The protective mechanism of action of plantamajoside on a rat model of acute spinal cord injury

HUA HU and XIAOFEI JIAN

Department of Orthopedics, The Central Hospital of Wuhan, Tongji Medical College, Huazhong
University of Science and Technology, Jiangan, Wuhan, Hubei 430014, P.R. China

Abstract. Acute spinal cord injury (ASCI) is a severe traumatic disease of the central nervous system, characterized by a high incidence and high morbidity, for which there are no effective drug therapies in the clinic. A rat model of ASCI was established to study the effects of plantamajoside (PMS) treatment on the expression of apoptotic factors, including caspase-3, caspase-9, poly (ADP-ribose) polymerase (PARP), Bax and Bcl-2. The Allen's weight hit rat ASCI model was used for the present study, and the rats were treated with various concentrations of PMS. The behavior of rats was assessed using the Basso-Beattle-Bresnahan locomotor rating scale (BBB), the histopathologic changes of spinal cord tissue were observed by hematoxylin and eosin staining, the survival of neurons was assessed by TUNEL staining and the expression levels of apoptotic proteins such as caspase-3, caspase-9, PARP, Bcl-2 and Bax was measured using western blot assays and RT-qPCR. It was observed that PMS could reverse the decrease in the BBB score after ASCI, improve the morphological characteristics of the spinal cord, reduce the degree apoptosis and affect the expression of caspase-3, caspase-9, PARP, Bax and Bcl-2 in a concentration dependent manner. In conclusion, PMS protected ASCI rats by inhibiting apoptosis; therefore PMS may be a potential candidate for ASCI therapy.

Introduction

Acute spinal cord injury (ASCI) is a fatal central nervous system disease, which usually causes paralysis below the contused spinal cord segment (1). ACSI not only brings great pain to patients, but also causes a serious socio-economic burden (2), with ~23 cases per million occurring every year, globally (3). The underlying pathological mechanisms of action behind ASCI are tissue edema after injury, which eventually lead to inflammation and apoptosis (4). However, the treatment of ACSI remains a major problem for researchers and clinicians, and there is no effective treatment for patients with ASCI (5). ASCI is divided into direct injury and secondary injury. Secondary injuries result from inflammation, altered Ca^{2+} homeostasis, oxidative stress and apoptosis (6). Apoptosis is one of the most important causes of spinal cord dysfunction and can dramatically impact the recovery for patients with ASCI (7). It has been reported that the expression levels of apoptosis relevant factors, including caspase-3, Bax and Bcl-2, are altered, accompanied with increasing neuronal apoptosis, after ASCI (8).

Plantamajoside (PMS; $C_{29}H_{36}O_{16}$) belongs to the phenyl-propanoid glycosides family, which is a unique component identified in Herba plantaginis (9). PMS has numerous beneficial pharmacological effects. PMS protects advanced glycation end-induced endothelial cells against inflammatory cellular dysfunction (10). In addition, PMS ameliorates lipopolysaccharide (LPS)-induced acute lung injury through improving pulmonary inflammation (11). PMS also inhibits LPS-induced mucin 5AC expression and inflammation through suppressing the PI3K/Akt and NF-κB signaling pathways (12). Moreover, it has been documented that PMS inhibits growth and metastasis of breast cancer by inhibiting the activity of MMP9 and MMP2 (13). Together, PMS has been shown to have anti-oxidant, anti-inflammatory, anti-cancer and anti-proliferative activities (14-16). However, to the best of our knowledge, there are no studies that have investigated the effects of PMS on apoptosis in rats after ASCI. Therefore, the present study aimed to investigate whether PMS could protect against apoptosis in ASCI rats and to elucidate the potential anti-apoptosis mechanisms of action that are involved in the expression of the Bcl-2 and Bax, as well as the caspase-3 signaling pathway.

Materials and methods

Experimental animals. A total of 36 adult male Sprague-Dawley rats (weight, 200-250 g; age, 9-11 weeks) were purchased from Hubei Provincial Institute of Science and Technology. Rats were raised in a suitable environment with 24±3˚C and 12-h light/dark cycle in separated cages (relative humidity 55-60%). All rats had free access to food and water and they

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Correspondence to: Dr Xiaofei Jian, Department of Orthopedics, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, 26 Shengli Street, Jiangan, Wuhan, Hubei 430014, P.R. China
E-mail: jianxiaofei125@126.com

were allowed to acclimate to the environment for at least for three days before the experimental procedure. All of the study protocols were approved by the Ethics Committee on Animal Experiments of Tongji Medical College, Huazhong University of Science and Technology.

Rat model of spinal cord injury. Rats were randomly assigned into six groups, namely: Sham, model, positive, PMS 80 mg/kg, PMS 40 mg/kg and PMS 20 mg/kg groups. The positive group was treated with methylprednisolone 30 mg/kg as a positive control (17). The ASCI model of rats was established according to Allen's weight hit model (18). Drinking was prohibited until the surgery had been finished. All rats were anesthetized with 3% chloral hydrate (450 mg/kg) by intraperitoneal injection and maintained in the prostrate position for surgery. Fur around the chest and abdomen of these rats was shaved. A 3 cm incision was performed at the position of the eighth thoracic vertebrae and subsequently the dura mater was exposed. A 25 g cm (10 g x 2.5 cm) injury to the spinal cord was set as the injury gravity, which induces a moderate injury (19). Following induction of the injury, the wound was sutured. The following standards were used to evaluate whether a successful rat model was made: i) Spinal cord ischemia and edema around the wound; ii) flicking of the body and legs as well as the appearance of the tail sway reflex, and iii) the above symptoms coupled with sluggish paralysis. To prevent infection of the wound, liquid ampicillin (8×10^5 U/kg; Pureone Bio Technology Co., Ltd.) was injected into the back, exterior muscles once every day, for three days. In order to keep each cage dry, the padding was changed daily. To establish the autonomic urinary reflex of the rats, the bladder was massaged twice per day. Three days after the surgery, all animals were intraperitoneally injected with pentobarbital (200 mg/kg; Beijing Huaye Huanyu Chemical Co., Ltd.) for euthanasia prior to further investigation.

Evaluation of neuronal function recovery. The neuronal function recovery after injury was scored in accordance with the 21-point Basso-Beattie-Bresnahan (BBB) scale, which was scored as 0-21 representing complete paralysis to normal locomotion, respectively. BBB scores categorize combinations of rat hindlimb movements; joint movement; weight support; fore/hindlimb coordination; trunk position and stability; stepping; paw placement; toe clearance; and tail position, representing sequential recovery stages that rats attain after ACSI (20). Rats were allowed to move randomly and scored over 4 min by two independent observers who were blinded to the experiments. The hindlimb movement ability was assessed at 24, 48 and 72 h after surgery. The ability of the hindlimb joints was firstly assessed with scores between 0-7. Subsequently the pace and coordination abilities of the hindlimbs were assessed (0-7 scores) and then the delicate abilities of paws during movement were assessed (0-7 scores).

Hematoxylin and eosin (H&E) staining. A total of 3 days after surgery, 0.9% NaCl solution was obtained to transcardially perfuse the rats, and subsequently followed by 4% paraformaldehyde (PFA) for 30 min. In order to post-fix spinal cords, they were dissected out and placed in 4% PFA for 12 h at 4˚C.

The spinal cords were then further embedded in paraffin at room temperature and 5-μm thick, serial transverse sections were made. These slices were subsequently stained with H&E dye for conventional morphological evaluation to evaluate the relative changes. Samples were stained with hematoxylin for 10 min and with eosin for 2 min, both at room temperature.

TUNEL staining. A TUNEL detection kit (Roche Diagnostics) was used to assess DNA fragmentation. The spinal cord specimens were preserved in 4% paraformaldehyde for 12 h at 4˚C and then washed with PBS, embed in paraffin and cut into 5-μm thick sections. The dewaxed sections were then incubated with 1:200 proteinase K for 10 min at 37˚C, followed by rinsing with PBS three times. The slides were then immersed in TUNEL assay reaction mixture and incubated at 37˚C for 1 h. Subsequently, 50 μl DAB substrate was added to the tissue and the reaction was carried out at room temperature for 10 min. Cell nuclei of apoptotic cells were distinguished by the presence of dark brown staining. The positive cells were counted in five arbitrarily selected fields in each slide (magnification, x400) using an optic microscope. Cell apoptosis (%) was calculated using the following formula: (the number of positive cells/the total cells) x 100%.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the spinal cord samples was extracted using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 600 ng of RNA was used for cDNA synthesis, at a temperature of 42˚C for 60 min and 75˚C for 5 min, using a RT First Strand kit (SA Biosciences LLC). The RT-PCR amplification was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on an ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling profiles were as follow: 95˚C for 1 min; 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and an extension at 72˚C for 30 sec. The primers used in the present study were as follows: Bcl-2, forward 3'-GCTGGGGATGACTTCTCTCG-5' and reverse 3'-CCACAATCCTCCCCCAGTTC-5'; Bax, forward 3'-CAC CAAGAAGCTGAGCGAGT-5' and reverse 3'-TAGAAA AGGGCAACCACCCG-5'; caspase-3, forward 3'-CGGACC TGTGGACCTGAAAA-5' and reverse 3'-CGTACAGTT TCAGCATGGCG-5'; caspase-9, forward 3'-TCTTGAGAC TCGAGGGAGGC-5' and reverse 3'-GGTCGTTCT TCACCTCCACC-5'; poly (ADP-ribose) polymerase (PARP), forward 3'-AGCCAATGTTCGAGTCGTGT-5' and reverse 3'-ACAGCATCCTCTTTGGACGG-5'; and GAPDH, forward 3'-TTTCGGTACGGTTAGTAG-5' and reverse 3'-TTTGACCTTGCCTTCCAC-5'. The relative expression levels of the genes were normalized to GAPDH. Data were comparatively analyzed using the $2^{-\Delta\Delta Cq}$ method (21).

Western blot analysis. Proteins from the spinal cord samples were extracted using RIPA lysis buffer kit (Omega Bio-Tek, Inc.) and the concentration was detected using a BCA protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of proteins (40 μg per lane) were loaded into 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane (EMD Millipore). The membranes were subsequently blocked with 5% skimmed milk for 1 h at room temperature and incubated with primary
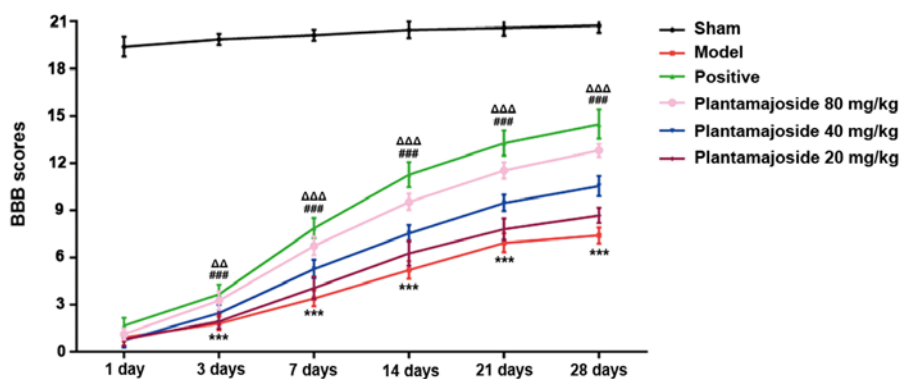
Figure 1. BBB score decreases in the ACSI rat model group and PMS improves the BBB score of ASCI rats. BBB score was measured at 1, 3, 7, 14, 21 and 28 days after ASCI among the six groups (n=6 for each group). ***P<0.001, PMS 80 mg/kg vs. the model group; ###P<0.001, PMS 40 mg/kg vs. the model group; ΔΔP<0.01, ΔΔΔP<0.001, PMS 20 mg/kg vs. the model group. ASCI, acute spinal cord injury; BBB, Basso-Beattie-Bresnahan; PMS, Plantamajoside.
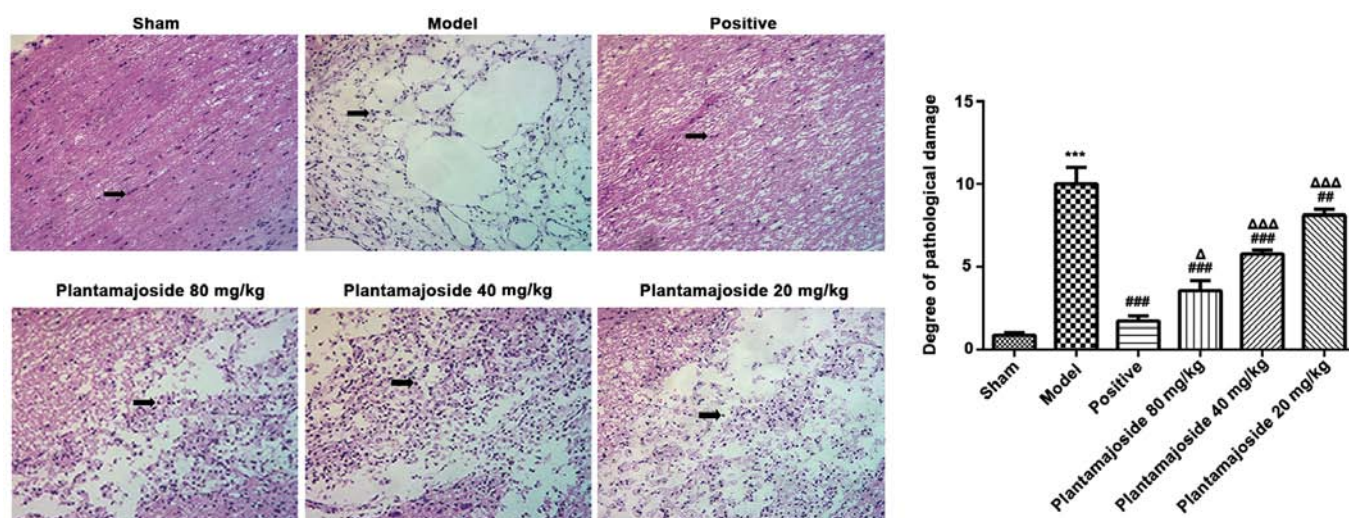

Figure 2. Plantamajoside alleviates the pathological morphology of the spinal cord in rats. The rat spinal cord samples were assessed using hematoxylin and eosin staining at 28 days after acute spinal cord injury and the degree of pathological damage was calculated (magnification, x100). The pathological changes are shown by the arrows. ***P<0.001 vs. the sham group; ##P<0.01, ###P<0.001 vs. the model group; ΔP<0.05, ΔΔΔP<0.001 vs. the positive group.

antibodies overnight at 4˚C, including: Anti-Bcl-2 (sc-56015; 1:1,000), anti-Bax (sc-20067; 1:1,000), anti-caspase-3 (sc-56053; 1:1,000), anti-caspase-9 (sc-81650; 1:1,000), anti-PARP (sc-56197; 1:1,000) and anti-GAPDH (sc-293335; 1:1,000) antibodies, which were purchased from Santa Cruz Biotechnology, Inc. The membranes were then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Enhanced chemiluminescence (Applygen Technologies, Inc.) was used for visualization. Band intensities were quantified using ImageJ software (v1.52r; National Institutes of Health). GAPDH was used as an endogenic control in all samples.

Statistical analysis. All results were confirmed in at least three independent experiments and analyses were performed using SPSS v14.0 software (SPPS, Inc.). All quantitative data are presented as the mean ± SD. The data graded by the scoring system was analyzed using a Kruskal-Wallis test, with post-hoc Dunn's test. Statistical comparisons with normally distributed data was made using ANOVAs followed by Turkey's post hoc

tests among the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

The rat ASCI model assessment. The rat model of ASCI was established using Allen's weight hit model (18). Rat functional deficits were evaluated using the BBB score until 28 days after ASCI. It was found that rats in the model group walked abnormally, with bilateral hind limb paralysis, and the BBB score of the model group was substantially lower than the sham group across the observed timeframe (Fig. 1). These results indicated that the rat ASCI model was established successfully.

PMS improves the behavioral performance of ASCI rats. According to the experimental result, it was found that the BBB score in sham group stayed at ~20 points which was the highest among these groups (Fig. 1). The BBB score of the rest of the groups increased with time, but the rate of increase varied between them. Overall, the groups stayed consistently in order, namely, from highest to lowest: The Sham, positive,
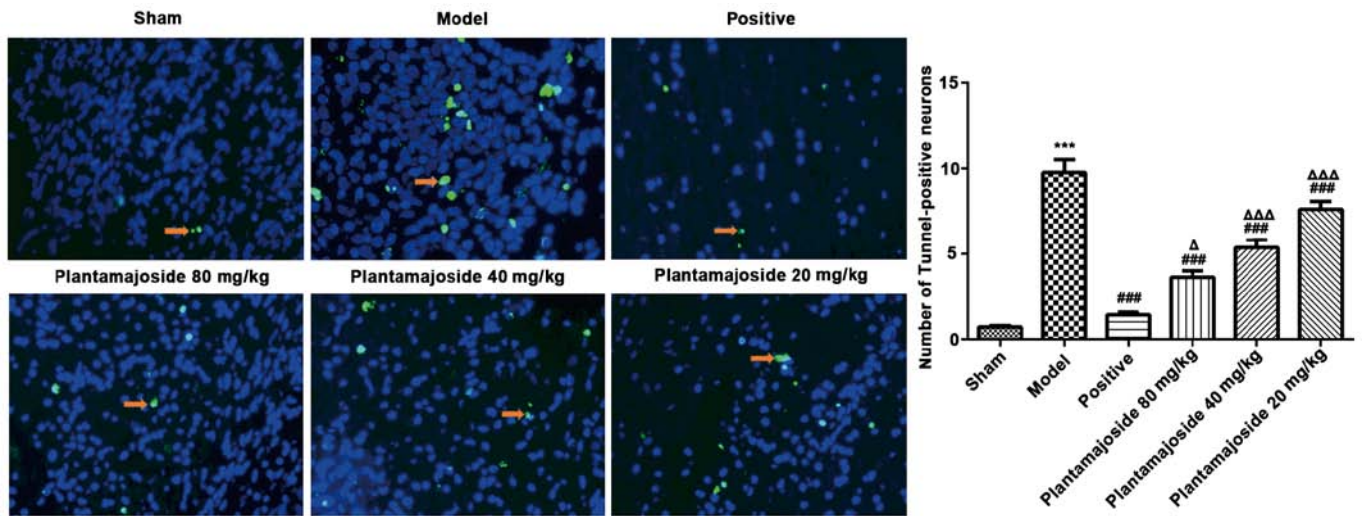
Figure 3. PMS inhibits neuronal apoptosis in the spinal cord in rats. The rat spinal cord samples were assessed using TUNEL staining, to analyze neuronal apoptosis at 28 days after acute spinal cord injury and the number of apoptotic cells was counted (magnification, x200). ***P<0.001 vs. the sham group; ###P<0.001 vs. the model group; ^P<0.05, ^^^P<0.001 vs. the positive group.

PMS 80 mg/kg, PMS 40 mg/kg, PMS 20 mg/kg and lastly, the model group (Fig. 1). This data suggested that PMS can improve the behavioral performance of ASCI rats in a concentration dependent manner.

PMS reduces the apoptosis of spinal cord cells. Histopathological alterations were subsequently investigated in ASCI rats. H&E staining of the spinal cord from rats at 28 days after ASCI showed that the rats in the model group had an unclear boundary between white and gray matter. The central canal displayed an abnormal morphology and some neurons were found with apoptotic bodies (Fig. 2). However, PMS improved the morphology in a concentration dependent manner. The neurons treated with PMS presented a better histologic characteristics relative to the model group, especially in the PMS 80 mg/kg group (Fig. 2), indicated that PMS could partially improve the morphology of the spinal cord structure. TUNEL staining was also performed which showed the extent of apoptosis induced neuronal damage. The number of TUNEL-positive cells notable increased in the model group, compared with the sham group (Fig. 3). Moreover, the number of apoptotic cells in the PMS treated groups had significantly decreased compared with the model group, with further decreases in a concentration dependent manner (Fig. 3). These results indicated that PMS may inhibit apoptosis and protect the spinal cord cells after ASCI.

The mechanisms of action behind the inhibition of apoptosis by PMS. To further confirm that PMS can inhibit apoptosis, the cells lysates of the spinal cords of rats at 28 days after ASCI were assessed by RT-qPCR and western blot assays, to measure the mRNA and protein expression levels of apoptosis related proteins, including caspase-3, caspase-9, PARP, Bax and Bcl-2 (Fig. 4). The RT-qPCR and western blotting data indicated that PMS significantly downregulated the expression levels of caspase-3, caspase-9, PARP and Bax compared with the model group after ASCI. In addition, Bcl-2 levels were significantly upregulated in the PMS treated rat ASCI models

at both the mRNA and protein level (Fig. 4). These results suggested that PMS plays a protective role against apoptosis through modulating the expression levels of apoptotic factors.

Discussion

Damage from ASCI is mainly caused by the direct injury itself and subsequent secondary injury (22). The secondary injury results in a pathological change in the normal tissue around the injured tissue (23). Secondary injury is accompanied by a series of changes at the molecular and cellular levels, including an inflammatory reaction, oxidative stress, internal flow of calcium ions and apoptosis, in which apoptosis is an important mechanism of action behind the damage observed from the secondary injury of ASCIs (24). Caspase-3, Bcl-2 and Bax are involved in apoptosis after spinal cord injury. A number of studies have suggested that the secondary injury is the key cause of dysfunction in the central nervous system (25,26). Therefore, inhibition of neuronal apoptosis provides an opportunity for a therapeutic strategy to improve spinal cord function after ASCI (27-29).

Since its discovery, PMS has been reported to possess broad pharmacological effects, which may exert beneficial functions for numerous therapies (14,30). Studies have indicated that PMS can regulate a variety of conditions, such as renal damage and breast cancer (13,31). PMS also possesses anti-oxidant, antibiotic and anti-inflammatory activities which has been found in a number of previous reports (32,33). However, the effect of PMS on apoptosis and its underlying mechanism of action remains unclear.

Apoptosis is a genetically programmed process resulting in cell death (34). It occurs during embryonic development, tissue reconstruction, immune regulation, and tumor degeneration. Apoptosis is critical for the development of multicellular organisms, but abnormal apoptosis can cause a variety of diseases (35). In damaged spinal cords, apoptosis causes neuronal losses (36). Apoptosis can be divided into two types of pathways, the external and internal pathways. The external pathways are induced by death receptors, such as Fas
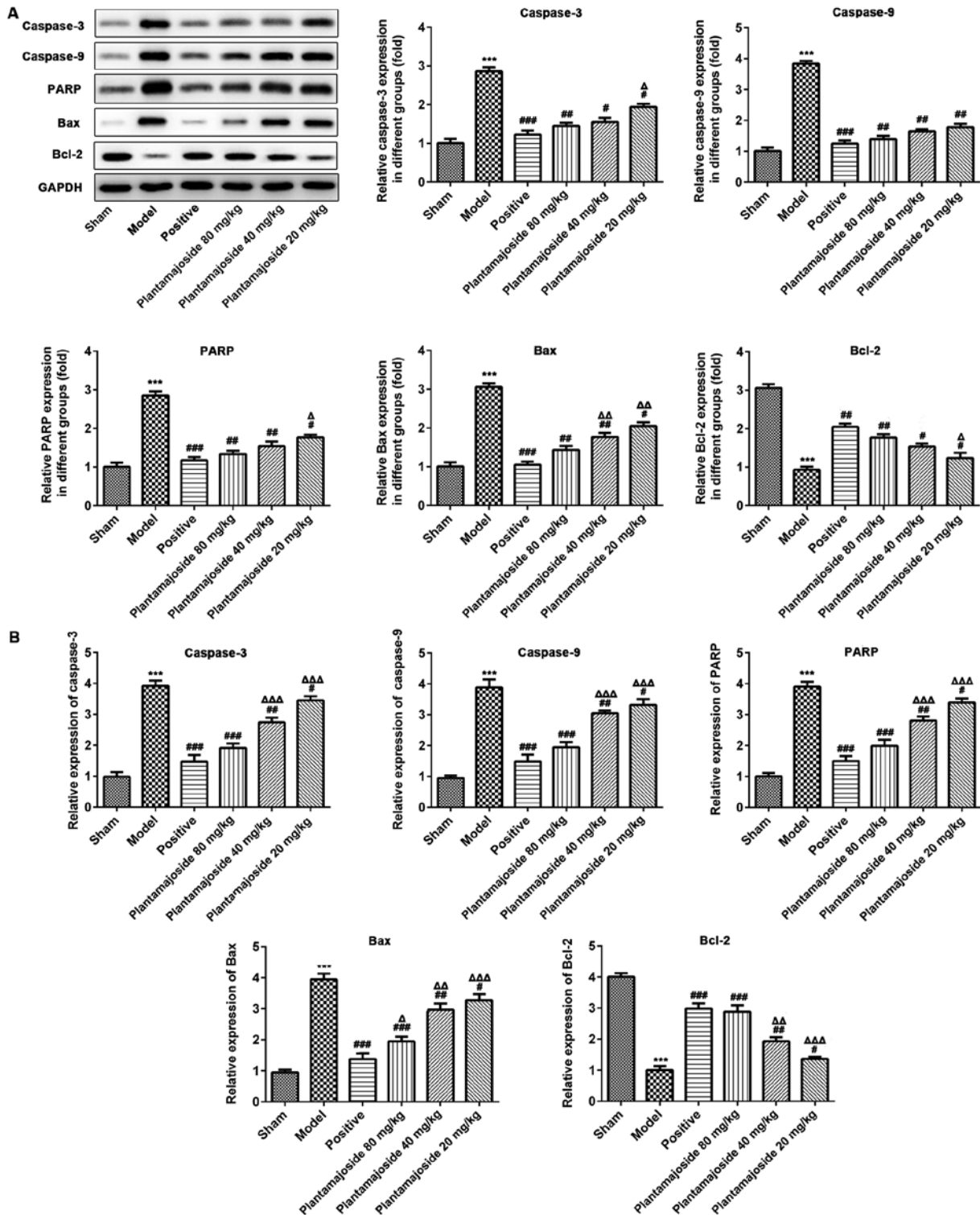
Figure 4. The protein and mRNA expression levels of apoptosis relevant genes in the various treatment groups in the spinal cord, 28 days after acute spinal cord injury. (A) Western blot assays were performed to identify the protein expression levels of caspase-3, caspase-9, PARP, Bax and Bcl-2. (B) The mRNA was extracted from the spinal cord samples of each group and the mRNA expression levels of caspase-3, caspase-9, PARP, Bax and Bcl-2 were assessed. Data are represented as the mean ± SEM from three independent experiments. n=3. ***$P<0.001$ vs. the sham group; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs. the model group; $^\Delta P<0.05$, $^{\Delta\Delta}P<0.01$, $^{\Delta\Delta\Delta}P<0.001$ vs. the positive group. PARP, poly (ADP-ribose) polymerase.

receptors (37). The internal pathways are triggered by various factors, such as DNA damage and endoplasmic reticulum stress (38). The internal pathway of the cell is regulated by the Bcl-2 protein family. The main anti-apoptotic members, Bcl-2 and Bcl-xl, play a key role in the mitochondrial outer membrane to maintain membrane integrity (39). Bcl-2, is an anti-apoptotic protein which can prevent apoptosis through regulating various signaling pathways after spinal cord injury (40). Bax protein is found in the cytoplasm of mitochondria. ASCI stimulation can activate Bax protein and alter the permeability of the mitochondrial membrane, which in turn can induce neuronal apoptosis (41).

The expression of Bcl-2 and Bax directly affects the apoptosis of spinal cord neurons (8). Bax disrupts the integrity of the mitochondrial membrane, causing apoptosis factors such as cytochrome c to leak into the cytoplasm (42). The cytochrome c complex can promote the formation and activation of caspase-9, then the activated caspase-9 is cut off and activates the downstream protease cascade such as caspase-3 (8). Caspase-3 is a cysteine protease, which can destroy a variety of proteases, decompose DNA, prevent the normal function of the calcium pump, cause calcium overload and eventually lead to apoptosis (43). It is considered to be the most important protease in the process of apoptosis (44). Studies have shown that caspase-3 plays an important role in ASCI and that caspase-3 positive cells are present in ischemic and traumatic spinal cord injury models (45,46). Therefore, caspase-3 expression levels may reflect the degree of apoptosis in spinal cord injuries. After caspase-3 activation, the ADP ribose polymerase, PARP-1, can also play a role in apoptosis, resulting in cell death (47,48).

In conclusion, the present study demonstrated that PMS promotes the recovery of neurological function and protects the tissue structure of the spinal cord after ASCI. The underlying mechanisms of action may be due to PMS interrupting apoptosis, thus enhancing the resistance to further damage of the spinal cord and rescuing the locomotive activity. Furthermore, it was revealed that PMS can efficiently inhibit apoptosis by regulating the expression levels of apoptotic factors, including caspase-3, caspase-9, PARP, Bax and Bcl-2.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH wrote the manuscript, analyzed the data and revised the manuscript. HH and XJ performed the literature search, designed the study and performed experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All of the study protocols were approved by the Ethics Committee on Animal Experiments of Tongji Medical College, Huazhong University of Science and Technology.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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