Aldehyde dehydrogenase 2 overexpression inhibits neuronal apoptosis after spinal cord ischemia/reperfusion injury

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Graphical Abstract

Aldehyde dehydrogenase 2 effects on apoptosis after spinal cord ischemia/reperfusion injury
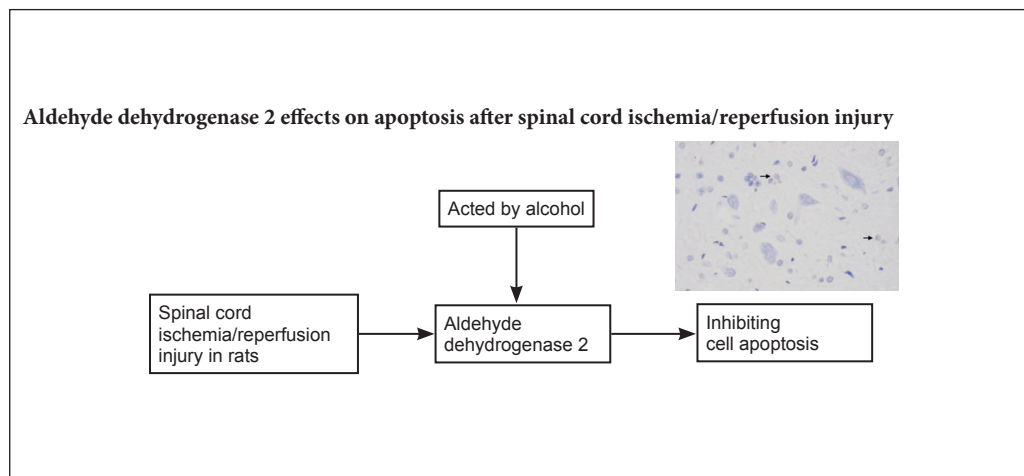
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Abstract

Aldehyde dehydrogenase 2 ($ALDH_2$) is an important factor in inhibiting oxidative stress and has been shown to protect against renal ischemia/reperfusion injury. Therefore, we hypothesized that ALDH2 could reduce spinal cord ischemia/reperfusion injury. Spinal cord ischemia/reperfusion injury was induced in rats using the modified Zivin's method of clamping the abdominal aorta. After successful model establishment, the agonist group was administered a daily consumption of 2.5% alcohol. At 7 days post-surgery, the Basso, Beattie, and Bresnahan score significantly increased in the agonist group compared with the spinal cord ischemia/reperfusion injury group. $ALDH_2$ expression also significantly increased and the number of apoptotic cells significantly decreased in the agonist group than in the spinal cord ischemia/reperfusion injury group. Correlation analysis revealed that $ALDH_2$ expression negatively correlated with the percentage of TUNEL-positive cells ($r = -0.485$, $P < 0.01$). In summary, increased $ALDH_2$ expression protected the rat spinal cord against ischemia/reperfusion injury by inhibiting apoptosis.

Key Words: *nerve regeneration; spinal cord ischemia/reperfusion injury; aldehyde dehydrogenase 2; alcohol; apoptosis; oxidative stress; terminal deoxynucleotidyl transferase dUTP nick-end labeling; neural regeneration*

Introduction

Decompression of a previously severely compressed region of the spinal cord could induce spinal cord ischemia/reperfusion injury (SCII), which could lead to further irreversible postoperative complications, such as paraplegia or paraparesis (Taher et al., 2013; Zhu et al., 2013; Yang et al., 2015). SCII includes two phases: 1) immediate spinal cord injury related to acute ischemia; and 2) delayed spinal cord injury involving ischemic cellular death and reperfusion injury. Although the molecular mechanisms of ischemia/reperfu-sion injury (IRI) pathogenesis remain poorly understood, evidence suggests that oxidative stress caused by excessive production of reactive oxygen species during the IRI process is a critical factor in direct and subsequent cellular damage (Noiri et al., 2001; Walker et al., 2001; Colón and Miranda, 2016). Lipid peroxidation, which is an important source of oxidative stress, is an autocatalytic mechanism resulting in oxidative destruction of cellular membranes associated with production of toxic, reactive, aldehydic metabolites and cell death (Niki et al., 2005). Reactive aldehydes, such

as 4-hydroxy-2-nonenal and malondialdehyde, the major end products of lipid peroxidation, are highly toxic and react with proteins to form various adducts, leading to dysfunctional proteins and subsequent cellular injury (Renner et al., 2005; Conklin et al., 2006; Marchitti et al., 2007). Previous studies of cardiac and renal IRI have suggested that preconditions with the effective activator aldehyde dehydrogenase 2 ($ALDH_2$) or physiological levels of ethanol provide protection for corresponding organs, which consistently correlates with the phosphorylation status of $ALDH_2$, a key metabolic enzyme in the oxidation and detoxification of reactive aldehydes in a range of organs and cell types, to inhibit oxidative stress (Chen et al., 2008; Yuan et al., 2011). $ALDH_2$ is a mitochondrial enzyme and belongs to the ALDH gene family. $ALDH_2$ not only catalyzes oxidation of acetaldehyde to acetic acid in ethanol metabolism, but also acts as a key metabolic enzyme in the detoxification of other reactive aldehydes such as hydroxynonenal (Vasiliou et al., 2005). However, to the best of our knowledge, it is unknown whether $ALDH_2$ has neuroprotective effects by reducing apoptosis under conditions of SCII. The objective of our study was to investigate the early effect of $ALDH_2$ on apoptosis through alcohol administration in rats subjected to SCII.

## Materials and Methods
Animals
Thirty adult, male, Sprague-Dawley rats, aged 8–12 weeks and weighing 250–270 g, were obtained from the Animal Laboratory of Second Military Medical University of China (license number: SCXK (Hu) 2013-0006). The study protocol was approved by the Animal Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University

School of Medicine, China. The experimental procedure followed the the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986), and "Consensus Author Guidelines on Animal Ethics and Welfare" produced by the International Association for Veterinary Editors (IAVE).

The rats were randomly divided into sham operation group (sham group, $n = 10$), SCII group (I/R group, $n = 10$), and SCII with alcohol group (agonist group, $n = 10$). Rats in the sham group underwent surgery without injury. Rats in the I/R and agonist groups suffered from SCII using Zivin's method (Zivin et al., 1980). After successful model establishment, the agonist group received daily consumption of 2.5% alcohol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); the other groups were fed water (30 mL per day) for 7 days post-surgery.

SCII modeling and alcohol intervention
The rat model of SCII was performed employing the previously described Zivin's method (Zivin et al., 1980). All rats were prohibited from drinking during the morning of the surgery. Rats were deeply anesthetized with 3% sodium pentobarbital (1 mL/kg, intraperitoneal injection) and placed in a supine position. After making a 4–5-cm medial incision, the abdominal aorta was exposed at the level of the left renal artery. A total of 400 U heparin was injected 4 minutes before aortic occlusion, and spinal cord ischemia was induced by clamping the aorta with a bulldog clamp just below the left renal artery. After occlusion, femoral artery pulsation ceased, and blood flow was obstructed for 40 minutes. The bulldog clamp was removed, and the abdominal wall was closed. Successful rat model of spinal cord ischemia criteria: cessation of abdominal aorta and double
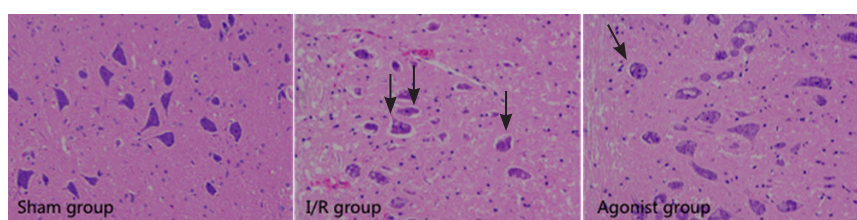
Sham group: Normal spinal cord tissues; I/R group: serious damage; agonist group: mild damage. Arrows refer to injured cells. I/R: Ischemia/reperfusion.

Figure 2 Aldehyde dehydrogenase 2 overexpression affected histomorphology in rats with spinal cord ischemia/reperfusion injury (hematoxylin-eosin staining, ×200).
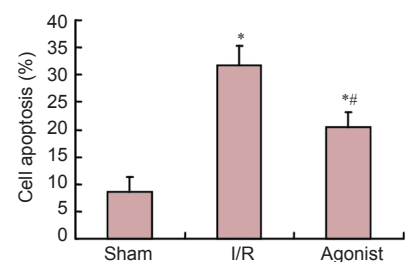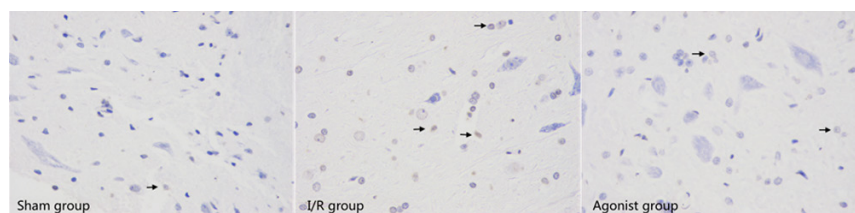

Figure 4 $ALDH_2$ overexpression affected apoptosis in rats with spinal cord ischemia/reperfusion injury (TUNEL assay, × 400).
Sham group: Few TUNEL-positive cells; I/R group: many TUNEL-positive cells (arrows) compared with the agonist group. *$P < 0.05$, *vs.* sham group; #$P < 0.05$, *vs.* I/R group (mean ± SD, $n = 10$, one-way analysis of variance and Student-Newman-Keuls test). $ALDH_2$: Aldehyde dehydrogenase 2; I/R: ischemia/reperfusion; TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling.

hindlimb skin cyanosis. Signs of recanalization blood flow: once the arterial clamp was loosened, abdominal aorta beating recurred and color in the skin of both hindlimbs was bright red.

Motor function assessment

Hind limb motor function assessment was performed at 7 days post-surgery using the Basso, Beattie, and Bresnahan (BBB) motor rating scale (Basso et al., 1995). This BBB scale was based on motor ability following SCII in a rat model. Briefly, the BBB scale was a 22-point scale from 0 to 21. The 0-point indicated no observable hind limb movement, and 21 points indicated consistent and coordinated gait with parallel paw placement of the limbs and consistent trunk stability. Locomotion was scored by two independent observers blind to experimental design.

Sacrifice time

All rats were sacrificed at 7 days post-surgery, and the spinal cords ($L_{2–5}$) were immediately removed to identify medullospinal pathologic changes, examine ALDH2 protein expression, and observe cell apoptosis, respectively, by hematoxylin-eosin staining, western blot assay, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

Hematoxylin-eosin staining

The first segment of the spinal cord ($L_{2–5}$) was dissected and post-fixed in 4% paraformaldehyde overnight. After dehydration, the spinal cords were embedded in paraffin and 5-μm-thick serial coronal sections were collected. To obverse histopathological changes, the sections were further subjected to hematoxylin-eosin staining. Sections (5-μm-thick)
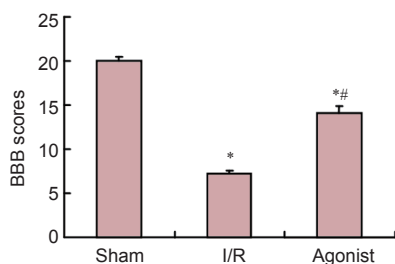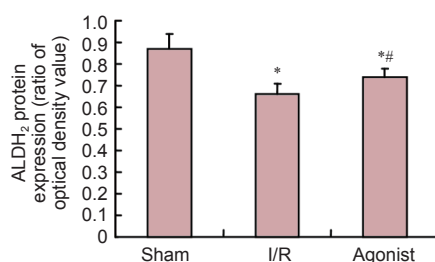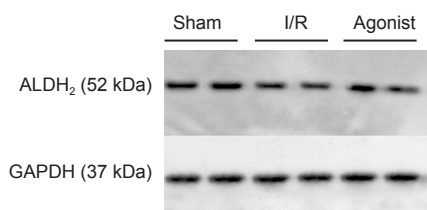

Figure 1 BBB scores in rats at 7 days after surgery.
*$P < 0.05$, *vs.* sham group, #$P < 0.05$, *vs.* I/R group (mean ± SD, $n = 10$, one-way analysis of variance and Student-Newman-Keuls test). BBB: Basso, Beattie, and Bresnahan; I/R: ischemia/reperfusion.

were stained with hematoxylin solution for 5 minutes followed by 5 dips in 1% acid ethanol (1% HCl in 70% ethanol) and then rinsed in distilled water. Afterwards, the sections were stained with eosin solution for 3 minutes, followed by dehydration in an alcohol gradient and cleared in xylene. An independent observer, blinded to the experimental design, collected images using an inverted fluorescence microscope (Nikon Eclipse T3-S, Tokyo, Japan).

Western blot assay

The second of spinal cord ($L_{2–5}$) sample was centrifuged at 1,500 r/min for 30 minutes, after which the supernatant was collected. Determination of protein concentration was performed using the bicinchoninic acid protein assay kit. After transferring 0,15, 30, 60, 120, 180, 240, and 300 μL of bovine serum albumin (500 μg/mL) into each 2-mL centrifuge tube, 1× PBS was added to each tube to reach a volume of 300 μL. The final concentrations of bovine serum albumin in each centrifuge tube were 0, 25, 50, 100, 200, 300, 400, and 500 μg/mL. Working reagent was prepared by mixing 50 parts bicinchoninic acid reagent A with 1 part bicinchoninic acid reagent B (50:1, reagent A:B). The protein extract (20 μL) was placed in an appropriately labeled test tube, and diluted with PBS 30 times. An additional 200 μL of working reagent was added to each tube and mixed well. The tubes were covered and incubated at 37°C for 30 minutes. The spectrophotometer measured at 550 nm and was set to zero using a cuvette filled only with 1 × PBS. Sample absorbance was then measured within 10 minutes. The sample was electrophoresed in a 5% stacking gel with 80 V constant voltage for 20 minutes, followed by 110 V constant voltage for 3.5 hours. A total of 20 μL was resolved on an electrophoresis gel and then transferred onto polyvinylidene difluoride membranes. Primary antibody specific for $ALDH_2$ (goat monoclonal antibody) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted in Tris-buffered saline and Tween 20 (TBST), added to the membrane, and incubated for 60 minutes at room temperature. The membrane was then washed in TBST and incubated in secondary antibody (rabbit anti-goat horseradish peroxidase-labeled polyclonal antibody, 1:10,000; Santa Cruz Biotechnology) for 2 hours at room temperature, followed by TBST washing (3 times) and TBS washing (10 minutes). Protein bands were visualized using enhanced chemiluminescence reagent (Beyotime, Haimen, China), followed by exposure and were photographed to save the experimental results. The ratio of optical density values between $ALDH_2$ and

Protein expression of $ALDH_2$ in the agonist group significantly increased compared with the I/R group. *$P < 0.05$, *vs.* sham group, #$P < 0.05$, *vs.* I/R group (mean ± SD, $n = 10$, one-way analysis of variance and Student-Newman-Keuls test). $ALDH_2$: Aldehyde dehydrogenase 2; I/R: ischemia/reperfusion; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Figure 3 Protein expression in the spinal cord of $ALDH_2$ in the sham, I/R, and agonist groups (western blot assay).

GAPDH bands, which was an indicator of ALDH$_2$ protein expression, was measured using the Gel-Pro analyzer 4.5 (Media Cybernetics, Sarasota, FL, USA).

TUNEL assay

The third spinal cord (L$_{2-5}$) segment was subjected to antigen retrieval proteinase K (Roche) for 30 minutes, and then washed three times with phosphate-buffered saline (PBS). TUNEL solution preparation and staining were performed according to the assay kit instruction manual (Roche, Basel, Switzerland). Cell quantification was obtained by two observers using an inverted fluorescence microscope at 400× magnification. Cell apoptosis (%) was equal to the number of positive cells/(positive cells + negative cells) × 100%.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, IL, USA) and all data are expressed as the mean ± SD. Intergroup differences were analyzed by one-way analysis of variance and Student-Newman-Keuls test. Correlation analysis was examined by Pearson correlation coefficient. Significance was set to $P < 0.05$.

Results

ALDH$_2$ overexpression affected behavior in SCII rats

Rats in the sham group did not show obvious symptoms of neurological impairments. BBB scores in the I/R (7.20 ± 0.40) and agonist groups (14.10 ± 0.80) significantly decreased compared with the sham group (20.00 ± 0.50) ($P < 0.05$), and BBB scores significantly improved in the agonist group compared with the I/R group ($P < 0.05$; **Figure 1**).

ALDH$_2$ overexpression affected histological changes in SCII rats

Hematoxylin-eosin staining results demonstrated well-maintained spinal cord tissues in the sham group, and neurons were morphologically normal with clear karyosomes, uniformly stained cytoplasm, and few vacuolar changes. In the I/R group, the number of normal neurons significantly decreased. Neurons were swollen and pyknotic, and the cytoplasm was stained dark with a great deal of vacuolization. In the agonist group, these pathological changes were markedly reduced compared with the I/R group, and the number of normal neurons was significantly greater than in the I/R group. Most neurons were multipolar with clear karyosomes, with few vacuolar changes (**Figure 2**).

ALDH$_2$ protein expression

ALDH$_2$ protein expression was significantly improved in the agonist group (0.74 ± 0.04) compared with the I/R group (0.66 ± 0.05) ($P < 0.05$; **Figure 3**).

ALDH$_2$ overexpression affected apoptosis in SCII rats

TUNEL staining results demonstrated few TUNEL-positive cells in the sham group (8.47 ± 2.80). The percentage of TUNEL-positive cells was significantly higher in the I/R (31.69 ± 3.66) and agonist groups (20.45 ± 2.72) than in the sham group ($P < 0.05$). However, the percentage of TUNEL-positive cells was markedly decreased in the agonist group compared with the I/R group ($P < 0.05$; **Figure 4**).

Correlation between ALDH$_2$ expression and apoptotic rate

The Pearson correlation coefficient between ALDH$_2$ expression (X) and apoptotic rate (Y) was −0.485 in the three groups. Thus, ALDH$_2$ expression and apoptosis rate were negatively correlated ($P < 0.01$).

Discussion

Although adequate surgical decompression is the preferred treatment for chronic severe compression of the spinal cord and results in favorable surgical outcomes, SCII patients experience unpredictable and disastrous postoperative complications (Taher et al., 2013; Zhu et al., 2013; Yang et al., 2015). Because of the serious consequences of SCII, groups around the world have focused on how to effectively alleviate SCII. The present results demonstrated that moderate alcohol administration following SCII induction provides early protection. After establishment of a rat model of SCII, alcohol-treated rats displayed significantly preserved neurological function characterized by high BBB scores and less histological damage and apoptosis. Results further demonstrated that the protective effect was associated with ALDH$_2$. ALDH$_2$ is a crucial enzyme involved in protecting the IRI by inhibiting oxidative stress. ALDH$_2$ is one of 19 members of the ALDH gene family and is localized within the mitochondria, a major site for reactive oxygen species and reactive aldehyde generation (Vasiliou et al., 2005). ALDH$_2$ not only catalyzes oxidation of acetaldehyde to acetic acid in ethanol metabolism, but also acts as a key metabolic enzyme involved in the detoxification of other reactive aldehydes such as hydroxynonenal (Vasiliou et al., 2005; Chen et al., 2008; Ma et al., 2011; Yuan et al., 2011; Sun et al., 2014). Increasing evidence indicates that ALDH$_2$ plays a protective role in IRI of the heart, kidney, and brain. He et al. (2012) confirmed that the cardioprotective effects of alpha lipoic acid on IRI took place through a mechanism involving ALDH$_2$ activation. Ji et al. (2016) reported that ALDH$_2$ inhibited excessive mitophagy and increased cardiomyocyte following IRI by reducing 4-hydroxy-2-nonenal and reactive oxygen species levels. Yuan et al. (2011) and Zhong et al. (2016) reported that ALDH$_2$ provides protection for kidneys against IRI by enhancing antioxidant capacity and preventing lipid peroxidation. Fu et al. (2014) believed ALDH$_2$ activation could reduce cerebral IRI by decreasing accumulation of reactive aldehydes concomitantly with improvements in brain injury and neurological function. Results from the study clearly showed that increased ALDH$_2$ expression alleviated SCII in rats, which was consistent with the above analysis in other organs. In our study, the agonist group rats, which expressed increased levels of ALDH$_2$, also exhibited significantly improved neurological functions as assessed by BBB scores compared with the I/R group at 7 days post-surgery. Additionally, the pathological changes were markedly reduced,

and the number of normal neurons was significantly greater in the agonist group compared with the I/R group. Most neurons were multipolar with clear karyosomes, and there were few vacuolar changes.

Although the underlying mechanisms involved in $ALDH_2$ protection against IRI, previous studies have shown detoxication of reactive aldehydes, reduced reactive oxygen species generation, and protection of mitochondrial function terminally inhibiting endoplasmic reticulum stress, apoptosis, and autophagy (Ma et al., 2011; Pang et al., 2015). The present study focused on the relationship between $ALDH_2$ and apoptosis during SCII.

Inhibition of apoptosis could reduce the extent of injury and preserve neurological function (Lee et al., 2003; Li et al., 2016), which is closely associated with reactive oxygen species accumulation during IRI (Ebert et al., 2014). The anti-apoptotic enzyme $ALDH_2$ has been shown to be involved in cell apoptosis induced by oxidative stress (Zhang et al., 2011; Fan et al., 2013). Ebert et al. (2014) reported that defective regulation of $ALDH_2$-dependent signaling events induces cardiomyocyte apoptosis resulting from increased 4-hydroxy-2-nonenal and reactive oxygen species. When $ALDH_2$ expression is stimulated, levels of 4-hydroxy-2-nonenal, reactive oxygen species, and apoptosis significantly decrease (Pang et al., 2015). Other studies also confirmed that increased $ALDH_2$ expression induced by alcohol or the effective activator Alda-1 reduces cardiomyocyte apoptosis (Chen et al., 2008; Ge et al., 2012; Gao et al., 2015). Zhong et al. (2016) reported that increased $ALDH_2$ expression reduces renal cell apoptosis during IRI. Following liver injury, decreased $ALDH_2$ expression also leads to apoptosis (Zhong et al., 2016). However, it remained to be shown whether $ALDH_2$ exhibits neuroprotective effects by reducing apoptosis in SCII. Results from the present study clearly showed that increased $ALDH_2$ expression stimulated by alcohol resulted in decreased SCII-induced apoptosis.

Although the molecular mechanisms involved in $ALDH_2$ and apoptosis remain poorly understood, increased $ALDH_2$ expression influences apoptosis, which might be associated with subsequent activation of heat shock protein 70, phosphorylation of c-Jun N-terminal kinase, and inhibition of p53 (Li et al., 2004; Harada, et al., 2005; Sun et al., 2014; Zhong et al., 2016). Further studies are needed to determine the relationship between these molecules.

Our study has several limitations that deserve special attention. First, it is necessary to improve accuracy in this experiment by increasing the number of animal models. Second, to determine long-term neurological protection by $ALDH_2$ through alcohol administration, future studies should increase the observation interval time after establishing the rat model of SCII. Third, this study preliminarily discussed the relationship between $ALDH_2$ and SCII, although further studies are needed to determine the involved mechanisms and contributions of $ALDH_2$ to SCII.

In summary, the neurological effects of alcohol on SCII took place through a mechanism involving, at least in part, $ALDH_2$ activation, which effectively inhibited apoptosis. Results from this study could provide a novel therapeutic target for treating spinal cord injury induced by ischemia/reperfusion.

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