

博 士 論 文

Neuroprotective effects of food-derived compounds against  
oxidative stress-induced cell death via mitochondrial enhancement

食品由来成分によるミトコンドリア増強作用を介した神経細胞保護効果

指導教員 佐々木 裕子 教授

甲子園大学大学院栄養学研究科

食品栄養学専攻 基礎栄養学部門

学籍番号・氏名 811851 伊藤 章悟

# Contents

|                          | Page  |
|--------------------------|-------|
| Abstract                 | 2-3   |
| 1. Introduction          | 4-6   |
| 2. Materials and Methods | 7-10  |
| 3. Results               | 11-13 |
| 4. Discussion            | 14-17 |
| 5. References            | 18-22 |
| 6. Figures               | 23-28 |

## Abstract

Oxidative stress is involved in most neurodegenerative diseases. Although it has been reported that the NAD<sup>+</sup> precursor nicotinamide mononucleotide (NMN) improves symptoms in some oxidative stress-associated disease models, the detailed mechanisms have yet to be elucidated. In this study, the author hypothesized that NMN attenuates oxidative stress-induced cell damage via sirtuin activation and increased numbers of mitochondria. To investigate whether NMN can protect neuronal cells from oxidative stress via the enhancement of mitochondrial function, the author used PC12 cells treated with 6-hydroxydopamine (6-OHDA) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as oxidative stress models. With regard to cytoprotective effects, pretreatment with NMN significantly reduced cell death induced by these stressors. These protective effects were attenuated by the sirtuin inhibitor, sirtinol, and knock-down of the peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α), which activates mitochondrial biogenesis. To clarify if NMN increased mitochondria, intracellular mitochondria were quantified using cytometric analysis. NMN clearly increased the numbers of intracellular mitochondria, and this increase was attenuated by sirtinol. Moreover, the author quantified the mitochondria-associated proteins Sirt3 and superoxide dismutase 2 (SOD2) and found that treatment with NMN increased these proteins. The author concludes that NMN protects neuronal cells from oxidative stress via sirtuin activation followed by

mitochondrial enhancement.

Keywords: NMN; oxidative stress; mitochondria; sirtuin; PGC1 $\alpha$

## 1. Introduction

Nicotinamide mononucleotide (NMN) is known to be a precursor of nicotinamide adenine dinucleotide (NAD<sup>+</sup>), and to activate sirtuins. NMN is biosynthesized *in vivo*, but also occurs in foods such as broccoli, cabbage and avocado [1]. Administration of NMN to cells activates intracellular sirtuins [2]. Sirtuins belong to the class III histone deacetylase (Hdac) family, and deacetylate not only histones but also some other intracellular proteins, as well as regulating their activities [3,4,5,6]. Thus, NMN affects intracellular signaling via sirtuin activation. Sirtuin activation has been reported to show anti-neurodegenerative functions in both *in vivo* [7] and *in vitro* [8] models. Therefore, the author hypothesized that NMN has a neuroprotective function via sirtuin activation.

In most neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, oxidative stress is involved in neuronal cell death. Many neurodegenerative diseases linked to oxidative stress have a long incubation period, in some cases lasting more than 20 years. However, diagnosis before onset is rarely achieved. Therefore, prevention many years prior to onset is important to reduce the risk of these diseases. One of the candidates for prevention is the ingestion of protective ingredients from food, with dietary components such as polyphenols having been reported to show neuroprotective effects against oxidative stress [9]. These

ingredients are considered to protect neuronal cells mainly via the reduction of reactive oxygen species (ROS). A few polyphenols, such as resveratrol and quercetin, have been reported to protect neuronal cells against oxidative stress [10] and are known to activate sirtuin [11]. This suggests that sirtuin activation could contribute to protection against oxidative stress. However, no neuroprotective function against oxidative stress by NMN has yet been reported. In this study, the author used hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 6-hydroxydopamine (6-OHDA) as oxidative stress models. 6-OHDA is also used as a stressor in Parkinson's disease models [12,13].

Mitochondria are where oxidative metabolism takes place. Mitochondria themselves produce ROS as by-product of their metabolic activity, and excess intracellular ROS are known to damage mitochondria. It was reported that mitochondrial numbers were reduced in neurodegenerative-diseased brain tissue [14]. Studies using a neuronal cell culture model have shown that oxidative stress induces mitochondrial damage [15]. However, mitochondria contain the antioxidant superoxide dismutase 2 (SOD2), which works to reduce ROS. This suggest that if there are sufficient mitochondria for eliminating ROS then this can reduce the risk of neurodegeneration. Therefore, natural products which increase mitochondria and SOD2 could attenuate oxidative stress and reduce the neurodegeneration.

Seven sirtuins (SIRT1–7) have been identified in mammals. Sirtuins belong to the class III Hdac family, which function to deacetylate intracellular proteins and

regulate their activity. PGC1 $\alpha$  is known to be a crucial factor for mitochondrial biogenesis, and its activity is regulated by the acetylation of lysine residues [16]. The cytosolic sirtuin Sirt1 deacetylates PGC1 $\alpha$  and stimulates mitochondrial biogenesis activity [6]. This suggests that Sirt1 activation by NMN induces mitochondrial biogenesis via PGC1 $\alpha$  deacetylation. Another sirtuin, Sirt3, is localized within mitochondria and regulates the activities of mitochondrial proteins by deacetylation. The mitochondrial antioxidative protein SOD2 has been proposed as one of the targets of Sirt3 [17]. Deacetylation of SOD2 by Sirt3 was reported to stimulate antioxidant activity [17], which suggests that Sirt3 activation by NMN increases mitochondrial antioxidant activity via the activation of SOD2.

In this study, the author focused on NMN as an ingredient in food for preventing neurodegenerative diseases caused by oxidative stress, and explored the mechanism for neuroprotection via sirtuin activation.

## 2. Materials and Methods

### 2.1. *Cell Culture*

PC12 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 10 U/ml penicillin, and 100 µg/ml streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. During treatment the cells were cultured in medium without FBS.

### 2.2. *Cell treatment*

PC12 cells were seeded in 96-well plates at a density of 3,000 cells/well. After 24 h culture, the cells were treated with 1 mM NMN ( $\beta$ -Nicotinamide mononucleotide) (Oriental Yeast, Tokyo, Japan) with or without 20 µM sirtinol (Wako, Osaka, Japan), a sirtuin inhibitor, for 2 h at 37°C. Then, an oxidative stressor was added, either 6-OHDA (SIGMA) (final concentration 100 µM) or H<sub>2</sub>O<sub>2</sub> (Wako, Osaka, Japan) (final concentration 50 µM). After incubation for 24 h at 37°C, treated cells were subjected to cell viability assays.

### 2.3. *Cell Viability Assay*



Cell viability was determined using the MTT assay and live/dead cell-staining with calcein AM and propidium iodide (PI, Dojindo, Kumamoto, Japan). Regarding the MTT assay, treated cell-medium was replaced with serum-free medium containing 0.25 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Dojindo, Kumamoto, Japan), and incubated at 37°C for 2 h, and then terminated by adding the same volume of 15% (w/v) sodium dodecyl sulfate (SDS) and 50% (v/v) dimethylformamide in water. After termination, absorbance was measured at 570 nm using a microplate reader (Molecular Devices, San Jose, USA). Data are shown as means  $\pm$  SD. Differences were analyzed using one-way ANOVA followed by a Tukey–Kramer *post hoc* test, with *p*-values <0.05 considered to be significant.

For live/dead cell staining, treated cells were stained with 1  $\mu$ g/ml calcein AM and 1  $\mu$ g/ml PI in medium without FBS at 37°C for 20 min. After staining, cells were observed under a fluorescent microscope (Bio-Rad, Hercules, USA).

#### 2.4. *siRNA transfection*

PC12 cells were transfected with 3 pmol PGC-1 $\alpha$  siRNA (SC-72151, Santa Cruz Biotechnology, Dallas, USA) using 10  $\mu$ l/ml Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific K. K., Tokyo, Japan). After culture for 24 h, the cells were

subjected to treatment.

### 2.5. *Mitochondrial analysis*

PC12 cells were seeded in 6-well plates at a density of 100,000 cells/well. After culture for 24 h, the cells were subjected to treatment. Treated cells were harvested and stained with 2  $\mu$ M 5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimidazolocarboyanine Iodide (JC-1) (SIGMA) at 37°C for 15 min. After incubation, cells were collected and resuspended in serum-free medium and analyzed using a Tali image-based cytometer (Life Technologies Japan Ltd., Tokyo Japan).

### 2.6. *Western blot analysis*

Treated cells were collected and lysed in 1% (v/v) Triton X-100 (MP Biomedicals, Santa Ana, USA) and 1% (v/v) protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) in PBS. The protein concentration was then measured using a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific K. K., Tokyo, Japan). Samples were subjected to 10% or 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After separation, the proteins were transferred electrophoretically

onto polyvinylidene fluoride (PVDF) blotting membrane. The membrane was blocked with 5% defatted milk in Tris-buffer containing 0.05% Tween 20 for 30 min at room temperature. Samples were then incubated at 4°C overnight with anti-SIRT1 (H-300) (1:500 rabbit polyclonal sc-15404, Santa Cruz Biotechnology, Dallas, USA), anti-SIRT3 (1:500 rabbit polyclonal ab75434, Abcam, Cambridge, UK), anti-SOD2/MnSOD (1:1,000 rabbit polyclonal ab13534, Abcam, Cambridge, UK), anti-PGC1 $\alpha$  (1:500 rabbit polyclonal GTX37356, GeneTex Inc., USA), or anti-GAPDH (1:1,000 rabbit polyclonal NB100-56875, Novus Biologicals, Centennial, USA) primary antibodies. Afterwards, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody anti-rabbit immunoglobulins from swine (1:1,000 polyclonal P0399, Dako, Santa Clara, USA). The bands were developed using ECL reagent (GE Healthcare Japan Ltd., Tokyo Japan) and analyzed using a C-DiGit Blot Scanner (LI-COR, Lincoln, USA). Data are shown as means  $\pm$  SD (n=3).

## 2.7. Statistical analysis

Data are shown as means  $\pm$  SD. Differences were analyzed using one-way ANOVA followed by a Tukey–Kramer *post hoc* test, with *p*-values <0.05 considered to be significant.

### 3. Results

#### 3.1. *NMN protects PC12 cells against H<sub>2</sub>O<sub>2</sub> and 6-OHDA-induced oxidative stress*

The author investigated whether NMN can protect PC12 cells from oxidative stress. PC12 cells were pretreated with 1 mM NMN for 2 h, and then an oxidative stressor was added (50  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 100  $\mu$ M 6-OHDA) for 24 h. After treatment, the number of live cells was quantified using an MTT assay. Both stressors were found to induce cell death, but NMN-pretreatment significantly reduced cell death (Fig. 1A). A live/dead cell-staining assay showed that oxidative stressors increased PI-positive dead cells, and that NMN-pretreatment clearly decreased the ratio of dead cells (Fig. 1B). These data indicate that NMN reduced both H<sub>2</sub>O<sub>2</sub> and 6-OHDA-induced cell death.

#### 3.2. *PC12 cells express Sirt1 and PGC1 $\alpha$ , and their expression is increased by NMN*

Before studying the contribution of Sirt1 and the mitochondrial biogenesis inducer PGC1 $\alpha$ , the author elucidated the expression of Sirt1 and PGC1 $\alpha$  in PC12 cells. As shown in Fig. 2, the PC12 cells used in this study expressed both. The author found that NMN treatment increased the expression of both Sirt1 and PGC1 $\alpha$ . This indicates that NMN not only activates Sirt1 followed by PGC1 $\alpha$  but also increases the expression of

both Sirt1 and PGC1 $\alpha$ , suggesting that NMN has a strong enhancing effect on Sirt1 and PGC1 $\alpha$  signaling.

### *3.3. Protective effects of NMN against oxidative stress were sirtuin activation-dependent*

To determine whether the protective effects of NMN were sirtuin activation-dependent, the author performed a PC12 cell-protection assay in the presence of the sirtuin inhibitor sirtinol. PC12 cells were pretreated with 1 mM NMN for 2 h in the presence or absence of 20  $\mu$ M sirtinol, then oxidative stressors (50  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 100  $\mu$ M 6-OHDA) were added and incubated for 24 h. After treatment, the number of live cells was quantified using an MTT assay. In the presence of sirtinol, the cell-protective effects of NMN against both H<sub>2</sub>O<sub>2</sub> and 6-OHDA were abolished (Fig. 3), indicating that the protective effect of NMN was sirtuin activation-dependent.

### *3.4. Protective effects of NMN against oxidative stress were PGC1 $\alpha$ activation-dependent*

To determine whether the protective effects of NMN were PGC1 $\alpha$  activation-dependent, the author performed a PC12 cell-protection assay using PGC1 $\alpha$  knock-down PC12 cells. PC12 cells were transfected with PGC1 $\alpha$  siRNA, incubated for 24 h, and then

subjected to a cell-protection assay. In siRNA-transfected cells, the protective effect of NMN against H<sub>2</sub>O<sub>2</sub> and 6-OHDA toxicity was abolished (Fig. 4), indicating that the protective effect is PGC1 $\alpha$  activation-dependent. This also suggests that mitochondrial biogenesis is involved in the protective effect.

### *3.5. NMN increased mitochondria in the presence or absence of oxidative stress via sirtuin activation*

PC12 cells were treated with NMN and oxidative stressors as a cell-protection assay in the presence or absence of sirtinol. Treated cells were harvested, stained, and subjected to cytometric analysis. As shown in Fig. 5, NMN increased the amount of mitochondria, but this effect was abolished by sirtinol, indicating that NMN increases mitochondrial numbers via sirtuin activation. This increase was observed even in the presence of both oxidative stressors (Fig. 5), suggesting that the increase in mitochondria is involved in the protective effect against oxidative stress.

The increase in mitochondria was also demonstrated by the expression levels of mitochondrial sirtuin Sirt3 and the mitochondrial antioxidative protein SOD2. As shown in Fig. 6, NMN treatment increased the expression of both proteins. Since Sirt3 can activate SOD2 [17], it is likely that NMN treatment not only increases the number of mitochondria but also enhances mitochondrial antioxidant activity.

#### 4. Discussion

NMN, a precursor of NAD<sup>+</sup>, was reported to have a protective role against amyloid- $\beta$  toxicity and cognitive impairment [18]. Oxidative stress has been linked to neurodegeneration in many neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. However, few studies have investigated whether NMN can protect neuronal cells from oxidative stress. Since NMN was reported to activate Nrf2 signaling, which is involved in cell protection, in an *in vivo* brain injury model [19], it seems likely that NMN would also protect neuronal cells against oxidative stress. In this study, the author focused on the protective function of NMN against oxidative stress-induced neurotoxicity. The author used H<sub>2</sub>O<sub>2</sub>- or 6-OHDA-induced cell death in PC12 cells as an oxidative stress-induced neurodegeneration model; 6-OHDA is also used as a stressor in Parkinson's disease models [12, 13].

First, the author analyzed the protective effects of NMN against H<sub>2</sub>O<sub>2</sub> and 6-OHDA toxicity. NMN significantly suppressed cell death induced by these oxidative stressors (Fig. 1). Although NMN can be converted to NADPH, which is known to be a cofactor of ROS-reducing proteins, such as thioredoxin and glutathione reductase, the author focused on the effects of NMN via sirtuin activation because the cytosolic

sirtuin Sirt1 was reported to suppress oxidative stress-induced cell damage [11]. Thus, the author analyzed whether this protective effect was sirtuin activation-dependent. In the presence of the sirtuin inhibitor sirtinol, the protective effect of NMN against both  $H_2O_2$  and 6-OHDA was abolished (Fig. 3). Since NMN is a precursor of  $NAD^+$ , the data suggest that  $NAD^+$  converted from NMN directly activates sirtuins followed by cell-protective signals. In addition, NMN treatment also increased the expression of Sirt1 (Fig. 2). This indicates that NMN strongly activates cellular Sirt1 activity not only via direct activation but also by increasing its expression.

Regarding the antioxidant function of Sirt1, Sirt1 activity was reported to be associated with mitochondrial biogenesis [20]. In some oxidative stress-induced cell death, mitochondrial damage was reported [21]. Since mitochondrial damage is known to cause cell death [22], any treatment that protects or enhances mitochondrial function should protect cells from oxidative stress. Sirt1 was reported to activate the mitochondrial biogenesis factor PGC1 $\alpha$  via its deacetylating activity [6]. Thus, the author analyzed the contribution of PGC1 $\alpha$  activation to the protective effects of NMN. In PC12 cells administered with PGC1 $\alpha$  siRNA, the protective effect of NMN against both  $H_2O_2$  and 6-OHDA was abolished (Fig. 4). In addition, NMN increased PGC1 $\alpha$  expression in PC12 cells. These data indicate that NMN stimulates mitochondrial biogenesis via the upregulation of PGC1 $\alpha$ , followed by the activation



of PGC1 $\alpha$  via Sirt1 activation. This has the effect of preventing a reduction in mitochondria caused by oxidative stress and helps to protect cells. Some polyphenols, such as resveratrol and quercetin, were reported to protect neuronal cells against oxidative stress [10] and to activate sirtuin [11]. Another, epigallocatechin-3-gallate (a green tea catechin), has been reported to activate Sirt1 [23], suppress oxidative stress, and increase the amount of PGC1 $\alpha$  mRNA in PC12 cells [24]. Thus, it is suggested that NMN could protect cells via similar mechanisms to food ingredients, such as epigallocatechin-3-gallate.

Next, the author investigated whether NMN treatment increased the number of mitochondria, using cytometric analysis. We found that NMN treatment clearly increased the number of cells containing high numbers of mitochondria, and that this effect was sirtuin activation-dependent (Fig. 5). In mitochondrial biogenesis, PGC1 $\alpha$  is known to be crucial [16]. PGC1 $\alpha$  is activated via Sirt1 activation [6], and the data suggest that Sirt1 activation following NMN treatment activates PGC1 $\alpha$ , which in turn induces mitochondrial biogenesis in PC12 cells. In addition, the expression of the mitochondrial proteins Sirt3 and SOD2 was also increased by NMN treatment (Fig. 6), supporting the cytometric analysis data. Mitochondrial Sirt3 can also be activated by NAD<sup>+</sup>, and SOD2 is activated by Sirt3 via deacetylation [16]. SOD2 is known to reduce intracellular ROS. This indicates that NMN-induced activation of Sirt3, followed by SOD2 activation, enhances mitochondrial antioxidant

activity. The increase and activation of SOD2 contributes to the protective effects of NMN against oxidative stress.

This study demonstrated the antioxidant effects of NMN via sirtuin activation and mitochondrial enhancement. The data for this study suggest that NMN or other molecules that activate sirtuins can, via mitochondrial enhancement, reduce the risk of neurodegenerative diseases. Furthermore, it is anticipated that the consumption of sirtuin-activating food ingredients, including NMN, could be a novel means of preventing neurodegenerative diseases caused by oxidative stress.

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## 6. Figures

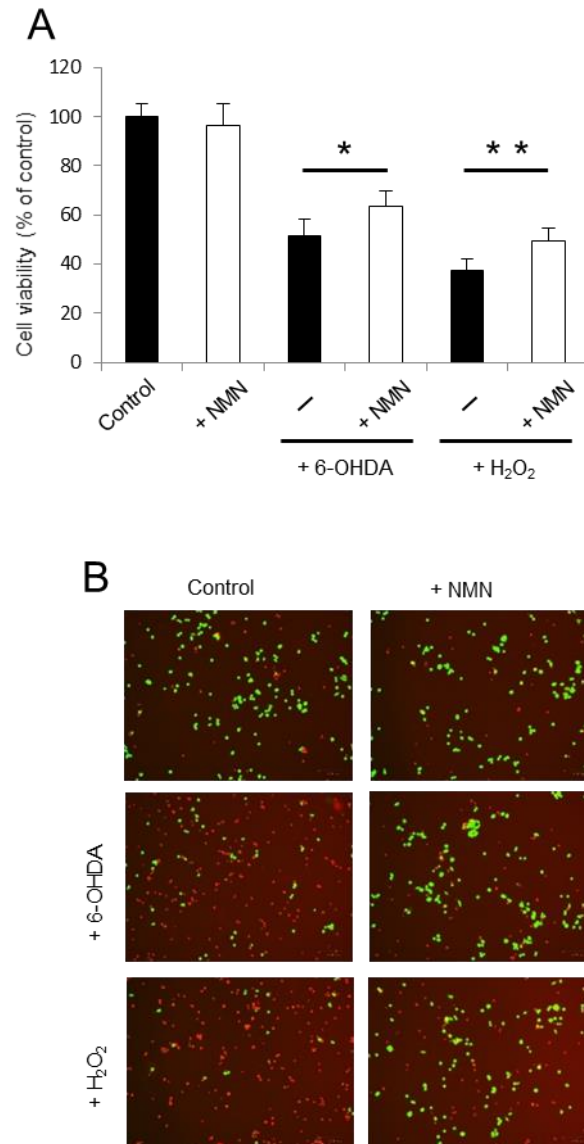


Fig. 1. **Protective effects of NMN against 6-OHDA or H<sub>2</sub>O<sub>2</sub> toxicity.** PC12 cells were pre-treated with NMN for 2 h, then 6-OHDA or H<sub>2</sub>O<sub>2</sub> was added and cells were incubated for 24 h. Cell viability was analyzed using an MTT assay (A). Data are presented as means  $\pm$  SD (n=6). \* $P$ <0.05, \*\* $P$ <0.01. For live/dead cell-staining, treated cells were stained with calcein AM and PI, and observed under a fluorescent microscope (B).



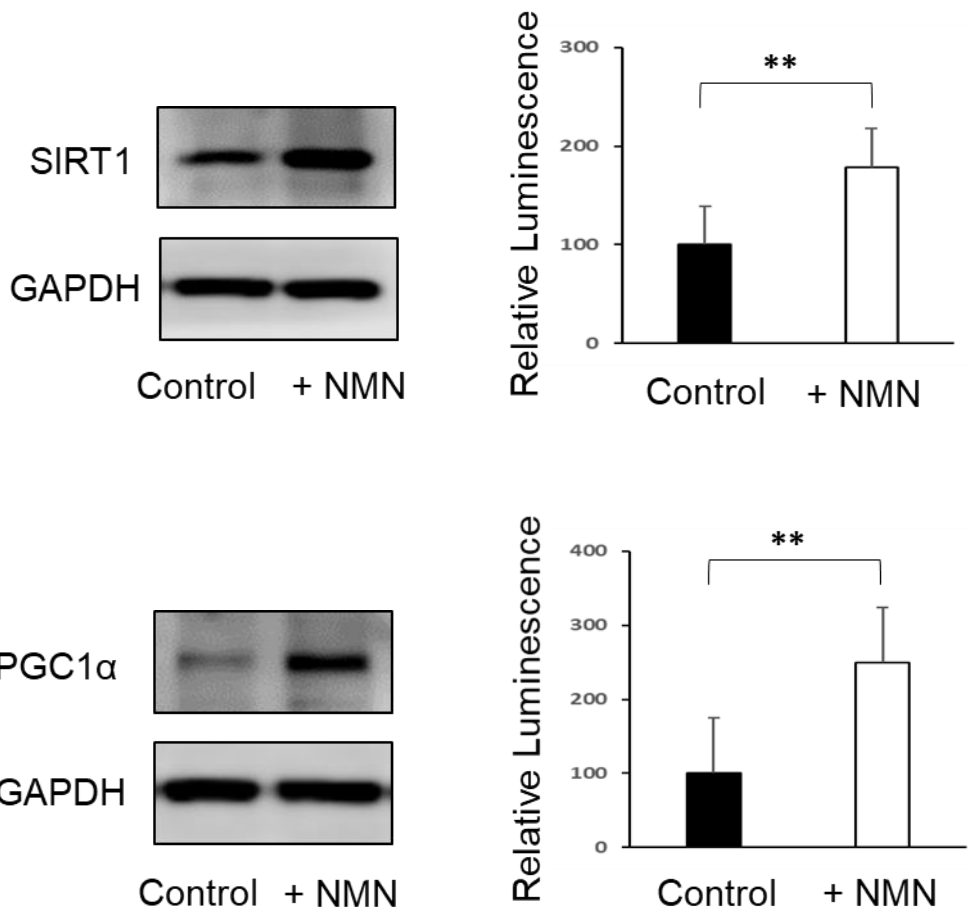
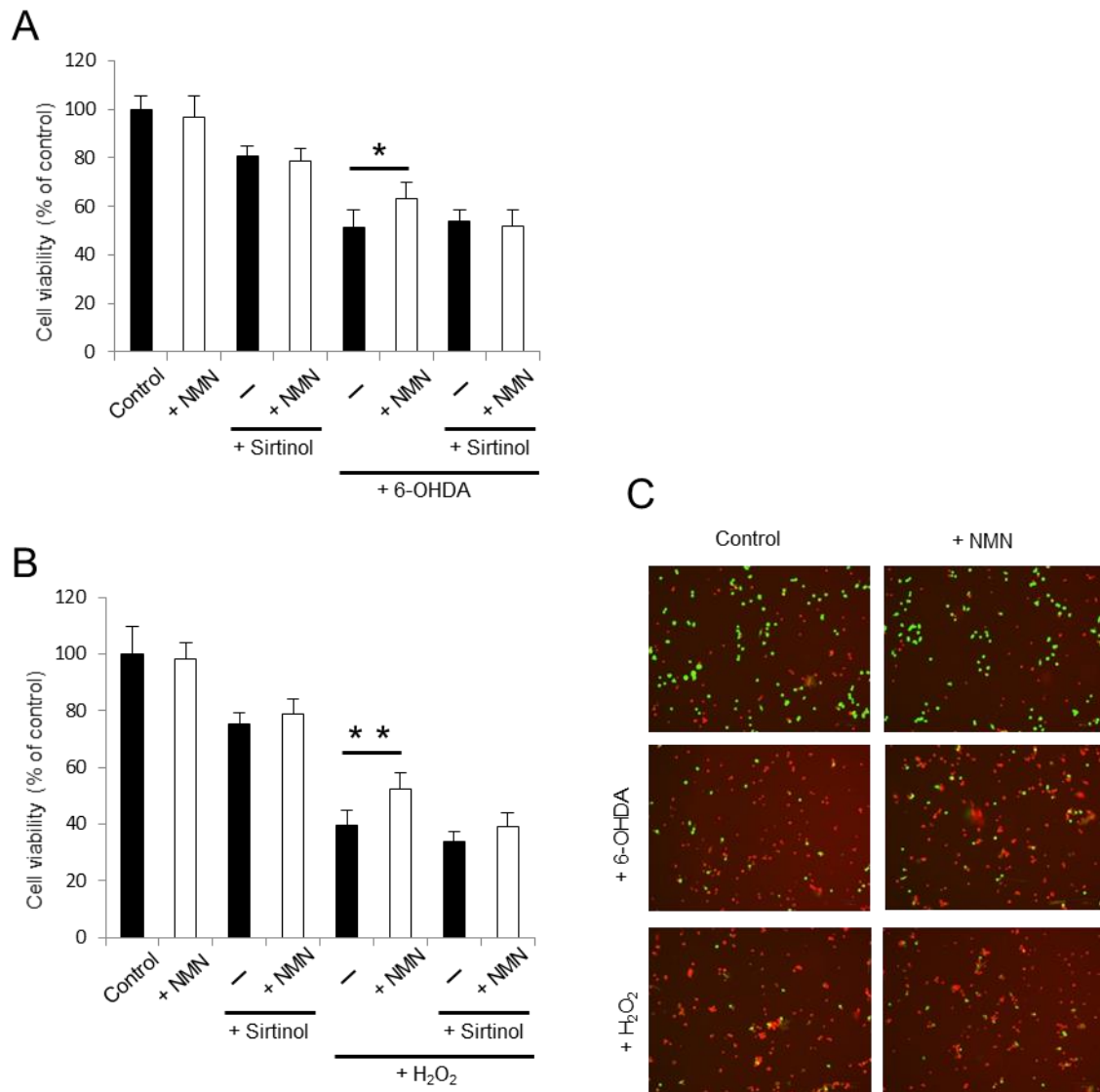
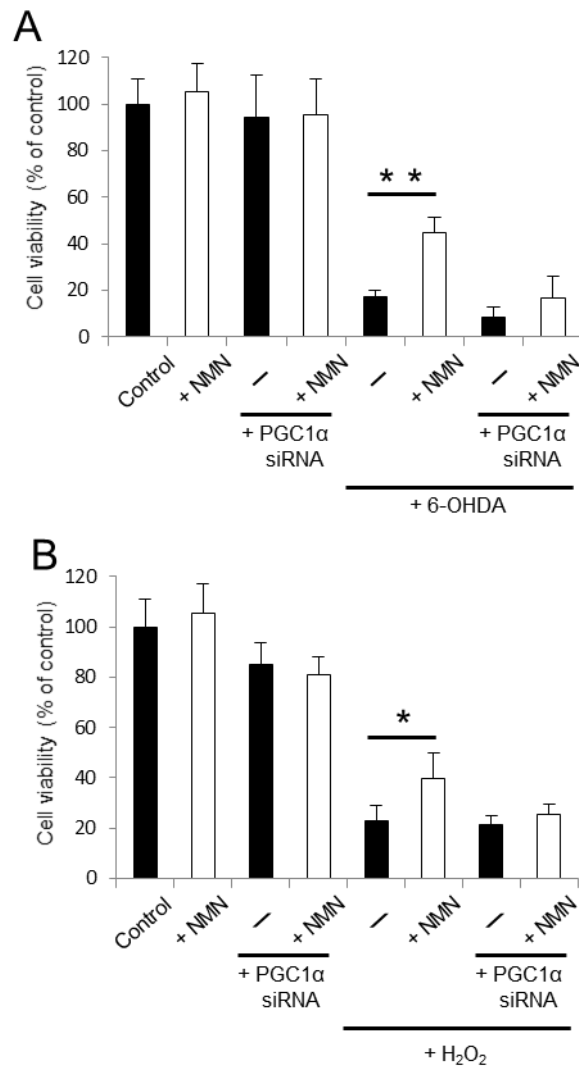


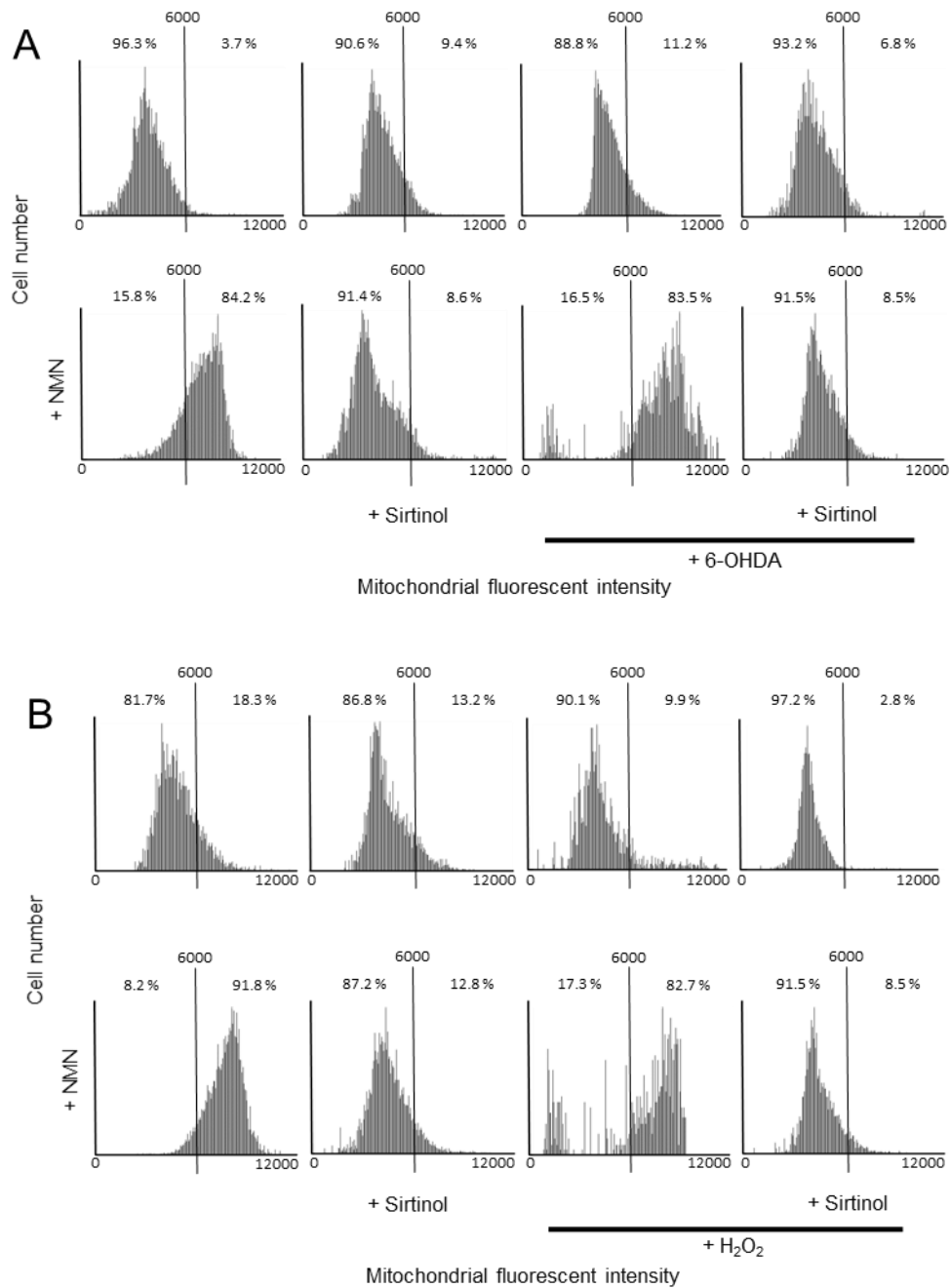
Fig. 2. **Effect of NMN on SIRT1 and PGC1α expression.** PC12 cells were treated with 1 mM NMN. After incubation for 24 h, cells were subjected to Western blot analysis. Expression levels were quantified by the intensity of ECL luminescence. Data are presented as means  $\pm$  SD (n=3).



**Fig. 3. Effect of sirtuin inhibition on the protective effects of NMN against 6-OHDA or H<sub>2</sub>O<sub>2</sub> toxicity.** PC12 cells were pre-treated with NMN for 2 h in the presence or absence of sirtinol, then 6-OHDA (A) or H<sub>2</sub>O<sub>2</sub> (B) was added and cells were incubated for 24 h. Cell viability was analyzed using an MTT assay. Data are presented as means  $\pm$  SD (n=6). \* $P$ <0.05, \*\* $P$ <0.01. For live/dead cell-staining, treated cells were stained with calcein AM and PI, and observed under a fluorescent microscope (C).



**Fig. 4. Effect of PGC1 $\alpha$  knock-down on the protective effects of NMN against 6-OHDA or H<sub>2</sub>O<sub>2</sub>.** PC12 cells were transfected with PGC-1 $\alpha$  siRNA and incubated for 24 h. Then, the cells were treated with NMN for 2 h, 6-OHDA (A) or H<sub>2</sub>O<sub>2</sub> (B) was added, then cells were incubated for 24 h. Cell viability was analyzed using an MTT assay. Data are presented as means  $\pm$  SD (n=6). \* $P$ <0.05, \*\* $P$ <0.01



**Fig. 5. Effect of NMN on the number of intracellular mitochondria in the presence or absence of 6-OHDA or H<sub>2</sub>O<sub>2</sub>, and their sirtuin dependence.** PC12 cells were treated with NMN in the presence or absence of sirtinol for 2 h, 6-OHDA (A) or H<sub>2</sub>O<sub>2</sub> (B) was added, then cells were incubated for 24 h. Mitochondria were stained with JC-1 and analyzed using image-based cytometry.

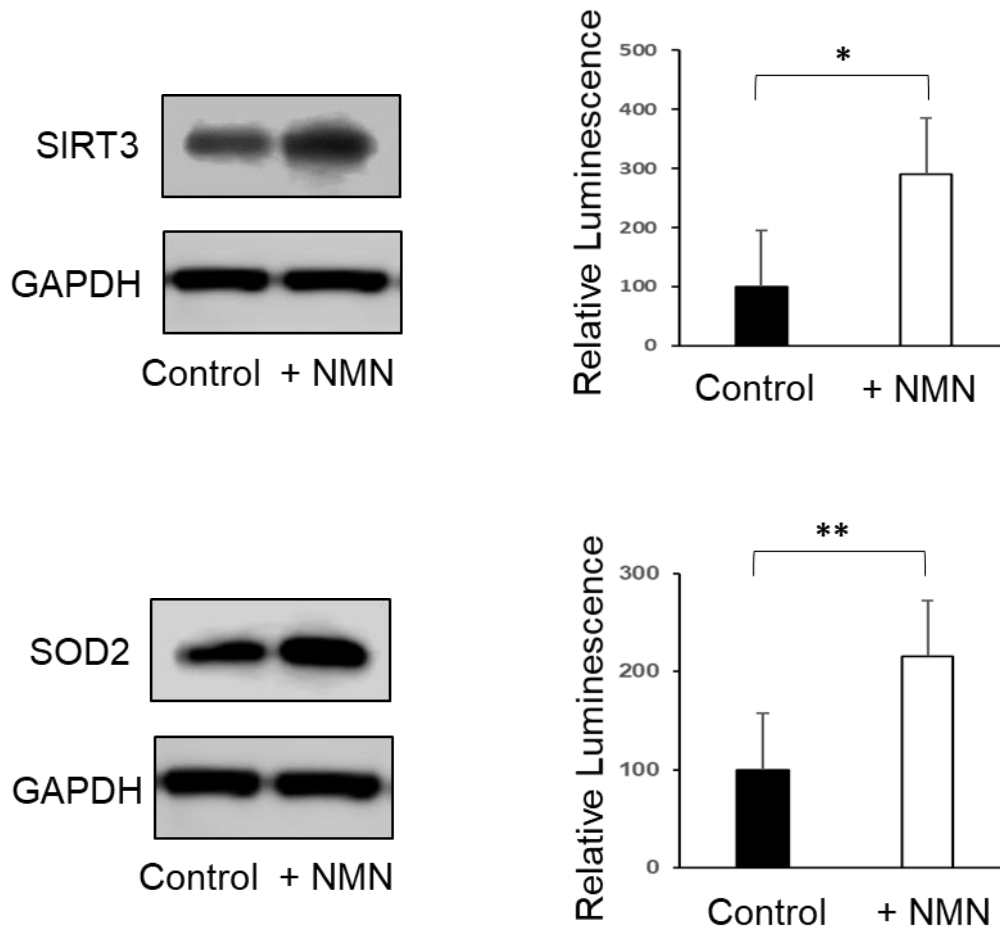


Fig. 6. **Effect of NMN on mitochondrial sirtuin and SOD2 expression.** PC12 cells were treated with NMN and subjected to Western blotting analysis. Expression levels were quantified by the intensity of ECL luminescence. Data are presented as means  $\pm$  SD (n=3).