Article

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Analgesic Mechanism of Dexmedetomidine and Esketamine in Rats with Spinal Cord Injury

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Background: Spinal cord injury (SCI) is usually caused by external direct or indirect factors, and with a high morbidity and mortality rate. The aim of this study was to observe the effects of Dexmedetomidine (DEX) combined with Esketamine (ESK) on pain behavior and potential analgesic mechanisms in rats with SCI. The goal was to provide a reliable multimodal analgesic medication regimen for SCI.

Methods: Thirty rats were divided into five groups with six rats in each group: Sham group, SCI group, DEX group, ESK group, and DEX+ESK group. The SCI model in rats was constructed, and the motor function of hind limbs of rats was measured using Basso Beattie Bresnahan (BBB) locomotor rating scale and inclined plate test. The levels of interleukin 18 (IL-18), interleukin 1β (IL-1β), and tumor necrosis factor-α (TNF-α) in the spinal cord were determined by enzyme-linked immunosorbent assay (ELISA). The expressions of substance P (SP), neurokinin-1 receptor (NK-1R), B cell lymphoma-2 (Bcl-2), and Bcl2-associated X protein (Bax) in the rats' spinal cord were measured by Western blot assay. The viability of spinal astrocytes was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: After 7 days, the BBB scores were significantly higher in the DEX, ESK, and DEX+ESK groups compared to the SCI group ($p < 0.01$). Additionally, the DEX+ESK group had significantly higher scores than both the DEX and ESK groups ($p < 0.01$). The maximum angle of the DEX ($p < 0.05$), ESK ($p < 0.05$), and DEX+ESK groups ($p < 0.01$) were higher than the SCI group, and the maximum angle of DEX+ESK group was higher than DEX and ESK groups ($p < 0.05$). The levels of IL-18, IL-1β, and TNF-α in the DEX, ESK, and DEX+ESK groups were lower than the SCI group ($p < 0.01$), while the DEX+ESK group had significantly lower IL-18, IL-1β, and TNF-α levels than the DEX and ESK groups ($p < 0.01$). The levels of SP ($p < 0.01$) and NK-1R ($p < 0.05$) were lower in the DEX, ESK, and DEX+ESK groups compared to the SCI group, and the levels of SP and NK-1R were lower in the DEX+ESK group compared to the DEX and ESK groups ($p < 0.01$). The DEX and ESK groups suppressed the activity of spinal astrocytes ($p < 0.01$), however, the DEX+ESK group had larger effects on spinal astrocytes than the ESK group ($p < 0.05$).

Conclusions: Treatment using DEX combined with ESK improves the motor function, inhibits inflammation and astrocyte activity, and exerts analgesic effects on rats with SCI. These findings can serve as a reference for the selection of multi-modal analgesics.

Keywords: Dexmedetomidine; Esketamine; spinal cord injury; multimodal analgesia

Introduction

Spinal cord injury (SCI) is a common and highly disabling central nervous system injury, mainly caused by falls, violent injuries, and traffic accidents, often leading to long-term physical and mental damage in patients [1]. Over the past 30 years, the highest incidence of SCI has been in high-income Asia Pacific, Western Europe, and North America [2]. Pain is one of the most persistent SCI complications and is mainly spontaneous pain that occurs beneath the skin [3]. The incidence of neuropathic pain after SCI is as high as 77%–86%, of which more than a third of patients have severe pain [4]. Poor analgesia can cause patients increased stress, increased risk of perioperative complications, and is not conducive to postoperative recovery. Therefore, a reasonable and effective analgesia program can greatly relieve pain and stress, thus improving patient quality of life after surgery.

Medication remains an important treatment for SCI pain. However, single-drug treatment is not ideal and has greater side effects. Combination medications may provide better pain relief than a single medication. Multimodal analgesia refers to the combination of multiple analgesic drugs and methods, each with different physiologic mechanisms, to mitigate adverse reactions caused by a single drug and exert the best analgesic effect [5]. At present, multimodal analgesia has been extensively used for clinical perioperative pain management [6,7]. However, clinicians still struggle to select multimodal analgesic drugs to achieve the ideal analgesic effect and avoid adverse drug reactions.

Dexmedetomidine (DEX) is a novel and highly selective α2-adrenergic receptor (α2-AR) agonist. At the spinal cord level, its primary mechanism involves inhibiting the signal transduction pathway of dorsal horn neurons by activating presynaptic α2-AR. This activation reduces the internal flow of calcium ions, decreases neurotransmitter release, and blocks the transmission of signals from peripheral nerve fibers, thus producing analgesic effects [8]. A previous study found that DEX reduces neuroinflammation and improves upper limb motor dysfunction after SCI in rats [9].

Esketamine (ESK) is a ketamine isomer known for its amplified analgesic and sedative effects, allowing for notable reductions in opioid dosage. ESK shows obvious advantages in the treatment of depression and epilepsy, establishing significant value in recent years. The primary mechanism of ESK involves blocking the calcium ion channel of the N-methyl-D-aspartate (NMDA) receptor, thus inhibiting the NMDA receptor-mediated activity of nitric oxide synthase, contributing to its analgesic effect. Sustained-release ESK regulates the activation of spinal astrocytes in spinal nerve ligation mice, inhibits the excitability of dorsal root ganglion (DRG) neurons, and has a safe and lasting analgesic effect [10]. However, the effect of DEX combined with ESK on SCI has not been studied.

This study used a rat model of SCI to investigate how combining DEX and ESK affects inflammatory factors, substance P (SP) and neurokinin-1 receptor (NK-1R) levels, and spinal cord astrocyte activity. It aimed to discuss the possible analgesic mechanisms and establish a more reasonable multi-mode analgesic medication regimen based on the existing regimen.

Materials and Methods

SCI Model in Rat

Thirty male SD rats (230–280 g, aged 4 months) were collected from the Animal Experimental Center of Heilongjiang provincial hospital. All the rats were fed in an animal house kept at 50% humidity and 25 °C, with ad libitum to food and water. Rats were intraperitoneally injected with 1% pentobarbital sodium (3.5 mL/kg, H31021724, Shanghai Pharma New Asia Pharma, Shanghai, China) prior to inducing the SCI model. Once the anesthesia took effect, the rats were placed in a prone position, and approximately 2 cm of skin was incised at the T10 spinous process to fully expose the dura. Subsequently, the spinal cord was impacted by a 5 g needle dropped from a height of 10 cm. The successful SCI model criteria in the studied rats were as follows: bleeding and edema at the injury site, tail wagging reflex, retraction of both lower limbs and body, and delayed paralysis of both lower limbs after anesthesia. Animal experiments adhered to the guidelines outlined in the revised Animals (Scientific Procedures) Act 1986 in the UK and complied with the regulations formulated by the Ethics Committee of Heilongjiang Provincial Hospital (2022092).

Experimental Grouping

Thirty rats were randomly divided into five groups with six rats in each group. In the sham operation group (Sham group), the rats did not sustain SCI and were sutured directly after operation; in the model group (SCI group), the rats sustained SCI without drug intervention; in the DEX group, the SCI model rats were given 2 µg DEX (H20213533, Sichuan Meida Kang Huakang Pharmaceutical Co., Ltd., Deyang, China); in the ESK group, the SCI model rats were given 100 µg ESK (H20193336, Jiangsu Hengrui Pharmaceutical Co., Ltd., Lianyungang, China); in the DEX+ESK group (DEX+ESK group), the SCI model rats were given 1 µg DEX and 50 µg ESK.

Neurological Function Evaluation

Hind limb motor function was assessed at 1, 4, and 7 days according to the Basso Beattie Bresnahan (BBB) locomotor rating scale [11]. The specific scoring criteria are as follows: 0–7 represents the activity degree of each joint in the hind limbs, with zero indicating no movement and seven indicating normal movement; 8–13 represents gait and coordination of the hind limbs, with eight indicating an uncoordinated gait and 13 indicating a mostly normal gait; 14–21 represents the fine movement degree of the claws during movement, with 14 indicating minimal claw movement and 21 indicating normal claw movement. The total possible score of the above three items is 21 points, of which total hind limb paralysis is 0 points. The neurological function evaluation followed a double-blind procedure, and each score was independently completed by two trained physicians.

Inclined Plane Test

The rats with SCI were placed on the experimental operating platform with an inclined plate, and the surface of the plate had certain friction to help the rats maintain their position. The maximum angle at which rats with SCI could maintain stability on the inclined plane for at least five seconds was observed and recorded.

Western Blot

The rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (3.5 mL/kg, H31021724, Shanghai Pharma New Asia Pharma, Shanghai, China) and decapitated. The spinal cord tissues were removed and lysed on ice with cell lysate (C1051, Applygen, Beijing, China) for 30 minutes. Spinal cord tissues were taken, and cleaved on ice for 30 min with cell lysate and protease inhibitor. Cells were collected and centrifuged at 4 °C, 12,000 r/min for 5 min. A bicinchoninic acid (BCA) protein quantification kit (BC201, Yise, Shanghai, China) was used to quantify the extracted proteins. A 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was performed using 50 µg protein. After

electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and sealed with 5% nonfat milk for 1 h. The proteins were incubated overnight with the primary antibodies (anti-SP: S1542, 1:1000, Sigma, Shanghai, China; anti-NK-1R: sc-365091, 1:500, Santa Cruz Biotechnology, Dallas, TX, USA; anti- B cell lymphoma-2 (Bcl-2): ab59348, 1:1000, Abcam, Cambridge, UK; anti-Bcl2-associated X protein (Bax): ab32503, 1:1000, Abcam, Cambridge, UK; anti-GAPDH: ab9485, 1:2500, Abcam, Cambridge, UK; anti-β-actin: ab179467, 1:5000, Abcam, Cambridge, UK) at 4 °C. The next day, the second antibody (ab205718, 1:2000, Abcam, Cambridge, UK) was incubated at 37 °C for 1 h. Finally, the image was developed using an efficient chemiluminescence (ECL) kit (P0018M, Beyotime, Shanghai, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the levels of interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin 18 (IL-18) in the spinal cord. The rat spinal cord tissue was crushed and lysated, and subsequently centrifuged at 12000 rpm for 10 min after ultrasonic homogenization. A detection reagent was added to the samples according to the manufacturer's instructions (CB10205 (IL-1β), CB11057 (TNF-α), CB10203 (IL-18), COIBO BIO, Shanghai, China). The absorbance value of each well was detected by an enzyme-labeled instrument (Victor X, PerkinElmer, Waltham, MA, USA).

Primary Spinal Cord Astrocyte Culture

After anesthesia, rats in each group underwent thoracotomy. Fixed specimens were injected with 40 g/L paraformaldehyde, and approximately 2 cm of spinal cord tissue at the T10 site was removed. The spinal cord tissues were washed in D-Hanks' solution to remove the blood, and subsequently digested with trypsin at 37 °C in a 5 % CO_2 incubator for 15 min. After adding 10 % fetal bovine serum (FBS) and allowing it to rest for 3–5 min, the supernatant was collected. Then, the supernatant was centrifuged at 1200 r/min and 4 °C for 5 min. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMDM-F12) (Invitrogen, Carlsbad, USA) medium containing 20% FBS was added, and cell suspension was prepared after passing through a 200-mesh screen. The cells were inoculated at a density of 1×10^5 in a culture flask. The fluid was then changed after 2 days, followed by subsequent changes every 3 days. Once the cells filled the bottom of the culture bottle, the bottles were shielded from light and left on a shaking bed overnight. The extracted astrocytes were tested by mycoplasma and used for follow-up tests.

Identification of Astrocytes

Cells were fixed with 4% paraformaldehyde (G1101, Servicebio,Wuhan, China) for 20 min, and treated with 0.1% TritonX-100 (GD-MY808J, Guduo Biotechnology, Shanghai, China) at room temperature for 10 min. After cleaning, 3% bovine serum albumin (BSA) (S12012, Shanghai yuanye Bio-Technology, Shanghai, China) was added and incubated at room temperature for 30 min. Then, the cells were exposed to the glial fibrillary acidic protein (GFAP) primary antibody (1:400, 12389, Cell Signaling Technology, Boston, MA, USA) and incubated at 4 °C overnight. Subsequently, the secondary antibody was added (1:500, A0516, Beyotime, Shanghai, China) and incubated at 37 °C for 1 h without light. Finally, 4',6-diamidino-2-phenylindole (DAPI) (C0060, Solarbio, Beijing, China) was added for staining for 5 min. The images were captured under a microscope (THUNDER Imager, Leica, Heerbrugg, Germany) using an antifluorescent attenuator seal.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Spinal cord astrocytes (2×10^4) were inoculated into 96-well plates. After culture for 3 days, each well was treated with a 20 µL MTT solution and incubated at 37 °C for 4 h. Then, 150 µL DMSO was added to each well and oscillated at room temperature for 10 min. After color development, the absorbance at 490 nm was detected using an enzyme-labeled instrument.

Statistical Analysis

Graph Pad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze the statistical data. Analysis of variance (ANOVA) and a subsequent Tukey's test were used to compare more than two sets of data. Two-factor ANOVA was used to analyze the BBB incline plate scores. Data was displayed as mean $\pm$ standard deviation, and the difference was statistically significant when $p < 0.05$.

Results

The motor function of rats was observed by BBB score and inclined plate test Motor function of rats in the Sham, SCI, DEX, ESK, and DEX+ESK groups were observed and evaluated. The BBB score in the sham group was 21 points. Compared with the Sham group, BBB scores in the SCI, DEX, ESK, and DEX+ESK groups were significantly lower ($p < 0.01$) on days 1, 4, and 7. Compared with the SCI group, BBB scores were significantly higher after 4 and 7 days ($p < 0.01$) in the DEX, ESK, and DEX+ESK groups. BBB scores were significantly higher on days 4 and 7 ($p < 0.01$, Fig. 1A) in the DEX+ESK group compared to the DEX and ESK groups. The maximum angle observed in the inclined plane test was significantly lower in the SCI, DEX, ESK, and DEX+ESK groups compared to the Sham group after 1, 4, and 7 days ($p < 0.01$). Compared with the SCI group, the maximum tilt angle of the DEX group ($p < 0.05$), ESK group ($p < 0.05$), and DEX+ESK group ($p < 0.01$) was significantly higher after 7 days. The maximum

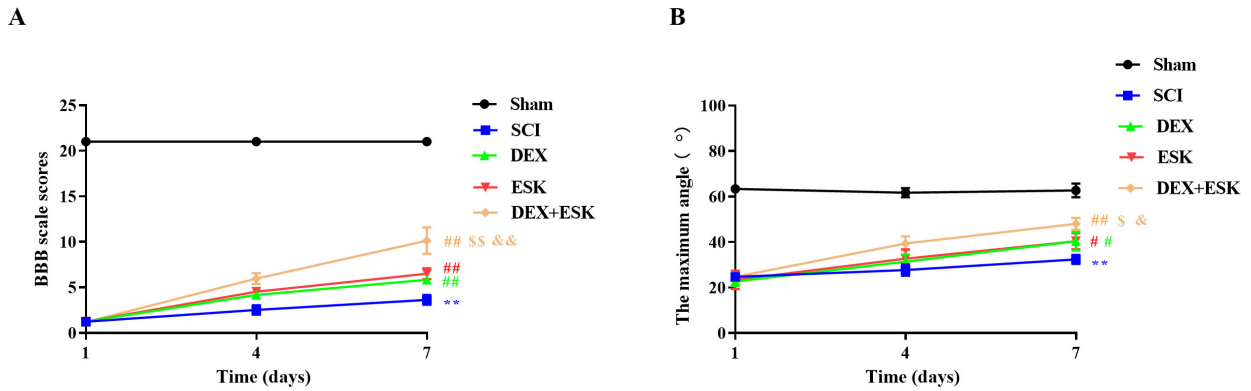

Fig. 1. The motor function of rats was observed by Basso Beattie Bresnahan (BBB) score and inclined plate test. (A) BBB scores of rats in each group. (B) The maximum angle of rates in each group. $^{**}p < 0.01$: compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: compared to SCI group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: compared to DEX group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$: compared to ESK group. SCI, Spinal cord injury; DEX, Dexmedetomidine; ESK, Esketamine.

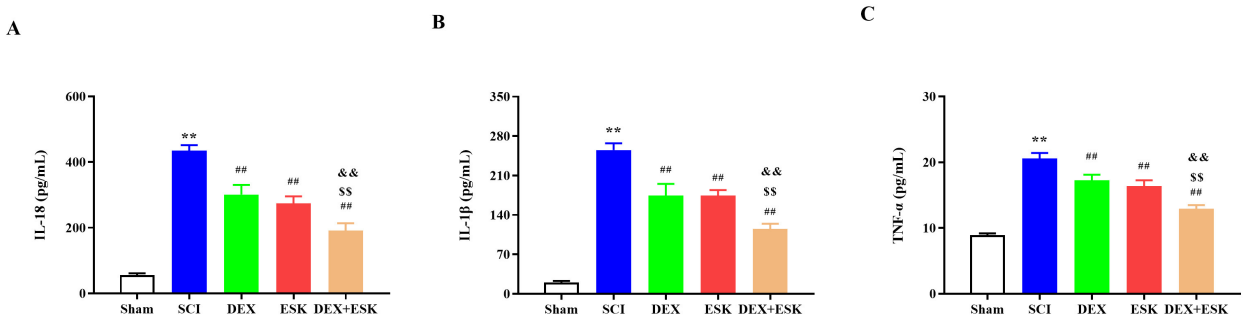

Fig. 2. Comparison of inflammatory cytokine levels in each group. (A) IL-18 content of rats in each group. (B) IL-1β content of rats in each group. (C) TNF-α content of rats in each group. $^{**}p < 0.01$: compared to Sham group; $^{\#\#}p < 0.01$: compared to SCI group; $^{\$\$}p < 0.01$: compared to DEX group; $^{\&\&}p < 0.01$: compared to ESK group. IL-18, interleukin 18; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α.

tilt angle in the DEX+ESK group was notably higher than the DEX and ESK groups after 7 days ($p < 0.05$, Fig. 1B). Over time, the improvement of hind limb motor function in the DEX+ESK group was more pronounced than that in the DEX and ESK groups.

Comparison of Inflammatory Cytokine Levels in Each Group

According to ELISA results, the levels of IL-18, IL-1β, and TNF-α were greater in the spinal cord tissue of SCI rats. However, their secretion levels exhibited a significant decrease following treatment ($p < 0.01$). The results showed that DEX and ESK can reduce the neuroinflammatory response after SCI, and that the combined treatment effect of DEX and ESK is better ($p < 0.01$, Fig. 2A–C).

Protein Content Changes of SP and NK-1R in Spinal Cord Tissues

Compared to the Sham group, the SCI group displayed significantly higher protein contents of SP and NK-1R. However, both DEX and ESK treatments decreased the protein content of SP and NK-1R ($p < 0.01$, Fig. 3). Notably, the influence of the DEX+ESK group on SP and NK-1R expression exceeds that of the DEX and ESK groups ($p < 0.01$, Fig. 3).

Effect of Multimodal Analgesia on Viability and Apoptosis of Spinal Astrocytes in Rats

As shown in Fig. 4A, GFAP was positively expressed in the cells, confirming the identity of astrocytes. After one week, the effects of different treatments on viability and apoptosis of spinal cord astrocytes were observed using MTT and Western blot assays. The SCI group exhibited enhanced cell viability, whereas DEX and ESK treatments resulted in inhibition. Moreover, the combined DEX+ESK treatment demonstrated superior effects compared to the ESK group (Fig. 4B). Similarly, Bcl-2 expression was upregulated and Bax expression was downregulated in the SCI group. Compared with the SCI group, both DEX and ESK reversed the expression of apoptosis-related proteins. In addition, the reverse effect of the DEX+ESK group was more obvious than that of the DEX and ESK groups ($p < 0.01$, Fig. 4C).

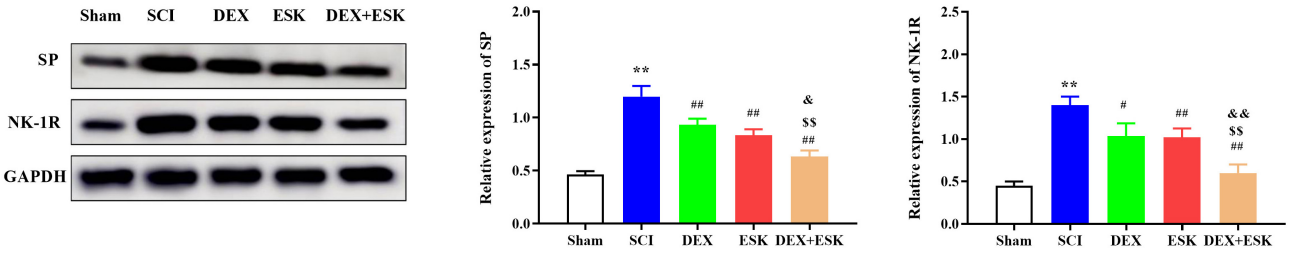

Fig. 3. Protein content of SP and NK-1R in spinal cord tissues. $^{**}p < 0.01$: compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: compared to SCI group; $^{\$\$}p < 0.01$: compared to DEX group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$: compared to ESK group. SP, substance P; NK-1R, neurokinin-1 receptor; GAPDH, glyceraldehyde phosphate dehydrogenase.

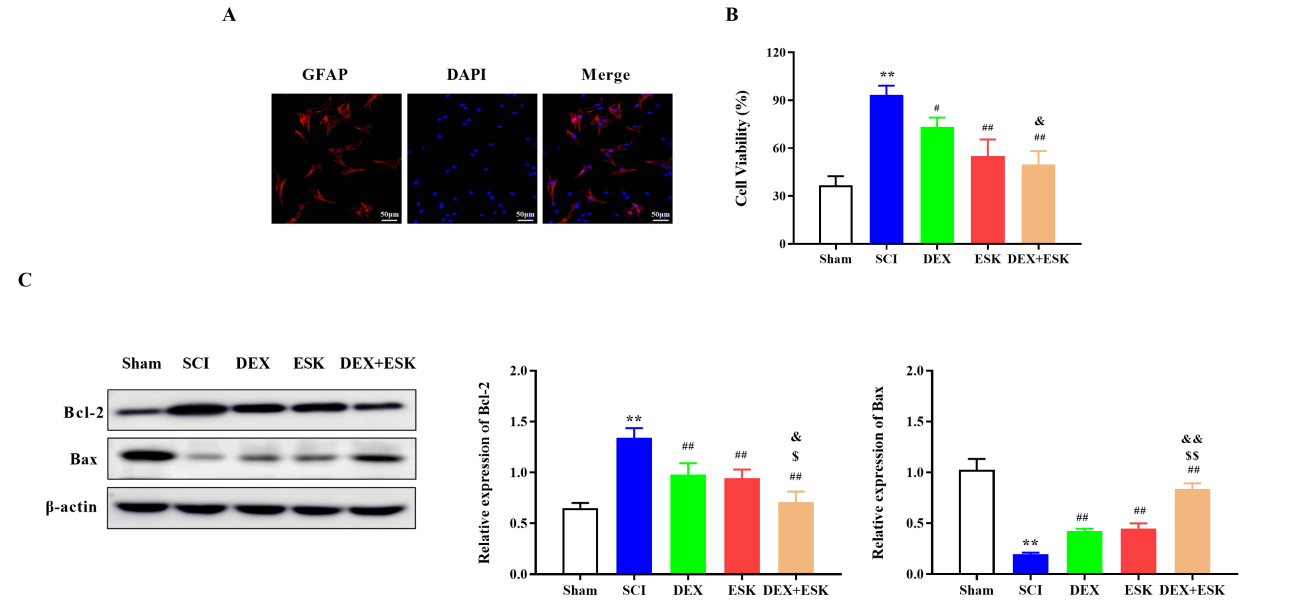

Fig. 4. Effect of multimodal analgesia on the proliferation and apoptosis of spinal astrocytes in rats. (A) Immunofluorescence identification of astrocytes. (B) Cell viability as measured by MTT assay. (C) The protein expressions of Bcl-2 and Bax as tested by Western blot assays. $^{**}p < 0.01$: compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: compared to SCI group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: compared to DEX group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$: compared to ESK group. GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; Bcl-2, B cell lymphoma-2; Bax, Bcl2-associated X protein; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Discussion

SCI is one of the most devastating traumatic events. Pain is one of the common complications of SCI and greatly reduces patients' quality of life and affects their postoperative recovery. Medication remains an important means of SCI treatment. However, no single drug or method can achieve optimal or complete pain relief with minimal side effects. Therefore, the concept of multimodal analgesia has been proposed to address these limitations [12]. Multimodal analgesia is the addition or coordination of drugs or analgesia with different mechanisms of action through the intervention of multi-layered pain perception or transmission, which plays an important role in perioperative pain management [13]. In this study, we established a model of SCI in rats to explore the possible analgesic mechanisms of combined DEX and ESK therapy. DEX has strong sedative, analgesic, and anti-sympathetic nerve excitation effects [14]. ESK can improve neuroplasticity, improve damaged nerve recovery, and has been used in the treatment of nerve damage diseases [15]. The results of this study confirmed that DEX and ESK independently improved the motor function of SCI mice, and that combined treatment exhibited superior effects to either group individually. After SCI, severe oxidative stress and inflammation will occur in the spinal cord tissue. ELISA results showed that IL-18, IL-1β, and TNF-α levels increased in the spinal cord tissue of SCI rats, and their secretion levels decreased after treatment

using DEX and/or ESK. The results showed that DEX combined with ESK could reduce the release of inflammatory factors and alleviate the neuroinflammatory response after SCI. Similar to our results, by mediating the nuclear factor erythroid-2-related actor 2/heme oxygenase 1 (Nrf2/HO-1) signaling pathway, DEX alleviates cellular inflammatory response and reduces oxidative stress injury after SCI [16]. In addition, DEX combined with ESK has been studied in anesthesia and analgesia for various diseases. Studies indicate that this combination can reduce nerve damage and inflammation in patients with laparoscopic total hysterectomy, potentially minimizing postoperative cognitive dysfunction [17]. Additionally, research suggests that the combined use of DEX and ESK can reduce the opioid dosage and adverse reactions [18].

Substance P (SP) is an important neurokinin transmitter and is considered to be an important messenger in pain transmission. SP acts by binding to its NK-1R receptor and is involved in signal transmission of neuropathic pain [19]. SP and NK-1R expression in the spinal cord of rats with incision pain decreased after treatment, suggesting that SP and NK-1R can be used as a detection of pain information [20]. Similarly, we found increased SP and NK-1R expression levels in SCI spinal cord tissue, suggesting that SP and NK-1R are involved in the generation and maintenance of postoperative pain. DEX and ESK treatment significantly reduced SP and NK-1R levels when compared to the SCI group, with the most pronounced effects established with combined treatment. Therefore, we concluded that the treatment of DEX combined with ESK could significantly reduce hyperalgesia in rats.

Astrocytes can separate and support neuronal cell bodies. After SCI, astrocytes undergo proliferation and activation to form glial scars. These scars represent the main obstacle to the inhibition of SCI the repair [21]. In this study, we investigated astrocyte activity after SCI, finding that promoted cell viability was inhibited after treatment, and the effect of combined treatment was better than that of treatment using DEX or ESK alone. The Bax/Bcl-2 pathway is an important signaling molecule that regulates apoptosis-dependent pathways [22]. The results of this study showed that combined therapy can promote Bax expression and inhibit Bcl-2 expression in astrocytes, suggesting that its neuroprotective effect may be related to the anti-apoptotic effect of the Bcl-2 family. It has been found that SCI-induced neuropathic pain can be alleviated by inhibiting the activation of astrocytes and microglia [23]. Similar to our study, DEX+ESK may play an analgesic role by inhibiting astrocyte activity and reducing the formation of glial scars. Previous studies have confirmed that astrocytes play an important role in neuropathic pain [24], but further research is necessary to understand the relevant mechanisms.

There are still shortcomings in this study. First, Haematoxylin and eosin (HE) staining and immunohistochemistry should be used to detect pathological spinal cord changes. Second, the dose-effect relationship between DEX and ESK was not discussed in this study. Additionally, future analyses should consider exploring alternative potential analgesic mechanisms, including the modulation of NMDA receptors and α2-AR. Finally, we only observed the neurological function and inflammatory response of 7-day-old rats post-SCI, and could not evaluate the long-term efficacy and safety of combination therapy.

Conclusions

In conclusion, the combination of DEX and ESK can reduce inflammatory cytokine levels, SP, NK-1R expression, and astrocyte activity, thereby reducing pain sensitivity and improving motor function in rats with SCI. While further verification is warranted, the combination of DEX and ESK treatment has the potential to counteract the adverse effects of SCI in rats.

Availability of Data and Materials

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

Author Contributions

HZ and ZL designed the research study; HZ performed the research; HZ collected and analyzed the data. HZ has been involved in drafting the manuscript and ZL has been involved in revising it critically for important intellectual content. HZ and ZL give final approval of the version to be published. HZ and ZL have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Animal experiments adhered to the guidelines outlined in the revised Animals (Scientific Procedures) Act 1986 in the UK and complied with the regulations formulated by the Ethics Committee of Heilongjiang Provincial Hospital (2022092).

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Conflict of Interest

The authors declare no conflict of interest.

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