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*Original Article*

# Intrathecal administration of dexmedetomidine attenuates the development of morphine tolerance via suppression of HMGB1-mediated neuro-inflammation in the spinal cord

Hongyan Gong[1], Shaomei Li[1], Jun Liu[1], Xiaoran Zhang[1], Jingjing Liu[1], Tieli Dong[2]

[1]*Department of Anesthesiology, First Affiliated Hospital of Xinxiang Medical University, Xinxiang 453000, Henan Province, China;* [2]*Department of Anesthesiology, Second Affiliated Hospital of Zhengzhou, Zhengzhou 450003, Henan Province, China*

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**Abstract:** Morphine loses analgesic potency after repetitive administration. Inflammation in the spinal cord has been highlighted as an important contributor to morphine tolerance. Dexmedetomidine, a α2-adrenoceptor agonist widely used as a sedative in clinical settings, has been demonstrated to possess anti-inflammation properties in various experimental models. Thus, it was speculated that intrathecal administration of dexmedetomidine would attenuate the development of morphine tolerance via suppression of neuroinflammation in the spinal cord. The present study found that intrathecal administration of dexmedetomidine (1.5 μg daily for consecutive seven days) maintained the anti-nociceptive effects of morphine, as assessed by the tail flick test. It also reduced pro-inflammatory cytokine (IL-1β and TNF-α) expression and suppressed microglia activation, as indicated by decreases of Iba-1 (a microglia marker) protein expression and Iba-1-positive cell densities in the spinal cords of morphine-tolerant rats. In addition, repetitive morphine injections resulted in a marked increase of cytosolic HMGB1 expression in the spinal cord, attenuated by dexmedetomidine administration. However, the intrathecal co-administration of rHMGB1 (0.5 μg daily) with dexmedetomidine blocked the negative effects of dexmedetomidine on IL-1β and TNF-α expression and microglial activation in the spinal cord. Moreover, behavioral tests showed that intrathecal rHMGB1 abrogated the effects of dexmedetomidine on maintaining the anti-nociceptive effects of morphine. In conclusion, intrathecal administration of dexmedetomidine is an effective approach for the prevention of tolerance to morphine analgesia. These effects may be related to the inhibitory action of dexmedetomidine on HMGB1-mediated neuroinflammation in the spinal cord.

**Keywords:** Dexmedetomidine, morphine tolerance, HMGB1, inflammation

## Introduction

Morphine is frequently used for management of moderate to severe pain. However, its analgesic potency fades with receptive or long-term administration, thereby limiting its use in clinical settings. Much progress has been made concerning the underlying mechanisms of morphine tolerance, including the endocytosis/desensitization of opioid receptors [1] and dysfunction of glutamatergic receptors [2]. Importantly, a growing body of evidence has highlighted neuro-inflammation as an important contributor to the development of morphine tolerance [3, 4]. Thus, prevention of inflammation has been thought as a potential target in attenuating the development of morphine tolerance.

Dexmedetomidine is a potent α2-adrenoceptor agonist with sedative and analgesic properties, widely used in clinical settings. A previous clinical study revealed that the pre-anesthetic administration of intravenous dexmedetomidine significantly reduces morphine consumption in postoperative patient-controlled analgesia [5]. Similarly, another recent study suggested that intrathecal treatment of dexmedetomidine reduces morphine consumption in patients suffering from refractory cancer pain [6]. In addition to sedative and analgesic properties, the anti-inflammation actions of dexmedetomidine

are supported by accumulating evidence, having been associated with protective effects in the models of endotoxin-induced shock [7, 8], brain ischemic injuries [9], and spinal cord injuries [10]. However, it is still unknown whether intrathecal administration of dexmedetomidine could reduce inflammation in the spinal cords of morphine-tolerant rats.

High-mobility group box 1 (HMGB1) is a nonhistone DNA binding protein with both intranuclear functions and extracellular cytokine-like effects [11, 12]. Normally, HMGB1 proteins are located in the nucleus where they binds to DNA, regulating transcription and stabilizing the nucleosomal structure [12]. However, under pathological conditions, HMGB1 can translocate to the cytoplasm, thereby triggering activation inflammatory cascades [11, 12]. This regulatory role of HMGB1 in inflammation has been demonstrated in a series of animal models, including ischemic spinal cord injuries [13]. Furthermore, the involvement of upregulation of spinal HMGB1 has been associated with tactile hyperalgesia in the model of neuropathic pain [14], suggesting a possible role of HMGB1-mediated inflammation in analgesic dysfunction. It has also been suggested that dexmedetomidine could suppress HMGB1 production or release in lipopolysaccharide-activated macrophages [15] and human whole blood [16], as well as in animal models of renal ischemic injuries [17] and sepsis [18, 19]. However, little is known about the relationship between dexmedetomidine and HMGB1 during the development of morphine tolerance. Therefore, the present study aimed to test the hypothesis that intrathecal administration of dexmedetomidine would attenuate the development of morphine tolerance via suppression of HMGB1-mediated neuro-inflammation in the spinal cord.

## Materials and methods

### *Animals*

Male adult Sprague-Dawley rats, weighing 220-260 g, were purchased from the Experimental Animal Center of Xinxiang Medical University. The animals were housed with food and water ad libitum for 1 to 2 weeks before experimentation. Behavioral tests were performed under artificial light, with a temperature of 23 ± 0.5°C and humidity of 60 ± 5%. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University.

### *Intrathecal catheterization*

Intrathecal catheterization was performed, as described previously [20]. Briefly, after anesthesia with ketamine (50 mg/kg; Bioniche, Belleville, ON, Canada), a sterile polyethylene (PE)-10 tube (Clay Adams, MD, USA) was implanted through the intervertebral space between L5 and L6 and extended to the subarachnoid space. Only animals with normal hind-limb motor function 3 days after intrathecal catheterization were included in the experiment. All animals were permitted to recover and habituate to the experimental environment for 1 week. Drugs or the vehicle (saline) (10 μL) were injected through the exited end of the tube, followed by a flush with saline (15 μL).

### *Drug treatment and experimental groups*

Tolerance to morphine analgesia was induced by intrathecal treatment of morphine (diluted in saline, 15 μg daily for consecutive 7 days). For corresponding experimental groups, dexmedetomidine (1.5 μg daily) or reconstitute HMGB1 (rHMGB1) were prepared with saline and intrathecally administered 30 minutes before each daily morphine injection. The dose and protocol of intrathecal dexmedetomidine administration was chosen based on a previous study [21].

Rats were randomly divided into four groups: (1) Saline group (Saline): rats were intrathecally administered with saline (10 μL) for daily for consecutive 7 days (D1 to D7); (2) Morphine group (Morphine): rats were intrathecally administered with 15 μg of morphine daily for consecutive 7 days; (3) Dex group (Dex): operations were the same as in the Morphine group, except that a 1.5 μg of dexmedetomidine was given intrathecally 30 minutes before each daily morphine injection; (4) Dex plus rHMGB1 group (Dex + rHMGB1): operations were the same as the Dex group, except that a combined intrathecal treatment of rHMGB1 (0.1, 0.2, or 0.5 μg) was given along with daily dexmedetomidine administration.

### *Behavioral tests*

The latency of tail flick was measured by hot-water tail flick tests 30 minutes after each mor-

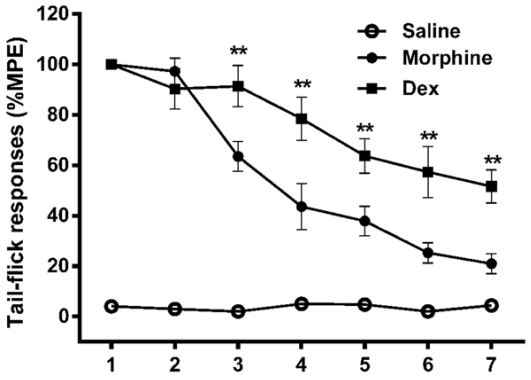


**Figure 1.** Intrathecal administration of dexmedetomidine attenuates the development of morphine tolerance, as assessed by the tail flick test. Dexmedetomidine (1.5 μg) was injected 30 minutes before daily morphine (15 μg) treatment and the behavioral test was performed 30 minutes after morphine injections. Data are expressed as mean ± SD (n = 7 per group). **P<0.01 vs the morphine-treated group at the corresponding time point.

phine injection, determining the development of morphine tolerance [22]. Briefly, rats were kept in a plastic container (23 cm × 10 cm) with the lower third of the tails immersed in the water at a temperature of 50 ± 0.3°C. Positive response was defined by a rapid removal of tails from the hot water. A cut-off time of 15 seconds was set to avoid tail damage. Three trials were performed for each animal with an interval of 10 minutes. The mean was recorded. The percentage of maximal possible antinociceptive effects (%MPE) was calculated using the following formula: [(post-drug latency-baseline latency)/(cut-off time-baseline latency)] × 100%.

*Western blot*

At set time points, rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), then euthanized with $CO_2$ asphyxiation. Spinal cord L4-6 segments were removed and total protein was extracted by homogenization in ice-cold RIPA lysis buffer (Beyotime, Nantong, China) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cytoplasmic proteins were extracted with the Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, USA), according to manufacturer instructions. Equal amounts of protein samples were resolved on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), and detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Signals of bands were detected using ChemiDoc XRS + System (Bio-Rad) with Image Lab 3.0 software. Primary antibodies were used in the present study, including mouse anti-β-actin monoclonal antibody (Santa Cruz, Santa Cruz, CA, Cat# sc-130300, 1:1000), rabbit anti-HMGB1 polyclonal antibody (Abcam, Cambridge, MA, Cat# ab18256, 1:1000), rabbit anti-Iba-1 (microglia marker) polyclonal antibody (Wako, Japan, Cat# 016-20001, 1:2000), rabbit anti-IL-1β polyclonal antibody (Santa cruz, Cat# sc-7884, 1:1000), rabbit anti-phospho-p38 MAPK (p-p38) monoclonal antibody (Abcam, Cat# ab178867, 1:500), and rabbit anti-TNF-α polyclonal antibody (Abcam, Cat# ab6671, 1:1000).

*Immunofluorescence*

Immediately after behavioral testing, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M PBS through the ascending aorta. After perfusion, the spinal cord L4-L6 segment was removed and post-fixed in 4% paraformaldehyde for 1 hour, then cyto-protected overnight with 30% sucrose. The spinal sections (20 μm) were cut in the frozen section machine (SLEE, Zeiss, Germany) and mounted on the slides. Next, the sections were permeabilized with 0.3% Triton X-100 for 8 minutes, followed by blocking with 2% normal goat serum for 30 minutes at room temperature. The sections were then incubated overnight at 4°C with rabbit anti-Iba-1 polyclonal antibody (Wako, Cat# 016-20001, 1:500), followed by incubation with Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch). After counterstaining with DAPI (4', 6-diamidino-2-phenylindole, 0.1 μg/mL), the sections were examined with a fluorescence microscope (Leica, Wetzlar, Germany). Four sections were selected randomly for each animal. The number of Iba-1-positive cells in the spinal dorsal horn were counted in six fields, at a magnification of 200 ×, by an investigator blinded to the experimental design.

*Statistical analysis*

Data are presented as mean ± SEM, except for behavioral tests which are expressed as mean

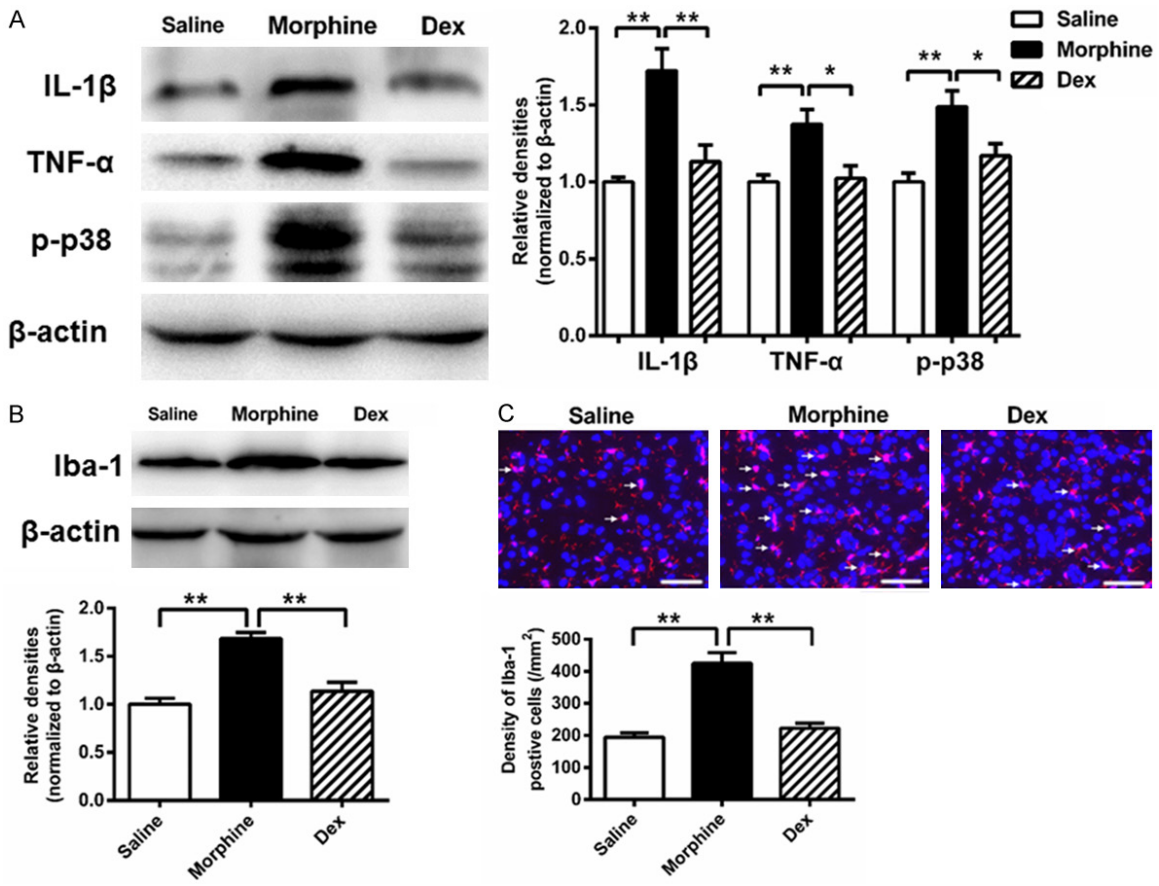


**Figure 2.** Dexmedetomidine reduces the production of pro-inflammatory cytokines and the density of microglia in the spinal cords of morphine-tolerant rats. A. The effects of intrathecal treatment of dexmedetomidine on protein levels of IL-1β, TNF-α and p-p38 in the spinal cord were detected by Western blot analysis at the seventh day of successive morphine treatment and normalized to β-actin (n = 5 per group). Data are expressed as mean ± SEM. *P<0.05 or **P<0.01. B. The effects of dexmedetomidine on the spinal Iba-1 (microglia marker) protein expression were assessed by Western blot at the seventh day of successive morphine treatment (n = 5 per group). Data are expressed as mean ± SEM. **P<0.01. C. The effects of dexmedetomidine on the spinal density of Iba-1-positive cells (arrows) were assessed by immunofluorescence at the seventh day of successive morphine treatment (n = 4 per group). Scale bars = 50 μm. Data are expressed as mean ± SEM and analyzed with Mann-Whitney U tests. **P<0.01.

± SD. Statistical analysis was performed with SPSS software version 13.0 (SPSS Inc., Chicago, IL). Differences in the densities of Iba-1-positive cells among groups were compared with one-way ANOVA followed by Mann-Whitney U-test. Other data were analyzed with one-way ANOVA followed by the Dunnett test. P<0.05 indicates statistical significance.

## Results

*Intrathecal administration of dexmedetomidine attenuates the development of morphine tolerance*

First, this study tested whether intrathecal treatment of dexmedetomidine could prevent the development of morphine tolerance. As shown in **Figure 1**, results showed that daily intrathecal treatment of morphine (15 μg) for 7 consecutive days lead to substantial tolerance to morphine analgesia (n = 7 per group, P<0.01). However, consecutive administration of dexmedetomidine significantly attenuated morphine antinociceptive tolerance (**Figure 1**, n = 7 per group, P<0.01).

*Dexmedetomidine reduces inflammation in the spinal cords of morphine-tolerant rats*

Inflammation has been demonstrated to be an important factor in the development of morphine tolerance [23]. Therefore, this study investigated the potential effects of intrathecal

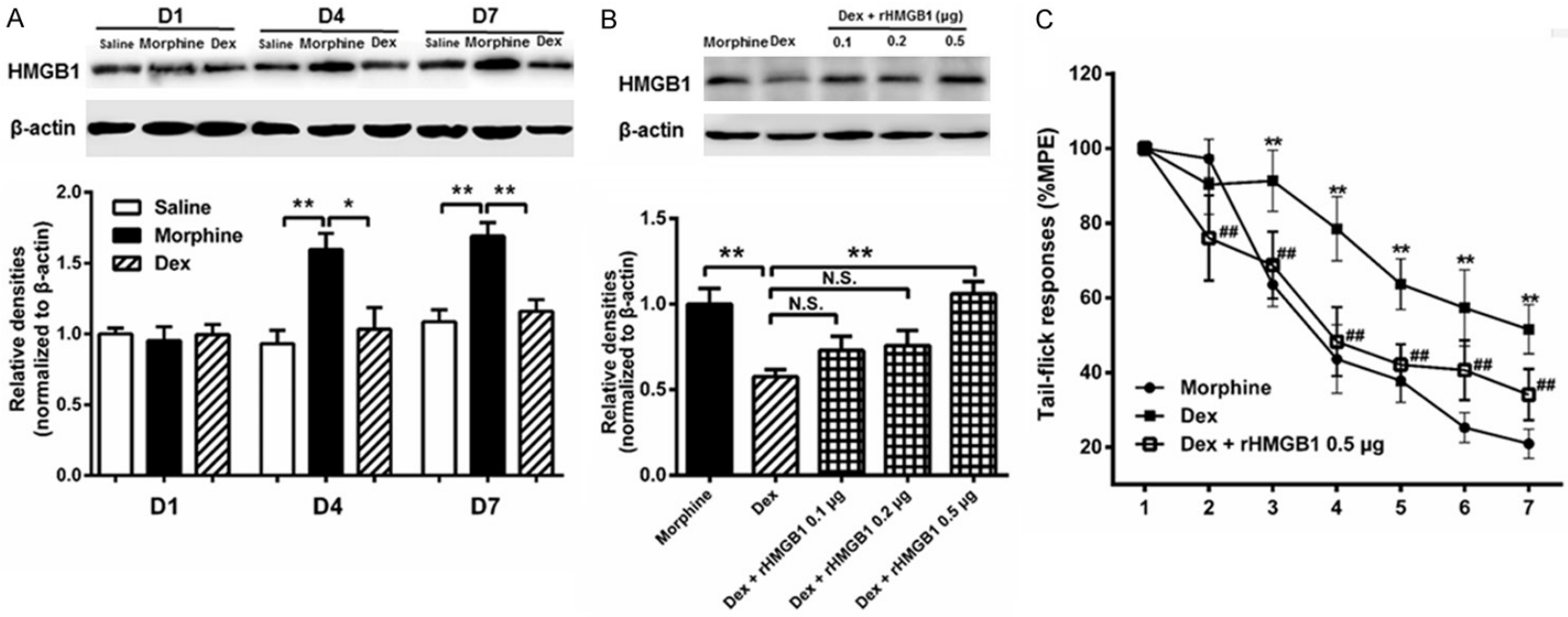


**Figure 3.** Dexmedetomidine reduces cytosolic HMGB1 protein expression in the spinal cords of morphine-tolerant rats. A. The effects of dexmedetomidine on the cytosolic HMGB1 protein expression were evaluated by Western blot analysis. Intrathecal administration of dexmedetomidine attenuated the increase of cytosolic HMGB1 protein expression in the spinal cords of morphine-tolerant rats at D4 and D7. Data are expressed as mean ± SEM (n = 4 per group). *P<0.05 or **P<0.01. B. The combined intrathecal treatment of rHMGB1 (0.5 μg) reversed dexmedetomidine-induced decrease of cytosolic HMGB1 protein expression in morphine-tolerant rats. Different doses of rHMGB1 (0.1, 0.2, or 0.5 μg) were diluted in saline and injected i.t. in combination with the daily dexmedetomidine treatment. Data are expressed as mean ± SEM (n = 4 per group). **P<0.01. C. The inhibitory effects of dexmedetomidine treatment on morphine tolerance were reversed in the presence of combined intrathecal treatment of rHMGB1 (0.5 μg). Data are expressed as mean ± SD (n = 6 or 7 per group). **P<0.01 vs the morphine-treated group and ##P<0.01 vs the Dex group at the corresponding time point.

dexmedetomidine administration on spinal inflammation caused by tolerance to morphine. Results showed that protein levels of IL-1β and TNF-α were increased in morphine-tolerant rats at D7, compared with saline-treated rats (**Figure 2A**, n = 5 per group, P<0.01). Importantly, the increase in spinal IL-1β and TNF-α protein expression was significantly alleviated by the intrathecal administration of dexmedetomidine (**Figure 2A**, n = 5 per group, P<0.05 or P<0.01).

The significant roles of p38 MAPK indicate that it is an essential pathway in microglia-mediated inflammation during the development of morphine tolerance [23]. As shown in **Figure 2A**, it was found that protein levels of phospho-p38 were elevated in morphine-tolerant rats at D7, compared with saline-treated rats (n = 5 per group, P<0.05), whereas this increase of p38 phosphorylation was relieved by treatment with dexmedetomidine (n = 5 per group, P<0.05). In addition, protein expression of Iba-1 (microglia marker) and the density of Iba-1-positive cells was higher in morphine-tolerant rats at D7, compared with saline-treated rats (**Figure 2B** and **2C**, n = 5 per group, P<0.01), whereas these effects were abrogated by treatment with dexmedetomidine (**Figure 2B** and **2C**, n = 4 per group, P<0.01).

*Dexmedetomidine attenuates HMGB1 release in the spinal cords of morphine-tolerant rats*

To investigate the involvement of HMGB1 in the development of morphine tolerance, this study first determined the time course of cytosolic HMGB1 protein expression during the development of morphine tolerance. Results showed that cytosolic HMGB1 protein expression was higher in morphine-treated rats at D4 and D7, compared with saline-treated rats (**Figure 3A**, n = 4 per group, P<0.01). Of note, these increases of HMGB1 protein expression were blocked by the treatment with dexmedetomidine (**Figure 3A**, n = 4 per group, P<0.05 or P<0.01). In contrast, the combined intrathecal administration of recombinant HMGB1 (rHMGB1) significantly increased HMGB1 protein expression in the spinal cords (**Figure 3B**, n = 4 per group, P<0.01). It was then asked whether combined intrathecal administration of rHMGB1 could affect the effects of dexmedetomidine on morphine tolerance? A tail flick test was conducted. As expected, behavioral tests showed that attenuation of morphine tolerance caused by dexmedetomidine was reversed by combined rHMGB1 treatment (**Figure 3C**, n = 6 or 7 per group, P<0.01).

*Treatment of rHMGB1 abrogates the inhibitory actions of dexmedetomidine on inflammation*

The present study aimed to confirm the roles of HMGB1 in the effects of dexmedetomidine on inflammation induced by morphine tolerance. Results showed that downregulation of IL-1β, TNF-α and p-p38 protein expression, induced by dexmedetomidine, disappeared in the presence of combined rHMGB1 administration (**Figure 4A**, n = 5 per group, P<0.05 or P<0.01). Similarly, decreases of Iba-1 protein expression, as well as Iba-1-positive cells in the spinal cord of dexmedetomidine-treated rats, were markedly reversed by intrathecal co-administration of rHMGB1 (**Figure 4B** and **4C**, n = 4 or 5 per group, P<0.01).

## Discussion

The present study reveals that intrathecal administration of dexmedetomidine maintained the anti-nociceptive effects of morphine, attenuated morphine-induced pro-inflammatory cytokines (such as IL-1β and TNF-α) expression, and suppressed microglia activation in the spinal cords of morphine-tolerant rats. In addition, it was found that repetitive morphine injections resulted in a marked increase of cytosolic HMGB1 expression in the spinal cord, attenuated by dexmedetomidine administration. However, intrathecal co-administration of rHMGB1 blocked the negative effects of dexmedetomidine on inflammation and morphine tolerance.

Dexmedetomidine has been widely used in clinical settings due to its potent effects of sedation and auxiliary analgesia. Accordingly, dexmedetomidine has been applied as an intravenous adjunction with morphine to relieve post-operative pain [5]. However, beyond the sedative and analgesic actions, systemic administration of dexmedetomidine could result in numerous side effects, such as bradycardia, hypotension, and even hypothermia [24-26], due to the wide distribution of α2-adrenoceptors. In contrast, another clinical trial reported that intrathecal administration of dex-

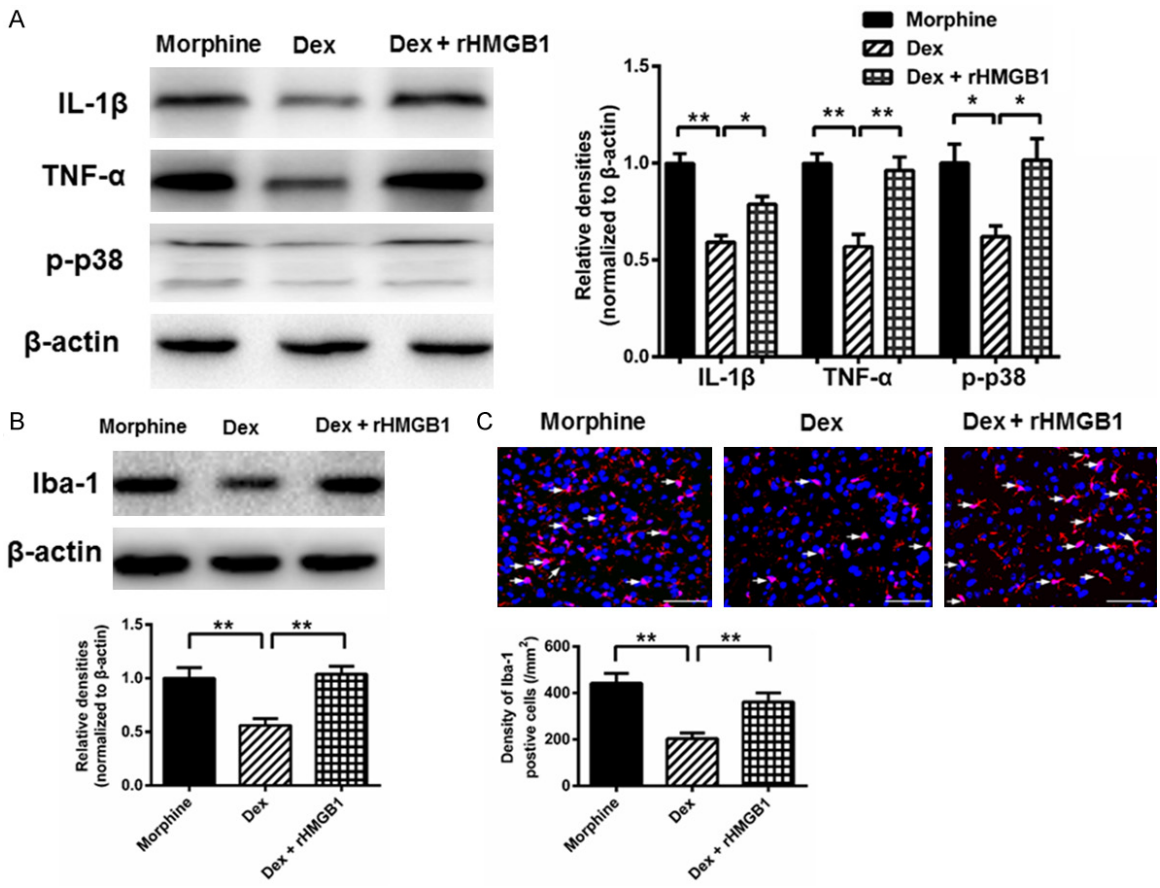


**Figure 4.** Combined treatment of rHMGB1 abrogates the inhibitory actions of dexmedetomidine on inflammation in the spinal cords of morphine-tolerant rats. A. The effects of combined treatment of rHMGB1 on protein levels of IL-1β, TNF-α, and p-p38 in the spinal cord (n = 5 per group). Data are expressed as mean ± SEM. *P<0.05 or **P<0.01. B. The effects of the combined treatment of rHMGB1 on the spinal Iba-1 protein expression (n = 5 per group). Data are expressed as mean ± SEM. **P<0.01. C. The effects of the combined treatment of rHMGB1 on the spinal density of Iba-1-positive cells (arrows) (n = 4 per group). Scale bars = 50 μm. Data are expressed as mean ± SEM and analyzed with Mann-Whitney U-tests. **P<0.01.

medetomidine with a lesser dose can similarly reduce morphine consumption without causing serious side effects [6]. Therefore, the present study used the intrathecal approach. Present data suggests that intrathecal administration of dexmedetomidine maintained the anti-nociceptive effects of morphine, as indicated by the tail-flick test, over the seven-day observation period. These results are generally consistent with previous studies [27, 28], demonstrating the inhibitory effects of systemic dexmedetomidine administration (intraperitoneally 20 [28] or 50 [27] μg/kg) on morphine tolerance. Thus, results suggest that the intrathecal approach with dexmedetomidine prevents analgesic tolerance to morphine and avoids side effects, due to lesser doses than the systemic approach.

Neuro-inflammation, including microglia activation and production of pro-inflammatory cytokines (such as IL-1β and TNF-α), has been considered a crucial factor in the development of morphine tolerance [3, 23, 29-31]. Consistent with previous studies, present results showed that repetitive morphine administration resulted in a significant increase in IL-1β and TNF-α protein expression, as well as microglia activation, indicated by increases in the Iba-1 (microglia marker) protein expression and the density of Iba-1-postive cells in the spinal cord. It has been reported that dexmedetomidine can reduce production of pro-inflammatory cytokines in the spinal cords of rats subjected to spinal cord injuries [10]. The present study found that intrathecal administration of dexmedetomidine attenuated the increase in protein

expression of IL-1β and TNF-α, suggesting an inhibitory action of dexmedetomidine on the production of pro-inflammatory cytokines induced by chronic morphine injections. Beyond the negative effects on pro-inflammatory cytokines, the inhibitory properties of dexmedetomidine on microglia activation were observed in the present study, as indicated by the reduction of Iba-1 protein expression and the density of Iba-1-postive cells. These effects were also supported by the attenuation of p38 MAPK phosphorylation induced by intrathecal dexmedetomidine administration, given the key role of p38 MAPK in mediating microglia activation during the development of morphine tolerance [23].

Another interesting question concerns the mechanisms underlying the anti-inflammation properties of intrathecal administrated dexmedetomidine. It was hypothesized that HMGB1 might be an essential mediator in this phenomenon based on the its core role in inflammation [11, 32]. Present results showed that the spinal cytosolic protein expression of HMGB1 increased with repetitive morphine injections. Furthermore, upregulation of HMGB1 was attenuated by the intrathecal administration of dexmedetomidine, indicating a direct inhibitory action of intrathecal dexmedetomidine on HMGB1. To further confirm the roles of HMGB1 in the anti-inflammation effects of dexmedetomidine during morphine tolerance, rHMGB1 was co-administrated intrathecally. Findings suggest that the consecutive co-administration of 0.5 μg rHMGB1 blocked the inhibitory effects of dexmedetomidine on pro-inflammatory cytokines and microglia activation. Further behavioral tests showed that maintenance of anti-nociceptive effects of morphine were blocked by the consecutive co-administration of rHMGB1, indicating that suppression of HMGB1 is an important mechanism underlying the negative effects of dexmedetomidine on inflammation and morphine tolerance.

In conclusion, intrathecal administration of dexmedetomidine is an effective approach for the prevention of analgesic tolerance to morphine. These effects may be related to the inhibitory actions of dexmedetomidine on HMGB1-mediated neuro-inflammation in the spinal cord.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Tieli Dong, Department of Anesthesiology, Second Affiliated Hospital of Zhengzhou, Zhengzhou 450003, Henan Province, China. Tel: +86-373-4403817; E-mail: newgenecn@163.com

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