae family) is a Chinese traditional medicinal herb with multiple therapeutic applications [9]. We reported that its aqueous extract accelerated peripheral nerve regeneration after crush injury to rabbit common peroneal nerve [7], and prevented primary cultured hippocampal neurons from glutamate-induced cell damage [19]. Many research groups have isolated and identified active constituents, such as sterones, polysaccharides, and polypeptides, from *A. bidentata* Blume. We have also isolated an ingredient, named *A. bidentata* polypeptides (ABPP), from the aqueous extract of the herb. Our previous studies showed that ABPP protected primary culture of rat hippocampal neurons against NMDA-induced excitotoxicity [13] and to promote regeneration of the injured nerve in a murine model of sciatic nerve crush [15,17].

The objectives of this study were to investigate the effects of ABPP on cultured dorsal root ganglia (DRGs) of rats and to evaluate the regenerative outcomes achieved by ABPP after crush injury to rabbit common peroneal nerve.

2. Materials and methods

2.1. Culture and treatment of DRG explants

DRG explants were harvested from spinal and peripheral roots of postnatal day 1 Sprague-Dawley (SD) rats, and plated on poly-L-lysine-coated cover slips to allow incubation in DMEM medium supplemented with 5% FBS, 5% horse serum, 2 mM L-glutamine, and

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antibiotics (Sigma, St. Louis, MO). Then, DRG explants were transferred to a serum-free neurobasal medium added with ABPP, which had been prepared from an aqueous extract of the roots of *A. bidentata* Blume as previously described [17], at the final concentration of 0.01, 0.1, or 1.0 μg/ml, to allow incubation for 72 h. The DRG explants cultured in the plain medium was used as control.

2.2. Immunohistochemistry

Following treatment, DRG explants were fixed in 4% paraformaldehyde, blocked with 2% goat serum/0.3% Triton X-100, and then immunostained with a mouse anti-GAP-43 monoclonal antibody (1:200, Sigma), followed by reaction with FITC-conjugated goat anti-mouse IgG. To quantify neurite outgrowth from DRG explants [2], non-overlapping images were taken at 5× magnification under fluorescence microscopy (Leica, Germany), and digitized using a CCD video camera. Two concentric circles were manually drawn firstly around the cell body and then around the halo of neurites to give the exclusion zones. The area occupied by neurites was measured by using a grain counting function in Leica image analysis software (Leica, Germany).

2.3. Culture and treatment of DRG neurons

The primary culture of DRG neurons was obtained by a differential adhesion technique as described previously [8]. In brief, DRG tissues were harvested from postnatal day 1 SD rats, and digested at 37 °C sequentially with 0.1% collagenase type III (Gibco, Grand Island, NY) and 0.25% trypsin/EDTA (Sigma). After mechanical dissociation, the procured DRGs were placed in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and antibiotics to allow incubation. The non-adherent DRG neurons were collected, re-suspended, and plated onto a pre-coated 6-well plate for culture in a serum-free neurobasal medium added with ABPP at the final concentration of 0.01, 0.1, or 1.0 μg/ml, or in the plain medium, respectively. The culture medium was renewed every 3 days.

2.4. Western blot analysis

DRG neurons were collected and homogenized in an ice-cold cell lysis buffer containing protease inhibitors. Total proteins were quantified by BCA analysis, and separated by SDS-PAGE, followed by transferring to a PVDF membrane, which was blocked with 5% non-fat powdered milk in Tris-buffered saline containing 0.05% Tween-20, and allowed to incubate overnight at 4 °C with following primary antibodies (Abcam, Cambridge, MA): mouse anti-GAP-43 monoclonal antibody (1:200), mouse anti-NF-68 monoclonal antibody (1:200), mouse anti-phosphorylated-ERK1/2 monoclonal antibody (1:10,000), rabbit anti-total-ERK1/2 polyclonal antibody (1:1000). HRP-labeled anti-mouse or anti-rabbit IgG (1:1000, Santa Cruz, CA) was used as secondary antibodies. After exposure to ECL hyperfilm, the bands were scanned by densitometry.

2.5. Animal surgery and treatment

New Zealand adult rabbits, weighing 2.2 ± 0.2 kg, were obtained from the Experimental Animal Center of Nantong University. They were anesthetized with an intravenous injection of 2% sodium pentobarbital (30 mg/kg body weight) before a skin incision was made in the left hindlimb and the underlying muscle was split. The common peroneal nerve was exposed, and 3-mm long nerve at 2-cm above the edge of gastrocnemius muscle was crushed by clamping with a smooth-jawed forceps at a constant force of 50 N for 30 s [6]. The distal end of crush site was marked with an 8–0 nylon suture, and the surgical incision was closed. The common peroneal nerve on the contralateral side was sham-operated.

After surgery, all animals were randomly divided into 3 groups ($n = 8$/group) to receive a daily intravenous injection of saline (vehicle, negative control), and ABPP at the concentration of 1.5 and 6.0 mg/kg, respectively. The treatment lasted for 5 weeks.

2.6. Electrophysiological tests

At the end of treatment, animals were anesthetized before the injured common peroneal nerve was re-exposed. Electrical stimuli were applied at 1.5 cm proximal and distal to the crush site of the common peroneal nerve, respectively. The compound muscle action potentials (CMAPs) were recorded from the belly of the tibialis posterior muscle by using a portable digital MYTO Electromyographic machine (Esaote, Italy) with Galileo NT System software (Esaote, Italy).

2.7. Histological and morphometric evaluation

Following electrophysiological assessments, animals were transcardially perfused, and the distal common peroneal nerve, the tibialis posterior muscle, and the enlarged lumbar spinal cord (L4–L6) were respectively harvested from 6 rabbits of each group. The tissue samples were fixed in buffered 4% paraformaldehyde, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, and then cut into transverse sections.

The nerve, muscle, and spinal cord sections were subjected to Meyer's trichrome staining, hematoxylin and eoxin (H&E) staining, and methylene blue staining, respectively, and then mounted with resinous mounting medium to obtain photographs with a CCD video camera plus a Qwin image analysis system (Leica, Germany). For each sample, 6 visual fields were randomly selected to undergo morphometric analysis to determine the density of regenerated myelinated nerve fibers (*i.e.* the number of regenerated myelinated nerve fibers per unit area), the cross-sectional area (CSA) of tibialis posterior muscle fibers, the number of anterior horn motor neurons on both the injured and uninjured sides, respectively.

2.8. Electron microscopy

The nerve samples were taken from the crushed side of the remaining 2 animals in each group, and fixed with pre-cooled 2.5% glutaraldehyde, post-fixed with 1% osmium tetraoxide solution, dehydrated in a graded ethanol serious, embedded in Epon 812 epoxy resin, and cut into transverse sections. The semi-thin sections were stained with toluidine blue for orientation by light microscopy, and the ultra-thin sections were stained with lead citrate and uranyl acetate for examination under transmission electron microscopy (JEOL, Japan). Ten random visual fields of each section were digitalized into a Q550IW image analysis system and analyzed with Leica Qwin package (Leica, Germany) to determine the diameter of myelinated nerve fibers and the myelin sheath thickness.

2.9. Statistical analysis

Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) plus *post hoc Scheffe's* tests by using SPSS 11.5 software package. Statistical significance was set at $P < 0.05$.

3. Results

3.1. ABPP encouraged neurite outgrowth from cultured DRG explants/neurons through activation of ERK1/2

Immunostaining with anti-GAP-43 showed that multiple neurites emanated radically from the edge of DRG explants after 72 h