Protective effects of extracellular polymeric substances from *Aphanizomenon flos-aquae* on neurotoxicity induced by local anesthetics

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Abstract. The neurotoxicity of local anesthetics has received an increasing amount of attention and more effective therapeutic agents are required. Extracellular polymeric substances from *Aphanizomenon flos-aquae* (EPS-A) are high molecular weight polysaccharides. The present study aimed to elucidate the protective effects of EPS-A on neurotoxicity induced by local anesthetics in an intraperitoneal injection bupivacaine rat model. The results of immunohistochemical staining inicated that following intraperitoneal injection of EPS-A the levels of apoptosis and caspase-3 decreased, and the expression levels of microtubule-associated protein 1A light chain 3 (LC3) and beclin1 increased. In order to further clarify the mechanism of the EPS-A-mediated protection, the expression of key proteins associated with autophagy was investigated by western blotting. The results suggested that the ratio of LC3-II/LC3-I and the expression level of beclin1 increased. Taken together, the results indicated that EPS-A induced neuroprotective effects on bupivacaine-induced neurotoxicity. The underlying mechanism may be associated with the inhibition of apoptosis, upregulation of autophagy and improvement of cell survival. The results suggested that EPS-A may be a candidate neuroprotective agent against neurotoxicity caused by local anesthetics.

Introduction

At present, the neurotoxicity of local anesthetics is receiving an increasing amount of attention (1), and therefore, it is particularly important to identify safe methods of prevention and treatment. One pharmacological strategy against the neurotoxicity of local anesthetics is the use of drugs to induce autophagy to treat the associated diseases (2,3). Studies have shown that certain drugs can directly or indirectly activate autophagy to prevent cell death (4,5). Based on these observations, it has been hypothesized that the regulation of autophagy could serve as the basis for the development of candidate neuroprotective agents against neurotoxicity caused by local anesthetics. However, the components of the autophagy signaling pathway remains at an initial stage (6), therefore, the major aims of the current study were to explore drugs for the prevention and treatment of nerve injury caused by local anesthetics via the autophagy signaling pathway.

In previous years, polysaccharides have been investigated in numerous research studies and serve roles in controlling cell division and differentiation, regulate cell growth and aging, and aid in maintaining the normal metabolism of an organism (7-9). Numerous studies have indicated that polysaccharides can activate autophagy via a non-serine/threonine-protein kinase mTOR pathway and serve a protective role in glomerular lesions and neurodegenerative diseases (10,11). It was also thought that polysaccharides, as natural products, were more safe. Therefore, polysaccharides have attracted increasing research attention (12). *Aphanizomenon flos-aquae,* a filamentous, heterocystous and dominant cyanobacterium, is common in nutrient-rich freshwater cyanobacterial blooms (13). During the process of growth until death, *A. flos-aquae* continuously secretes extracellular polymeric substances (EPS) into the surrounding environment (13). EPS are high molecular weight polysaccharides consisting of three or four different monosaccharides arranged in groups of £10 to form repeating units (14). Numerous studies have indicated that EPS exhibit numerous bioactivities, including antitumor, immune-adjusting, antithrombotic, ameliorating endothelial cell injury, antioxidant and antiviral (14,15). Although several studies on the biological activities of EPS have been performed, with a particular focus on their anti-apoptotic activity (16-18), little is known about their neuroprotective effects *in vivo*. Therefore, in the present study, rats received intraperitoneal injections of EPS from *A. flos-aquae* (EPS-A), and apoptosis and autophagy were observed in spinal cord neurons. The aim was to investigate the protective effects of EPS-A on neuronal injury and the possible underlying mechanisms.

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Materials and methods

EPS-A. A strain of *A.flos-aquae*, isolated from Lake Dianchi in China, was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan city of China. The culture of *A.flos-aquae* was performed according to the methods of Zhang *et al* (19), and extraction and purification of EPS-A were performed as previously described by Hu *et al* (20).

Animal selection and housing. The present *in vivo* study was approved by the Medical Ethics Committee of the First Hospital of Lanzhou University (Lanzhou, China). A total of 18 healthy adult male Sprague-Dawley rats (age, 5-7 weeks; weight, 180-220 g), were supplied by the Animal Breeding and Research Center of Lanzhou University (Lanzhou City, China). Rats were housed in separate cages with food and water freely available until the time of testing and kept in temperature-controlled rooms (20-24˚C; relative humidity, 50-60%) with a 12-h light/dark cycle (6:00 a.m.-6:00 p.m.).

Groups and treatment. A total of 18 rats were randomly divided into groups A, B and C (n=6/group). In groups A and B, rats received intraperitoneal injections of 1 ml 0.9% NaCl, whole group C received an intraperitoneal injection of EPS-A (100 mg/kg), once a day for 3 days. Subsequently, the group A rats were subjected to 2% isoflurane inhalation and intrathecal injection of 0.9% NaCl 50 μl. Groups B and C received 2% isoflurane inhalation and intrathecal injection of 1% bupivacaine 50 μl (2.5 mg/kg animal body weight).

Bupivacaine in lumbar anesthesia. When optimal flexion of the rat lumbar spine was achieved in a prone position, a 27-gauge needle attached to a 100 μl syringe (model, KL-34; Hamilton Medical, Inc., Reno, NV, USA) was inserted into the midline of the lumbar 4-5 (L4-5) intervertebral space and 50 μl of the respective drug was injected. While a tail-flick indicated entrance into the intrathecal space, rats were subsequently observed for paralysis of the hind limbs, indicative of a spinal blockade. During the selection process, rats were excluded if they lacked a healthy appearance or required more than one spinal puncture. A total of 6 rats were included in each group following this.

Spinal cord section specimen. Rats in each group were sacrificed at 6 h following the aforementioned anaesthesia and the spinal cord was rapidly collected, then sections were transported in fixative (10% neutral buffered formalin) at room temperature for 36-48 h. A section of tissue was frozen in liquid nitrogen and stored at -70˚C until further use.

Hematoxylin & eosin (H&E) staining. H&E staining is a standard method used for detecting morphological alterations. First, the spinal cord sections were sliced into 5 μm sections using a microtomer, deparaffinized at 40˚C in a water bath and rehydrated. Samples were then washed with distilled water and dried. Hematoxylin was added for 5 min and rinsed with water. Subsequently 1% HCl ethanol solution (1 ml HCl added to 99 ml 70% ethanol) was added for 10 sec in triplicate to remove excess haematoxylin. Following this, sections were washed using distilled water for 25 min, 0.5% eosin was added for 2 min and slices were dehydrated with 95 and 100% ethanol. Dimethylbenzene (Absin Bioscience, Inc., Shanghai, China) was added for 5 min twice and incubated at 37˚C for 24 h. Finally, sections were kept in a special slide container at room temperature and observed under a light microscope.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL). TUNEL staining was performed to examine apoptosis. The spinal cord samples were fixed in 10% neutralized formalin at room temperature for 20 min and embedded in paraffin. Sections were deparaffinized, rehydrated and incubated for 15 min at 37˚C with proteinase K working solution (Shanghai Xiangsheng biotechnology Co., Ltd., Shanghai, China). Sections were rinsed twice with PBS (pH 7.4), blocked with hydrogen peroxide for 15 min, and 50 μl TUNEL reaction mixture was added and incubated for 60 min at 37˚C in a humidified atmosphere in the dark. Following rinsing with PBS (pH 7.4) three times, sections were incubated for 30 min at 37˚C with 50 μl converter peroxidase (Shanghai Xiangsheng biotechnology Co., Ltd.). A total of 50 μl diaminobenzidine [5 μl 20xDAB, 1 μl 30% H_2O_2, 94 μl PBS (pH 7.4)] was added and incubated for 10 min at 25˚C. Sections were rinsed with PBS (pH 7.4) three times and cover slips were mounted onto 50% glycerol treated slides. Samples were analyzed under a light microscope (magnification, x400). Slices were photographed continuously and six visual fields were randomly selected under an optical microscope. Pictures were numbered and random fields were obtained using random numbers generated by a computer. The final value of a slice was obtained by a mean value from random high-power fields. The optical density was quantified using Image-Pro Plus (version 7.0; Media Cybernetics, Inc., Rockville, MD, USA).

Immunohistochemical (IHC) staining. IHC is widely used in research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of cells or tissues. In the present study, spinal cord samples were replaced with ethanol in decreasing concentrations (100, 90 and 70%), washed with PBS (pH 7.4) three times for 5 min each and heated in a water bath (95-98˚C) in 0.01 M trisodium citrate (pH 6.0) for 15 min for antigen retrieval. Subsequently, the sections were incubated with a blocking reagent (3% milk and 5% fetal bovine serum; Absin Bioscience, Inc.) for 1 h at room temperature and further incubated with anti-caspase-3 (cat. no. ab20816; dilution 1:500; Abcam, Cambridge, UK), anti- microtubule-associated protein 1A light chain 3 (LC3) polyclonal rabbit antibody (cat. no. L8918; dilution 1:200; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or anti-beclin1 (cat. no. SAB2103299; dilution 1:500; Sigma-Aldrich; Merck KGaA) at 4˚C overnight. Following washing with PBS (pH 7.4), the tissue samples were exposed to biotinylated anti-rabbit immunoglobulin G (cat. no. A0239; dilution 1:1,000; Beyotime Institute of Biotechnology, Fuzhou, China) and horseradish peroxidase conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA, USA). After the slides were sealed, the sections were imaged with a

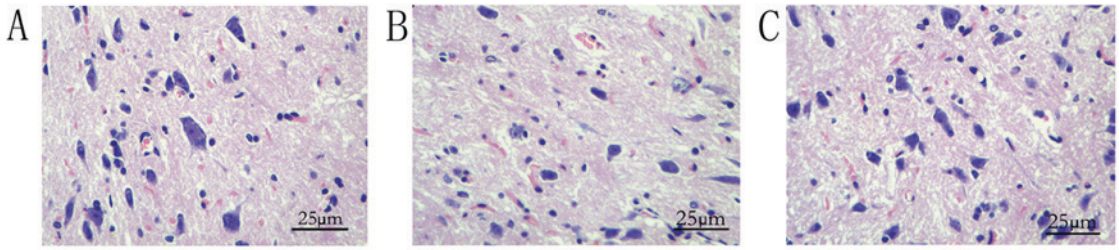

Figure 1. Pathological alteration of spinal cord neurons in (A) control, (B) bupivacaine and (C) EPS-A group rats were examined by hematoxylin & eosin staining (magnification, x400).

confocal microscope. The optical density was quantified using Image-Pro Plus version 7.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis. Western blotting is used for the detection and quantification of specific proteins in a sample of tissue. Protein levels of two autophagy markers, LC3 and beclin1, were detected. To investigate autophagy activation, the ratio of LC3-I to LC3-II, a marker of autophagic vacuole formation, was measured. The spinal cord samples were cut into small pieces (2 mm long; 2 mm thick) and homogenized in lysis buffer [50 mM Tris HCl (pH 7.6), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Triton-X, 5 units.ml^{-1} aprotinin, 5 μg.ml^{-1} leupeptin, 5 μg.ml^{-1} pepstatin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride]. Lysate protein levels were determined using a BCA protein assay kit (Shanghai Qcbio Science&Technologies Co., Ltd., Shanghai, China). Equal amounts of proteins (~30 μg) were loaded per lane and subjected to 10% SDS-PAGE, and subsequently transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline with Tween 20 (TBST; 137 mM sodium chloride, 20 mM Tris, 0.1% Tween 20; Absin Bioscience, Inc.) for 1 h at room temperature. Primary antibodies specific to LC3 (cat. no. bsm-33309M; dilution 1:1,000; Biosynthesis Biotechnology Co., Ltd., Beijing, China), beclin-l (cat. no. bs-1353R; dilution 1:200; Biosynthesis Biotechnology Co., Ltd., Beijing, China), caspase-3 (cat. no. ab20816; dilution 1:500; Abcam) and β-actin (cat. no. bsm-33139M; 1:1,000; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) were diluted in TBST overnight at 4˚C. Membranes were incubated with Goat-anti-mouse immunoglobulin G secondary antibodies conjugated to alkaline phosphatase (cat. no. A32723; dilution 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature. Reactive bands were detected by incubating with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich; Merck KGaA) for 5 min. Band densities were detected with an imaging densitometer (GS-800; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the optical density was quantified using Image-Pro Plus (version 7.0; Media Cybernetics, Inc.), which was calibrated against β-actin.

Statistical analysis. Statistical analysis was performed using SPSS software (verison 20.0; IBM Corp., Armonk, NY, USA). Data were presented as the mean ± standard deviation. All experiments were performed in triplicate. The differences between groups were evaluated by one-way analysis of variance and multiple group comparisons were performed by using Dunnett's tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Pathological alterations. As demonstrated by light microscopy, in group A, spinal cord neurons in the rats were uniformly distributed, the morphology was normal, nissl bodies were clear, cell membranes were intact, the neural fibers of the white matter were arranged in an orderly manner and the intercellular matrix was uniform (Fig. 1A). However, following bupivacaine injection, the number of spinal cord neurons was reduced, neurons were shrunken and darkly stained and the nuclei were condensed (Fig. 1B). However, following administration of EPS-A, injury was ameliorated markedly (Fig. 1C). Therefore, the rat models were successfully established.

Alterations in apoptosis. TUNEL staining was used to observe neuronal apoptosis under a light microscope. The results indicated that neuronal apoptosis in the spinal cord was altered. The apoptosis level in group B increased compared with group A. Compared with group B, the level of apoptosis in group C decreased significantly (Table I; Fig. 2).

Alterations in the expression levels of caspase-3. The expression of casepase-3 was observed by immunohistochemical staining. Caspase-3-positive neurons were stained brown. The expression of caspase-3 in group B increased compared with group A. Compared with group B, the expression of caspase-3 in group C significantly decreased (Table I; Fig. 3).

Alterations in the expression levels of LC3 and beclin1. Immunohistochemical staining was performed to investigate alterations in the expression levels of LC3 and beclin1. The LC3- and beclin1-positive neurons were stained brown. LC3 and beclin1 levels in group B increased compared with group A. Compared with group B, the LC3 and beclin1 levels in group C significantly increased (Table I; Figs. 4 and 5).

Western blotting. In order to further determine the level of autophagy, western blot analysis was performed to observe the expression levels of LC3 and beclin1. The results of the present study indicated that the expression level of beclin1 and the ratio of LC3-II/LC3-I differed between the groups. Compared with group A, the level of beclin1 and the ratio of LC3-II/LC3-I

Table I. Comparison of apoptosis and autophagy of spinal cord neurons in rats treated with bupivacaine and extracellular polymeric substances from *Aphanizomenon flos-aquae* as determined by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling and immunohistochemistry.

Group	Apoptosis	Casepase-3	Beclin1	LC3
A	0.137±0.004	0.157±0.002	0.182±0.006	0.141±0.008
B	0.164±0.005[a]	0.194±0.004[a]	0.199±0.003[a]	0.178±0.010[a]
C	0.146±0.006[a,b]	0.157±0.002[b]	0.238±0.003[a,b]	0.208±0.012[a,b]
Sum	0.149±0.012	0.169±0.018	0.206±0.025	0.175±0.030
F	41.06	314.47	260.87	68.27
P	0.000	0.000	0.000	0.000

[a]P<0.05 vs. group A; [b]P<0.05 vs. group B. LC3, microtubule-associated protein 1A light chain 3; Sum, sum of the mean ± standard deviations. F and P represent the differences between groups which were evaluated using one-way analysis of variance; a and b represent the multiple group comparisons that were confirmed using the Dunnett's test.

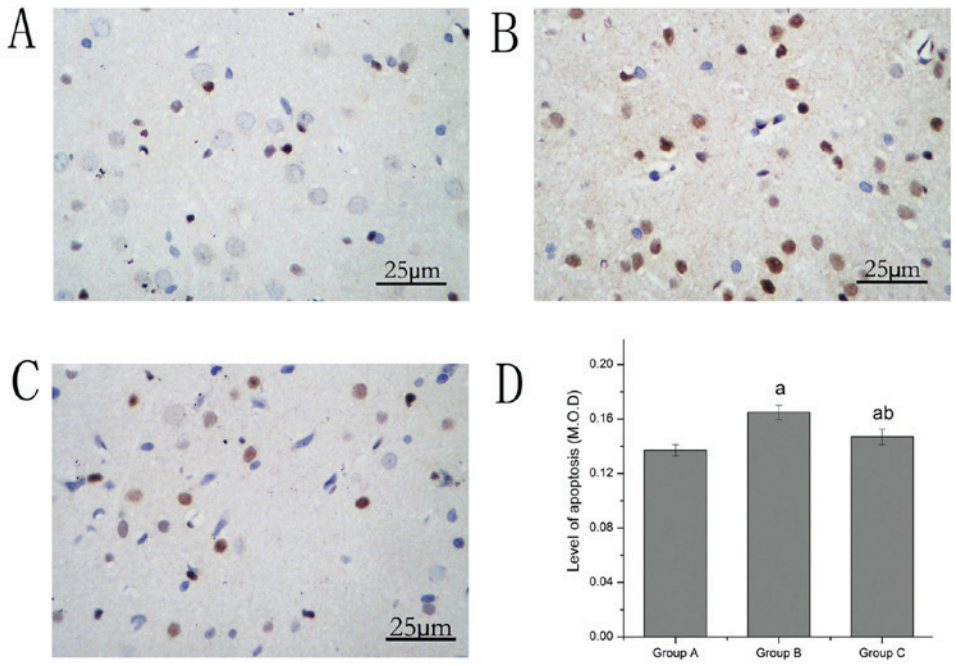

Figure 2. Comparison of the level of apoptosis of spinal cord neurons in rats. Apoptotic alterations of spinal cord neurons are observed in (A) control, (B) bupivacaine and (C) EPS-A group rats measured by the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (magnifcation, x400). (D) Quantitative comparisons of the level of apoptosis. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B. M.O.D, mean optical density.

increased significantly in group B. Compared with group B, the level of beclin1 and the ratio of LC3-II/LC3-I increased even further in group C, and these results were consistent with those of the immunohistochemical staining (Table II; Fig. 6).

Discussion

Local anesthetics are widely used in clinical anesthesia and pain treatment (21). When their effects end, in general, neurological function can be restored; however, their neurotoxicity has attracted an increasing amount of attention from clinicians (22,23). Identification of novel drugs which serve a protective role in neuronal injury may provide theoretical basis for further research to protect damaged neurons. Previously, studies have shown that polysaccharides can reduce the swelling and shrinking of neurons, promote nervous regeneration, reduce neurological defects and exert protective effects on damaged neurons (24-26). Therefore, the present study aimed to evaluate the beneficial effects of EPS-A on neurotoxicity induced by local anesthetics in an intraperitoneal injection bupivacaine rat model.

Morphological alteration is the primary way to detect injury. H&E is one of principal histological stains and the most widely used staining method in medical diagnosis (27). The results of the present study indicated that following injection of bupivacaine, the number of spinal cord neurons in rats was reduced. However, upon administration of EPS-A, injury was ameliorated markedly. The results suggested that EPS-A markedly attenuated neuronal injury compared with bupivacaine induced neuronal injury. However, EPS-A was able to attenuate these pathological alterations and, therefore,

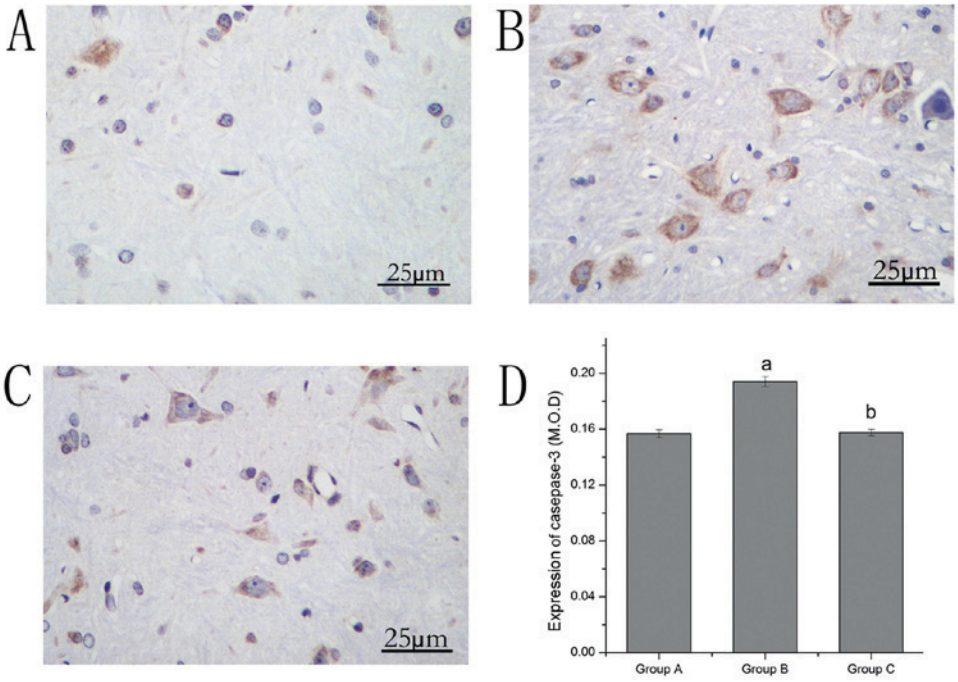

Figure 3. Comparison of the expression levels of caspase-3 in spinal cord neurons in rats. The expression of caspase-3 observed by immunohistochemical staining (magnification, x400) in (A) control, (B) bupivacaine and (C) EPS-A group rats. (D) Quantitative comparisons of the expression levels of caspase-3. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B. M.O.D, mean optical density.

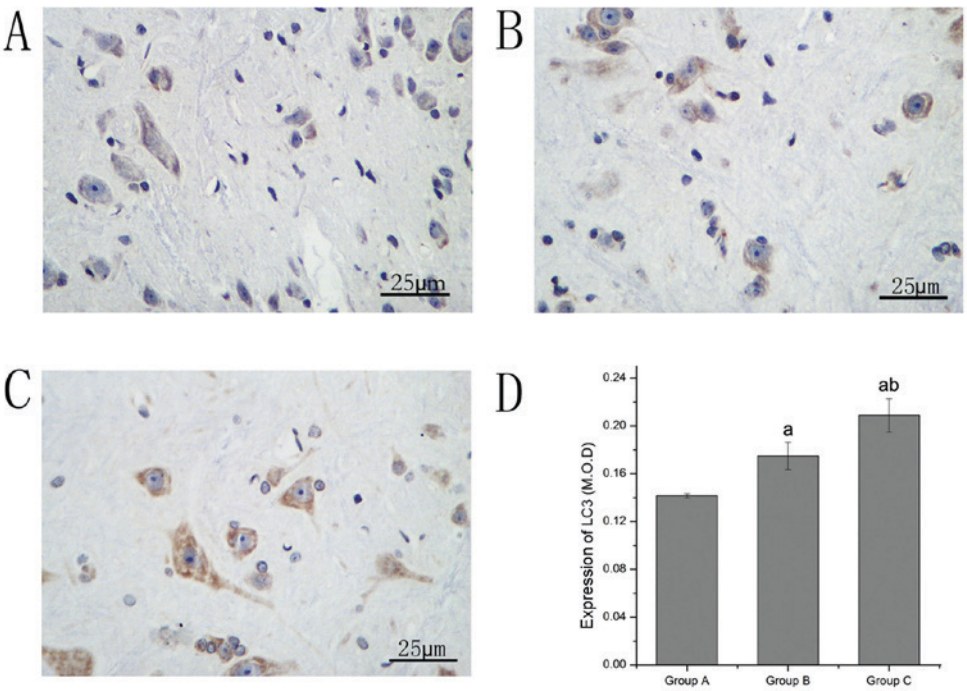

Figure 4. Comparison of the expression levels of LC3 of spinal cord neurons in rats. The alterations of LC3 expression were observed using immunohistochemical staining (magnification, x400) in (A) control, (B) bupivacaine and (C) EPS-A group rats. (D) Quantitative comparisons of the expression levels of LC3. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B. M.O.D, mean optical density. LC3, microtubule-associated protein 1A light chain 3.

the effect of EPS-A on the level of pathological alterations may be involved in the reduction of neuronal injury in rats treated with bupivacaine.

Apoptosis leads to the loss of a large number of neurons, which is an important mechanism of secondary spinal cord injury (28,29). It was also hypothesized to be one mechanism of neurotoxicity caused by local anesthetics (30). A previous study suggested that apoptosis is more common in the neurotoxicity of local anesthesia as induced by local anesthetics (31). In the present study, the results indicated that the apoptosis rate in spinal cord neurons increased following intrathecal injection of bupivacaine, however, the pre-administration

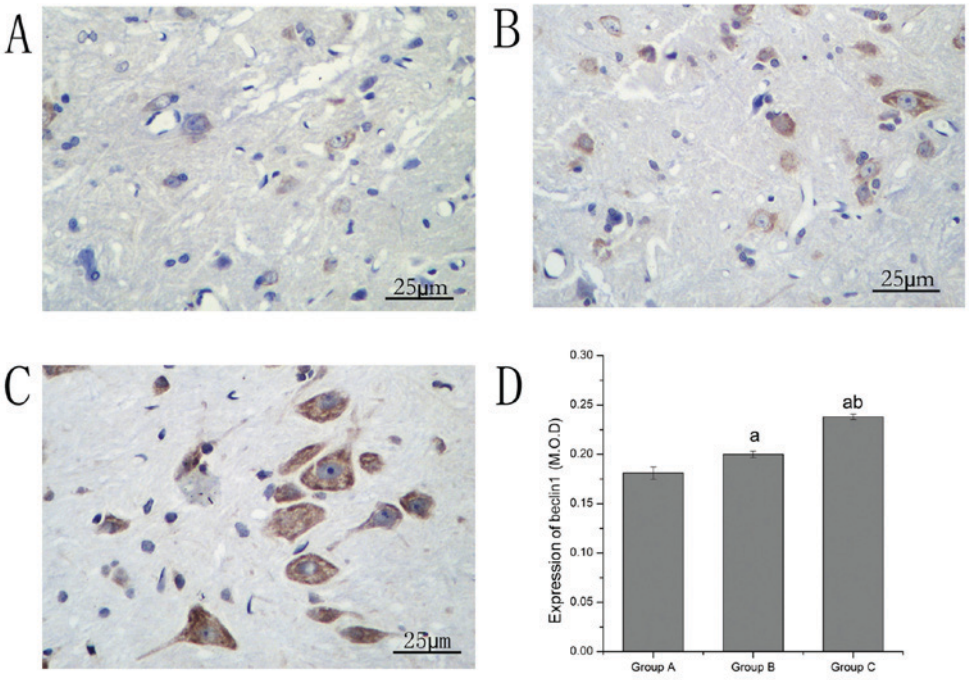

Figure 5. Comparison of the expression levels of beclin1 of spinal cord neurons in rats. The alterations of beclin1 were observed using immunohistochemical staining (magnification, x400) in (A) control, (B) bupivacaine and (C) EPS-A treated rats. (D) Quantitative comparisons of the expression levels of beclin1. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B. M.O.D, mean optical density.

Table II. Comparison of the expression levels of beclin1 and LC3 of spinal cord neurons in rats treated with bupivacaine and extracellular polymeric substances from *Aphanizomenon flos*-aquae as determined by western blotting.

Group	Beclin1	LC3
A	0.428±0.099	0.141±0.008
B	0.712±0.064[a]	0.178±0.010[a]
C	0.849±0.129[a,b]	0.208±0.012[a,b]
Sum	0.663±0.203	0.175±0.030
F	27.112	1005.334
P	0.000	0.000

[a]P<0.05 vs. group A; [b]P<0.05 vs. group B. LC3, microtubule-associated protein 1A light chain 3; Sum, sum of the mean ± standard deviations. F and P represent the differences between groups which were evaluated using one-way analysis of variance; a and b represent the multiple group comparisons that were confirmed using the Dunnett's test.

of EPS-A resulted in a significant decrease in apoptosis. Furthermore, the results indicated that EPS-A could ameliorate the increase of apoptosis rate, thus the neuroprotective effects of EPS-A may be mediated by its capacity to reduce neuronal apoptosis.

The expression of caspase-3 is closely associated with the level of neuronal apoptosis following spinal cord injury (32). The expression of caspase-3 may also reflect the degree of neuronal damage to some extent (33). In the present study, the results indicated that the injection of bupivacaine significantly increased the expression of caspase-3. However, following treatment with EPS-A, the expression of caspase-3 significantly decreased. The results suggested that the neurotoxic effects caused by bupivacaine were associated with casepase3-dependent apoptosis. EPS-A pre-administration was able to alleviate the neurotoxic effects caused by bupivacaine and significantly reduced casepase3-dependent apoptosis, which was consistent with the results of the TUNEL staining. Therefore, the role of EPS-A in the expression of caspase-3 may be involved in the attenuation of apoptosis in rats treated with bupivacaine. The results implied that EPS-A may affect the level of apoptosis, and this may aid in understanding the beneficial neuroprotective effects of EPS-A against local anesthetics.

Autophagy serves an important role in death and survival of neuronal cells, which may influence several neurodegenerative disorders (34,35). Basal autophagy acts as one of the cytoprotective mechanisms and participates in maintaining homeostasis (36). Additionally, in disease conditions of myocardial ischemia-reperfusion and neuronal ischemic anoxia, autophagy enhancement serves a role as a protective mechanism (37). Recent studies (38,39) have shown that drug-induced autophagy is characterized by the altered expression of autophagy-associated proteins LC3 and beclin1. LC3 and beclin1 are two pacemakers in the autophagic cascade. LC3, exists in cytosolic (LC3-I) and membrane bound forms (LC3-II) (40). The ratio of conversion from LC3-I to LC3-II is closely associated with the extent of autophagosome formation (41). Beclin1 is essential for the recruitment of other autophagic proteins during the expansion of the pre-autophagosomal membrane (42,43). The results of the present study indicated that bupivacaine increased the protein expression levels of beclin1 and LC3; however, with pre-administration of EPS-A, the LC3 and beclin1 protein expression levels improved further. The present study also suggested that the

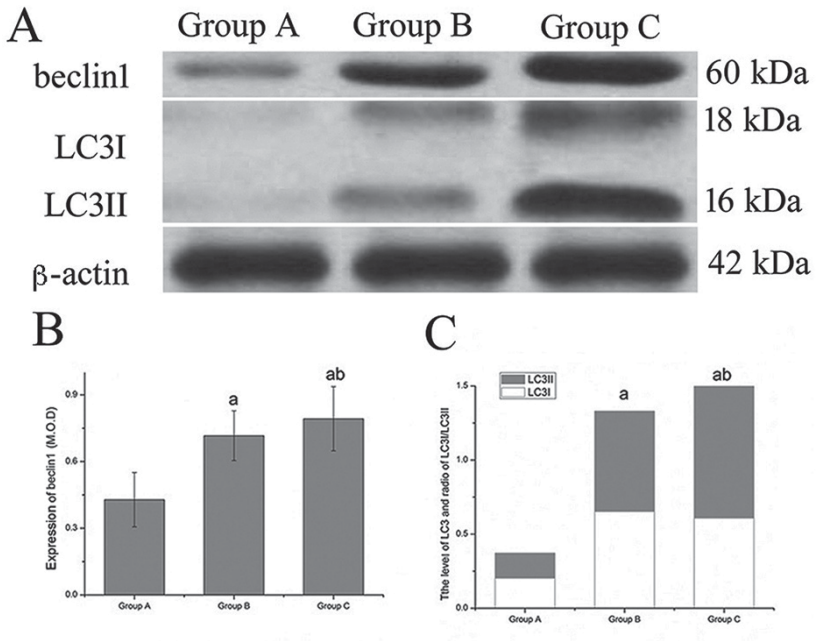

Figure 6. Comparison of the expression level of LC3 and beclin1 of spinal cord neurons in rats. (A) Comparison of the expression levels of LC3 and beclin1 detected by western blotting of groups A, B and C. (B) Quantitative comparisons of the levels of beclin1 of groups A, B and C. (C) Quantitative comparisons of the level of LC3-II/LC3-I of groups A, B and C. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B. LC3, microtubule-associated protein 1A light chain 3; M.O.D, mean optical density.

induction of autophagy was associated with the alteration of the LC3II/LC3I ratio. In addition, further data confirming the protective effects of EPS-A were obtained from the western blot analysis of beclin1 and LC3. The results demonstrated that bupivacaine increased the ratio of LC3II/LC3I and the expression levels of beclin1; however, following pre-administration of EPS-A, the ratio of LC3II/LC3I and the beclin1 expression level increased further. The results implied that EPS-A may affect the autophagy level, and this may aid in understanding the beneficial neuroprotective effects of EPS-A against local anesthetics by inducing the expression of autophagy.

Autophagy is associated with apoptosis in a number of ways, which are as complex as the roles of autophagy in cell survival and death (44). It has been suggested that autophagy may be a trigger for apoptotic cell death (45), while others have argued that autophagy protects against apoptosis and inflammation; apoptosis and autophagy may cooperate, coexist or antagonize each other to balance death and survival signaling (46). In the present study, the results suggested that autophagy and apoptosis exhibited cross-inhibitory interactions; the activation of autophagy could inhibit apoptosis and autophagy could promote cell survival against apoptosis, which was consistent with a previous report (47).

In conclusion, EPS-A exhibited neuroprotective effects against bupivacaine-induced neurotoxicity in an intraperitoneal injection bupivacaine rat model. The mechanisms may be attributed to inhibiting apoptosis, inducing autophagy and improving cell survival. These results suggested that EPS-A may be a candidate neuroprotective agent against the neurotoxicity of local anesthetics. The promotion and utilization of autophagy induced by drugs has attracted an increasing amount of attention in the field of medicine. However, further details on the beneficial mechanisms of EPS-A in apoptosis and autophagy in an intraperitoneal injection model are required, and this remains to be evaluated in the future.

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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

XX wrote the manuscript. XX, YLv and YLe conceived and designed the present study. XX and YaZ performed the majority of the experimental procedures. YLv analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the First Hospital of Lanzhou University in (Lanzhou, China).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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