Baicalin relieves neuropathic pain by regulating α_2-adrenoceptor levels in rats following spinal nerve injury

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Received October 26, 2018; Accepted October 2, 2019

DOI: 10.3892/etm.2020.9019

Abstract. In the present study, the ability of baicalin to relieve neuropathic pain due to spinal nerve ligation in rats was explored, and the relationship between baicalin and α_2-adrenoceptors (α_2-AR) was determined. The neuropathic pain model was established by ligating the L5-L6 spinal nerves in Sprague-Dawley rats. Several α_2-AR antagonists were injected into the intramedullary sheath to evaluate the role of baicalin in neuropathic pain. The antagonists included nonselective α_2-AR antagonist idazoxan, α_{2a}-AR antagonist BRL 44408, α_{2b}-AR antagonist ARC 239 and α_{2c}-AR antagonist JP 1302. The rats were divided into an untreated control group, saline group, baicalin group and baicalin + α_2-AR antagonist groups. Paw withdrawal threshold (PWT) was tested to assess the level of pain felt by the rats. The levels of α_2-AR mRNA were tested by reverse transcription-quantitative PCR. Inflammatory factors, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-17 and IL-1β, were analyzed by ELISA. The histopathological changes were assessed by hematoxylin and eosin staining. Flow cytometry was used to examine the percentage of $CD4^+$ peripheral blood mononuclear cells (PBMCs). Compared with the saline group, the PWT value increased after treating with baicalin. However, intrathecal injection of α_2-AR antagonist reversed the antinociceptive effects of baicalin. Compared with the saline group, the expression of α_{2a}-AR and α_{2c}-AR mRNA was upregulated significantly in the baicalin group ($P<0.05$). Levels of α_2-AR mRNA were also decreased in the baicalin + idazoxan group compared with the baicalin group ($P<0.05$). The levels of TNF-α, IL-6, IL-17 and IL-1β were raised after treatment with baicalin. In addition, baicalin treatment ameliorated the histological damage in the spinal cord. The percentage of $CD4^+$ PBMCs was increased in the saline group compared with the control group ($P<0.05$). Compared with the baicalin group, the percentage of CD4+ PBMCs was raised after treatment with the α_2-AR antagonists. In conclusion, intrathecal injection of baicalin produced an antiallodynic effect in a spinal nerve ligation-induced neuropathic pain model. The mechanism may be related to the regulation of a_2-AR expression.

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Key words: baicalin, neuropathic pain, α_2-adrenoceptors, spinal nerve ligation

Introduction

Neuropathic pain is one of the most common categories of chronic pain and sensory dysfunction affecting a considerable proportion of the global population, negatively influencing their emotional health and overall quality of life (1). The prevalence of trauma related to the peripheral nervous system in the United States is 1.3-2.8% (2). Neuropathic pain is characterized by a response to non-noxious stimuli (tactile allodynia), spontaneous pain (exaggerated pain response to normal painful stimulus), hyperalgesia and loss of sensation in local areas (3,4). It can be triggered or initiated in the peripheral or central nervous system (5). Owing to the complex etiology of neuropathic pain, it is considered to be one of the most challenging pathologies to treat in clinical practice (6). At present, most pain medications to treat neuropathic pain are not satisfactory, and cause undesirable side effects. Therefore, it is necessary to find an effective therapy with minimal side effects.

It is well known that α_2-adrenoceptor (α_2-AR) agonists have antinociceptive effects in the spinal cord (7). α_2-ARs are located not only in the central nervous system, but also in the peripheral nervous system (8). The complex processes involved in α_2-AR pain regulation have been extensively researched. A previous study demonstrated that α_2-AR agonists could relieve nerve injury-induced pain by binding to α_2-AR in patients and animal models with neuropathic pain (9). Blockade of spinal 5-hydroxytryptamine (HT)3 receptors reduced α_2-AR-mediated anti-hypersensitivity by reducing total GABA release (10). Furthermore, it is well known that α_2-AR agonists can enhance the analgesic effects of morphine (11). Recently, the α_2-AR antagonists atipamezole and idazoxan have been shown to block the induction of tolerance to morphine, which was verified through intrathecal atipamezole in opioid naïve and tolerant rats weakening the anti-nociceptive effect of morphine (12).

Baicalin is a common flavonoid substance isolated from the root of *Scutellaria baicalensis* Georgi. Previous studies have demonstrated that baicalin possesses anti-inflammatory, antioxidant, antitumor and antiallergy properties (13-15). Furthermore, Chou *et al* (16) established the model of carrageenan-evoked thermal hyperalgesia in rats and found that baicalin had a clear analgesic effect. A previous study also demonstrated that baicalin helps relieve pain in patients suffering from osteoarthritis of the knee (17).

In this study, it was determined whether baicalin may reduce pain in a spinal nerve ligation rat model of neuropathic pain, and the roles of the peripheral α_2-AR subtypes in the mechanism of action of baicalin were investigated.

Materials and methods

Animals. Male Sprague Dawley rats [Beijing Vital River Laboratory Animal Technology Co., Ltd.; Charles River Laboratories, Inc.; license no. SCXK9 (Beijing) 20160006; weight, 150-200 g; age, 3 months] were housed at 22-24˚C and 50-65% humidity on a 12 h light/dark cycle and were provided with free access to food and water. All experiments in this study were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The procedures were all approved by the Animal Ethics Committee of China Pharmaceutical University (Nanjing, Jiangsu, China), where the study was carried out.

Neuropathic pain model. Animals were anesthetized with halothane, 1-3% in oxygen, with spontaneous ventilation. A 3 cm paramedian incision was made in the left L4-sacral area, and a bundle of paraspinal muscles was removed to visualize the left L6 transverse process. Using small scissors, the left L6 transverse process was removed completely and the L4-L5 spinal nerves were exposed. After the L4 spinal nerve was separated, the L5 spinal nerve was cut and spread laterally. The fascia and skin were closed using sutures, and the animals were allowed to recover for 10 days prior to the epidural catheterization. Paw withdrawal mechanical threshold (PWT) <4 g after surgery was recognized as the standard of neuropathic pain induction.

Drugs administration. A total of 70 rats were randomly divided into the following groups: Control group (n=10); saline group (n=10); baicalin group (n=10); baicalin combined α_2-AR antagonist groups (n=40). The α_2-AR antagonist group was subcategorized in to four groups based on the antagonist used, which included the nonselective α_2-AR antagonist idazoxan (n=10); α_{2a}-AR antagonist BRL 44408 (n=10); α_{2b}-AR antagonist ARC 239 (n=10); and α_{2c}-AR antagonist JP 1302 (n=10). The rats were treated with 20 mg/kg baicalin by intrathecal injection. The drugs used in the study were purchased from Tocris Bioscience. Idazoxan was dissolved in distilled water, and the other drugs were dissolved in physiological saline. All drugs were delivered in a volume of 2 μg/20 μl administered by intrathecal injection. In the control group, the rats did not undergo any surgery. The rats in saline group were injected with physiological saline (10 μl). Drugs were administered once per day for 7 days.

Behavioral tests. The PWT was measured by the up and down method (18). A series of von Frey filaments (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5 and 15 g) in a perpendicular fashion were used to stimulate the surface of the lateral paw. Each was applied until slightly bent and held for approximately 5 sec. Reponses in the form of sharp withdrawal or paw licking were regarded as a positive response. Only rats with marked allodynia (withdrawal threshold <4 g) after spinal nerve ligation were studied.

Expression of α_2a-AR, α_2b-AR, α_2c-AR mRNA in the spinal cord. When the final test was completed, three rats from each experimental group were sacrificed by cervical dislocation whilst anesthetized to obtain the L4-L5 dorsal spinal cord. Tissue samples were frozen immediately at -80˚C. Total RNA in spinal cord tissues was extracted using an RNeasy kit (cat. no. 74104; Qiagen GmbH) following the manufacturer's instructions. RNA quality and quantity were measured using a Nanodrop Spectrophotometer (UL-2000; Macylab Instruments, Inc.), while RNA integrity was assessed by gel electrophoresis. A total of 500 ng RNA was used to generate cDNA with a reverse transcription (RT) kit (Takara Biotechnology Co., Ltd.). The temperature conditions for the RT procedure were 65˚C for 10 min, 37˚C for 10 min, 75˚C for 15 min and 37˚C for 20 min. A Mastercycler® nexus X2 (Eppendorf) was used for RT-quantitative (q) PCR (Power SYBR™ Green RNA-to-C_T™ 1-step kit, cat. no. 4389986; Thermo Fisher Scientific, Inc.). The thermocycling conditions were 95˚C for 15 sec followed by 35 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative levels of target mRNAs were standardized to the reference gene β-actin gene. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (19). Primers for RT-qPCR in this study were as follows: α_{2a} forward 5'-GCG CCCCAGAACCTCTTCCTGGTG-3', reverse 5'-CCAGCG CCCTTCTTCTCTATGGAG-3'; α_{2b} forward 5'-AAACGC AGCCACTGCAGAGGTCTC-3', reverse 5'-ACTGGCAAC TCCCACATTCTTGCC-3'; α_{2c} forward 5'-CTGGCAGCC GTGGTGGGTTTCCTC-3'; reverse 5'-GTCGGGCCGGCG GTAGAAAGAGAC-3'; and β-actin forward 5'-CGGGAA ATCGTGCGTGACAT-3', reverse 5'-GAAGGAAGGCTG GAAGAGTG-3'.

Quantification of inflammatory mediators in serum. Blood was collected via the tail vein at 72 h after drug injection. Serum inflammatory mediators, including tumor necrosis factor (TNF)-α (cat. no. DY510), interleukin (IL)-6, (cat. no. DY506), IL-17 (cat. no. DY4437) and IL-1β (cat. no. DY401) were tested with ELISA kits supplied by R&D Systems China Co., Ltd. according to the manufacturers' instructions.

Histological evaluation. Spinal cord tissues were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 24 h, dehydrated and then embedded in paraffin wax. The tissues were then cut into 5-μm-thick sections, and stained with hematoxylin and eosin solution (Fuzhou Maixin Biotech Co., Ltd.) at 37˚C for 5 min. Pathological changes were observed under a light microscope (magnification, x400; Nikon Corporation).

Flow cytometry analyses. The peripheral blood mononuclear cells (PBMC) were separated from blood following the Ficoll 400 and uropolinum density centrifugation method (20). The T-lymphocyte subset $CD4^+$ was identified

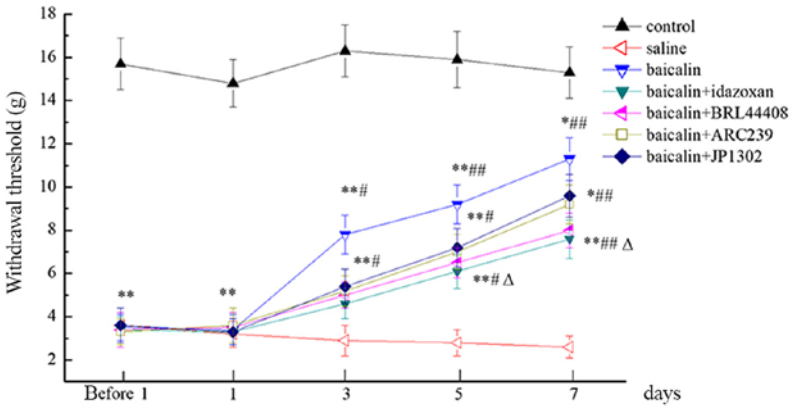

Figure 1. Baicalin treatment increases the paw withdrawal threshold, and the antiallodynic effects of baicalin are antagonized by intrathecal injection of α_2-adrenocetor antagonists. Data are presented as the mean ± SD (n=5). *P<0.05, **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the saline group, $^{\Delta}$P<0.05 vs. the baicalin group.

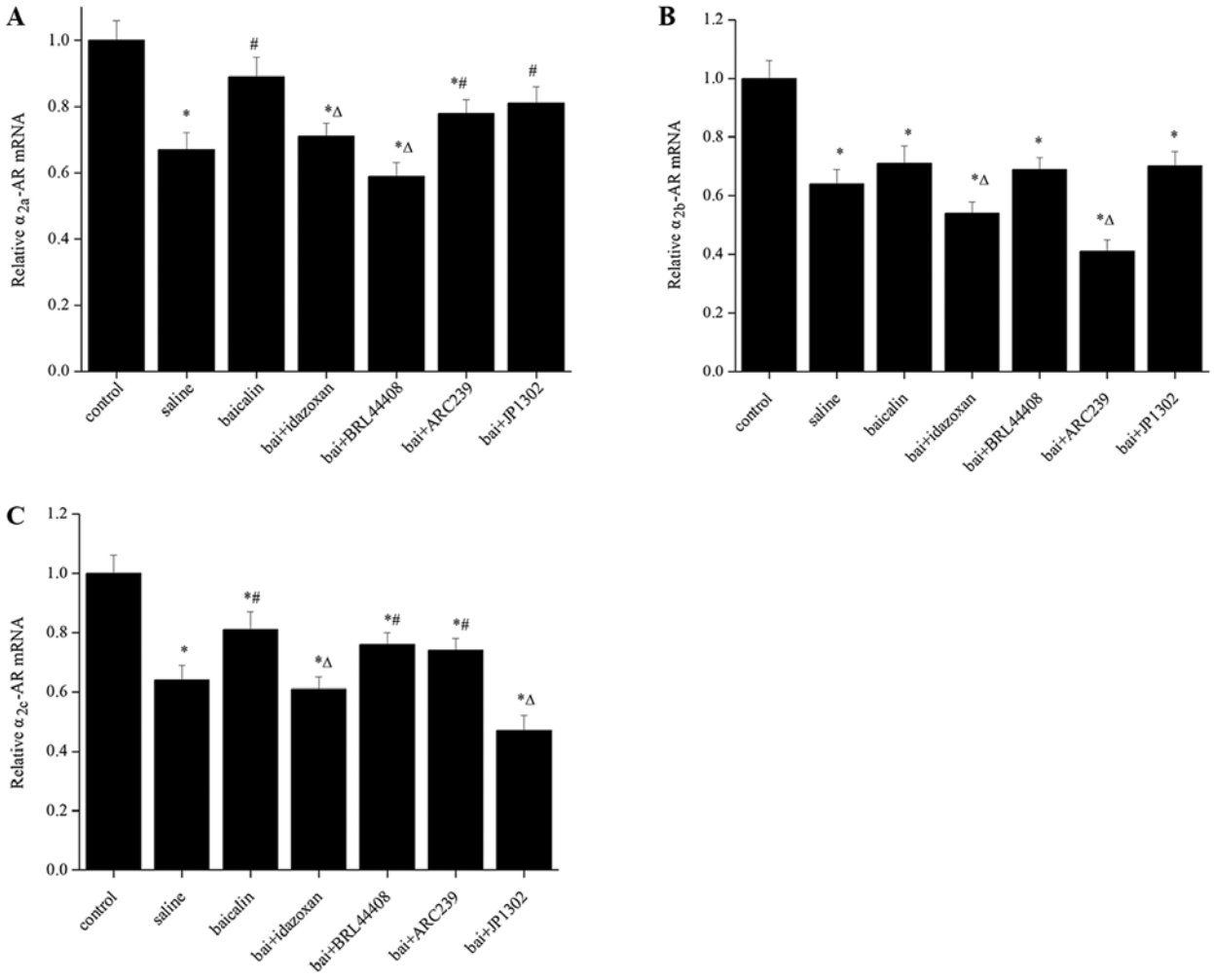

Figure 2. Effects of baicalin and α_2-AR antagonists on α_2-AR mRNA. Baicalin increased the expression of (A) α_{2a}-AR mRNA, (B) α_{2b}-AR mRNA and (C) α_{2c}-AR mRNA. Data are presented as the mean ± SD (n=5). *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the saline group; $^{\Delta}$P<0.05 vs. the baicalin group. AR, adrenoceptor.

by flow cytometry analysis of cells isolated from the PBMCs. PBMCs adjusted to a density of $1x10^6$ cells/ml in complete medium were used for analysis. Phytohemagglutinin solution (25 μl; Cylex, Inc.) was added to block non-specific binding and the cells were incubated for 15-18 h at 37˚C and 5% CO_2. The frequency of the T-lymphocyte subset was evaluated after staining with the FITC-conjugated mouse anti-rat CD4 (1:100; cat. no. FAB554F, BD Biosciences) at 4˚C for 25 min. After washing, cells were incubated with the pacific blue-A fluorochrome-conjugated isotype control (1:100; cat. no. A10478,

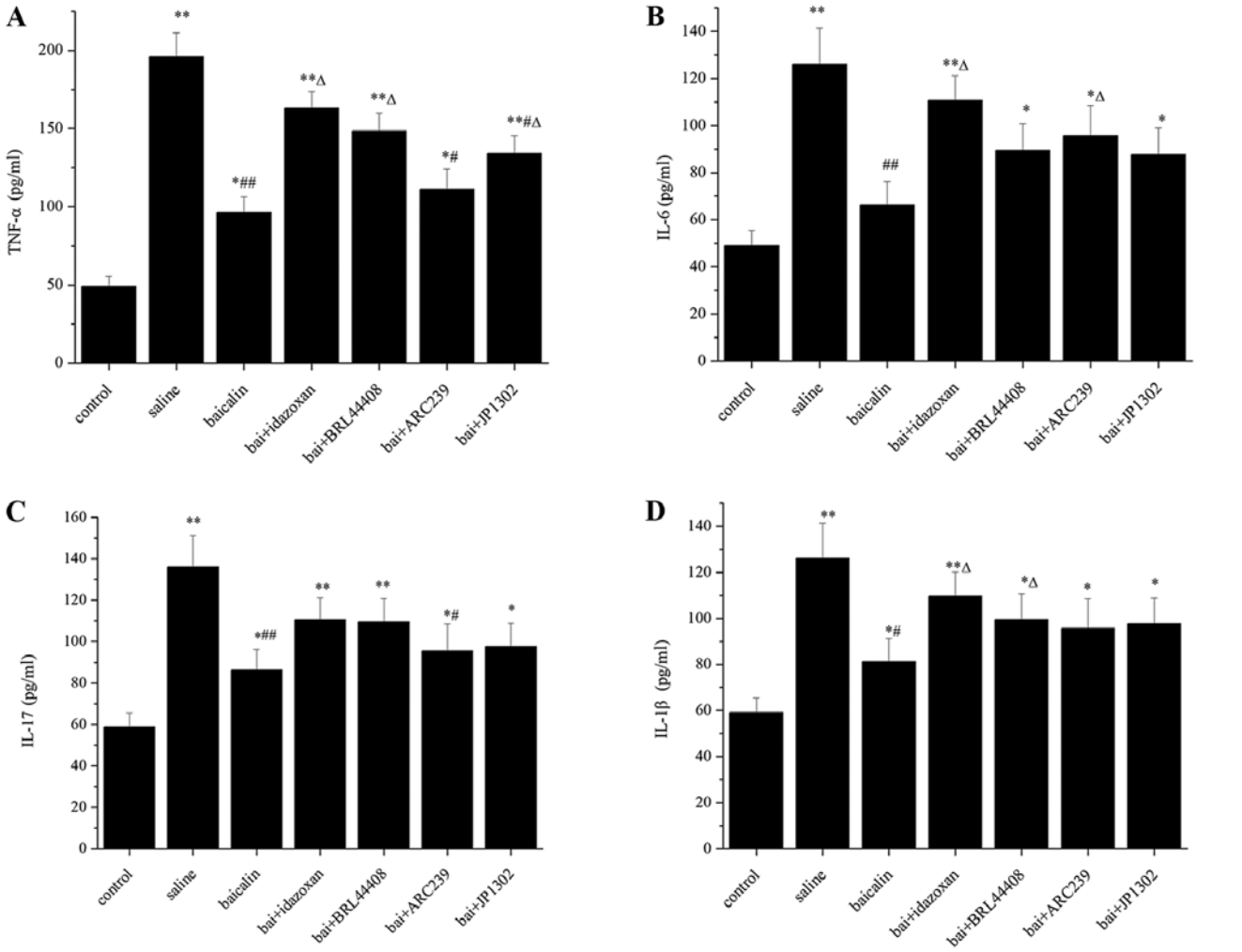

Figure 3. Effects of baicalin and α_2-AR antagonists on the serum levels of (A) TNF-α, (B) IL-6, (C) IL-17 and (D) IL-1β. Data are presented as the mean ± SD (n=5). *P<0.05, **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the saline group; $^{\Delta}$P<0.05 vs. the baicalin group. AR, adrenoceptor; IL, interleukin; TNF, tumor necrosis factor.

Thermo Fisher Scientific, Inc.), to gate nonspecific fluorescence signals, at 4˚C for 25 min. Data were analyzed using FlowJo software (version 7.2.5; FlowJo LLC).

Statistical analysis. Statistical analysis was implemented using SPSS 20.0 (IBM Corp.). Statistical comparisons between groups were analyzed using one-way ANOVAs followed by Duncan multiple range post hoc tests. All results are reported as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference. Each test was repeated 3 times.

Results

Baicalin increases the PWT. Compared with the control group, a significant decrease in PWT was observed in all treatment groups (P<0.05; Fig. 1). After baicalin treatment, PWT increased, but the antiallodynic effect of baicalin was antagonized by intrathecal injection of α_2-AR antagonists. PWT was reduced after 5 days of idazoxan administration when compared to baicalin group (P<0.05).

Baicalin contributes to the increase in α_2-AR mRNA. The expression of α_{2a}-AR, α_{2b}-AR and α_{2c}-AR mRNA were significantly downregulated in the saline group, compared with the control group (P<0.05; Fig. 2). Intrathecal administration of baicalin upregulated the levels of α_2-AR mRNA, especially the levels of α_{2a}-AR and α_{2c}-AR mRNA (P<0.05; Fig. 2A and C). Compared with baicalin group, the levels of α_{2a}-AR, α_{2b}-AR and α_{2c}-AR mRNA were significantly reduced after the administration of idazoxan (P<0.05). The antagonist BRL 44408 markedly reduced α_{2a}-AR mRNA expression when compared with the baicalin group (P<0.05; Fig. 2A). The antagonist ARC239 markedly reduced α_{2b}-AR mRNA when compared with the baicalin group (P<0.05; Fig. 2B). The antagonist JP1302 markedly reduced α_{2c}-AR mRNA expression compared with the baicalin group (P<0.05; Fig. 2C).

Baicalin decreases the levels of TNF-α, IL-6, IL-17 and IL-1β in the serum. As shown in Fig. 3, the levels of TNF-α, IL-6, IL-17 and IL-1β in the saline group were all markedly higher than those in the control group (P<0.05). Intrathecal administration of baicalin significantly reduced the levels of TNF-α, IL-6, IL-17 and IL-1β when compared to the saline group (P<0.05). TNF-α, IL-6 and IL-1β release was significantly increased with idazoxan treatment compared with baicalin group (P<0.05; Fig. 3A, B and D). TNF-α release was also

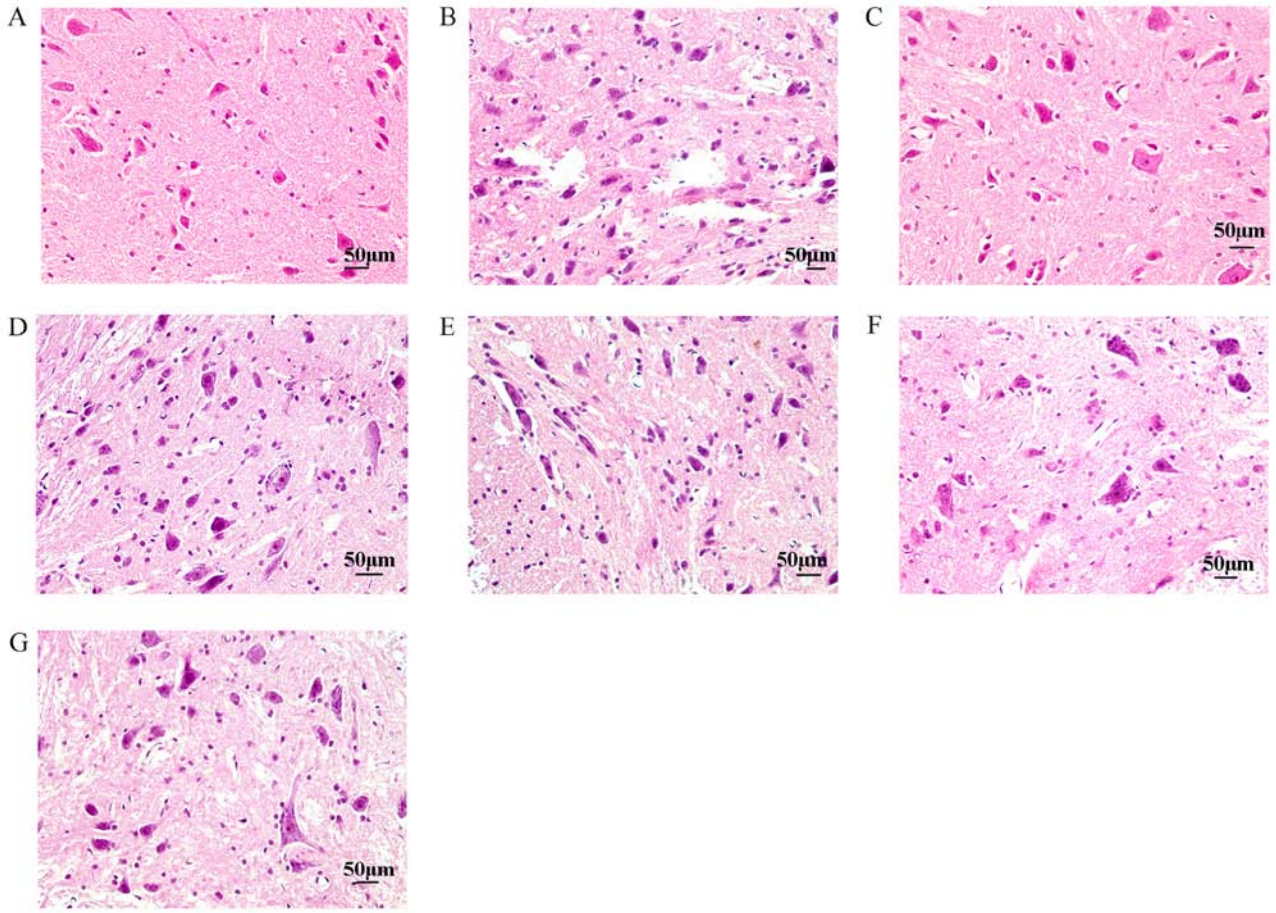

Figure 4. Baicalin treatment decreases the neuronal apoptosis and reverses the pathomorphology, as observed from spinal cord sections from the (A) control group, (B) saline group, (C) baicalin group, (D) baicalin + idazoxan group, (E) baicalin + BRL 44408 group, (F) baicalin + ARC 239 group and (G) baicalin + JP 1302 group.

significantly increased after treatment with BRL44408 and JP1302 ($P<0.05$; Fig. 3A). Treatment with ARC239 also significantly increased IL-6 release ($P<0.05$; Fig. 3B). Treatment with BRL44408 also increased IL-1β release ($P<0.05$; Fig. 3C).

Histopathological changes in spinal cord tissue. As shown in Fig. 4A, the distribution of spinal cord neurons was orderly, and the nuclei were clearly visible in the control group. However, the number of neurons decreased and the distribution of neurons was uneven in the saline group (Fig. 4B). Compared with the saline group, baicalin treatment decreased neuronal apoptosis and reversed the pathomorphology (Fig. 4C). Intrathecal administration of different α_2-AR antagonists reversed the effects of the baicalin treatment (Fig. 4D-G).

Baicalin decreases the expression of CD4⁺ cells. To analyze the effects of α_2-AR expression on $CD4^+$ T cells, the percentage of $CD4^+$ PBMCs was measured by flow cytometric analysis (Fig. 5). The results showed that the frequency of $CD4^+$ cells was significantly increased in rats following spinal nerve injury ($P<0.05$). Compared with the saline group, baicalin treatment suppressed the frequency of $CD4^+$ cells ($P<0.05$). The administration of different α_2-AR antagonists appeared to increase the number of $CD4^+$ cells compared with that in baicalin group but this difference was not significant.

Discussion

Previous studies have shown that peripheral administration of an α_2-AR agonist attenuates nociceptive responses in both control animals and hypersensitive animals under neuropathic conditions (20,21). The results of this present study showed that intrathecal injection of baicalin attenuated neuropathic pain induced by spinal cord ligation, and the antiallodynic effects of baicalin were attenuated by α_2-AR antagonists. This present study revealed that baicalin relieved pain by reducing inflammation, and this beneficial effect may be associated with the expression of α_2-AR in spinal cord.

A previous study reported that norepinephrine and other α_{2a}-AR agonists decreased the release of glutamate in healthy rat dorsal horn synaptosomes, and had analgesic as well as anti-sympathetic effects (22). α_{2c}-AR was recognized to contribute to spinal cord analgesia induced by α_2 adrenoreceptor agonists (23). Blockade of spinal 5-HT3 receptors reduced α_2-adrenoceptor-mediated anti-hypersensitivity via reducing total GABA release (24). α_2-AR stimulation induces Gs-mediated acetylcholine release in the dorsal horn after peripheral nerve injury (25). In this present study, the expression levels of α_2-ARs were changed in spinal nerve injury rats, compared to untreated rats. Notably, intrathecal administration baicalin increased α_{2a} and α_{2c}-AR mRNA. These results indicated that baicalin relieved the pain by regulating α_2-AR mRNA levels.

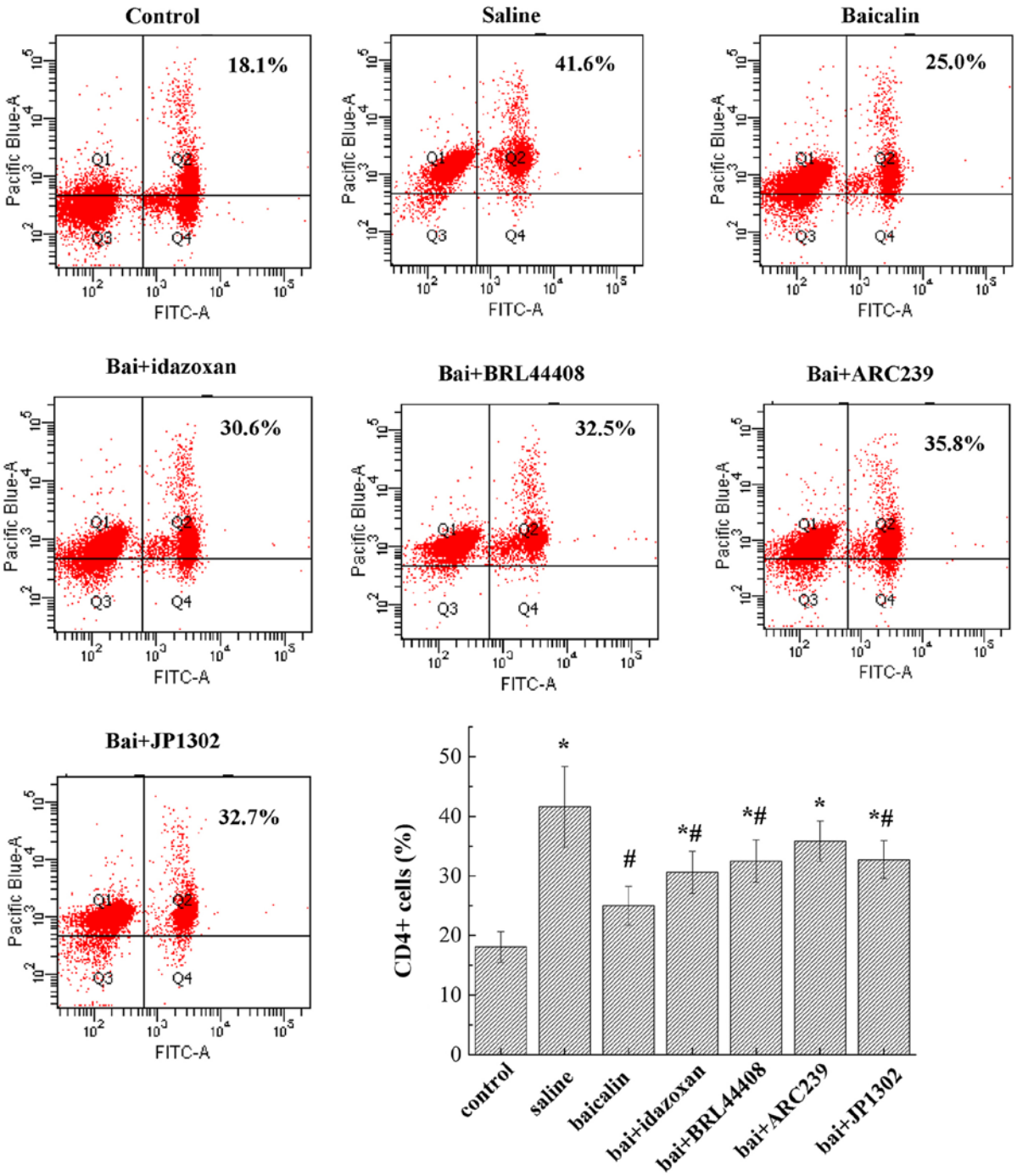

Figure 5. Effects of baicalin and α_2-adrenoceptor antagonists on the percentage of $CD4^+$ peripheral blood mononuclear cells. Baicalin reduced the percentage of $CD4^+$ cells (n=5). $^*P<0.05$ vs. the control group; $^\#P<0.05$ vs. the saline group. Bai, baicalin.

There is increasing evidence demonstrating that neuro-inflammation is one of the pivotal contributors to the development of neuropathic pain. Some pro-inflammatory cytokines produced by microglia in the spinal cord, such as IL-6, IL-17 and IL-1β, play an important role in inflammatory processes (26). TNF-α is also a biomarker of acute neuro-inflammatory responses (27). The present study showed that the serum levels of TNF-α, IL-6, IL-17 and IL-1β increased after spinal nerve ligation; however, the release of TNF-α, IL-6 IL-17 and IL-1β was reduced by intrathecal administration of baicalin. Furthermore, baicalin treatment appeared to improve the order of nerve fibers and reduced the percentage of $CD4^+$ PBMCs. These data suggested that the baicalin was capable of reducing neuropathic pain by regulating the inflammatory response.

In conclusion, this present study indicated that intrathecal administration of baicalin relieved neuropathic pain following spinal nerve ligation in rats. The mechanisms of action may be through upregulating the expression of α_2-ARs in the spinal cord. This may suggest that baicalin has therapeutic potential for neuropathic pain.

Acknowledgements

Not applicable.

Funding

This work was supported by Nature Science Foundation of Shandong Province (grant no. ZR2015HL029) and Science and Technology Planning Project of Yantai Urban (grant nos. 2014WS046 and 2015WS070).

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

LJH and SSJ participated in the design of the study and XHS, XYL, FFW, WL and QSJ carried out the study and performed statistical analysis. LJH, and SSJ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments in this study were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The procedures were all approved by animal Ethics Committee of China Pharmaceutical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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