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Cordycepin rescues lidocaine-induced neurotoxicity in dorsal root ganglion by interacting with inflammatory signaling pathway MMP3

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ABSTRACT

Application of local anesthetic reagents, such as lidocaine (Lid), could cause significant neurotoxicity in spinal cord dorsal root ganglia neurons (DRGNs). In this study, we investigated the potential rescuing effect of cordycepin (CDC) in an *in vitro* explant model of lidocaine-induced apoptosis in DRGNs. Explant of rat neonatal DRGNs was prepared, and treated with Lid *in vitro* to induce neuronal apoptosis. Prior to Lid treatment, DRGN explant was pre-incubated with various concentrations of CDC to evaluate its rescuing effect on Lid-induced apoptosis. We found that, in cultured DRGNs, Lid caused significant neuronal apoptosis whereas pre-incubation of CDC rescued Lid-induced neurotoxicity. In addition, gene and protein expressions of Caspase-9 and Matrix metalloproteinase 3 (MMP3), a key component of inflammatory signal pathway, were both upregulated by Lid but downregulated by CDC pre-incubation. We further overexpressed MMP3 in cultured DRGNs. And discovered that forced MMP3 overexpression upregulated endogenous MMP3 and caspase-9, and reversed the rescuing effect of CDC on Lid-induced neurotoxicity in DRGNs. Therefore, we concluded that CDC has protective effect on local anesthesia induced spinal cord neurotoxicity, possibly through the inverse regulation on inflammatory signaling pathway.

1. Introduction

Both clinical and animal studies have shown that, general anesthesia, though in rare cases, may cause severe and irreversible neural damage in young and developing brains (Davidson, 2011; Karnwal and Lippmann, 2017; Rappaport et al., 2015; Stratmann, 2011). Specifically, local anesthetic reagent, lidocaine (Lid), was demonstrated to induce neurotoxicity in developing or immature neuronal lineage, such as spinal cord dorsal root ganglion neurons (DRGNs), by inducing neuronal apoptosis, neuroinflammation, growth cone collapse and neurite degeneration (Hiruma et al., 1999; Puljak et al., 2009; Radwan et al., 2002b). Although studies had demonstrated that, several molecular signaling pathways, including inflammatory signaling pathways and epigenetic microRNAs may be involved in the genesis or protection of anesthesia-induced neurotoxicity (Li et al., 2015; Puljak et al., 2009), there have been little knowledge on whether pharmaceutical reagents may directly affect or rescue anesthesia-induced neurotoxicity in developing neurons.

Cordycepin (CDC) is a derivative of fungal genus Cordyceps militaris, and has long been an active pharmacological ingredient in traditional Chinese medicine to treat a variety of diseases, such as cancers or inflammatory diseases (Molassiotis et al., 2009; Park et al., 2009; Paterson, 2008; Won and Park, 2005). Specifically, CDC has been shown to yield anti-inflammatory effect by reducing oxidative stress, inhibiting reactive oxygen specie, and regulating key transcriptional factors in inflammatory signaling pathways (Hwang et al., 2008; Won et al., 2009). In neuronal lineage, CDC was shown to protect ischemia/reperfusion neuronal damage in cortical neurons both *in vitro* and *in vivo* (Cheng et al., 2011; Hwang et al., 2008). However, there has been no study on whether CDC has protective effect in anesthesia-induced neurotoxicity.

Gene of matrix metalloproteinase 3 (MMP3) is one of the major component in inflammatory signaling pathway (Parks et al., 2004). In spinal cord DRGNs, MMP3 was demonstrated to be upregulated in an *in vitro* neuropathic pain model and inducing significant macrophage production (Nishida et al., 2008). In another *in vitro* cortical injury model caused by oxygen-glucose deprivation, MMP3 was demonstrated to be inversely regulated by CDC (Cheng et al., 2011). In addition, CDC-induced MMP3 inhibition was shown to reduce oxidative stress, thus rendering significant protective function in inflammatory brain tissues

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(Cheng et al., 2011). Yet, it is unknown whether MMP3 may be associated with CDC to regulate anesthesia-induced neurotoxicity in spinal cord DRGNs.

2. Materials and methods

2.1. Ethic approval

In this work, all protocols were approved by the Animal Research and Ethic Committees in the Cancer Hospital of Jilin Province and Jilin Provincial People's Hospital in Changchun, China. All experiments were conducted in accordance with the guideline of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Cheng et al., 2011).

2.2. Explant of neonatal rat dorsal root ganglion neurons

The method of preparing *in vitro* explant of neonatal rat dorsal root ganglion neurons (DRGNs) was performed according to previous publications (England et al., 1996; Li et al., 2015). Briefly, one-day old Sprague-Dawley rats were killed, and L5-L6 segment of spinal cord dorsal root ganglion was quickly extracted. Tissues were digested in a 0.25% trypsin solution (Gibco, USA) at 37 °C for 30 mins. After stopping digestion with Dulbecco's modified Eagle medium (DMEM, Gibco, USA) plus 10% fetal bovine serum (FBS, Gibco, USA), dorsal root ganglion was centrifuged at 200 × *g* for 5 mins at room temperature. After discarding supernatant, the pellet was re-suspended in neurobasal medium (Gibco, USA) including B-27 serum-free supplement (Gibco, USA) penicillin and streptomycin (Sigma Aldrich, USA), and plated in a 6-well tissue culture plate (VWR, USA) at 37 °C with 5% CO_2. 24 h later, floating non-neuronal cells were discarded. The attached dorsal root ganglion neurons (DRGNs) were lifted off, plated in a new 6-well tissue culture plate in fresh culture medium and maintained for 3 ~ 5 days to be used in further experiments.

2.3. In vitro treatment of cordycepin and lidocaine

DRGN explant was treated with cordycepin (CDC, Sigma Aldrich, USA) and lidocaine (Lid) *in vitro*. Firstly, DRGN explant was incubated with various concentrations of CDC (□M) at 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2 for 24 h. On second day, CDC was removed and 0.2 mM Lid was added into culture for 24 h, according to previous published method (Li et al., 2015). Then, the explant was maintained in regular culture medium, without CDC or Lid, for another 24 h, prior to further assessments.

2.4. Apoptosis assay

The apoptosis of DRGN explant was evaluated using a Click-iT TUNEL Alexa Fluor 594 imaging kit (Invitrogen, USA) according to the manufacturer's instruction. Also, an auto-fluorescent anti-NeuN antibody (Alexa Fluor® 647) (Abcam, USA) was applied to identify nucleus of DRGNs in the explant. After immunohistochemistry was completed, the 6-well tissue culture plate was moved onto an Olympus IX-70 inverted microscope (Olympus, Japan). Fluorescent images were taken using TRITC and Cy5 filters. In each well, apoptotic DRGNs were identified as those positive to both TUNEL and NeuN stainings. Then, non-apoptotic DRGNs were identified as those negative to TUNEL but positive to NeuN stainings. The averaged percentage of non-apoptotic DRGNs for each experimental condition was then calculated.

2.5. RNA extraction and quantitative real-time PCR (qRT-PCR)

For DRGN explant, RNA extraction was performed using a Trizol RNA purification Kit (Qiagen, USA) according to the manufacturer's instruction. Reverse Transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, USA) according to the manufacturer's instruction. Relative mRNA levels of rat Caspase-9 and Matrix metalloproteinase 3 (MMP3) genes were detected using a Power SYBR™ Green PCR Master Mix (Applied Biosystems, USA) (Qiagen, USA) and calculated against internal control of GAPDH gene using the $2^{-\Delta CtCt}$ method.

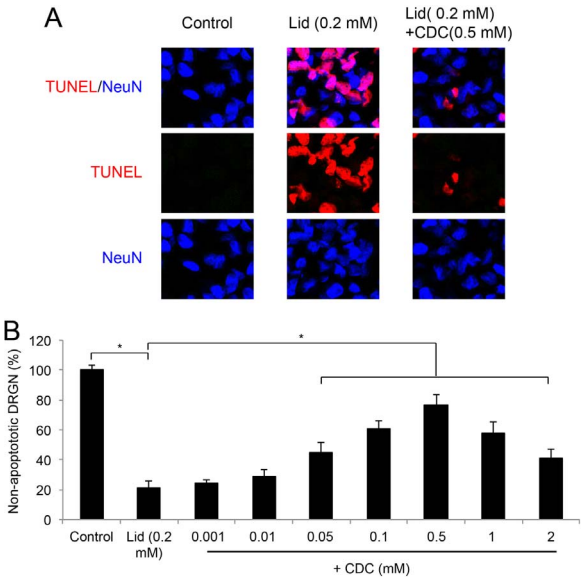

Fig. 1. Effect of cordycepin on lidocaine-induced neuronal apoptosis in DRGNs. (A) Explant of neonatal rat dorsal root ganglion neurons (DRGNs) was prepared *in vitro*. The explant was treated without (Control) or with 0.2 mM lidocaine (Lid) for 2 h. In addition, prior to Lid treatment, DRGN explant was pre-incubated with cordycepin (0.5 mM) for 24 h. Then, 24 h after Lid treatment, Lid-induced neuronal apoptosis was evaluated by TUNEL staining (TRITC/Red). The nucleuses of DRGNs were stained with a NeuN antibody (Cy5/Blue). **(B)** Based on TUNEL immunostaining, the averaged percentages of non-apoptotic DRGNs were calculated for various experimental conditions, including Control, Lid (0.2 mM), Lid (0.2 mM) with additional CDC pre-incubation at concentrations (□M) of 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2 (*, $P < .05$).

2.6. Western blot assay

Cultured DRGNs were collected and digested in a 0.4% NP40 cell lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% Glycerol and protease inhibitor (Roche, USA). Equal protein (30 μg) from each experimental sample was electrophoresed at 120 V for 2 h on a 15% SDS–PAGE gel (Invitrogen, USA) and then transferred to a PVDF membrane. Incubation of primary antibodies, including rabbit-against-rat polyclonal Caspase 9 antibody (1: 500, Abcam, USA) and rabbit-against-rat polyclonal MMP3 antibody (1: 500, Abcam, USA), was conducted at 4 °C for 24 h. After washing 3 times in tris-buffered saline (TBS, Invitrogen, USA), incubation of horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was conducted at room temperature for 2 h. The imaging and densitometric quantification on blots were then conducted using an enhanced chemiluminesence system (Amersham Biosciences, USA) according to the manufacturer's instruction.

2.7. MMP3 overexpression assay

The full sequence of rat MMP3 gene was extracted and amplified using a rat cDNA library. It was then inserted into the XhoII and EcoRV restriction sites of a pcDNA/3.1 overexpression plasmid (Promega, USA) to generate the MMP3 overexpressing vector, pcDNA/MMP3. An empty pcDNA/3.1 plasmid, pcDNA/+, was used as control vector in