

# Anti-oxidative effects of nicotinamide mononucleotide, a regulator of aging, on rat high glucose-induced tenocytes in vitro

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## Research article

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# Abstract

**Background** Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) plays an important role in energy metabolism, mitochondrial function, aging, and cell death. Nicotinamide mononucleotide (NMN) is one of the key precursors of NAD<sup>+</sup>. The purpose of this study is to evaluate the oxidative stress effects of NMN on rat tenocytes *in-vitro*.

**Methods** Tenocytes from normal Sprague–Dawley rats were cultured in regular glucose (RG) and high-glucose (HG) conditions with or without NMN, and were divided into four groups: RG NMN<sup>-</sup>, RG NMN<sup>+</sup>, HG NMN<sup>-</sup>, and HG NMN<sup>+</sup>. Cell viability, reactive oxygen species (ROS) production, apoptosis, and messenger RNA (mRNA) expressions of NADPH oxidase (NOX) 1, NOX4, interleukin (IL)-6, SIRT1, and SIRT6, were determined *in-vitro*.

**Results** The NMN groups led to significantly higher cell viabilities compared with the other groups. The mRNA expressions of NOX1, NOX4, and IL6, in the HG NMN<sup>+</sup> group were significantly lower compared with those of the HG NMN<sup>-</sup> group. Conversely, the corresponding expressions of the SIRT1 and SIRT6 levels in the HG NMN<sup>+</sup> group were significantly higher compared with those of the HG NMN<sup>-</sup> group. Both the accumulation of ROS and apoptosis in the HG NMN<sup>-</sup> group were significantly higher compared with those in the RG NMN<sup>-</sup> group at 48 h.

**Conclusion** The expression levels of NOX1, NOX4, IL6, and ROS were significantly reduced by NMN. These results suggest that NMN could effectively reduce the oxidative stress by activating SIRT1 and SIRT6, and by inhibiting the activity of NOX and apoptosis in the tenocytes.

## Introduction

Musculoskeletal disorders of the hand and shoulder are more common in diabetic than nondiabetic patients [1]. Diabetes mellitus is associated with many musculoskeletal disorders, such as tendinitis, joint stiffness, tendon ruptures, carpal tunnel syndrome, Dupuytren's disease, and adhesive capsulitis [2, 3, 4, 5, 6, 7]. An *in vivo* study showed hyperglycemia-impaired tendon-bone healing in a rat model of a rotator cuff tear [8]. Another study showed that patients with rotator cuff tears had significantly higher fasting plasma glucose levels within the normoglycemic range than patients with meniscal tears, and concluded that increasing plasma glucose levels may be a risk factor in rotator cuff tear cases [9].

The suggested pathogenesis is related to the excessive generation of oxidative stress caused by hyperglycemia [8]. Oxidative stress induced by hyperglycemia is triggered by reactive oxygen species (ROS) and is controlled by antioxidant enzymes, such as superoxide dismutase and catalase. Accordingly, increases in ROS cause both deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein damages, while alterations in antioxidant enzyme levels lead to cellular and tissue damages and to organ dysfunction [10, 11, 12, 13]. Other previously published studies reported that the main source of ROS was NADPH oxidase (NOX) and its activation increased ROS production [14, 15, 16].

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) was discovered more than a century ago as a low-molecular weight substance in boiled yeast extracts, and was shown to be capable of stimulating fermentation and alcohol production in-vitro [17]. Recently, it has become clear that the cellular role of NAD<sup>+</sup> extends far beyond its classic participation in redox reactions given that it also acts as a substrate for several families of regulatory enzymes [18, 19, 20, 21]. A number of studies have demonstrated that NAD<sup>+</sup> declines during the aging process and plays an important role in energy metabolism, mitochondrial function, aging, and cell death [22, 23, 24]. One of the key precursors of NAD<sup>+</sup> is nicotinamide mononucleotide (NMN) that is converted to NAD<sup>+</sup> by nicotinamide mononucleotide adenylyltransferase [23]. It has been recently shown in various animal models that NMN mitigates age-associated physiological changes in liver, adipose tissue, muscle, pancreas, kidney, and in the central nervous system [25, 26, 27, 28]. However, previous studies have not evaluated the antioxidative effect of NMN on tenocytes. The purpose of this study is to evaluate the oxidative stress effects of NMN on rat tenocytes.

## Materials And Methods

All animal procedures were performed in accordance with our approved experimental protocol and the guidance of the Animal Care and Use Committee of our institution.

### Cell culture

Sprague–Dawley (SD) female rats (age = eight weeks) were used in this study. Achilles tendons were excised from SD rats and were washed twice with phosphate buffered saline (PBS). Tissues were cut in small pieces with approximate sizes in the range of 1.5–2.0 mm<sup>3</sup>. Several pieces were placed on a culture plate and cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan, UT, USA) mixed with 10% fetal bovine serum (FBS, Cansera, Rexdale, Ontario, Canada), 100 U/ml penicillin, and 100 µg/ml streptomycin. The explants were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells from the tendons were subcultured after trypsin digestion. The media were changed every five days. In this study, cells at passage 2 were used.

### Cell proliferation assay

Cell proliferation was measured by a water-soluble tetrazolium salt (WST) assay with a cell counting kit-8 (Dojindo, Kumamoto, Japan). All of the wells in the 96-well plates were seeded with 2,000 cells and were filled with 100 µl DMEM. The 96-well plates were then placed in a CO<sub>2</sub> incubator at 37 °C before the WST assay evaluation. The cells were exposed to DMEM in regular glucose (RG) concentrations (12 mM) with four different NMN concentrations (0, 10 µM, 100 µM, and 1 mM) for 48 h. For the WST assay, each well was supplemented with 10 µl of WST for 4 h at 37 °C in a CO<sub>2</sub> incubator before spectrophotometric evaluation. The conversion of WST to formazan was measured at 450 nm spectrophotometrically.

### Experimental protocol

Tenocytes were seeded in twelve-well culture plates at a density of  $1 \times 10^5$  cells per well. These were then incubated in DMEM at two different glucose concentrations, namely, at 12 mM in the regular glucose (RG) group and at 33 mM in the high-glucose (HG) group without FBS to avoid overgrowth. NMN (Oriental Yeast Co, Tokyo, Japan) was dissolved in PBS, and the final concentration of NMN was 100  $\mu$ M according to the cell proliferation assay outcome. In brief, tenocytes were divided into four groups: a) RG group without NMN (RG NMN<sup>-</sup>), b) RG group with NMN (RG NMN<sup>+</sup>), c) HG group without NMN (HG NMN<sup>-</sup>), and d) HG group with NMN (HG NMN<sup>+</sup>).

## Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

At 48 h, the total amount of RNA from all tenocytes was extracted with a RNeasy Mini Kit (Qiagen, Valencia, CA). With the use of a high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems, Foster City, CA), the total RNA was reverse transcribed in a single strand cDNA. Real-time PCR was performed in triplicate on the cDNA with an Applied Biosystems 7900HT fast real-time PCR system and SYBR Green reagents (Applied Biosystems). Housekeeping gene expression levels were normalized and expressed relative to the control (untreated) culture levels using the  $2^{-\Delta\Delta C_t}$  method.

## ROS measurements

At 48 h, the accumulation of intracellular ROS levels in tenocytes was detected by the oxidation-sensitive fluorescent probe 2',7'-Dichlorofluorescein Diacetate (DCFH-DA) using the total ROS/Superoxide detection kit (Enzo life Sciences, Farmingdale, New York) according to the manufacturer's protocol. Tenocytes ( $1 \times 10^4$ ) were incubated with a final DCFH-DA concentration of 10  $\mu$ M for 60 min at 37 °C in the dark. Next, they were washed three times with PBS, trypsinized, and resuspended. For quantitative analysis of ROS accumulation, fluorescence intensity was calculated by Adobe Photoshop CC 2020 software (Adobe Systems Incorporated, San Jose, USA) and normalized to cell number as determined by 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) in randomly selected fields.

## Immunofluorescence staining for analysis of apoptotic cells

Nuclear fragmentation was detected by TUNEL staining with an APO-DIRECT™ kit (PHOENIX FLOW SYSTEMS, San Diego, CA) with fixed cells (4% paraformaldehyde/PBS) and DAPI according to the manufacturer's protocol. Apoptotic cells and DAPI-positive cells in four rectangular areas (0.75 mm  $\times$  1.0 mm) were counted in each slide, and the average values were calculated for quantitative measurements. The percentage of apoptotic cells were calculated using the formula (number of apoptosis-positive nuclei/ number of DAPI-positive nuclei)  $\times$  100.

## Statistical analyses

All data were expressed as the mean  $\pm$  standard deviation (SD). Cell proliferation was analyzed with one-way analysis of variance (ANOVA). For a comparison of the four groups, two-way ANOVA was performed.

Post-hoc analyses were performed by the Fisher's protected least-significant difference test. A p-value < 0.05 was considered statistically significant.

## Results

### Cell proliferation assay

Cell proliferation in the groups with NMN (10  $\mu$ M, 100  $\mu$ M, and 1 mM) was significantly higher than that in the groups without NMN (Fig. 1). The proliferation in the NMN group (100  $\mu$ M) was the highest among all groups. However, there were no significant differences in the three groups with NMN. Thus, the middle concentrations (100  $\mu$ M) were selected in the following experiments.

### Quantitative RT-PCR

The mRNA expressions of NOX1, NOX4, and IL6 in the HG NMN<sup>-</sup> groups were significantly higher than those in RG NMN<sup>-</sup> group at 48 h (Fig. 2). The mRNA expressions of these markers in the HG NMN<sup>+</sup> groups were significantly lower compared to those in the HG NMN<sup>-</sup> group. Conversely, the mRNA expressions of SIRT1 and SIRT6 in the HG NMN<sup>-</sup> groups were significantly higher than those in the RG NMN<sup>-</sup> group. The mRNA expressions of these markers in the HG NMN<sup>+</sup> groups were significantly higher compared with those in the HG NMN<sup>-</sup> group.

### ROS analyses

The intracellular ROS levels were detected using DCFH-DA staining. Fluorescence staining showed ROS accumulation (green) in tenocytes and DAPI (blue) (Fig. 3). The accumulation of intracellular ROS levels in the HG NMN<sup>-</sup> group was significantly larger compared to those in the RG NMN<sup>-</sup> group at 48 h (Fig. 4). The accumulation in the HG NMN<sup>+</sup> group was significantly smaller compared to that in the HG NMN<sup>-</sup> group, but there was no difference within the RG groups.

### Apoptotic cell analyses

Fluorescence staining showed abnormal nuclear morphology, such as nuclear fragmentation in apoptotic cells (green) and DAPI (blue) (Fig. 5). The numbers of apoptotic cells in the HG NMN<sup>-</sup> group were higher compared with those in the RG NMN<sup>-</sup> group at 48 h, but there were no differences between the RG NMN<sup>-</sup> group and HG NMN<sup>-</sup> group. The apoptotic cells in the HG NMN<sup>+</sup> group were significantly lower compared with those in the HG NMN<sup>-</sup> group (Fig. 6).

## Discussion

It has been previously suggested that diabetes mellitus increases susceptibility to tendinopathy [29, 30]. The previously published reports have suggested several pathological mechanisms regarding tendon

lesions with diabetes [2, 3, 4, 5], but the molecular mechanisms underlying tendinopathy are still unknown. One report has described that HG concentrations upregulate the expressions of matrix metalloproteinases (MMPs) in tenocytes. Furthermore, the potential combination of increased local matrix degradation evoked by enhanced MMP expressions and decreased ECM may predispose patients with diabetes to tendinopathy or tendon injuries [31]. Another report showed that immunohistochemistry analyses identified both the higher density of type 1 collagen and an increase in the expression of vascular endothelial growth factor, and increased immunostaining for NFκB p50 nuclear localization in the nucleus in the Achilles tendons of the diabetic group [32].

Furthermore, a number of studies have demonstrated that hyperglycemic conditions induce oxidative stress and cytokine production, and they lead to inflammation and tissue damage in various organs [33, 34, 35]. Ueda et al. reported the upregulation of the expression of mRNA for NOX1 and IL-6, and the production of ROS in HG conditions in tenocytes of the Achilles tendons in rats [36]. Previously published reports showed the main source of ROS is NOX and its activation increased ROS production [14, 15, 16]. It has also been shown that HG levels stimulate ROS production through protein kinase C-dependent activation of NOX in cultured aortic smooth muscle cells and in endothelial cells [37]. In addition, mitochondrial dysfunction caused by hyperglycemia leads to cell apoptosis [38]. In the present study, the expressions of NOX1, NOX4, and IL-6, ROS production, and cell apoptosis, were significantly higher in HG compared with RG conditions. These results are in agreement with those reported previously.

Cumulative evidence has suggested that NAD<sup>+</sup> plays significant roles in a variety of biological processes, including energy metabolism, mitochondrial functions, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death [23]. NMN is a major precursor of NAD<sup>+</sup> in the salvage pathway of NAD<sup>+</sup> synthesis where it is converted to NAD<sup>+</sup> in cells by nicotinamide mononucleotide adenylyltransferase. These findings strongly suggest that enhancing NAD<sup>+</sup> biosynthesis by administering NMN is an efficient therapeutic intervention against many disease conditions [39]. The enhancement of NAD<sup>+</sup> leads to the upregulation of key NAD<sup>+</sup>-consuming enzymes, such as sirtuins, poly-adenosine diphosphate-ribose polymerases, and CD38/157 ectoenzymes that play critical roles in many biological processes [23, 40]. In the present study, the administration of NMN upregulated significantly cell proliferation and increased significantly the expressions of SIRT1 and SIRT6 in HG conditions.

Many studies have demonstrated that SIRT1 is a potent intracellular inhibitor of oxidative stress and inflammatory responses [41, 42]. SIRT1 regulates immune responses via NF-κB signaling. The NF-κB signaling is a crucial regulator of the immune defense system and an inducer of inflammatory responses [43]. The NF-κB system is also involved in many housekeeping and survival functions during cellular stress by controlling apoptosis, proliferation, and energy metabolism [44, 45]. Yeung et al. reported that SIRT1 could inhibit the transactivation capacity of the NF-κB complex by deacetylating the Lys310 residue of the RelA/p65 component [46]. NF-κB signaling is a potent inducer of the expression of NOX components, such as gp91 phox and p22 phox [47]. The present study showed that the administration of NMN reduced significantly the expressions of NOX1, NOX4, and IL-6, in HG conditions. Quantitative

analysis of ROS production also showed that treatment with NMN reduced ROS production in HG conditions. These results showed that inhibition of the activity of NOX led to reduced ROS production.

SIRT6 is associated with diverse enzymatic activities, including deacetylation and ribosylation [48]. SIRT6-dependent deacetylation is essential to the regulation of DNA repair, cellular glucose/lipid metabolism, telomere maintenance, cellular senescence, and life span [49]. Fan et al. showed that SIRT6 alleviated HG induced podocyte apoptosis by activating 5' adenosine monophosphate protein kinase (AMPK) [50]. Furthermore, the previous report showed that the overproduction of ROS causes cellular damage and promotes the process of apoptosis based on the activation of caspase and the regulation of the expressions of the Bcl-2 family proteins [51]. In the present study, the quantitative analysis of apoptosis showed that treatment with NMN reduced apoptosis in HG conditions.

There are several limitations associated with this study. Firstly, the *in-vitro* results were preliminary. Therefore, clinical applications in humans will require additional detailed research including in vivo animal studies. Secondly, the monolayer culture of tenocytes *in-vitro* never reproduced the actual physiological conditions. Previous studies have demonstrated that primary tenocytes maintained the phenotypical stability until passage 5 when the cultures in prior passages were in subconfluent states [52]. In the present experiments, tenocytes were cultured after passage 2. Finally, while there are several apoptotic pathways involved in HG conditions, the mediator of the apoptotic signaling pathways was not investigated in this study.

In conclusion, HG concentrations upregulated the mRNA of NOX1, NOX4, and IL-6 expressions, and the production of ROS and apoptosis. NMN significantly reduced ROS production and cell death based on the activation of SIRT1 and SIRT6 and the inhibition of the activity of NOX. NMN is thus a potential prodrug in the treatment of diabetic tendinopathy.

## Abbreviations

ROS

reactive oxygen species, NOX:NADPH oxidase

NAD

Nicotinamide adenine dinucleotide, NMN:Nicotinamide mononucleotide

SD

Sprague–Dawley, PBS; phosphate buffered saline

DMEM

Dulbecco's Modified Eagle's Medium, FBS:fetal bovine serum

## Declarations

## Ethics approval and consent to participate

The research protocol was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (Permission number P190204).

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Funding

Funding information is not applicable.

## Authors' contributions

YM and AI contributed to the conception and design of the study. TKA, TKU and SM performed the experiments and collected the data. Data and statistical analysis was done by YM and AI. Manuscript preparation was done by YM, AI and TN. Supervising was done by RK. All authors read and approved the final manuscript.

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Figures

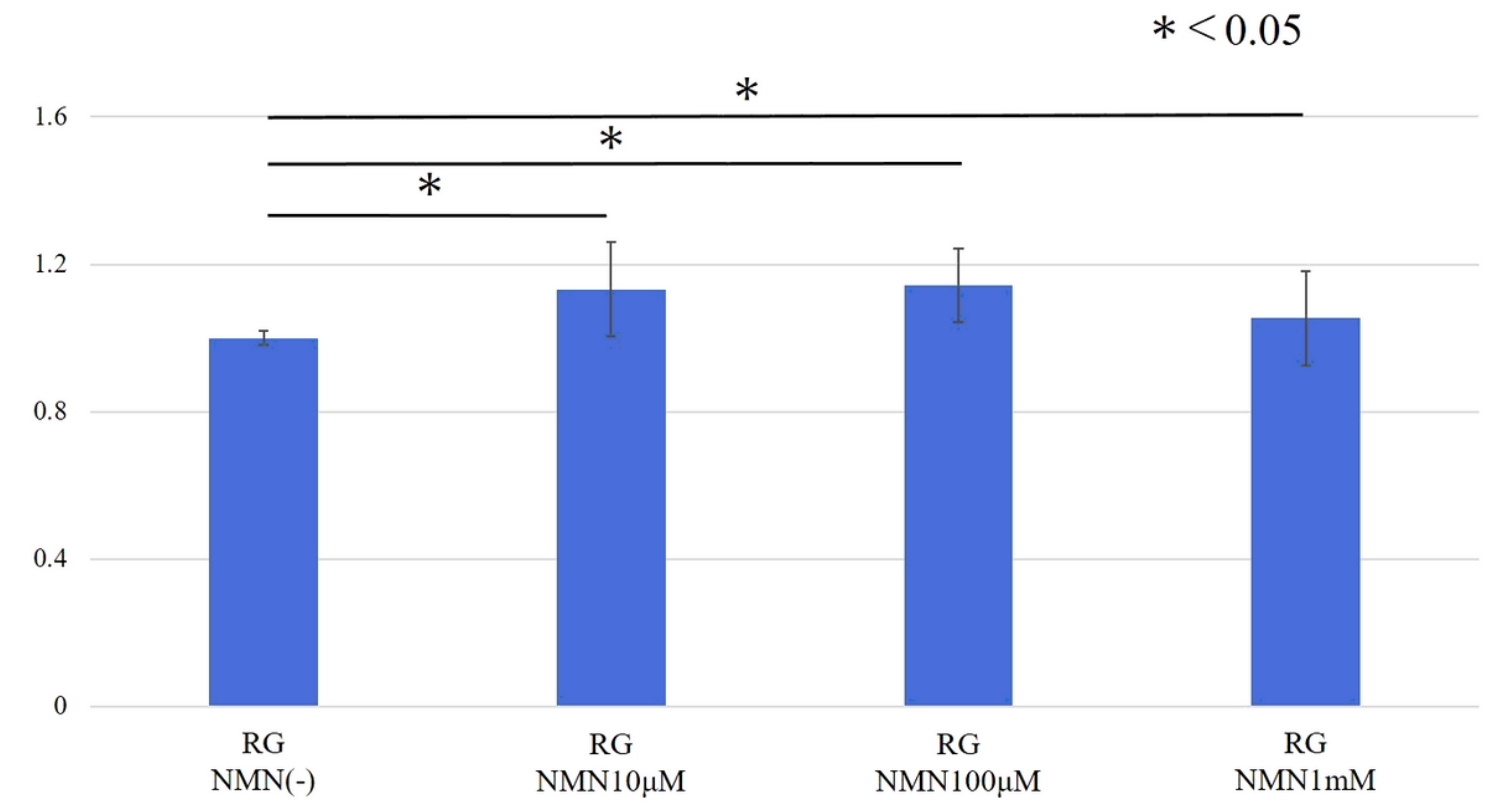


Figure 1

Cell proliferation Cell proliferation was measured by a water-soluble tetrazolium salt (WST) assay using a cell counting kit-8 (Dojindo, Kumamoto, Japan). The cells were exposed to DMEM in regular glucose concentrations (12 mM) with four different NMN concentrations (0, 10  $\mu$ M, 100  $\mu$ M, and 1 mM) for 48 h. Cell proliferations in the groups with NMN (10  $\mu$ M, 100  $\mu$ M, and 1 mM) were significantly higher than that in the groups without NMN (\* $p$  < 0.05). The proliferation in the group with 100  $\mu$ M NMN was the highest among all groups.

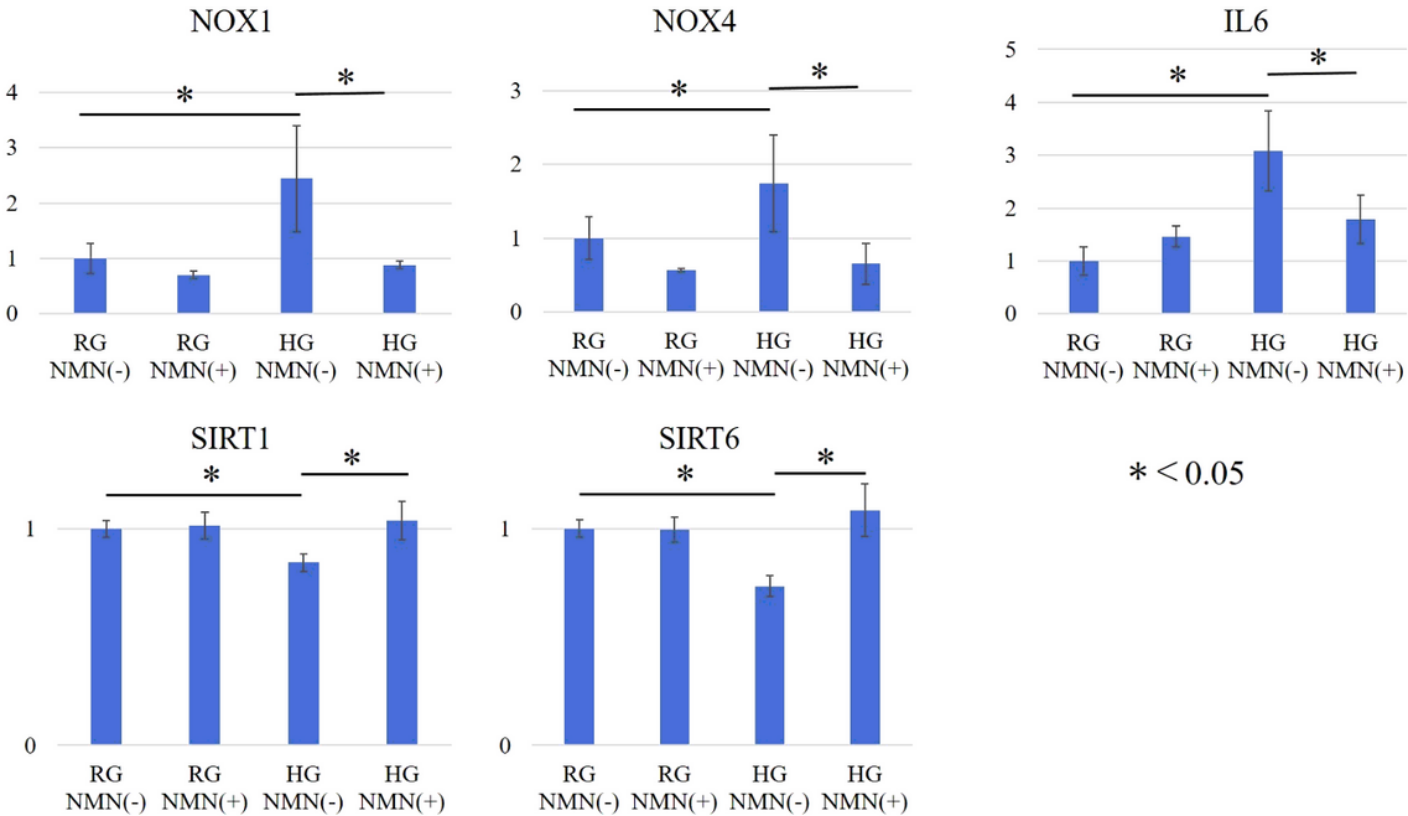
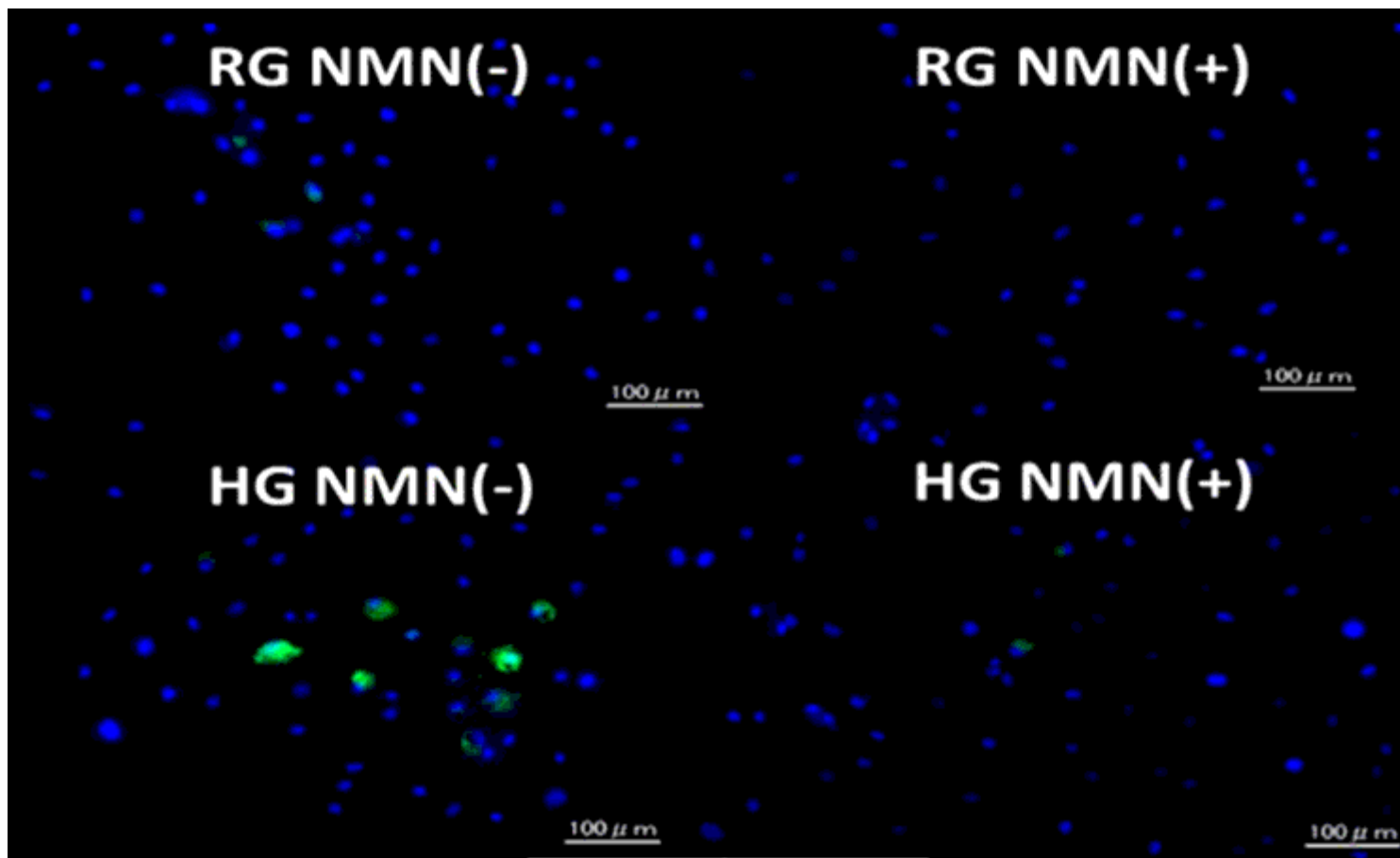


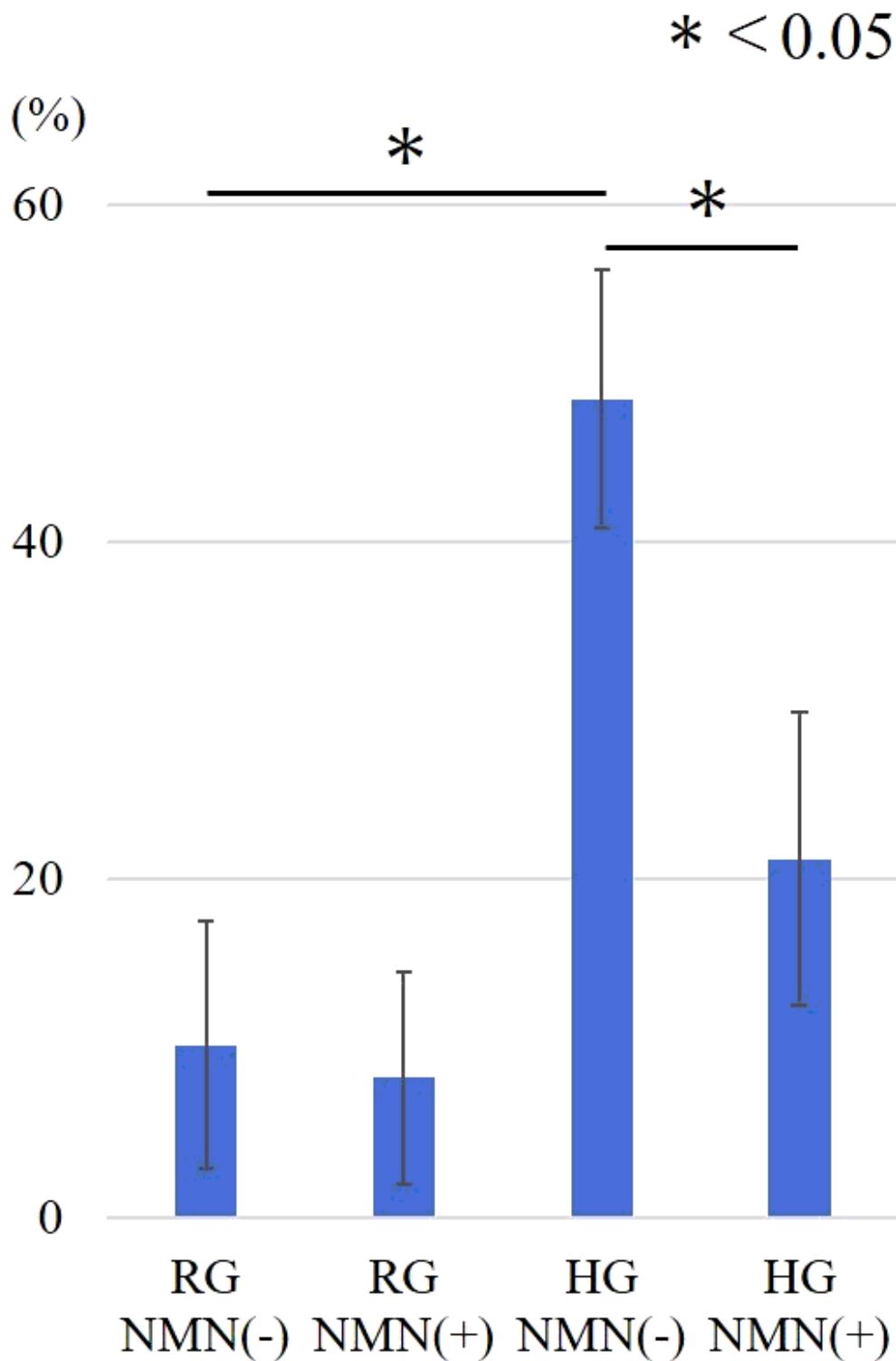
Figure 2

Quantitative reverse transcription PCR The mRNA expressions of NOX1, NOX4, and IL6 in the HG NMN- groups were significantly higher than those in the RG NMN- group at 48 h. The mRNA expressions of these markers in the cases of the HG NMN+ groups were significantly lower compared to those of the HG NMN- group. Conversely, the mRNA expressions of SIRT1 and SIRT6 in the HG NMN- groups were significantly higher than those in the RG NMN- group. The mRNA expressions of these markers in the HG NMN+ groups were significantly higher compared to those of the HG NMN- group.



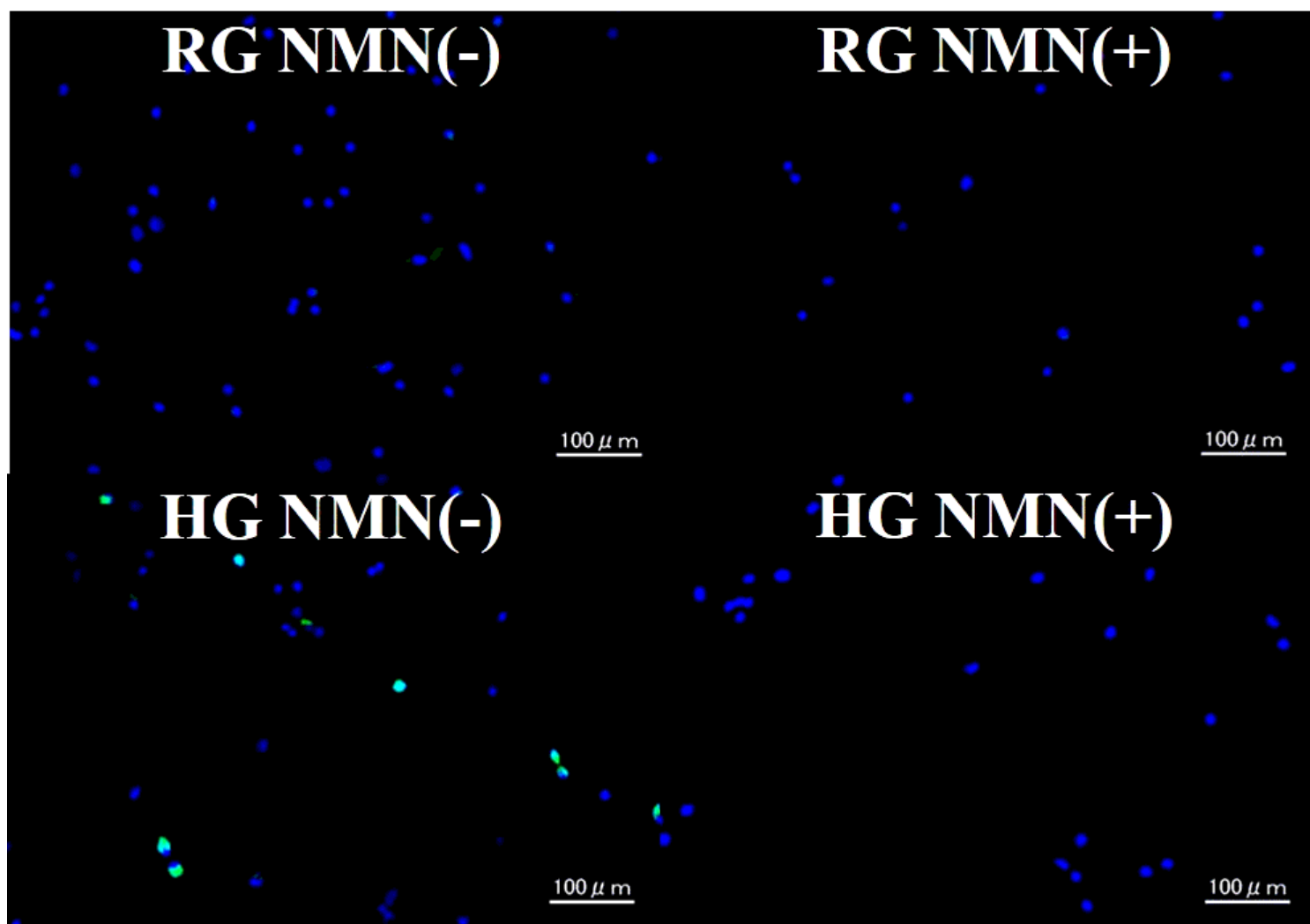
**Figure 3**

ROS evaluation Intracellular ROS levels were detected with DCFH-DA staining. Fluorescence staining showed ROS accumulation (green) in tenocytes and DAPI (blue).



**Figure 4**

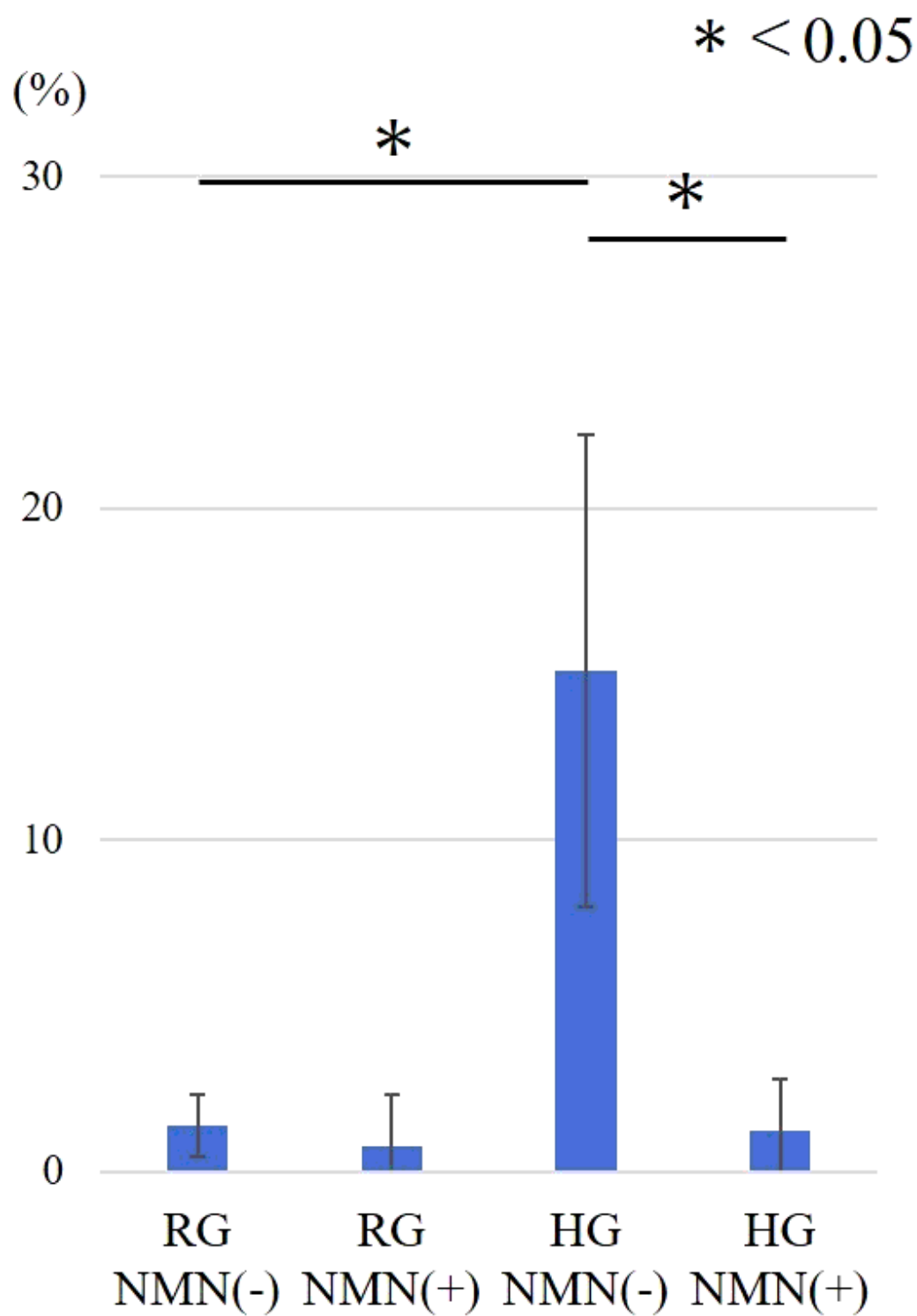
Quantification of ROS accumulation The accumulation of intracellular ROS levels in the HG NMN- group was significantly larger compared with that in the RG NMN- group at 48 h. The accumulation associated with the HG NMN+ group was significantly smaller compared with that of the HG NMN- group, but there was no difference within the RG groups.



**Figure 5**

Apoptosis examination Fluorescence staining showed abnormal nuclear morphology, such as nuclear fragmentation in apoptotic cells (green) and DAPI (blue).





**Figure 6**

Quantification of apoptotic accumulation Apoptotic cell number associated with the HG NMN- group was higher compared with that of the RG NMN- group at 48 h, but there were no differences between the RG NMN- and HG NMN- groups. The number of apoptotic cells associated with the HG NMN+ group was significantly lower compared with that of the HG NMN- group.