Chinese Journal of Physiology 59(2): 78-86, 2016
DOI: 10.4077/CJP.2016.BAD331

Roles of NAD in Protection of Axon against Degeneration *via* SIRT1 Pathways

Jing Zhang[1, #], Wei-hua Guo[2, #], Xiao-xia Qi[1], Gui-bao Li[1], Yan-lai Hu[1], Qi Wu[1], Zhao-xi Ding[1], Hong-yu Li[1], Jing Hao[3], and Jin-hao Sun[1]

[1]*Key Laboratory of the Ministry of Education for Experimental Teratology and Department of Anatomy, Shandong University School of Medicine*
[2]*Department of Radiology, The Second Hospital of Shandong University*
and
[3]*Department of Histology and Embryology, Shandong University School of Medicine*
Jinan 250012, Shandong, People's Republic of China

Abstract

Axonal degeneration is a common pathological change of neurogenical disease which often arises before the neuron death. But it had not found any effective method to protect axon from degeneration. In this study we intended to confirm the protective effect of nicotinamide adenine dinucleotide (NAD), investigate the optimal administration dosage and time of NAD, and identify the relationship between silence signal regulating factor 1 (SIRT1) and axonal degeneration. An axonal degeneration model was established using dorsal root ganglion (DRG) neurons injured by vincristine to observe the protective effects of NAD to the injured axons. In addition, the potential contribution of the SIRT1 in axonal degeneration was also investigated. Through the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, immunochemistry staining, axons counting and length measuring, transmission electron microscope (TEM) observation, we demonstrated that NAD played an important role in preventing axonal degeneration. Further study revealed that the expression of SIRT1 and phosphorylated Akt1 (p-Akt1) was up-regulated when NAD was added into the culturing medium. Taking together, our results demonstrated that NAD might delay the axonal degeneration through SIRT1/Akt1 pathways.

Key Words: axonal degeneration, DRG, NAD, protection, SIRT1, vincristine

Introduction

Axonal degeneration is a common pathological change of neurogenical disease, which often occurs in diseases such as nerve injury, neurotoxic injury, neurodegeneration and amyotrophic lateral sclerosis (17). As a presymptom, axonal degeneration often arises before the neuron death. In progressively neurodegenerative diseases, axonal degeneration can activate the upstream mechanism of the neuron death, and cause the functional disturbance of neurons (29). Several studies demonstrated that inhibiting or delaying the axonal degeneration might promote the survival and regeneration of neuron and the re-establishment of nerve function (7), suggesting that the methods to prevent or delay axonal degeneration would provide a new therapy strategy for nerve injuries and neurodegenerative diseases.

Increasing evidence has demonstrated that axonal degeneration was not a passive process induced by the loss of the trophic effect from the neuron soma, but a unique self-destructive process (2). The dis-

Corresponding author: Jin-hao Sun, M.D., Ph.D., Key Laboratory of the Ministry of Education for Experimental Teratology and Department of Anatomy, Shandong University School of Medicine, Jinan 250012, Shandong, People's Republic of China. Tel: +86 531 88382093, Fax: +86 531 88382093, E-mail: sunjinhao@sdu.edu.cn
[#]These authors contributed equally to this work.
Received: October 30, 2014; Revised: March 2, 2015; Accepted: April 14, 2015.
 ISSN : 0304-4920. http://www.cps.org.tw

covery of Wallerian degeneration slow mice (Wld^s mice) further evidenced that axonal degeneration was an active process determined by axon immanent procedure. As gene natural mutation mice, when axons of Wld^s mice are cut off, the axonal degeneration time is longer than the normal mice and the axons exhibit prolonged survival (4, 17). Although large amount of works have focused on it, the mechanism of axonal degeneration is still not clear to date. The effective methods of protecting axon and inhibiting degeneration still have not been found yet. Vincristine is a frequently-used cancer chemotherapeutic reagent, which also commonly used to establish axon degeneration model (4, 30).

Since the discovery of the Wld^s mice, the studies of the mechanisms of axonal degeneration focused in them. It was evidenced that the mutant gene of Wld^s mice was mediated by Nmnat1, which was a key enzyme of Nicotinamide Adenine Dinucleotide (NAD) (1). NAD is an essential coenzyme participating in the electron transfer system of the mitochondria and could delay axonal degenerations caused by various neurodegenerative injuries (31). In mammalian cells, NAD is synthesized from multiple precursors, such as tryptophan, nicotinic acid, nicotinamide, and nicotinamide riboside (NmR), and plays determinant roles in a number of biological functions, including cell differentiation, apoptosis and chromatin stability (8, 30). In the mitochondria, NAD involves in energy metabolism, while in the nucleus NAD could regulate DNA repair and transcription (19). NAD also has significant effects on other biology processes, such as antioxygen, oxidative stress, cell senescence and so on (21). Interestingly, it was also reported that exogenous application of the NAD precursors could also delay the axonal degeneration (27). Therefore, much of effort has been focused on the neuroprotective properties of NAD.

Silence signal regulating factor 1 (SIRT1) is an NAD dependent deacetylase participating in the regulation of genetic transcription, energy metabolism and cell aging progress (11, 15). Through deacetylating protein targets, SIRT1 could influence the activity of plenty transcription factors, such as forkhead-box transcription factors (FOXOs), peroxisome proliferator-activated receptor γ (PPAR γ) and nuclear factor-κB (NF-κB), control inflammatory processes, stress responses, apoptosis, axonal degeneration, and delay the development of Alzheimer's disease (AD) (9, 32). But the evidences about whether SIRT1 participates in the protective process of NAD remain poor. Emerging evidence suggests that SIRT1 can modulated protein kinase B (PKB/Akt), a kind of serine/threonine kinase regulating through phosphatidylinositol-3-kinase (PI3K)-mediated signaling, which can inhibit neuron apoptosis (35), accelerate axonal regeneration (24), and promoting axon elongation and branching (20).

Herein, in this study we intend to confirm the axonal protective effect of NAD, investigate the optimal administration dosage and time of NAD, and identify the relationship between SIRT1 and axonal degeneration on an axonal degeneration model established using dorsal root ganglion (DRG) neurons injured by vincristine.

Materials and Methods

Animals and Materials

Polylysine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F12, 1:1), fetal bovine serum were provided by Hyclone Company (Logan, UT, USA). B27 supplement was purchased from Invitrogen (Grand Island, NY, USA); NF200 antibody (mouse anti-NF200) was purchased from Sigma (St. Louis, MO, USA).

De novo Wistar rat aged 1~3 days, were obtained from laboratory animal center of Shandong university (Jinan, Shandong, China). All procedures involving animal handling employed in this study were performed following the Guidelines of the Animal Care and Use Committee of Shandong University and under a valid institutional animal protocol.

Primary Cultures of DRG Neurons

DRG neuron were isolated from *de novo* Wistar rat aged 1~3 days as described previously (13, 16). Briefly, rats were degermed with 75% alcohol and killed by decapitation with sharp scissors. Ganglia were dissected from the thoracic and lumbar regions, plated into 24-well plates coated with poly-D-lysine, and cultured in DMEM/F12(1:1) media with 5% fetal bovine serum, 20 μl/ml 1 × B-27, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C with 5% CO_2 with media change the second day. Nonneuronal cells were removed from the cultures by adding 10 μM cytarabine to the culture media 24 h after incubation.

Establishment of Axonal Degeneration Model

Vincristine sulfate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in culture medium, filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA), and stored at –20°C. After 5 days of culture, DRG neurons were incubated with vincristine at different concentrations (5, 10, and 50 μM) and examined under an invert microscope at 4, 8, 12, and 24 h

after the treatment. In normal control group, DRG neurons were treated with nothing.

Protective Effect of NAD on DRG Neurons

DRG neurons were assigned into five groups and treated [1] with 10 μM vincristine in vincristine injury group [2] with 0.5 mM NAD 24 h prior to treatment with 10 μM vincristine [3] with 1.0 mM NAD 24 h prior to treatment with 10 μM vincristine, [4] with 2.0 mM NAD 24 h prior to treatment with 10 μM vincristine, and [5] with nothing in normal control group. 12 or 24 h later, the DRG neurons in all groups were collected and subjected to various examinations.

In the following experiment, we use 1.0 mM NAD preincubated with 24 h as the protect group.

MTT Assay

DRGs harvested from each rat were treated with 0.25% trypsin for 1 h at 37°C and dissociated physically by pipetting. Cells were resuspended and plated into 96-well plates coated with poly-D-lysine at a density of 3×10^5 cells/cm^2. The plates were placed in a humidified 5% CO_2 incubator, 37°C for 5 days. Then DRG neurons were treated as described above. Viability of DRG neurons was determined using MTT assay. Briefly, DRG neurons in each well of 96-well plates were incubated with 0.5 mg/ml MTT at 37°C for 4 h. After gently aspirating medium, 100 μL of DMSO was added into each well and the plates were shaken at room temperature for 10 min. The absorbance of each well was determined using a microplate reader (Multiskan MK3, Thermo Labsystems, Philadelphia, PA, USA) at a wavelength of 570 nm (A570) to quantify cell viability.

Neurofilament (NF) Immunocytochemical Staining

Immunocytochemical staining was performed according to the manufacturer's instructions. Firstly, cells and spheres attached to coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature. Then the cells were washed with 0.1 M PBS for 5 min and permeabilized with 0.5% Tritonx-100-PBS for 30 min at room temperature. After washed with PBS for 2 times, the cells were blocked with 10% normal serum equinum for 60 min, and incubated overnight with rabbit anti-neurofilament medium NF200 (diluted 1:200, Boster Biological Technology, Wuhan, Hubei, PRC) at 4°C. After three times washes with PBS, cells were treated with fluorescein isothiocyanate (fitc),which was labeled anti-rabbit IgG for 2 h at 37°C, then observed with a Zeiss 780 laser scanning confocal microscope (Carl Zeiss SAS, Jena, Germany).

Measurement of Neurite Number

After NF Immunocytochemical staining, images of the cultured DRG neurons in the normal group, the injury group and NAD protection groups were obtained with an inverted phase contrast microscope. For counting the numbers of survival neurites, six randomly selected fields from three different wells at each time point were observed. The numbers of survival neurites on each coded photomicrographs were measured using IPP image analysis software. The results were analyzed statistically.

Transmission Electron Microscopy (TEM) Morphological Analysis

The DRG cells were scraped, centrifugated at 30,000 g for 10 min at room temperature, and fixed with 2.5% glutaraldehyde for 24 h, then washed with phosphorus acid for three times, followed by fixed with 1% osmium acid for 2 h at 4°C. The cells were dehydrated with ethanol in increments of 10% from 50% up to 90%, for 10 min of each concentration. After these, the specimens were further dehydrated twice with 100% ethanol for 10 min respectively. Following dehydration, the DRG cells were infiltrated with increasing amounts of epon to ethanol (from 1:1 to 3:1), each for 30 min. After imbedded with pure epon, semi- thin slice ultrathin sections were cut and dyed with uranium acetate and lead citrate. The TEM images of the nanocrystals were acquired on a Philips CM electron microscope operating at 10000 kV.

Semiquantitative Reverse Transcripase-Polymerase Chain Reaction (RT-PCR) for Detection of the mRNA Levels of SIRT1

After treated with different drugs for 24 h, the cultured DRG neurons were collected, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Next the same amount of total RNA in each sample was used as the template for cDNA synthesis using cDNA synthesis kit (Takara) according to the manufacturer's instructions. The primers sequence for SIRT1 and β-actin were as follows: SIRT1 5′-ACTGGAAAATAGATTGTCTTC-3′ (antisense) and 5′-TCTGTGATTGCTACACTTGTA-3′ (sense). β-actin 5′-GAGGCGTACAGGGATAGCAC-3′ (antisense) and 5′-GAGGGCATGGGTCAGAAG-3′ (sense). The predicted size of the amplified SIRT1 and β-actin DNA products were 734 bp and 302 bp, respectively.

The PCR was performed at the conditions of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final 50 sec at 72°C. The PCR products were electrophoresed through

a 2% agarose gel. Gels were stained with ethidium bromide, and photographed under ultraviolet light. Then the photographs and electrophoresis gel image were analysized using Totallab image software.

Western Blotting Analysis for Detection of the Protein Levels of SIRT1 and Akt1

The protein levels of SIRT1 at different experimental conditions were analyzed using Western blot assay, with β-actin as an internal control. Western blot was performed using a standard procedure as suggested by Cell Signaling Technology. The dilutions of the primary antibodies were as follows: 1:1,000 for anti-SIRT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies, and 1:1,000 for anti-phophorylated Akt1 (Cell Signaling Technology, Danvers, MA, USA) antibodies.

Statistical Analysis

Results were expressed as means ± S.E.M. for three or more independent experiments. Statistical significance was estimated by analysis of variance (ANOVA) followed by Student-Newman-Keuls test for comparison of several groups. $P < 0.05$ was considered statistically significant.

Results

Establishment of Axonal Degeneration Model

As shown in Fig. 1A, five days after culturing, DRG cells exhibited great quantity axons of different length. The axons appeared straight and upright. While when treated with vincristine of different concentrations (5, 10, and 50 μM), the axons of DRG neurons exhibited varying degrees of damages. Exposed to 5 μM vincristine, the neuron morphology exhibited little changes, while when treated with 10 μM vincristine, the axons started swelling from distal end, then retracting and mutilating after 4 h (Fig. 1B). 8 h after exposed to 10 μM vincristine, the axons were significantly insulted and condensed into different size of separating bullules (Fig. 1C). The axons morphology had severity destruction after 12 h. After exposed to 50 μM vincristine for 8 h, nearly all axons degenerated and disappeared. There were large amount of debris could be found in the medium. Therefore, 10 μM concentration groups have the manifest axonal degeneration.

The Protection of NAD Preventing the Axonal Degeneration

To assess the impact of NAD on vincristine-induced axonal degeneration, the DRG explants were pre-incubated with NAD of different concentrations (0.5, 1.0, and 2.0 mM) for 24 h, before the addition of 10 μM vincristine. 0.5 mM NAD treatment had no obvious protective effect, as the axons swelled, retracted and condensed into many bullules 12 h after adding vincristine. Pre-incubated with 1.0 mM NAD could delay the axonal degeneration. The axons still kept integrity 24 h after adding vincristine with few bullules (Fig. 2B). Interestingly, 2.0 mM NAD exhibited little protective effect. The distal end of the axon suffered little degeneration 12 h after vincristine treatment, but after 24 h the axons started subject to critical breakdown (Fig. 2C), and after 36 h there were nearly no intact structure left. The results of MTT assays further verified that 1.0 mM NAD pre-incubate for 24 h increased cell viability and attenuated vincristine-induced axonal degeneration ($P < 0.01$, Fig. 2D).

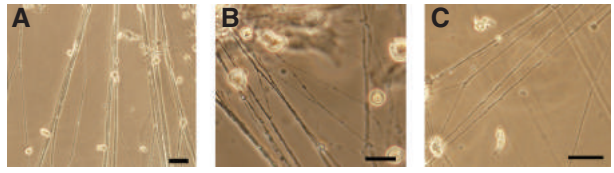

Fig. 1. Vincristine induced damages to DRG neurons. (A) DRG neurons cultured in DMEM/F12 (1:1) media exhibited great quantity axons of different length. The axons appeared straight and upright. (B) Four hours after treated with 10 μM vincristine, the axons started swelling from distal end and bullules could be observed along the axons. (C) Eight hours after exposed to 10 μM vincristine, the axons were significantly insulted and condensed into amounts of separating bullules. Scale bars: A-C, 50 μm.

NAD Antagonizes the Damage of Vincristine on Axon Outgrowth

In order to further detect the growth and degeneration of axons injured by vincristine and confirm the protective effects of NAD, NF200 axonal specific immunofluorescence dyeing (IMF) was performed, and the number of survival neuron axons was measured in each group (Fig. 3). In normal control group, DRG explants gave out a large number of radial projections to the peripheral area. The axons of DRG neurons exhibited straight and upright, and arranged orderly. In the distal segment, axons gathered to form lace-like network (Fig. 3A). When treated with vincristine, the DRG explants sent few axons which were much shorter and rarefaction arranged (Fig. 3B). While pre-incubated with NAD could evidently improve these changes (Fig. 3C). As shown in Fig. 3D, in vincristine injury group the axonal number was obviously decreased as compared with control group ($P < 0.01$).

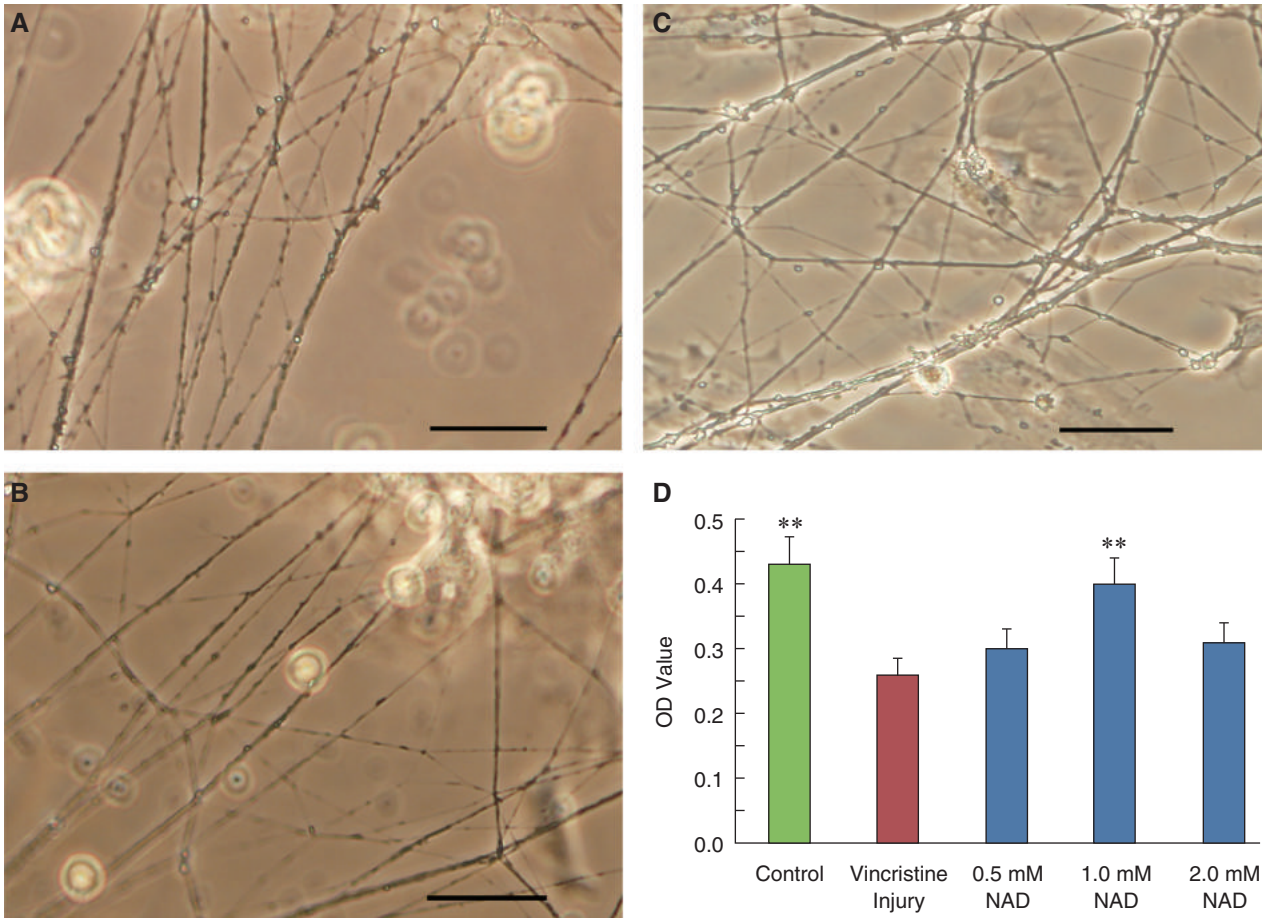

Fig. 2. NAD protected DRG neurons from vincristine induced axonal degeneration. (A) DRG explants of vincristine injury group with amounts of bullules along the axons. (B-C) The DRG explants treated with 1 mM (B), 2 mM (C) NAD, respectively. 1 mM NAD exerted protective effects against vincristine damage. The axons still kept integrity 24 h after adding vincristine with few bullules (B). But 2.0 mM NAD exhibited little protective effect (C). Cell viability was further assessed by MTT assays (D). OD values represent means ± SD from five independent experiments. $^{**}P < 0.01$ *vs.* vincristine injury group. Scale bars: A-C, 100 μm.

While pre-incubate with NAD could enhance axonal number ($P < 0.01$).

The Results of TEM

TEM detection showed that compared with DRG explants five days after culturing (Fig. 4A), the smooth endoplasmic reticulum (SER) of axons exposed to 10 μM vincristine dilated and formed vacuoles, the normal vesicular framework elongated, and formed compact accumulation as the increase of the vacuoles. Meanwhile, the number of normal neurofilament decreased and some of them turned to be granular fragments (Fig. 4B). While pre-incubated for 24 h with 1.0 mM NAD, there were less myelin bodies could be found, the neurofilament arranged parallelly, and axons swelling became lighter (Fig. 4C).

NAD Averts Axonal Degeneration through SIRT1/Akt1 Pathway

To explore the protective mechanisms of NAD against vincristine induced axonal degeneration, Semiquantitative RT-PCR and Western blotting were carried out to detect the expression of SIRT1 in DRG neurons treated with vincristine after pre-incubation with or without NAD. The results of RT-PCR showed that the expression of SIRT1 mRNA was obviously decreased in vincristine injury group, and these decreases were markedly reversed by pre-incubation with NAD (Fig. 5A, $P < 0.01$). Consistent with the change of mRNA, Western blot showed that levels of SIRT1 protein and p-Akt1 were downregulated significantly in DRG neurons treated with vincristine and these decreases were reversed by pre-incubation with NAD (Fig. 5, B, C and D, $P < 0.01$).

Discussion

It had been widely accepted that the self-destruction of axons occur in many physiological and

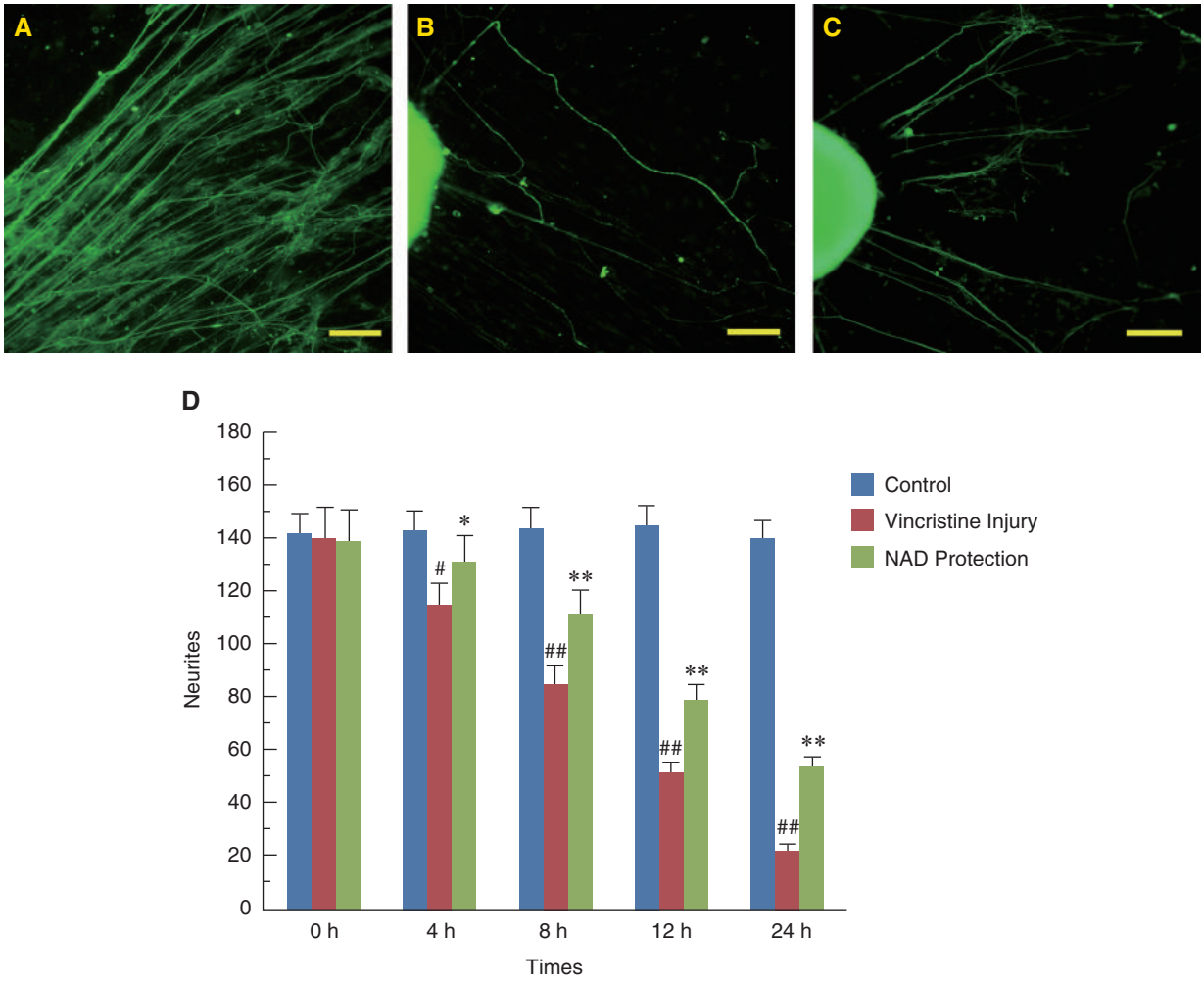

Fig. 3. Effects of vincristine and NAD on neurite outgrowth. (A-C) The photomicrographs of DRG neurons exposed to different drugs with NF200 axonal specific immunofluorescence dyeing. (A) In normal control group, DRG explants gave out large numbers of radial projections to the peripheral area. The axons were straight and upright, and arranged orderly. (B)When incubated with vincristine, the DRG explants sent few axons which were short and arranged sparsely. (C) This negative effect to neurons was dramatically blocked by pre-incubated with NAD. (D) Exposed to vincristine, the axonal number was obviously decreased as compared with control group. While pre-incubate with NAD could enhance axonal number. $^{\#}P < 0.05$ *vs.* control group, $^{\#\#}P < 0.01$ *vs*. control group, $^{*}P < 0.05$ *vs*. vincristine group, $^{**}P < 0.01$ *vs*. vincristine injury group, (n = 5). Scale bars: A-C, 50 μm.

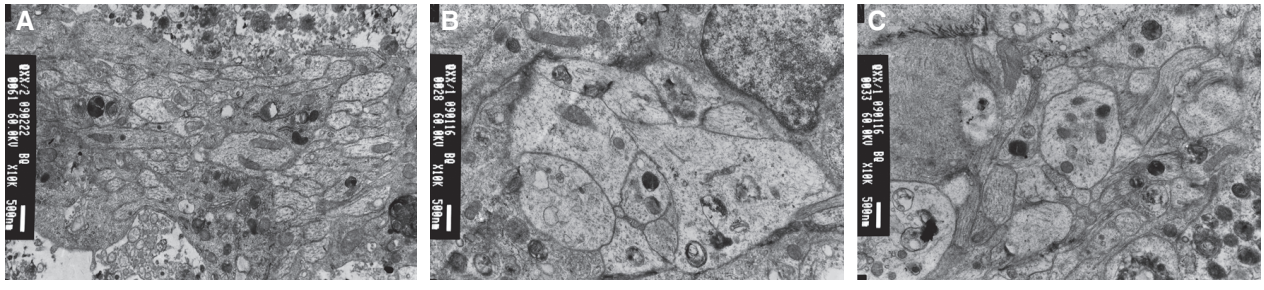

Fig. 4. Changes of the ultrastructures of DRG neurons treated with vincristine and NAD. (A) Five days after culturing, the neurofilament arranged parallelly and organelles could be found in the axoplasm DRG explants in control group. (B) Exposed to 10 μM vincristine, the SER of axons dilated and formed vacuoles, the normal vesicular framework elongated, and formed compact accumulation as the increase of the vacuoles. Meanwhile, the number of normal neurofilament decreased and some of them turned to be granular fragments. (C) 24 h after pre-incubated with 1.0 mM NAD, there were less myelin bodies could be found, the neurofilament arranged parallelly, and axons swelling became lighter.

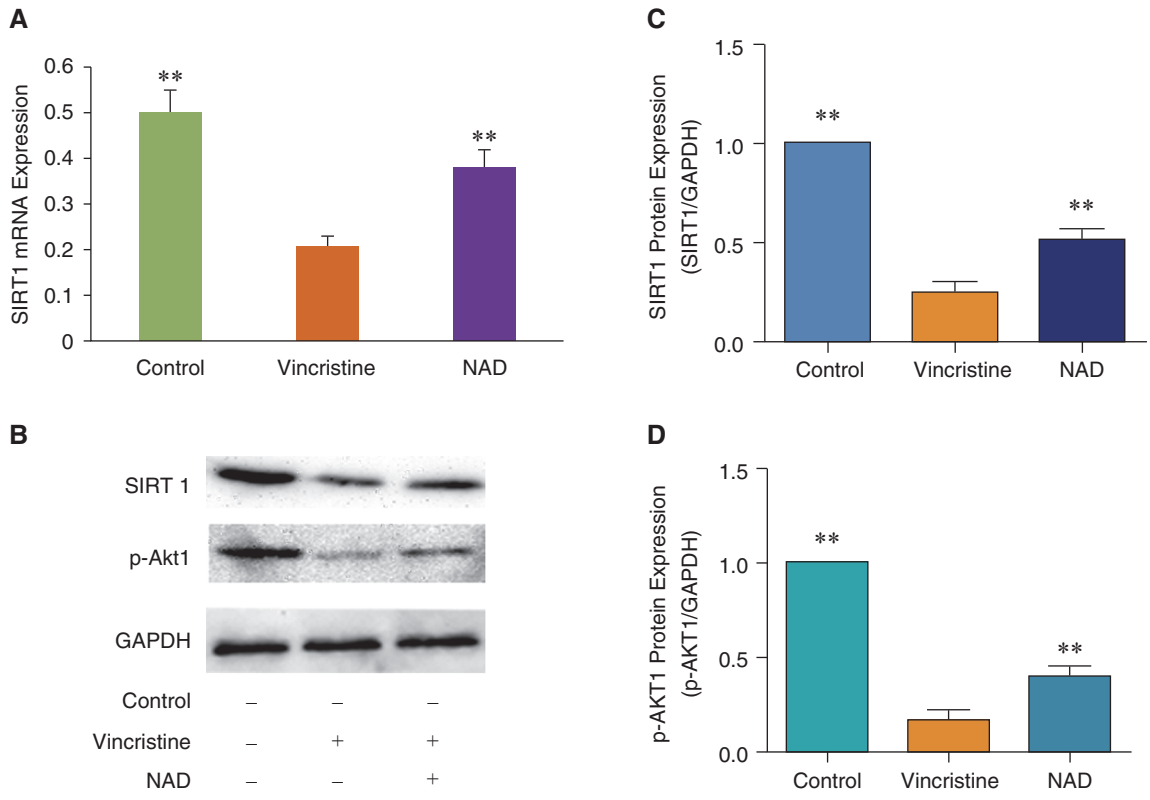

Fig. 5. NAD increased SIRT1 and p-Akt1 expressions against vincristine treatment. Semiquantitative RT-PCR (A) and Western blotting (B, C, D) were used to determine the expression. As shown in A, the mRNA expression of SIRT1 was significantly decreased in neurons treated with vincristine. When pre-incubated with NAD, the down-regulated expressions were markedly reversed. In B, C and D, the levels of SIRT1 protein and p-Akt1 were significantly decreased in neurons treated with vincristine and these decreases were reversed by pre-incubation with NAD. $^{**}P < 0.01$ *vs*. vincristine injury group, (n = 3).

pathological settings, such as during development or after injury (18, 26). This self-destruction is characterized by breakdown of axonal microtubules (MTs) and neurofilaments (NFs), and axonal fragmentation (10). Given NAD^+ could delay axonal degeneration after neuron injury *in vitro* (1, 27). Nevertheless, these observations did not exactly reveal the mechanisms acted in this process. Thus in the present study, we used an axonal degeneration cell model to explore the feasible function of NAD in axonal degeneration and demonstrate that NAD played an important role in preventing axonal degeneration. Further study found that the expression of SIRT1 was up-regulated when NAD was added into the culturing medium. All results indicated that NAD might delay the axonal degeneration through SIRT1 pathways.

Although vincristine is a well-known cancer chemotherapeutic reagent, it also has well-characterized axonal toxicity. Thus previous experiments had used 0.5 μM vincristine for up to 9 days to establish the model of axonal injury (34). In this study we also used vincristine of different concentration to establish axonal degeneration cell model but found that exposed to 5 μM vincristine had little effect on the neuron morphology, even treated for 48 h. We speculate that the difference with previous study might because the time of duration was different. Our results also showed that, when treated with 10 μM vincristine, the axons started swelling from distal after 4 h and after 8 h the axons were significantly insulted and obvious axon loss could be observed. These results indicated that 10 μM vincristine was the suitable concentration, so we established stable axonal degeneration with 10 μM vinblastine.

As metabolic cofactor, NAD, as well as its precursors, its derivatives, and its metabolic enzymes, played central roles in cellular metabolism and energy production (23, 33). It was involved in electron-transport processes of energy metabolism, and could regulate DNA repair and transcription in nucleus (1). Numerous studies have suggested that NAD treatment could prevent not only necrosis but also apoptosis and autophagy (12, 25, 36). In this study, we observed the effect of NAD in vincristine-induced nerve axonal degeneration, and confirmed that NAD treatment could increase cell viability and attenuated the axonal degeneration in a time-dependent manner. We also found that pre-incubate with NAD could promote average

axonal length and enhanced axonal number. NAD is famous for its protective functions, one of which is to delay axonal degenerations caused by various neurodegenerative injuries (31). In neurons, nicotinamide mononucleotide adenylyltransferase 2 (NMAT2), the NAD-synthesizing enzyme, is transported along the axon, and its catalytic activity participates in maintaining axon viability. So synthesis and maintenance of NAD concentrations is crucial to axon integrity (1). It is also becoming clear that NAD is used as a substrate of poly (ADP-ribose) polymerase (PARP) and Sir2, and the levels of NAD can regulate SIRT1 activity (8).

SIRT1, a member of silent information regulator 2 protein family, is a deacetylase whose activity is dependent on NAD and also is regulated by NAD (14, 22). As a 'NAD-consuming' enzyme, SIRT1 is involved in several cellular processes, including lifespan regulation, transcriptional silencing, DNA repair and recombination (8). It was reported that SIRT1 was a critical protein for cell survival and could help cells to resistant to oxidative stress or radiation-induced stress (11). To further explore the underlying mechanisms of NAD against vincristine induced axonal degeneration, the expressions of SIRT1 and its downstream molecule Akt1 were examined in this study. Our results showed that treatment with vincristine obviously reduced SIRT1 and p-Akt1 expressions, while, when pre-incubated with NAD, the down-regulated expressions were markedly reversed. SIRT1 can be activated by several small-molecule compounds, including resveratrol (3), quercetin, curcumin, and catechins (6). Moreover, neurons treated with resveratrol prior to axotomy exhibited a decrease in axonal degradation (1). Another recent study also demonstrated the protective effect of resveratrol against Wallerian degeneration by activating SIRT1 (5). Recent studies demonstrated that SIRT1 could play a critical role in survival of degenerative human nucleus pulposus (NP) cells through activating Akt anti-apoptotic signaling pathway (28). Our finding also indicated that SIRT1 might participate in the axonal protection mediated by NAD through Akt anti-apoptotic signaling pathway.

Although NAD, SIRT1 and Akt1 participate in the axonal degeneration, but the precisely mechanism of NAD-SIRT1-Akt1 pathway has not been shown. Further study about the NAD-SIRT1-Akt1 pathway will help to provide insight into the mechanism of neuropathies.

Acknowledgments

This work was supported by grants of the National Natural Science Foundation of PRC (No. 81371471), Natural Science Foundation of Shandong Province (No. ZR2010HM051, No. ZR2012HM026 and No. ZR2014HM109), Shandong Provincial Science and Technology Development Plan (No. 2011GSF11810).

References

1. Araki, T., Sasaki, Y. and Milbrandt, J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 305: 1010-1013, 2004.
2. Barrientos, S.A., Martinez, N.W., Yoo, S., Jara, J.S., Zamorano, S., Hetz, C., Twiss, J.L., Alvarez, J. and Court, F.A. Axonal degeneration is mediated by the mitochondrial permeability transition pore. *J. Neurosci.* 31: 966-978, 2011.
3. Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., Prabhu, V.V., Allard, J.S., Lopez-Lluch, G., Lewis, K., Pistell, P.J., Poosala, S., Becker, K.G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K.W., Spencer, R.G., Lakatta, E.G., Le Couteur, D., Shaw, R.J., Navas, P., Puigserver, P., Ingram, D.K., de Cabo, R. and Sinclair, D.A. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 444: 337-342, 2006.
4. Belenky, P., Bogan, K.L. and Brenner, C. NAD^+ metabolism in health and disease. *Trends Biochem. Sci*. 32: 12-19, 2007.
5. Calliari, A., Bobba, N., Escande, C. and Chini, E.N. Resveratrol delays Wallerian degeneration in a NAD^+ and DBC1 dependent manner. *Exp. Neurol.* 251: 91-100, 2014.
6. Chung, S., Yao, H., Caito, S., Hwang, J.W., Arunachalam, G. and Rahman, I. Regulation of SIRT1 in cellular functions: role of polyphenols. *Arch Biochem. Biophys.* 501: 79-90, 2010.
7. Coleman, M.P. and Perry, V.H. Axon pathology in neurological disease: a neglected therapeutic target. *Trends Neurosci.* 25: 532-537, 2002.
8. Di Stefano, M. and Conforti, L. Diversification of NAD biological role: the importance of location. *FEBS J.* 280: 4711-4728, 2013.
9. Figarska, S.M., Vonk, J.M. and Boezen, H.M. SIRT1 polymorphism, long-term survival and glucose tolerance in the general population. *PLoS One* 8: e58636, 2013.
10. Griffin, J.W., George, E.B. and Chaudhry, V. Wallerian degeneration in peripheral nerve disease. *Baillieres Clin. Neurol.* 5: 65-75, 1996.
11. Hisahara, S., Chiba, S., Matsumoto, H. and Horio, Y. Transcriptional regulation of neuronal genes and its effect on neural functions: NAD-dependent histone deacetylase SIRT1 (Sir2alpha). *J. Pharmacol. Sci.* 98: 200-204, 2005.
12. Hong, Y., Nie, H., Wu, D., Wei, X., Ding, X. and Ying, W. NAD^+ treatment prevents rotenone-induced apoptosis and necrosis of differentiated PC12 cells. *Neurosci. Lett.* 560: 46-50, 2014.
13. Jackson, J.G., Usachev, Y.M. and Thayer, S.A. Bradykinin-induced nuclear factor of activated T-cells-dependent transcription in rat dorsal root ganglion neurons. *Mol. Pharmacol.* 72: 303-310, 2007.
14. Kauppinen, T.M., Gan, L. and Swanson, R.A. Poly (ADP-ribose) polymerase-1-induced NAD^+ depletion promotes nuclear factor-κB transcriptional activity by preventing p65 de-acetylation. *Biochim. Biophys. Acta* 1833: 1985-1991, 2013.
15. Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Geny, B., Laakso, M., Puigserver, P. and Auwerx, J. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 127: 1109-1122, 2006.
16. Liu, N., Varma, S., Shooter, E.M. and Tolwani, R.J. Enhancement of Schwann cell myelin formation by K252a in the Trembler-J mouse dorsal root ganglion explantculture. *J. Neurosci. Res.* 79: 310-317, 2005.
17. Lunn, E.R., Perry, V.H., Brown, M.C., Rosen, H. and Gordon, S. Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. *Eur. J. Neurosci.* 1: 27-33, 1989.

18. Luo, L. and O'Leary, D.D. Axon retraction and degeneration in development and disease. *Annu. Rev. Neurosci.* 28: 127-156, 2005.
19. Mack, T.G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L., Fernando, F.S., Tarlton, A., Andressen, C., Addicks, K., Magni, G., Ribchester, R.R., Perry, V.H. and Coleman, M.P. Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat. Neurosci.* 4: 1199-1206, 2001.
20. Markus, A., Zhong, J., and Snider, W.D. Raf and akt mediate distinct aspects of sensory axon growth. *Neuron* 35: 65-76, 2002.
21. Massudi, H., Grant, R., Guillemin, G.J. and Braidy, N. NAD^+ metabolism and oxidative stress: the golden nucleotide on a crown of thorns. *Redox Rep.* 17: 28-46, 2012.
22. Min, S.W., Sohn, P.D., Cho, S.H., Swanson, R.A. and Gan, L. Sirtuins in neurodegenerative diseases: an update on potential mechanisms. *Front Aging Neurosci.* 5: 53-62, 2013.
23. Mouchiroud, L., Houtkooper, R.H. and Auwerx, J. NAD^+ metabolism: a therapeutic target for age-related metabolic disease. *Crit. Rev. Biochem. Mol. Biol.* 48: 397-408, 2013.
24. Namikawa, K., Honma, M., Abe, K., Takeda, M., Mansur, K., Obata, T., Miwa, A., Okado, H. and Kiyama, H. Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. *J. Neurosci.* 20: 2875-2886, 2000.
25. Pittelli, M., Felici, R., Pitozzi, V., Giovannelli, L., Bigagli, E., Cialdai, F., Romano, G., Moroni, F. and Chiarugi, A. Pharmacological effects of exogenous NAD on mitochondrial bioenergetics, DNA repair, and apoptosis. *Mol. Pharmacol.* 80: 1136-1146, 2011.
26. Raff, M.C., Whitmore, A.V. and Finn, J.T. Axonal self-destruction and neurodegeneration. *Science* 296: 868-871, 2002.
27. Sasaki, Y., Araki, T. and Milbrandt, J. Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. *J. Neurosci.* 26: 8484-8491, 2006.
28. Wang, D., Hu, Z., Hao, J., He, B., Gan, Q., Zhong, X., Zhang, X., Shen, J., Fang, J. and Jiang, W. SIRT1 inhibits apoptosis of degenerative human disc nucleus pulposus cells through activation of Akt pathway. *Age (Dordr)* 35: 1741-1753, 2013.
29. Wang, J. and He, Z. NAD and axon degeneration: from the Wlds gene to neurochemistry. *Cell Adh. Migr.* 3: 77-87, 2009.
30. Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M.W. and He, Z. A local mechanism mediates NAD-dependent protection of axon degeneration. *J. Cell Biol.* 170: 349-355, 2005.
31. Wang, L., Ding, D., Salvi, R. and Roth, J.A. Nicotinamide adenine dinucleotide prevents neuroaxonal degeneration induced by manganese in cochlear organotypic cultures. *Neurotoxicology* 40: 65-74, 2014.
32. Yang, T. and Sauve, A.A. NAD metabolism and sirtuins: metabolic regulation of protein deacetylation in stress and toxicity. *AAPS J.* 8: E632-E643, 2006.
33. Ying, W. Roles of NAD^+, PARP-1, and Sirtuins in cell death, ischemic brain injury, and synchrotron radiation X-ray-induced tissue injury. *Scientifica (Cairo)* 2013: 691251, 2013.
34. Zhai, R.G., Cao, Y., Hiesinger, P.R., Zhou, Y., Mehta, S.Q., Schulze, K.L., Verstreken, P. and Bellen, H.J. Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. *PLoS Biol.* 4: e416, 2006.
35. Zhang, J., Feng, X., Wu, J., Xu, H., Li, G., Zhu, D., Yue, Q., Liu, H., Zhang, Y., Sun, D., Wang, H. and Sun, J. Neuroprotective effects of resveratrol on damages of mouse cortical neurons induced by β-amyloid through activation of SIRT1/Akt1 pathway. *Biofactors* 40: 258-267, 2014.
36. Zheng, C., Han, J., Xia, W., Shi, S., Liu, J. and Ying, W. NAD^+ administration decreases ischemic brain damage partially by blocking autophagy in a mouse model of brain ischemia. *Neurosci. Lett.* 512: 67-71, 2012.