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The therapeutic effect of dexmedetomidine on rat diabetic neuropathy pain and the mechanism

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Summary
Diabetic neuropathy pain (DNP) is a common chronic complication of diabetes characterized by spontaneous pain, hyperalgesia and allodynia. Dexmedetomidine is a selective $\alpha_2$ adrenergic agonist that relieves sympathetic nervous tension and reduces the release of glutamate. Thus, it is possible that dexmedetomidine may relieve DNP as well. In this study, we examined the effect of dexmedetomidine on DNP in the presence or absence of the $\alpha_2$ adrenergic antagonist yohimbine in rats utilizing a streptozotocin (STZ)-induced diabetes as a model of DNP. To examine DNP, we examined behavior using the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) tests, and microglia and astrocyte activation was examined by immunofluorescence staining. The levels of pro-inflammatory cytokine TNF-$\alpha$ and IL-1$\beta$ in the spinal cord were measured by ELISA. Cell apoptosis in spinal cord was examined by TUNEL assay. Glutamate production in caudal lumbar was measured by high performance liquid chromatography (HPLC). We found that STZ-treated rats had decreased pain threshold, elevated activation of microglia but not astrocytes, increased level of pro-inflammatory cytokines, increased apoptosis and glutamate production compared to control animals, and these effects were ameliorated by dexmedetomidine treatment. Pretreatment of yohimbine abolished almost all of the protective effects of dexmedetomidine except for glutamate production. In conclusion: our data confirmed that dexmedetomidine can relieve hyperalgesia in diabetic neuropathy pain, and protect spinal cord cells from apoptotic death. The mechanism may be related to dexmedetomidine-mediated inhibition of microglia activation, reduction of inflammatory reaction in the spinal cord, and suppression of glutamate production.

Keywords: dexmedetomidine; diabetic neuropathy pain; microglia; inflammatory reaction; glutamate
INTRODUCTION

Diabetic neuropathy pain (DNP) is one of the most common chronic complications of diabetes, characterized by spontaneous pain, hyperalgesia and allodynia, seriously affecting patient quality of life\(^1\). Peripheral nerve damage due to long-term high blood glucose causes ectopic discharge and activation of the sympathetic nervous system, which plays an important role in the cause and persistence of DNP. In addition, it has been shown that high blood glucose activates spinal cord glial cells which subsequently trigger neuropathic pain by releasing proinflammatory cytokines \(^2\). Glutamate, an excitatory amino acid, is an important neurotransmitter mediating transmission of pain information\(^3\). Guirimand \textit{et al.} \(^4\) suggested that long-lasting harmful stimulation causes the release of glutamate in the dorsal horn of the spinal cord. Ionotropic glutamate receptors are divided into the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and \textit{N}-methyl-D-aspartate (NMDA) receptors. NMDA receptors are voltage-dependent ion channel receptors located on the cell membrane of projection neurons of the spinal dorsal horn that allow \(\text{Ca}^{2+}\) and \(\text{Na}^+\) to pass through the cell membrane when activated, and are in a closed state when bound with \(\text{Mg}^{2+}\) \(^5\). Peripheral nerve injury induces the transmission of pain sensation, resulting in a large amount of glutamate and substance P release. Glutamate activation of AMPA receptors on dorsal horn neurons causes the inflow of \(\text{Na}^+\). The degree of depolarization of dorsal horn neurons causes the detachment of \(\text{Mg}^{2+}\) from the NMDA receptors. Glutamate binds to NMDA receptors and causes the inflow of extracellular \(\text{Ca}^{2+}\) \(^6\). Therefore, glutamate is an important target for the control of pathological pain.

Dexmedetomidine is a highly selective \(\alpha_2\) adrenergic agonists that reduces sympathetic...
nervous tension and thus indirectly improves vagus nerve tension, activates the anti-inflammatory channel of the parasympathetic nerve, and inhibits the inflammatory reaction. Recent studies have shown that dexmedetomidine could induce analgesia by down-regulating the expression of the P2X3 receptor in the dorsal horn of spinal cord in DNP rats.

Studies have confirmed that dexmedetomidine interacts with the $\alpha_{2A}$ adrenergic receptor in cerebral cortex nerve synapses, activate glutaminase, enhance the ability of astrocytes on glutamate oxidative metabolism and reduce excitatory toxicity. In addition, dexmedetomidine also inhibits voltage-gated calcium ion channels, thereby inhibiting the release of glutamate. Thus, it is quite possible that dexmedetomidine can be used to treat DNP via inhibiting the release of glutamate, although this has not been reported.

This study aims to explore the influences of dexmedetomidine on the activation of spinal cord glial cells and expression of inflammatory factors in a rat model of diabetes. Results could pave a road for further mechanistic study and clinical applications for dexmedetomidine treatment of DNP.

MATERIALS AND METHODS

Ethics approval and consent to participate

The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, approved protocols of the Institutional Animal Ethics Committee of the Guangzhou Medical University (2016-016).

Animals and experimental groups
Six- to eight-week-old male Sprague-Dawley (SD) rats, weighing 180-200 g, were obtained from the Guangdong Medical Laboratory Animal Center, Foshan, Guangdong, China (Animal license No. SCXK 2013-0002). The animals were housed individually in plastic boxes at a controlled room temperature of 23-25°C. Animals had free access to water and food. Forty-eight rats were randomly assigned into four groups of 12 animals per group, including control group (C group), diabetic neuropathic pain group (DNP group), dexmedetomidine group (DEX group) and yohimbine group (YOH group).

Establishment of the rat model of DNP was performed as previously described 12). Food was removed from rats for 16 h while water was removed for 4 h for all groups prior to injection. The DNP, DEX and YOH groups were injected intraperitoneally with 70 mg/kg of streptozotocin (STZ, Sigma, USA) for one time while C group was given intraperitoneal injection of saline as control. Three days later, rat caudal vein fasting glucose ≥16.7 mmol/L was measured to confirm type I diabetes. Rat mechanical withdrawal threshold (MWT) was measured at day 21 and MWT < 4 g referred to successful establishment of DNP. The DEX group was given daily intraperitoneal injections of dexmedetomidine (50 μg/kg, Jiangsu Hengrui Medicine Co., Ltd., China) from day 28 after STZ injection for 7 consecutive days. The dosage of dexmedetomidine was determined as reported previously 13). The YOH group was given daily intraperitoneal injections of the selective α2-adrenoceptor antagonist yohimbine (0.1 mg/kg, Sigma, USA) followed by the injection of dexmedetomidine (50 μg/kg) 30 min later for 7 consecutive days started from day 28 after STZ injection.

**Behavioral tests**

Mechanical withdrawal threshold (MWT) tests were carried out to assess the response of the
paw to a mechanical stimulus. In brief, rats were placed in a Plexiglas chamber, and the MWT test was performed by stimulating the plantar surface of the left hind paw with von Frey filaments (Stoelting Company, USA). Each von Frey filament was held for a 3-5 s period. Each trial was started with the application of 0.6 g force following consecutive stimuli with an ascending or descending pattern. A positive response was defined as a quick withdrawal of the hind paw upon stimulation. If a positive response was elicited, reduced force was applied. If a negative response was elicited, increased force was applied. Stimuli were continued until the minimal force that evoked withdrawal was identified. A cut off value of 15 g was set. The MWT test was repeated ten times, and average values of the 50% withdrawal threshold were calculated.

In order to measure the pain sensitivity, thermal withdrawal latency (TWL) tests were performed 29-35 days post-STZ injection. A BME-410C full-automatic plantar analgesia tester (Youer Equipment Scientific Co., Ltd., Shanghai, China) was used to measure the sensitivity of the paw to thermal stimuli. Animals were placed on the surface of a 3 mm-thick glass plate covered with a Plexiglas chamber. The radiant heat source was positioned at a fixed distance below the glass plate. Heat stimuli were directed at the exposure site on the left hind paw. TWL was defined as the elapsed time (in seconds) to withdraw the paw from the heat source. Each test session included the delivery of five thermal stimuli at 5 min intervals, and the average latency was calculated. A cut-off time of 60 s was set to avoid tissue damage.

Tissue preparation

After completion of the last behavioral assessment, the rats were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg). The rats were perfused
transcardially with 250 mL of normal saline (n = 7 rats per group). Next, the spinal cord between the L3 and L5 segments was removed. The tissues were stored at -80°C and used for HPLC and Western blotting (n = 3 rats per group). The other tissues were post-fixed in 4% paraformaldehyde for 24 h and embedded in paraffin using routine histological preparation. The paraffin-embedded tissue blocks were horizontally serial sectioned into 5 μm slices with a microtome and mounted on poly-lysine-coated slides for HE staining and TUNEL staining (n = 4 rats per group).

**Immunofluorescent staining of glial cells**

Specimens were fixed with 4% paraformaldehyde at 4°C overnight, dehydrated in 30% sucrose and embedded with optimal cutting temperature (OCT) embedding agent. The tissues were frozen and cut into sections with 10 μm thick, followed by rinsing and blocking. Samples were incubated with mouse-anti-rat microglia Iba1 antibody (1:200, sc-53086, Santa Cruz, USA) and rabbit-anti-rat astrocyte GFAP antibody (1:200, ab7260, Abcam, USA). After washing, samples were incubated with goat-anti-rabbit fluorescein isothiocyanate (FITC) 488-conjugated secondary antibody (1:200, A22110, Abbkine, USA) followed by donkey-anti-mouse AlexaFluor 532-conjugated secondary antibody (1:200, A34213, Abbkine, USA) incubation. After nuclei staining, patching and sealing, the sections were scanned with a confocal laser microscope (Leica SP2, Wetzlar, Germany). Microglia activation index was obtained with the following method: microglia cells at resting state were small, with fine process and shallow dyeing, while microglia cells at activation state were large, with thick process and dense dyeing. Five viewing-fields of high magnification were randomly selected from each section to count total microglia and activated microglia and calculate the activation
rate of microglia (the number of activated microglia / the total number of microglia×100%).

The astrocyte activation index was obtained in similar way: five view fields of high magnification were randomly selected from each section to count total astrocyte and activated astrocyte (as defined for microglial cells) and calculate the activation rate of astrocyte (the number of activated astrocyte / the total number of astrocyte×100%).

**Determination of TNF-α and IL-1 levels**

The levels of TNF-α and IL-1 in the spinal cord were measured by ELISA. The caudal lumbar (L3–5) segments were dissected, ground with a grinder, and homogenized with an ultrasonic tissue homogenizer. Samples were centrifuged and the supernatant was collected for analysis. TNF-α and IL-1 production were evaluated using ELISA kits (Boster Biological Technology Co., Ltd., Wuhan, China). OD values at 490 nm were recorded using a microplate reader (NK3; Ladsystems, Helsinki, Finland). The average levels of TNF-α and IL-1 were calculated based on the standard curves as directed by the kit.

**Sample Preparation for HPLC**

Samples of caudal lumbar (L3–5) segments were weighed and frozen (-80 ºC) until analysis by HPLC. Immediately prior to HPLC, tissue samples were sonicated in 0.1 M HClO₄ solution containing 0.4 mM Na metabisulfite and centrifuged at 12,000 rpm for 5 min at room temperature. Supernatants were filtered (Spartan 3/0.2 PA nylon syringe filter, Schleicher & Schuell) and kept on ice until injection into the HPLC system. Prior to injection into the apparatus, samples (20 µL) were automatically derivatized with orto-phtaldialdehyde (Merck, Darmstadt, Germany) and β-mercaptoethanol (Sigma-Aldrich) in 0.5 M borate buffer with methanol (1:9) and injected with an autosampler (Merck-Hitachi, LaChrom, L-7250).
Measurements of glutamate (Glu) were performed using the LC-2010CHT SHIMADZU system with an ultraviolet detector. The separation was performed using a VP-ODS column and a mobile phase that was a binary eluent of 50 mM CH$_3$COONa (pH 6.5) and methanol under gradient conditions (CH$_3$OH from 26% to 40% during 0.5 h). The column temperature was maintained at 40°C. The flow rate was 1.0 mL/min. All measurements were performed in quadruplicate. Data were collected and processed using Chromatography Data Station Software (version 3.1.1, Merck-Hitachi Model D-7000).

**TUNEL staining**

Samples of caudal lumbar (L3–5) segments were used for apoptosis analysis. After deparaffinization, rehydration and treatment with 3% H$_2$O$_2$ for 10 min at room temperature, sections were incubated with proteinase K (1:200 in Tris-buffered saline (TBS) for 30 min at 37°C. TUNEL staining was performed using the *In Situ* Cell Death Detection Kit (Roche, USA). The sections were then incubated with TdT and dUTP-digoxigenin in a humidified chamber at 37°C for 2 h, followed by three washes in TBS. The sections were then incubated with streptavidinbiotin complex (SABC, Sigma-Aldrich Co. Ltd., USA) at 37°C for 60 min and colorized with DAB for 10 min. Sections were counterstained with hematoxylin, then examined under a light microscope, and analyzed with Image-pro plus 6.0 software. Four sections were selected from each rat in each group. For each section, five fields were randomly selected in the injured area at a high-power field (×400 magnification). For each section, the stained cells were counted, and the apoptotic ratio was calculated according to the following formula: apoptotic ratio = the number of TUNEL-positive cells / the total number of cells. The average of four sections was used for comparison among groups.
Western blotting

To detect the protein expression of cleaved caspase 3 in caudal lumbar (L3–5) segments, total protein was extracted using lysis buffer. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were then blocked and incubated with rabbit monoclonal anti-cleaved caspase 3 antibody (1:500 dilution; Santa Cruz, USA), followed by incubation with goat anti-rabbit IgG secondary antibody (1:2000 dilution; Santa Cruz, USA). β-actin was used as a loading control. The expression bands of target proteins were analyzed by Scion Image software (version 4.0.3; Scion Co., USA). The densitometric values were used to conduct the statistical analysis. The relative protein expression was calculated over β-actin.

Statistical analysis

Analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Values are presented as the mean ± standard deviation (SD) or number appropriate. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed by the least significant difference test. A P value of < 0.05 was considered statistically significant.

RESULTS

Changes of body weight and blood glucose in each group

After injection of STZ, all rats in groups DNP, DEX and YOH showed polydipsia, polyphagia and polyuria. Compared with group C, these rats had significant weight loss and increased fasting blood glucose levels. However, there was no statistical significance between groups DNP, DEX and YOH in body weight and fasting blood glucose (Fig. 1), suggesting that the subsequent changes were unlikely to be caused by blood glucose levels.
Dexmedetomidine increases pain threshold of DNP rats

Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were performed in all groups. Compared with the control group, both MWT and TWL in the DNP group were significantly decreased at every postoperative time point. In contrast, MWT and TWL in the DEX group were elevated at each postoperative time point (P<0.05) (Fig. 2), suggesting that dexmedetomidine may alleviate the pain of DNP rats, probably via activation of α2-adrenoceptor, as blocking this receptor by yohimbine abolished the effect of dexmedetomidine (Fig. 2).

Dexmedetomidine inhibits the microglia activation in the spinal cord rats with DNP

Compared with the control group, rat spinal microglia were significantly activated in the DNP and YOH groups, while the activation index was significantly decreased in the DEX group. No statistically significant difference of astrocyte activation was found among the four groups (Fig. 3), suggesting that dexmedetomidine can effectively inhibit activation of rat spinal microglia but has influence on astrocytes.

Dexmedetomidine inhibits spinal cord inflammation in DNP rats

Compared with group C, the content of TNF-α and IL-1 in the spinal cord was significantly increased in the DNP group. Injection of dexmedetomidine significantly downregulated the concentrations of these two pro-inflammatory cytokines (Fig. 4). However, this protective effect of dexmedetomidine was abolished in the presence of yohimbine (Fig. 4). Our results suggested that dexmedetomidine could effectively inhibit spinal cord inflammation of DNP rats, via activation of the α2-adrenoceptor.

Dexmedetomidine reduces the glutamate levels in the spinal cord of DNP rats
Compared with group C, rat spinal glutamate was elevated in group DNP. Dexmedetomidine abolished this increase even in the presence of yohimbine (Fig. 5), suggesting that dexmedetomidine can reduce the glutamate release in the spinal cord of DNP rats, independent of α2-adrenoceptor activation.

Dexmedetomidine inhibits cell apoptosis in the spinal cord of DNP rats

We further used the TUNEL assay to examine the apoptosis rate of cells in spinal cord. DNP rats showed increased apoptosis rate when compared with group C (Fig. 6). Dexmedetomidine effectively protected cells from apoptosis (Fig. 6, group DEX); however, this protective effect was abolished in the presence of yohimbine (Fig. 6, group YOH). In addition, the expression of cleaved caspase 3 in the spinal cord was elevated in DNP rats, which could be reversed by the treatment of dexmedetomidine (Fig. 7). These results suggest that dexmedetomidine could inhibit the cell apoptosis in the spinal cord of DNP rats via activation of α2-adrenoceptor.

DISCUSSION

Dexmedetomidine acts mainly by activation of α2A adrenergic receptors (α2AR). Three subtypes of α2AR have been identified, including α2A, α2B and α2C. α2A receptors are the major subtype in the brain, associated with a variety of physiological functions such as the regulation of pain, anxiety, sympathetic activity, temperature and behavioral responses. α2B receptors are distributed on vascular smooth muscle and mediate vasoconstriction to control high blood pressure. α2C receptors regulate various behavioral responses including dopaminergic nerve conduction and low temperature. It is possible that the neuroprotective effects of dexmedetomidine are mainly produced by activating α2aR. To support this hypothesis, Ma et al. suggest that dexmedetomidine generates a nerve-protective role by
binding to α2A adrenal receptors, which is reversed by the administration of the corresponding antagonists. However, dexmedetomidine does not play a nerve protective role in transgenic rats with limited α2AR expression, and the possible mechanisms mainly include: reducing the excitatory toxicity of glutamate\textsuperscript{11}, inhibiting catecholamine release, improving blood flow to perfusion of an ischemic area\textsuperscript{16, 17} and regulating neuronal apoptosis\textsuperscript{17}. This study confirmed that intervention with dexmedetomidine suppressed the cell apoptosis in spinal cord of DPN rats. Accumulative evidence indicate that neuropathic pain is pathologically characterized by the neuronal apoptosis and glial activation\textsuperscript{18-20}. Hence, we speculate that majority of TUNEL-positive cells should be neurons, and the administration of dexmedetomidine prevented the apoptosis of neurons in DPN spinal cord. Dexmedetomidine reversed the apoptosis induced by DNP, and the anti-apoptotic effect of dexmedetomidine was antagonized by the α2A receptor antagonist yohimbine. Note that yohimbine slightly antagonized the effects of dexmedetomidine on reversing the c-caspase 3 activation. These data suggest that the intrinsic apoptosis, characterized by the cytochrome c from the mitochondria and caspase 3 activation, may not be the only way which executes cell apoptosis in this model.

Dexmedetomidine can produce analgesia in multiple regions, including the brainstem locus coeruleus and spinal cord\textsuperscript{21}, probably due to its strong anti-inflammatory effect\textsuperscript{22, 23}. This study showed that for chronic neurological pain induced by diabetes, intraperitoneal injection of dexmedetomidine could produce analgesia action, which could be antagonized by yohimbine. Moreover, the occurrence of DPN can be effectively reduced by controlling blood glucose. This study also confirmed that there were no differences of body weight or blood glucose among untreated DNP rats or those treated with dexmedetomidine or yohimbine, but the pain threshold was clearly varied. This observation indicates that dexmedetomidine or yohimbine failed to ameliorate diabetic symptoms.
A large number of experiments have confirmed that glial cells also play an important role in the generation and development of pain\(^{24-26}\). Nerve injury can activate glial cells, which generate and secrete pro-inflammatory factors to trigger consistent neuropathic pain. Activated glial cells produce and release many excitatory neurotransmitters, including cytokines, inflammatory mediators and neural active substances. Excessive accumulation of these substances can sensitize neurons and glial cells, resulting in further development of pathological pain\(^{27}\). This study confirmed the dexmedetomidine can effectively inhibit spinal microglia activation in DNP rats, thus alleviating spinal inflammation, through presynaptic \(\alpha_2\) receptors, which can then be inhibited by yohimbine.

In conclusion, our data confirmed that dexmedetomidine can relieve hyperalgesia and inhibit cell apoptosis in spinal cord of rats with diabetic neuropathy pain. The mechanism may be related to the ability of dexmedetomidine in the inhibition of microglia activation, reduction of inflammatory reaction in the spinal cord, and suppression of glutamate production. However, we could not rule out such ameliorative effects mediated by dexmedetomidine may also be attributed to its peripheral action in addition to its efficacy in the activation of \(\alpha_2\) adrenergic receptor.

Conflicts of interest: The authors declare no conflict of interest
References


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Figure legends

Figure 1. Changes of rat body weight and blood glucose in rats. The body weight (A) and blood glucose (B) in animals was determined on day 21 after intraperitoneal injection of STZ. *P < 0.05 DNP. The groups are control (C), diabetic neuropathic pain (DNP), dexmedetomidine (DEX) and yohimbine (YOH), n=12 for each group.

Figure 2. Mechanical and thermal hypersensitivity in rats. Mechanical and thermal hypersensitivity in animals was determined by measuring the mechanical withdrawal threshold (MWT) (A) and the thermal withdrawal latency (TWL) (B) on day 29 and 35 post-operation, respectively. *P < 0.05 vs. DNP. N=12 for each group.

Figure 3. Immunohistochemical analysis of the microglia and astrocyte activation in the spinal cord. On day 35 post-injection, sections of the spinal cord derived from control (A), diabetic neuropathic pain (B), dexmedetomidine (C) and yohimbine (D) groups, were stained with anti-Iba1 (red) antibody and anti-GFAP (green) antibody. Nuclei were counterstained with DAPI. Representative micrographs were presented. Scale bars, 20 μm. (E) Activation of the percentage of Iba1-labeled microglia. (F) Activation of the percentage of GFAP-labeled astrocytes. N = 4 for each group. *P < 0.05.

Figure 4. The inflammation content of TNF-α and IL-1 levels in the spinal expression. On day 35 post-injection, content of TNF-α and IL-1 in the spinal cord were analyzed by ELISA. (A) ELISA results show content of TNF-α each group; (B) ELISA results show content of IL-1 each groups; N = 4 for each group. *P < 0.05.

Figure 5. Glutamate release in the spinal cord. On post-injection day 35, glutamate release in the spinal cord derived from control (A), diabetic neuropathic pain (B), dexmedetomidine
(C) and yohimbine (D) groups, was analyzed by HPLC. Peak to valley profile was shown. (E) Content of the glutamate release. N = 4 for each group. *$P < 0.05$.

**Figure 6. TUNEL staining of rat spinal tissue slices.** TUNEL staining was performed using the In Situ Cell Death Detection Kit and samples were counterstained with hematoxylin. Representative samples derived from control (A), diabetic neuropathic pain (B), dexmedetomidine (C) and yohimbine (D) groups were shown. Magnification 400x. Scale bars, 50 μm. For each section, the number of positive cells was counted, and (E) the apoptotic ratio was calculated. N = 4 for each group. *$P < 0.05$.

**Figure 7. Expression of cleaved caspase 3 rat spinal tissues.** (A) Proteins were extracted from the C, DNP, DEX or YOH group on day 35 post-injection. Western blotting analysis was conducted using anti-cleaved caspase 3 antibody. Representative results are presented. (B) The relative expression of cleaved caspase 3 was calculated. N = 4 for each group. *$P < 0.05$.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.

A

![Western Blot Image]

- c-caspase 3
- β-actin

B

![Bar Graph]

- cleaved caspase 3/β-actin (fold)
- C, DNP, DEX, YOH
- * indicates significant difference