

EXPERIMENTAL TRIALS

Autophagy activation attenuates the neurotoxicity of local anesthetics by decreasing caspase-3 activity in rats



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Received 23 October 2018; accepted 18 April 2020

Available online 9 November 2020

KEYWORDS

Neurotoxicity;
Autophagy;
Local anesthetics;
Apoptosis

Abstract

Background and objectives: The mechanisms by which local anesthetics cause neurotoxicity are very complicated. Apoptosis and autophagy are highly coordinated mechanisms that maintain cellular homeostasis against stress. Studies have shown that autophagy activation serves as a protective mechanism *in vitro*. However, whether it also plays the same role *in vivo* is unclear. The aim of this study was to explore the role of autophagy in local anesthetic-induced neurotoxicity and to elucidate the mechanism of neurotoxicity in an intrathecally injected rat model.

Methods: Eighteen healthy adult male Sprague-Dawley rats were randomly divided into three groups. Before receiving an intrathecal injection of 1% bupivacaine, each rat received an intraperitoneal injection of vehicle or rapamycin (1 mg.kg⁻¹) once a day for 3 days. The pathological changes were examined by Haematoxylin and Eosin (HE) staining. Apoptosis was analysed by TdT-mediated dUTP Nick-End Labelling (TUNEL) staining. Caspase-3, Beclin1 and LC3 expression was examined by Immunohistochemical (IHC) staining. Beclin1 and LC3 expression and the LC3-II/LC3-I ratio were detected by western blot analysis.

Results: After bupivacaine was injected intrathecally, pathological damage occurred in spinal cord neurons, and the levels of apoptosis and caspase-3 increased. Enhancement of autophagy with rapamycin markedly alleviated the pathological changes and decreased the levels of apoptosis and caspase-3 while increasing the expression of LC3 and Beclin1 and the ratio of LC3-II to LC3-I.

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PALAVRAS-CHAVE

Neurotoxicidade;
Autofagia;
Anestésicos locais;
Apoptose

Conclusions: Enhancement of autophagy decreases caspase-3-dependent apoptosis and improves neuronal survival *in vivo*. Activation of autophagy may be a potential therapeutic strategy for local anaesthetic-induced neurotoxicity.

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A ativação autofágica atenua a neurotoxicidade dos anestésicos locais ao diminuir a atividade da caspase-3 em ratos

Resumo

Introdução e objetivos: Os mecanismos de neurotoxicidade dos anestésicos locais são complexos. A apoptose e a autofagia são mecanismos altamente organizados que mantêm a homeostase celular durante o estresse. Estudos revelam que a ativação da autofagia atua como mecanismo de proteção *in vitro*. Não está claro se a autofagia também desempenha essa função *in vivo*. O objetivo deste estudo foi analisar o papel da autofagia na neurotoxicidade induzida por anestésico local e esclarecer o mecanismo dessa neurotoxicidade utilizando um modelo de injeção intratecal em ratos.

Métodos: Dezoito ratos Sprague-Dawley machos adultos saudáveis foram divididos aleatoriamente em três grupos. Antes de receber a injeção intratecal de bupivacaína a 1%, cada rato recebeu injeção intraperitoneal de veículo ou rapamicina ($1 \text{ mg} \cdot \text{kg}^{-1}$) uma vez ao dia durante 3 dias. As alterações patológicas foram examinadas por coloração com Hematoxilina e Eosina (HE). A apoptose foi analisada por coloração com o método dUTP Nick-End Labeling (TUNEL) mediado por TdT. A expressão de caspase-3, Beclin1 e LC3 foram examinadas por coloração Imunohistoquímica (IHQ). A expressão de Beclin1 e LC3 e a razão LC3-II/LC3-I foram detectadas por análise de *western blot*.

Resultados: Após a injeção intratecal de bupivacaína, ocorreu lesão patológica nos neurônios da medula espinhal e os níveis de apoptose e caspase-3 aumentaram. A ativação da autofagia causada pela rapamicina mitigou de forma expressiva as alterações patológicas e diminuiu os níveis de apoptose e caspase-3, aumentando a expressão de LC3 e Beclin1 e a razão LC3-II/LC3-I.

Conclusões: O aumento da autofagia diminui a apoptose dependente da caspase-3 e melhora a sobrevivência neuronal *in vivo*. A ativação da autofagia pode ser uma estratégia terapêutica potencial para a neurotoxicidade induzida por anestésicos locais.

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Introduction

Local anesthetics produce reversible regional loss of sensation and have been used to relieve acute and chronic pain. In general, they are accepted as safe. However, studies have shown that local anesthetics administered intrathecally may induce cell swelling, atrophy, edema, axonal degeneration, the appearance of myelin ovoids, and macrophage infiltration,¹ and continuous application of not only high concentrations but also normal clinical doses² of local anesthetics can cause neurotoxicity.³⁻⁵ Although the potential neurotoxicity of local anesthetics has been investigated for many years, the mechanisms by which they induce neuronal injury are not fully understood. Therefore, it is necessary to identify these mechanisms to develop effective clinical strategies with which to prevent adverse outcomes after local anesthetic administration.

Studies have shown that bupivacaine may induce neurotoxicity primarily through apoptosis and autophagy.⁶ Apoptosis has been suggested to be one of the main

contributors to neuronal injury in different models of neurodegenerative disorders.⁷ For example, strong evidence of neuronal apoptosis has been observed in numerous animal models.⁸ In recent years, autophagy has been proposed as an important intracellular degradation system that delivers cytoplasmic constituents to lysosomes and helps to maintain a balance between synthesis and degradation.⁹⁻¹¹ Increases in the numbers of autophagosomes have been observed in the context of a variety of physiological and pathological conditions in the nervous system.^{12,13} Although the autophagy pathway is a stress adaptation pathway that promotes cell survival under most circumstances, a growing number of studies have demonstrated that it can trigger cell injury.⁶ However, many studies have indicated that apoptosis and autophagy have a complicated relationship; for example, autophagy can precede apoptosis and play a protective role.¹⁴ It can also promote apoptosis under some circumstances.¹⁵ Therefore, the contribution of autophagy to cell survival remains controversial.

Studies have shown that autophagy activation serves as a protective mechanism *in vitro*.¹⁶ However, whether it also

plays the same role *in vivo* is unclear. The present study was designed to investigate the role of autophagy and its relationship to apoptosis *in vivo*. Bupivacaine, an amide-type local anesthetic, is widely used in clinics and has been found to be potentially neurotoxic when applied to neural tissues at clinical concentrations in animal and cellular models.¹⁷ Rapamycin (RAP), a lipophilic macrolide antibiotic, enhances autophagy by inhibiting mammalian target of RAP (mTOR), helps maintain normal cellular metabolism, and exhibits neuroprotective properties.¹⁸ In the current study, we examined bupivacaine-induced neurotoxicity in an intrathecally injected rat model. The principal aim was to investigate bupivacaine-induced apoptosis and autophagy *in vivo* and to elucidate the underlying mechanism.

Materials and methods

This *in vivo* study was approved by the Ethics Committee of the First Hospital of Lanzhou University, and the procedures were carried out according to routine animal care guidelines. Healthy adult male Sprague-Dawley rats (180–220 g) were supplied by the Centre of Experimental Animals at Lanzhou University. The rats were housed in separate cages with freely available food and water until the time of testing in temperature-controlled rooms (20–24 °C, relative humidity 50–60%) on a 12-hours light/12-hours dark cycle (light from 6:00 AM to 6:00 PM).

Groups and treatments

Eighteen rats were randomly divided into three groups: the vehicle treatment group (Group A, n=6), the bupivacaine treatment group (Group B, n=6) and the RAP treatment group (Group C, n=6). In group A, all experimental rats received the same volume of vehicle. In Group B, each rat was intrathecally injected with 1% bupivacaine. In Group C, each rat was intraperitoneally injected with 1 mg.kg⁻¹ RAP (Sigma, St. Louis, MO, U.S.A., diluted with DMSO) once a day for 3 days and was then intrathecally injected with 1% bupivacaine.

Intrathecal injection of bupivacaine

After 2% isoflurane inhalation, the rats were placed in a prone position to achieve optimal flexion of the lumbar spine. A 27G needle attached to a 100 μ L syringe (KL-34, Hamilton Medical, Inc., Reno, NV, USA) was inserted into the midline of the lumbar 4–5 (L4–5) intervertebral space, and 1% bupivacaine (0.25 μ L.g⁻¹) was injected. A tail-flick indicated the entrance of the syringe into the intrathecal space. The rats were then observed for paralysis of the hind limbs, which was indicative of spinal blockade.

Spinal cord section specimens

The rats from each group were sacrificed 6 hours following the aforementioned anesthesia, and the spinal cords were rapidly collected. A section of the tissue from each rat was frozen in liquid nitrogen and stored at -70 °C until further use.

Histological observation

The spinal tissue was stained using a standard Haematoxylin and Eosin (HE) procedure. A pathologist blinded to the experimental procedure analysed the slices under a light microscope (400 \times). The extent of apoptosis was determined using a TdT-mediated dUTP Nick-End Labelling (TUNEL) kit as indicated by the kit instructions (Xiang Sheng Biotech Co., Ltd., Shanghai, China). After the tissues were stained, they were washed three times and analysed under a light microscope (400 \times). The optical density was quantified using Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD).

Immunohistochemical (IHC) staining

The expression of caspase-3, Light Chain 3 (LC3) and Beclin1 was measured by IHC staining. The samples were first washed in PBS (Ph 7.4) three times for 5 minutes each and boiled in 0.1% trisodium citrate for 15 minutes for antigen retrieval. Next, the sections were incubated with blocking reagent (3% milk and 5% fetal bovine serum; Absin Bioscience Inc., Shanghai, China) for 1 hour at room temperature and further incubated with anti-caspase-3 (1:500 dilution; Abcam, Cambridge, UK), anti-LC3 (1:200 dilution, Sigma, USA) and anti-Beclin1 (1:500 dilution, Sigma, St. Louis, MO, USA) antibodies at 4 °C overnight. Finally, after washing with PBS (pH 7.4), the tissues were exposed to a biotinylated anti-rabbit Immunoglobulin G (IgG) antibody (1:1000 dilution; Beyotime, Fuzhou, China) and a streptavidin peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). After the slides were sealed, the sections were imaged with a confocal microscope. The optical density was quantified using Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD).

Western blot analysis

Western blot analysis was employed to determine the protein levels of LC3 and Beclin1 and the ratio of LC3-II to LC3-I. Equal amounts of protein (approximately 30 μ g) were loaded into the lanes of 10% sodium dodecyl sulfate-polyacrylamide gels and subjected to gel electrophoresis. The separated proteins were then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline with Tween 20 (137 mM sodium chloride, 20 mM Tris, 0.1% Tween 20; Absin Bioscience Inc., Shanghai, China) for 1 hour at room temperature and then incubated with primary antibodies specific to LC3 (1:1000 dilution; Biosynthesis Biotechnology Co., Ltd., Beijing, China), Beclin1 (1:200 dilution; Biosynthesis Biotechnology Co., Ltd., Beijing, China), and β -actin (1:1000 dilution; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) diluted in Tris-buffered saline with Tween 20 overnight at 4 °C. The membranes were then incubated with goat-anti-mouse IgG secondary antibodies conjugated to alkaline phosphatase (1:1000 dilution; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 hour at room temperature, and the reactive bands were detected after incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO, USA) for

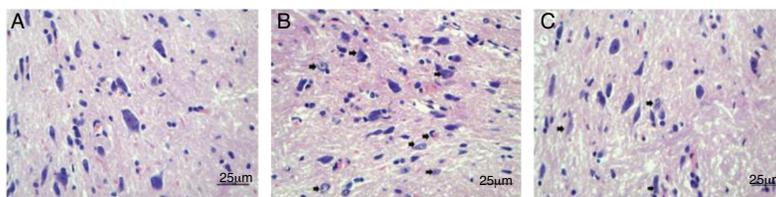


Figure 1 Pathological changes in the spinal cord neurons of the rats were examined by HE staining (magnification, 400 \times). (A) shows the normal structures of spinal cord neurons. In Group B (B), the numbers of spinal cord neurons were reduced, the neurons were shrunken and darkly stained, and the nuclei were condensed (black arrow). In addition, the tissues seemed disorderly and irregularly arranged. In Group C (C), the pathological changes were attenuated.

5 minutes. The band densities were scanned with an imaging densitometer (GS-800, Bio-Rad, USA), and the optical density was quantified using Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD) with normalization to β -actin.

Statistics

Statistical analysis was performed using SPSS 20.0 software (IBM SPSS, Armonk, NY). The data are presented as the mean \pm standard deviation. The significance of the differences (p -value) was evaluated by one-way ANOVA, and multiple-group comparisons were performed using Dunnett's tests, $p < 0.05$ was considered to indicate statistical significance.

Results

Pathological changes

Under light microscopy, the spinal cord neurons of the rats from group A were uniformly distributed with normal morphology, clear Nissl bodies, and intact cell membranes; in addition, the neural fibres of the white matter were arranged in an orderly manner, and the intercellular matrix was uniform (Fig. 1A). However, after bupivacaine injection, the numbers of spinal cord neurons were reduced, the neurons were shrunken and darkly stained, and the nuclei were condensed. Moreover, the tissues seemed disorderly and irregularly arranged (Fig. 1B). After RAP administration, the pathological changes were significantly alleviated (Fig. 1C). These findings indicated that the rat models were successfully developed.

Apoptotic changes

The results showed that neuronal apoptosis in the spinal cord differed among the groups. The apoptosis ratio was higher in Group B than in Group A ($p < 0.05$) and lower in Group C than in Group B ($p < 0.05$) (Fig. 2).

Changes in caspase-3

The expression of caspase-3 was observed by IHC staining under an ordinary optical microscope; the caspase-3-positive neurons were stained brown. The results showed that the expression of caspase-3 was higher in group B than

in Group A ($p < 0.05$) and lower in Group C than in Group B ($p < 0.05$) (Fig. 3).

Changes in LC3 and Beclin1

IHC staining was performed to investigate changes in LC3 and Beclin1. Under an ordinary optical microscope, the neurons that were positive for these proteins were stained brown. The LC3 and Beclin1 levels were higher in Group B than in Group A ($p < 0.05$) and noticeably lower in Group C than in Group B ($p < 0.05$) (Figs. 4 and 5).

To further determine the levels of autophagy, we performed western blot analysis to measure Beclin1 levels and the LC3-II/LC3-I ratio, and the results indicated that these parameters were altered by bupivacaine injection. Compared with those in Group A, the Beclin1 levels and the LC3-II/LC3-I ratio were noticeably increased in Group B ($p < 0.05$); in addition, compared with those in Group B, the Beclin1 levels and the LC3-II/LC3-I ratio were further increased in Group C ($p < 0.05$). These results were consistent with the immunohistochemistry results (Fig. 6).

Discussion

In this experiment, we found that bupivacaine induced caspase-3-mediated neuronal apoptosis and activated autophagy. Furthermore, RAP regulated the bupivacaine-induced apoptosis and autophagy. These results suggest that autophagy inhibits bupivacaine-induced apoptosis, and that autophagy enhancement is neuroprotective against bupivacaine-induced neurotoxicity. Thus, manipulation of autophagy may be an alternative approach for prevention of bupivacaine-induced neuronal damage.

Apoptosis is typically accompanied by cell morphological changes¹⁹ and is a critical pathway in morphological and pathological processes.²⁰ HE staining is a basic observation method commonly used in biological and medical studies; pathological diagnoses made with the aid of HE staining are very valuable and can serve as the foundations for clinical diagnoses.²¹ In the present study, morphological changes occurred in the spinal cord neurons of rats 6 hours after intrathecal bupivacaine injection. In addition, bupivacaine injection reduced the numbers of spinal cord neurons. However, RAP administration significantly alleviated these pathological changes. The data suggest that pre-administration of RAP significantly improves neurologic scores and increases neuronal survival in rats subjected to bupivacaine treatment.

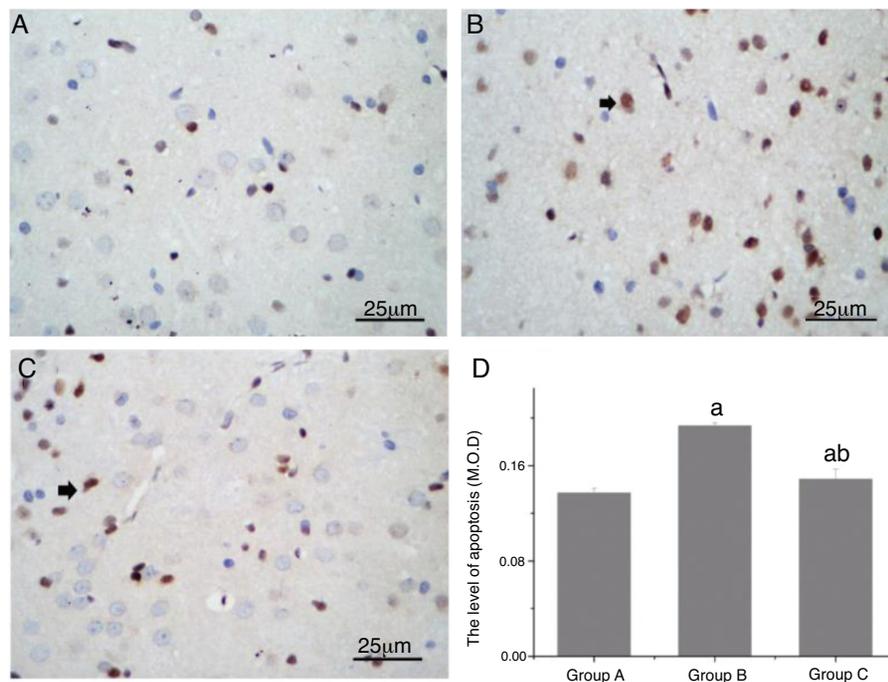


Figure 2 Comparison of the levels of apoptosis in the spinal cord neurons of the rats. (A, B, and C) show apoptotic changes (black arrow) in the spinal cord neurons of the rats as measured by TUNEL staining (magnification, 400×). (D) shows the quantitative comparison of the levels of apoptosis. M.O.D., Mean Optical Density; a, $p < 0.05$ compared with Group A; b, $p < 0.05$ compared with Group B.

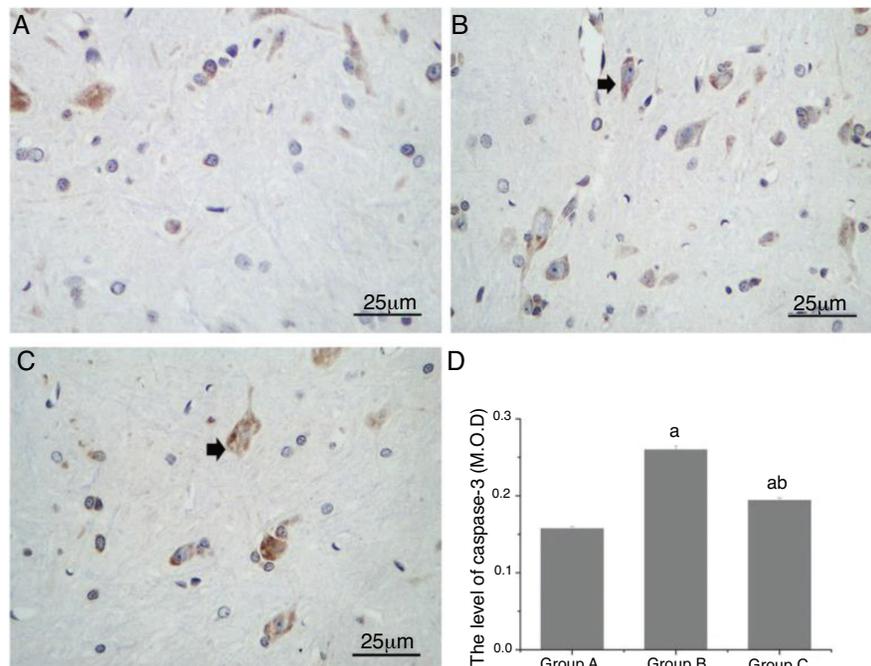


Figure 3 Comparison of the expression of caspase-3 in the spinal cord neurons of the rats. (A, B and C) show the expression of caspase-3 (black arrow) as observed by IHC staining (magnification, 400×). D shows the quantitative comparison of the expression of caspase-3. M.O.D., Mean Optical Density; a, $p < 0.05$ compared with Group A; b, $p < 0.05$ compared with Group B.

Apoptosis is an evolutionarily conserved form of cell death that is essential for the development and maintenance of tissue homeostasis. Dysregulation of apoptosis has been

implicated in several pathological conditions, including neurodegenerative disorders.²² Apoptosis is also a pathologic feature of amyotrophic lateral sclerosis, ischaemic brain

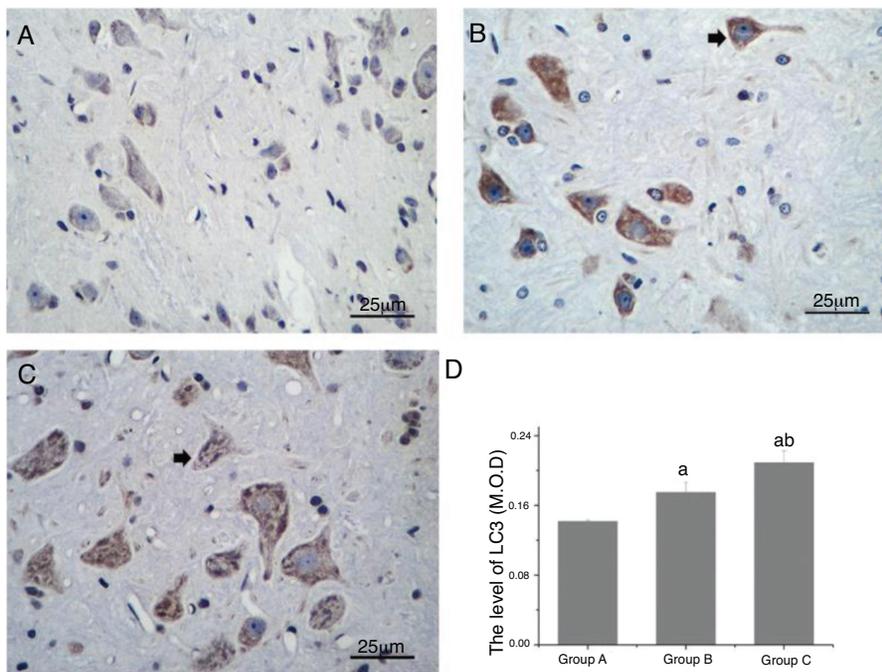


Figure 4 Comparison of the levels of LC3 in the spinal cord neurons of the rats. (A, B and C) show changes in LC3 (black arrow) as observed by IHC staining (magnification, 400 \times). (D) shows the quantitative comparison of the levels of LC3. M.O.D., Mean Optical Density; a, $p < 0.05$ compared with Group A; b, $p < 0.05$ compared with Group B.

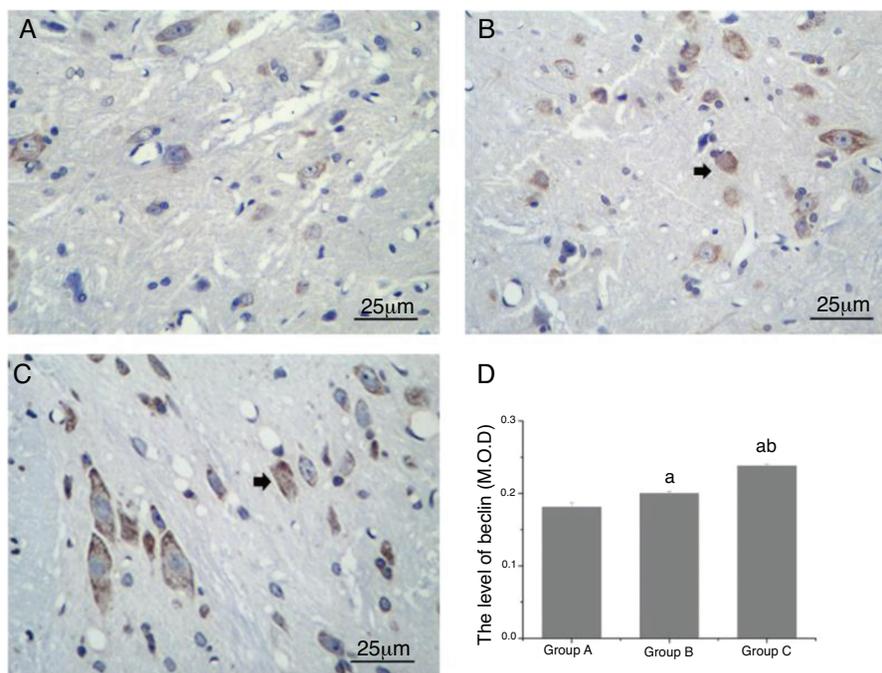


Figure 5 Comparison of the levels of Beclin1 in the spinal cord neurons of the rats. (A, B and C) show changes in Beclin1 (black arrow) observed by IHC staining (magnification, 400 \times). (D) shows the quantitative comparison of the levels of Beclin1. M.O.D., Mean Optical Density; a, $p < 0.05$ compared with Group A; b, $p < 0.05$ compared with Group B.

injury, certain brain inflammatory diseases and Central Nervous System (CNS) infections.^{23,24} In this study, the results showed that intrathecal injection of bupivacaine increased the apoptosis rates among spinal cord neurons, while administration of RAP significantly decreased the apoptosis rates.

These results imply that RAP prevents apoptotic neuronal death in rats.

Apoptosis can occur via a caspase-independent route or a route in which caspase is activated. It has been confirmed that caspases are essential for the execution of

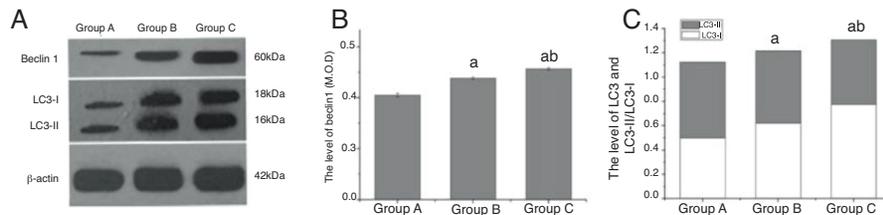


Figure 6 Comparison of the LC3 and Beclin1 levels and the LC3-I/LC3-II ratios in the spinal cord neurons of the rats. (A) shows the comparison of the levels of LC3 and Beclin1 as detected by western blotting. (B) shows the quantitative comparison of the levels of Beclin1. (C) shows the quantitative comparison of the LC3-I/LC3-II ratios. M.O.D., Mean Optical Density; a, $p < 0.05$ compared with Group A; b, $p < 0.05$ compared with Group B.

the apoptotic process;²⁵ specifically, caspase-3 is a frequently activated death protease that plays a pivotal role in the terminal or execution stage of apoptosis.²⁶ In the present study, the injection of bupivacaine significantly increased the expression of caspase-3; however, treatment with RAP significantly decreased the expression of caspase-3. These results suggest that bupivacaine induces caspase-3-mediated neuronal apoptosis. Furthermore, RAP pre-administration was able to regulate neuronal apoptosis via a caspase-3-dependent mechanism, consistent with the results of TUNEL staining. Taken together, these results indicate that RAP exerts a neuroprotective effect in rats treated with bupivacaine by inhibiting neuronal apoptosis.

Autophagy is the major pathway involved in long-lived protein and organelle degradation, cellular remodelling, and cellular survival during nutrient starvation.²⁷ Autophagy is also a normal physiological phenomenon that occurs at basal levels in most cells. Previous studies have shown that many neurological disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy, are related to disruption of the autophagic process.⁹ In addition, research has shown that LC3 and Beclin1 are two pacemakers in the autophagic cascade. LC3 is the mammalian equivalent of yeast Atg8. The two forms of LC3, LC3-I and its proteolytic derivative LC3-II (18 and 16 kDa, respectively), are localized in the cytosol and autophagosomal membranes, respectively. LC3-II can thus be used to estimate the abundance of autophagosomes prior to their destruction through fusion with lysosomes. Activation of autophagy in response to various stressors stimulates the conversion of LC3-I into LC3-II and upregulates LC3 expression. Beclin1, the mammalian orthologue of yeast Atg6, plays a critical role in autophagosome formation.¹⁹ In the present study, the results showed that bupivacaine increased LC3 and Beclin1 protein levels; however, RAP administration attenuated these changes, indicating that autophagy was triggered by bupivacaine. Western blot analysis of Beclin1 and LC3 levels further confirmed the induction of autophagy; the results showed that bupivacaine increased the LC3II/LC3I ratio and Beclin1 levels, while RAP attenuated the bupivacaine-induced changes. These results imply that the enhancement of autophagy may be a protective mechanism for neuronal survival. This finding is consistent with the findings of a previous report demonstrating that autophagy is a mechanism that protects cells from the effects of necrosis and apoptosis.²⁸

Autophagy and apoptosis are two types of cell death that occur through different mechanisms but are functionally linked; accumulating evidence has revealed that autophagy

and apoptosis may cooperate with, antagonize or assist each other, thus differentially influencing the fates of cells.⁷ Autophagy and apoptosis may sometimes work toward the same outcome, while under different conditions, they may exhibit a different relationship. Research into this relationship may have significant influences on the prevention and treatment of many diseases.²⁹

Summary

The results of our study show that autophagy and apoptosis engage in cross-inhibitory interactions. In addition, the overall results suggest that improved neuronal survival due to significant reductions in neuronal apoptosis are mediated by caspase-3 and the activation of autophagy. Enhancement of autophagy is a protective process that may promote cell survival against neuronal apoptosis *in vivo*. However, the mechanisms that lead to overactivation of autophagy are still unclear; thus, further research is needed in the future.

Conflicts of interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

This study was funded by the Gansu Province Health Industry Plan (China, project number: GSWKY2017-18).

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