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Panax quinquefolius saponin inhibits endoplasmic reticulum stress-mediated apoptosis and neurite injury and improves functional recovery in a rat spinal cord injury model

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ABSTRACT

The treatment goal in spinal cord injury (SCI) is to repair neurites and suppress cell apoptosis. *Panax quinquefolius* saponin (PQS) is the major active ingredient of American ginseng and has been demonstrated to have anti-inflammatory and anti-apoptotic roles in various diseases. However, the potential effect of PQS on the pathological process of acute SCI remains unknown. This work tested the effects of PQS on acute SCI and clarified its potential mechanisms. PQS treatment ameliorated the damage to spinal tissue and improved the functional recovery after SCI. PQS treatment inhibited endoplasmic reticulum (ER) stress and the associated apoptosis after acute SCI. PQS further abolished the triglyceride (TG)-induced ER stress and associated apoptosis in neuronal cultures. PQS appears to inhibit the ER-stress-induced neurite injury in PC12 cells. Our results suggest that PQS is a novel therapeutic agent for acute central nervous system injury.

1. Introduction

Acute spinal cord injury (SCI) is severe trauma resulting in permanent disability. The pathology of SCI has two phases: the primary injury results from the direct disruption of spinal cord tissue, and the secondary injuries include axonal disruption, neuronal death, inflammation, disruption of the blood–brain barrier, hypoxia, and ischemia [1,2]. These events during the secondary injury cause neuronal death and dysfunction, which contribute to the limited functional recovery after SCI [3]. Therefore, the treatment of central nervous system (CNS) injury focuses on treating the secondary injury by reducing neuronal death and repairing the functional neural circuitry [3,4].

Neurons, which are the most polarized cells in the human body, transmit information. The axon degeneration and neuronal cell death around the primary lesion lead to the permanent loss of vital functions after CNS injury [5]. For axon regeneration of the surviving neurons around the primary injury to occur, it is important to enhance the intrinsic growth ability and reduce inhibitory factors [6,7]. Cytoskeleton remodeling in the axon, including microtubule assembly, is essential for the formation of a growth cone and repair of the injured axon [8]. Stabilizing microtubules contribute to axon regeneration via activating autophagy after SCI [9]. The therapy for CNS injury is based on understanding the molecular mechanism that triggers the apoptotic cascade and axonal degeneration in injured neurons. Endoplasmic reticulum (ER) stress exerts critical effects on the pathological process of numerous CNS diseases [10,11]. The ER was originally regarded as an intracellular organelle responsible for maintaining cellular homeostasis and for countering potential injury triggered by the existence of misfolded protein on ER [12]. However, the unfolded protein response (UPR) extends beyond the control of protein folding, and the final effects differ from those originally thought to be induced by ER stress [13]. Excessive ER stress is seen in the area near the injured section during the secondary phase of SCI [14]. The accumulation of unfolded proteins in the ER initiates the UPR, which activates three signal pathways regulated by IRE1, ATF6, and PERK; this increases the expression of apoptotic protein, leading to neuronal death [15]. Meanwhile, molecular chaperone, like GRP78, s a member of the heat shock protein 70 (Hsp70) family, regulates protein folding and facilitates protein translocation in the ER ischemic preconditioning treated neural cells [16]. Neuronal ER stress contributes to axon degeneration and neuronal death during glaucomatous neurodegeneration [17]. Several studies have demonstrated that CHOP knockdown promotes neuronal survival and protects motor neuron axons [17,18].

Panax quinquefolius saponin (PQS), a major active component of *Panax quinquefolius* L. (American ginseng), which is extracted from the leaves, has anti-inflammatory and anti-apoptotic effects in various diseases. PQS reduced cardiomyocyte apoptosis during ischemia/reperfusion injury by inhibiting ER stress [19]. PQS also decreased the

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inflammatory cytokines released by activated macrophages *in vitro* [20].

However, little is known about the effects of PQS in acute SCI. Therefore, we examined the potential roles of PQS after acute SCI. We also explored the effects of PQS on apoptosis and neurite repair after acute SCI and examined its specific mechanisms. We hypothesized that PQS might suppress ER-stress related apoptosis and axon injury in acute SCI. Our results reveal that PQS could be a novel treatment for acute SCI.

2. Materials and methods

2.1. Chemicals and reagents

PQS was obtained from Jilin Yisheng Pharmaceutical ((ginsenoside-Re ≥ 75%), Jilin, China). HPLC and LC–MS of PQS demonstrated that PQS mainly contained ginsenoside-Re (≥75%), ginsenoside-Rb2, ginsenoside-Rd, ginsenoside-Rb1, ginsenoside-Rg1, and ginsenoside-Rb3 [21,22]. The herbal drugs were authenticated and standardized on main active compounds according to the Chinese Pharmacopoeia 2005. PQS was treated as showed below: crude powder of leaves of radix panacis quinquefolii were dissolved in water for filtration and evaporation. After dissolution in ethanol, the specimens were fractionated on a macro-porous adsorption resin D 101 column with water and 80% ethanol, PQS was acquired after vacuum drying at 60 ∘C. To minimize the purity and dose variability of PQS among different batches, the species, origin, harvest time, medicinal parts, and concocted processes were strictly controlled. Antibodies against acetyl-α-tubulin, CHOP, cleaved caspase12, and cleaved caspase3 were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-NEUN, anti-GRP78, and anti-PDI antibodies were obtained from Abcam (Cambridge, MA, USA). Antibodies including anti-Bax and anti-Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Unless otherwise noted, triglyceride (TG) and other agents were obtained from Sigma (St. Louis, MO, USA).

2.2. SCI

The animal experiments complied with the guide from the National Institutes of Health. The Adult female Sprague–Dawley rats (220–250 g, n = 45) were randomly divided into three group and anaesthetized by chloral hydrate (3.5 mL/kg) intraperitoneally, and T9 laminectomies were performed in SCI group and PQS group. Moderate crushing injury was induced with a vascular clamp (Oscar, China), with a 30-g force for 1 min. The sham group underwent only a laminectomy at the T9 level. Postoperative care included twice daily urinary bladder emptying and the intra-peritoneal injection of cefazolin sodium (50 mg/kg). Postoperatively, one group was given PQS (200 mg/kg) by gavage each day [23]. The sham group was given an equal volume of saline. All the mice were killed at 3, 7 and 28 days after operation for the further experiments.

2.3. Behavioral analysis

To examine the functional deficit in each group, the Basso–Beattie–Bresnahan (BBB) scores and inclined plane tests were used by two blind independent examiners 1, 3, 7, 14, 21, and 28 days after the operation [24].

2.4. HE and Nissl staining

To evaluate the structural damage and surviving neurons near the injury zone after the operation, each group was sacrificed at 28 days, and the T7–T10 tissue was excised and embedded in paraffin. Transverse sections (5-μm thick) were cut onto slides for histopathological assays. The slides were stained with hematoxylin and eosin (HE) and cresyl violet (Nissl staining) according to the manufacturer's protocols.

2.5. PC12 cell culture

The PC12 cells (Shanghai Institute of Cell Biology, shanghai, China) were expanded and cultured in RPMI 1640 medium with 10% FBS, 100 U/L penicillin/streptomycin. The cells were treated with PQS (50, 150 ng/mL), accompany with or without TG (10 μM) for 12 h.

2.6. TUNEL method

To evaluate the extent of apoptosis by detecting DNA fragmentation, spinal tissues were collected, and TUNEL staining was performed 1 week after surgery. 5 μm-thick transverse sections were deparaffinized with xylene and rehydrated using ethanol. The PC12 cells were incubated in 4% paraformaldehyde for 20 min. All samples were incubated by Triton X-100 (0.2%) for 20 min. The samples were incubated with the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) and DAPI [25]. Images were captured with a Nikon ECLIPSE Ti microscope (Nikon, Japan).

2.7. Western blot assay

The spinal tissues at the T8–T10 levels were removed 3 day after the operation. Briefly, the samples including cell were homogenized with RIPA including a protease inhibitor cocktail. The extracts were analyzed using bicinchoninic acid reagents (Thermo, Rockford, USA). Equal amounts of protein were fractionated on 10% SDS–PAGE gel and loaded on PVDF membrane (Bio-Rad, CA). The membranes were incubated by 5% non-fat milk and then primary antibodies overnight, following with the secondary antibodies. The membranes were imaged under the ChemiDoc™ XRS + Imaging System (Bio-Rad), and signals were analyzed with Image Lab 3.0 (Bio-Rad). Each experiment was repeated at least three times.

2.8. Statistical analysis

All results are expressed as the mean ± SD. Statistical differences were tested using one-way analysis-of-variance (ANOVA) and Tukey's test with GraphPad Prism. $P < 0.05$ means significantly different.

3. Results

3.1. PQS treatment attenuates structural damage and neuronal death and improves locomotion recovery after SCI

To demonstrate the potential roles of PQS in the functional recovery after the operation, the BBB and inclined plane tests were performed for 4 weeks. The BBB scores did not differ between the SCI group and PQS-treated SCI groups (Fig. 1A). However, the PQS group showed significantly higher BBB scores than the SCI group at 2, 3, and 4 weeks after the operation, indicating that PQS promoted neurological function. The results of the inclined plane test were similar (Fig. 1B) and suggested that PQS treatment contributed to functional recovery after the operation. The SCI group showed marked structural damage histopathologically (HE staining at 28 days). In comparison, the PQS group had less necrotic tissue near the injury (Fig. 1C, D). Using Nissl staining 4 weeks after the operation, the PQS group had less neurons loss in anterior horn, compared with the SCI group (Fig. 1E, F). Therefore, PQS has a therapeutic effect on SCI *in vivo*.

3.2. PQS treatment inhibits apoptosis and improves neurite repair in acute SCI

To examine whether PQS exerts anti-apoptotic effects in SCI, the