Role of Mitofusin 1 in Mitochondrial Quality Control and the Anti-Inflammatory Effects in Nucleus Pulposus Cells During Inflammation-Induced Degeneration

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Introduction

Mitochondrial dysfunction is a key driver of intervertebral disc (IVD) degeneration. Therefore, maintaining mitochondrial dynamics, governed by mitofusin (MFN) proteins, is essential for cellular health. Despite the recognized potential of antioxidant therapies, the specific mechanisms influencing mitochondrial function in nucleus pulposus cells (NPCs) under inflammatory stress remain unclear.

Objectives

To evaluate the roles of MFN1 and MFN2 in mitochondrial quality control (MQC) and their responses to inflammation and antioxidant treatments, focusing on grades I and III disc NPCs.

Methods

NPCs were isolated from human IVD tissue and treated with tumor necrosis factor-α (TNF-α) to induce inflammation. The antioxidant effects of Vitamin E and saponin were assessed via quantitative PCR, western blotting, immunocytochemistry, and transmission electron microscopy. For in vivo analysis, MFN1 knockdown NPCs were subcutaneously transplanted into BALB/c nude mice to evaluate the effects of MFN1 suppression on the integrity of extracellular matrix (ECM) under physiological conditions. Statistical analysis was performed to determine significance. All statistical analyses were conducted using the paired t-test and the Mann–Whitney U test for parametric and nonparametric data, respectively, with a significance threshold of $p < 0.05$.

Results

TNF-α treatment significantly increased the expression of MFN1 and MFN2, with MFN1 showing a slightly stronger response in grade III disc NPCs than in grade I disc NP cells. Antioxidant treatments with Vitamin E or saponin effectively reduced the expression of these proteins, with Vitamin E exhibiting a more potent anti-inflammatory effect than saponin. Importantly, MFN1 was identified as a key regulator of mitochondrial fusion and ECM integrity under inflammatory conditions. The alterations in mitochondrial morphology and ECM levels were notably more pronounced in grade III disc NPCs than in grade I disc NPCs, reflecting the advanced degeneration in these cells.

Conclusions

MFN1 and MFN2 contribute to mitochondrial dynamics during inflammation, with MFN1

being particularly responsive to inflammatory and anti-inflammatory conditions. Vitamin E showed superior efficacy in mitigating inflammation-induced damage compared to saponin. Therefore, these findings suggest that targeting mitochondrial dynamics, particularly through MFN1 modulation, provides novel therapeutic approaches for degenerative disc disease.

Key words: Mitochondrial Quality Control, Nucleus, Pulposus Cells, Disc degeneration, Mitofusin

1. Introduction

In healthy intervertebral discs (IVDs), maintaining the balance between extracellular matrix (ECM) anabolism and catabolism is crucial for preserving structural integrity. [1] However, aging and mechanical stress can disrupt this balance, leading to ECM degradation and IVD degeneration. These stressors affect the mitochondria, which are essential for energy production and apoptosis regulation, thereby resulting in mitochondrial dysfunction. Specifically, this dysfunction is characterized by disrupted membrane potential and reactive oxygen species (ROS) accumulation, which intensifies apoptosis and contributes to IVD degeneration. Therefore, preserving mitochondrial function is key to delaying the degenerative processes in IVD. [2,3]

Mitochondria regulate their number, size, and distribution through continuous fission and fusion. Mitochondrial fission, where one mitochondrion is divided into two mitochondria, involves several steps facilitated by proteins, such as dynamin-related protein 1, fission 1 (Fis1), and mitochondrial fission factor (MFF). In contrast, fusion involves the merging of the outer mitochondrial membranes of two mitochondria, mediated by mitofusin (MFN) proteins (MFN1 and MFN2), progressing to the inner mitochondrial membrane through optic atrophy 1 (OPA1). [4,5] In addition to mitochondrial function, balanced fission and fusion are associated with cellular metabolism and nutritional status. Mitochondrial dynamics also include the repair of damaged mitochondria; however, if repair is unsuccessful, dysfunctional mitochondria are selectively removed via mitophagy, an autophagic mechanism. [6]

During IVD degeneration, mechanical loading, pro-inflammatory cytokines, oxidative stress, and glucose stress contribute to mitochondrial dysfunction, creating a vicious cycle of ROS production and damage. Mitochondrial dynamics, particularly the balance between fusion and fission, are disrupted under stress conditions. [7] Oxidative stress induces mitochondrial fission while inhibiting fusion, thereby altering the expression of key proteins, e.g., MFF, Fis1, MFN1, MFN2, and OPA1. For instance, preventing fission with mitochondrial division inhibitor 1 reduced mitochondrial damage and apoptosis, highlighting the critical roles of MFN1 and MFN2 in maintaining mitochondrial integrity during IVD degeneration. [8]

Research has focused on the use of antioxidant agents to reduce inflammation, lower pro-inflammatory cytokine levels, and alleviate stress to prevent IVD degeneration. Although studies have shown the potential of antioxidant agents to mitigate inflammation, the exact mechanisms by which they affect mitochondrial function in nucleus pulposus cells (NPCs) are not fully understood. [9-11] Therefore, this knowledge gap highlights the need for further investigation into the specific mitochondrial pathways involved in the protection of NPCs from degeneration via antioxidant therapy.

While MFN1 and MFN2 are important proteins for mitochondrial fusion, the precise mechanisms and roles of membrane fusion are not fully understood. A fusion step that is dependent on the GTP-binding domain of MFN has been identified; however, whether this step is related to GTPase activity remains unclear. MFN1 exhibited a homotypic interaction in the GTP-dependent mitochondrial tethering reaction that was 100-fold stronger than that of MFN2, indicating that these

proteins have distinct functions during the fusion process. [12]

Therefore, this study aimed to understand mitochondrial quality control (MQC) in disc NPCs by investigating the roles of mitofusion, mitofission, and mitophagy during inflammation. We also explored the distinct roles of MFN1 and MFN2 in these processes.

2. Materials and methods

2.1 Ethics statements

The Institutional Review Board of the College of Medicine at Yonsei University (approval number: 4-2022-0167) approved all experimental procedures, and informed consent was obtained from all individuals participating in the study. This study complied with the standard reporting guidelines outlined in the Strengthening the Reporting of Observational Studies in Epidemiology statement to ensure the transparency, accuracy, and reproducibility of its findings.

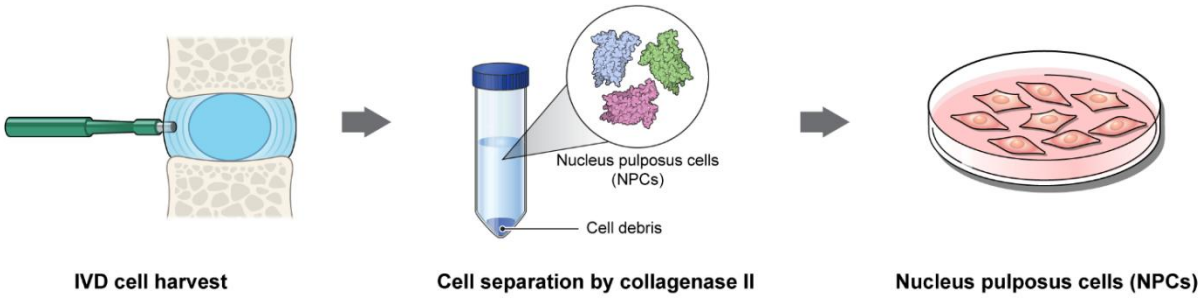
2.2. Patient samples

NPC samples were obtained from 15 patients diagnosed with disc-related conditions, including scoliosis. The degree of IVD degeneration was assessed using magnetic resonance imaging and classified according to a modified version of the Pfirrmann grading system. Samples from patients with grades I (n = 5), II (n = 3), III (n = 5), and IV (n = 2) IVD were obtained. The experiments were primarily conducted using grade I and III disc NPCs. This study's exclusion criteria included patients with a history of spinal surgery, active infection, inflammatory disorders, malignancies, or systemic diseases that could influence the inflammatory process or mitochondrial function. Patients who received antioxidant treatments before surgery were also excluded.

2.3 Isolation and expansion of human NPCs

IVD tissues obtained during surgery were finely chopped using a scalpel. The minced tissues were digested for 2 h at 37 °C with gentle agitation in a medium comprising equal parts of Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DMEM/F12, 1:1; Gibco™, Life Technologies Corporation, Grand Island, NY, USA) supplemented with collagenase type II (2.5 mg/mL; Sigma, St. Louis, USA). After digestion, the cells were passed through a sterile nylon mesh filter with a pore size of 100 μm (Falcon®, Durham, NC, USA) and seeded into T25 flasks (EasYFlask™, Nunc™, Roskilde, Sjælland, Denmark) at a density of approximately 1×10^5 cells/mL. Primary cultures were maintained for 2–3 weeks in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 1% v/v penicillin, streptomycin, and nystatin (Gibco™, Life Technologies Corporation) at 37 °C in a humidified incubator (Thermo Fisher Scientific, Marietta,

OH, USA) with 5% carbon dioxide. The culture medium was refreshed three times per week (Figure 1).



<Figure 1> Isolation and expansion of human NPCs

IVD: Intravertebral disc

2.4 Cell culture

Only NPCs, with the annulus tissue excluded, were used in this study. The cells exhibited typical NP morphology, and the expression of paired box-1 (PAX1) and ovo-like zinc finger 2 (OVOL2) was analyzed using quantitative polymerase chain reaction (qPCR). Next, the cells were cultured in DMEM/F12 containing 10% FBS and ascorbic acid (250 μg/mL) at passage two. The cells were seeded in 100-mm culture dishes (TPP®, Switzerland, EU) at a density of 2 × 105 cells/mL during culture incubation. They were treated with 4 mg/mL of tumor necrosis factor-α (TNF-α) for 24 h after 16 h of serum starvation. Additionally, the cells were also treated with 200-mM saponin (SPN) and vitamin E (Vit E) for 24 h after TNF-α treatment. The following groups were studied: control, TNF-α-induced inflammation, anti-inflammation by Vit E, and SPN-induced inflammation.

2.5 Sulfated glycosaminoglycan assay

Sulfated glycosaminoglycan (GAG) was isolated from cells lysed using a papain-based buffer composed of 0.1 M sodium acetate, 0.01 M sodium ethylenediaminetetraacetic acid (EDTA) (pH 6.4), and 5 mM cysteine hydrochloride. The extracted GAG was concentrated using the sulfated GAG assay protocol and quantified at 650 nm using a Blyscan instrument (Biocolor Ltd., Carrickfergus, County Antrim, UK). Finally, the concentration of sulfated GAG was calculated based on a standard curve generated with bovine tracheal chondroitin 4-sulfate provided in the assay kit.

2.6 RNA extraction and real-time polymerase chain reaction analysis

Total RNA was extracted from chondrocyte-like cells using the QIAGEN RNeasy® Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the Maxime RT Premix Kit (QIAGEN®). Genes of interest, including MFF, MFN1, MFN2, calcium-binding and coiled-coil domain 2 (NDP52), optineurin (OPTN), vimentin (VIM), tissue inhibitor of metalloproteinases-1 (TIMP-1), phosphatase and tensin homolog-induced kinase 1 (PINK-1), and type II collagen (Col II), were amplified using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative gene expression levels were normalized to beta-actin as the reference gene.

Table 1. Details of the primers

Target gene	**Oligonucleotide sequence**	**Annealing temperature (°C)**	**Product size**
β-actin	5′-GGC TGC TTC CAG CTC CTC CC-3′	60	99
	5′-AAG AGT GCC TCA GGG CAG CG-3′		
Col II	5′-CCT GGT CCC CCT GGA AAG CC-3′		95
	5′-CCA CGA GCA CCC TGA GGA CC-3′		
MFF	5′-AAC CCC TGG CAC TGA AAA CA-3′		120
	5′-TGC CAA CTG CTC GGA TTT CT-3′		
MFN1	5′-AGT CCA GGC ACA GAT GTC AC-3′		137
	5′-CGT TGC TGG AGT GGT AGG AG-3’		
MFN2	5′-GAA GTG GAG AGG CAG GTG TC-3′		148
	5′-GTC CTT CCT CTA TGT GGC GG-3′		
NDP52	5′-TTC ACC CAG CAT TTC ATC CCT-3′		115
	5′-TCT GTC CTT GGC TCC TCC AT-3′		
OPTN	5′-ACA AGC AGA GAA GGC AGA CC-3′		115

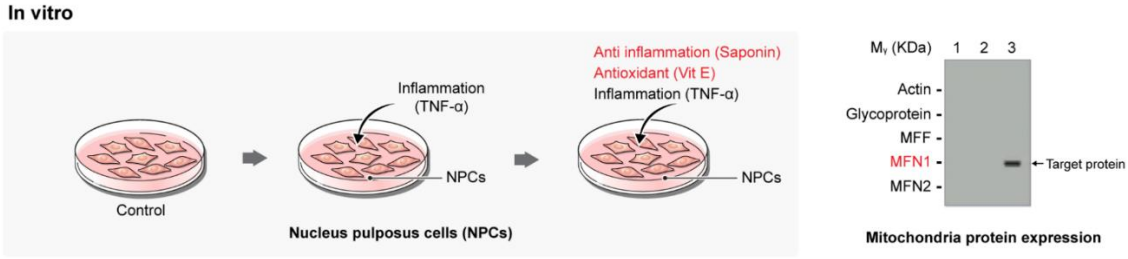
	5′-TGC TTC TCC TTC AGC CAT CC-3′		
VIM	5′-CCT CCT ACC GCA GGA TGT TC-3′		80
	5′-GGT GGA CGT AGT CAC GTA GC-3′		
TIMP-1	5′-GGC ATC CTG TTG TTG CTG-3′		133
	5′-GGT TGA CTT CTG GTG TCC CC-3′		
PINK-1	5′-TGG GGA GTA TGG AGC AGT CA-3′		76
	5′-GAT GAT GTT GGG GTG AGG GG-3′		

MFF: Mitochondrial fission factor, *MFN*: Mitofusin, *OPTN*: Optineurim, *VIM*: Vimentin, *TIMP-1*: Tissue inhibitor of metalloproteinases-1, *PINK*-1: Phosphatase and tensin homologue-induced kinase 1, *Col II*: Type II collagen, *NDP52*: Calcium binding and coiled-coil domain 2

2.7 Protein extraction and western blot analysis

IVD tissues were lysed in lysis buffer containing 150 mM NaCl, 5 mM EDTA (pH 8.0), 10 mM Tris-Cl (pH 7.4), 1% Triton X-100, and 0.2% sodium dodecyl sulfate (SDS), supplemented with protease and phosphatase inhibitors (Pierce® Mini Tablets; Thermo Fisher Scientific). The culture medium was collected to detect soluble proteins. Cells treated with TNF-α and vit E or TNF-α and SPN for 24 h were lysed in radioimmunoprecipitation lysis buffer (ATTO Corporation, Tokyo, Japan) containing protease and phosphatase inhibitors. Next, the lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Pierce®; Thermo Fisher Scientific) using the Mini Trans-Blot® Cell transfer system (Bio-Rad).

The membranes were probed with primary antibodies against TIMP-1 (Abcam, Milton, UK), P38, phospho-P38, Col II, MFF, MFN1, MFN2, NDP52, OPTN, VIM, PINK-1 (Cell Signaling Technology Inc., Danvers, MA, USA), and TIMP-1. Immunoreactive bands were visualized using the EzWestLumiPlus western blot detection system (ATTO Corp., Tokyo, Japan) after incubation with the secondary antibodies. The membranes were stripped and re-probed with anti-actin antibodies (Abcam) to normalize protein loading (Figure 2).



<Figure 2> Cells cultured with TNF-α and Vit E or TNF-α and saponin for 24 h

Results of western blot analysis of mitofusion-, mitofission-, and autophagy-related proteins are shown. TNF-α: Tumor necrosis factor-α, Vit E: Vitamin E, MFF*:* Mitochondrial fission factor, MFN1: Mitofusin 1, MFN2: Mitofusin 2

2.8 Immunocytochemistry

The cells were seeded into glass-bottomed culture dishes at a density of 3 × 10^4 cells per well (MarTek® Corporation, Ashland, MA, USA) and incubated for 24 h. After treatment, they were fixed in 4% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 (v/v) in phosphate-buffered saline (PBS) for another 10 min, and finally blocked with 10% FBS in PBS for 1 h. Subsequently, the cells were incubated overnight at 4 °C with primary antibodies against MFN1 and MFN2 (Cell Signaling Technology® Inc.), as well as with NG2 chondroitin sulfate proteoglycan (Merck®, Temecula, MA, USA). Next, the cells were washed and treated with anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (green) at a 1:200 dilution (Invitrogen®; Thermo Fisher Scientific) and 10 μM 4',6-diamidino-2-phenylindole (DAPI, Abcam®, Milton, UK) for nuclear staining at room temperature (20–22 °C) for 1 h. The samples were examined using a confocal microscope (Zeiss LSM700; Carl Zeiss, Berlin, Germany).

The cells for in vivo assay were seeded in hydrogel (TrueGel3D Hydrogel Kot; Merck®, St. Louis, MO, USA) and implanted in BALB/c nude mice for 1 and 7 days. After sacrificing the experimental animals, the hydrogels were fixed in 4% formaldehyde for 1 day, permeabilized with 0.5% Triton X-100 (v/v) in PBS for another 10 min, and blocked with 10% FBS in PBS for 1 h. Subsequently, the cells were incubated overnight at 4 °C with primary antibodies against MFN1 and MFN2 (Cell Signaling Technology® Inc.), as well as with NG2 chondroitin sulfate proteoglycan (Merck®, Temecula, MA, USA). Next, the cells were washed and treated with anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (green) at a 1:200 dilution (Invitrogen®; Thermo Fisher Scientific), anti-mouse Texas red conjugated secondary antibodies (red) at a 1:200 dilution (Vector Laboratories, Inc. Burlingame, CA, USA), and 10 μM DAPI (Abcam®, Milton, UK) for nuclear staining at room temperature (20–22 °C) for 1 h. Finally, the samples were examined using a confocal microscope (Zeiss LSM700; Carl Zeiss, Berlin, Germany).

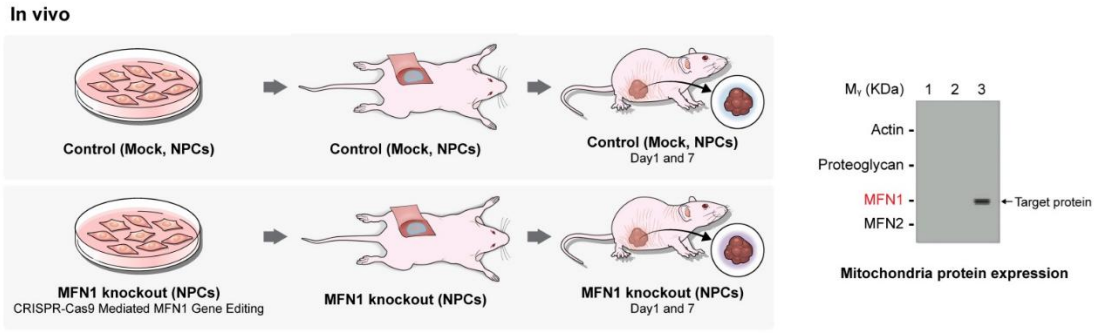
2.9 Transmission electron microscopy

The cells treated with SPN or Vit E for 24 h after TNF-α treatment were collected using 0.5% trypsin EDTA (Gibco™; Life Technologies Corporation) in a 1.5-mL Eppendorf tube, centrifugated, and washed with Dulbecco's PBS (Gibco™; Life Technologies Corporation). Next, the cells were fixed in Karmpvslu fixative, prefixed with 2% osmium tetroxide, dehydrated in a graded series of ethanol solutions, and embedded in araldite. Ultrathin sections were cut using an LKB Ultratome (LKB, Broma, Sweden) and collected on formvar-coated grids. Finally, the sections were stained with uranyl acetate and lead citrate and evaluated using transmission electron microscopy (TEM) (HT7800; Hitachi, Tokyo, Japan) operated at 80 kV.

2.10 Establishment of MFN 1-knockout cells subcutaneous xenograft model (*in vivo* study)

Candidate single guide RNAs (sgRNAs) targeting human MFN1 were designed using the Cas-designer tool. Seven sgRNAs were evaluated for their knockdown efficiency in HEK293T cells. These sgRNAs were individually cloned into the LentiArray™ Lentiviral sgRNA system (#35533; Thermo Fisher Scientific) to generate the lenti-Cas9-sgRNA vectors. Subsequently, HEK293T cells were co-transfected with these lenti-Cas9-sgRNA vectors and lentivirus packaging plasmids (MDLg, VSVG, and Rev). After 72 h, the lentiviral particles were harvested and used to infect the HEK293T cells. Genomic DNA was subsequently extracted from infected cells and analyzed using Surveyor assays. Among the seven sgRNAs tested, one sgRNA (5'-GTTATATGGCCAATCCCACT-3') demonstrated the highest targeting efficiency and was selected for further experiments. NPCs were transduced with lenti-Cas9-sgRNA or lenti-Cas9 alone, followed by puromycin selection over 7 days, resulting in the establishment of stable MFN1-knockdown human NPCs and corresponding negative control cells.

BALB/c nude mice were housed under specific pathogen-free conditions in the barrier facility of the Department of Laboratory Animal Resources at Yonsei Biomedical Research Institute, Yonsei University College of Medicine. After 1 week of acclimatization, 1.3×10^6 MFN1-knockout cells were subcutaneously implanted into the central region of the right flank of each mouse. The mice were observed 1 day and 1 week post-implantation, after which they were euthanized using a carbon dioxide euthanasia apparatus (LC500, Yuyan Scientific Instruments Co.). Next, the implanted cells were collected for analysis (Figure 3). All animal experiments were approved by the Yonsei University Health System Institutional Animal Care and Use Committee and adhered to the Guide for the Care and Use of Laboratory Animals (National Research Council, USA) (approval no. 2023-0292).



<Figure 3> *In vivo* analysis of MFN1-knockout nucleus pulposus cells (NPCs) and MFN1, MFN2, and proteoglycan expressions.

Schematic representation of the experimental procedure and outcomes. Control (Mock) and MFN1-knockout (generated using CRISPR-Cas9-mediated MFN1 gene editing) NPCs were subcutaneously implanted into BALB/c nude mice. Mice were observed at days 1 and 7 post-implantation, after which implanted cells were collected for analysis. Western blot analysis of mitochondrial protein expression confirmed MFN1 absence and MFN2 presence, along with actin and proteoglycan levels. MFN1: Mitofusin 1, MFN2: Mitofusin 2

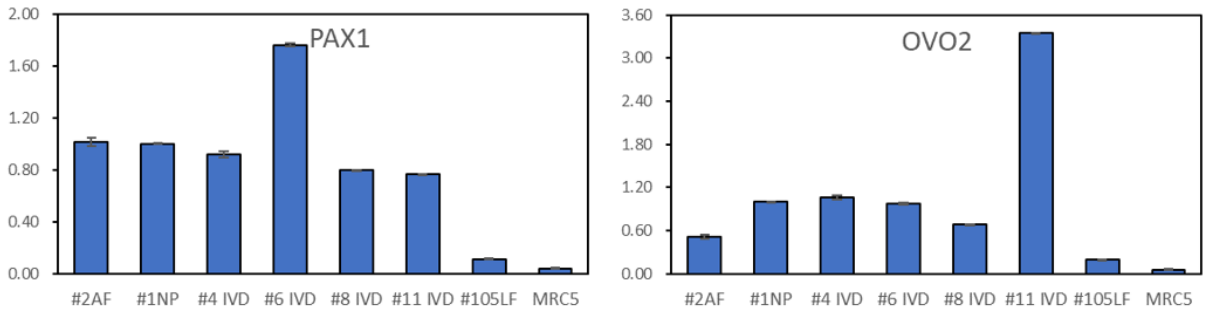
2.11 Statistical analysis

Data were obtained from three independent experiments, each conducted in triplicate, using chondrocyte-like cell cultures derived from 15 different donors. Results are expressed as mean ± standard deviation. Statistical comparisons for biochemical assays between two groups were performed using the paired t-test, while nonparametric data were analyzed with the Mann–Whitney U test. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA), with statistical significance considered at $p < 0.05$.

3. Results

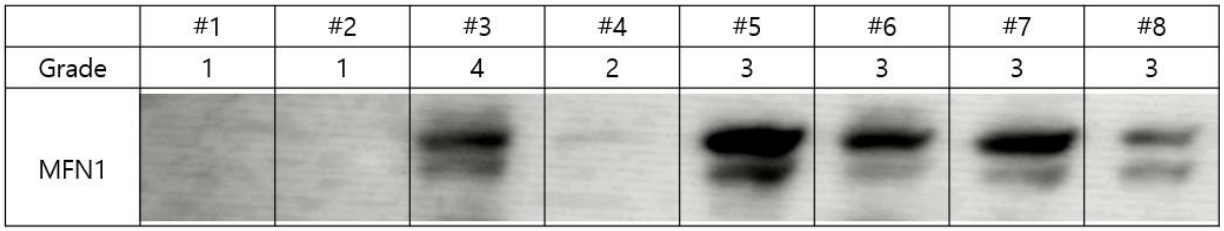
3.1 Patient and cell characteristics

This study included seven males and eight females with a mean age of 38.4 ± 4.2 years. The expressions of OVO2 and PAX1 were confirmed through qPCR in cultured cells, verifying these cells as the IVD cells used in the experiment (Figure 4). MFN1 expression in NPCs was significantly higher in Pfirrmann grades III and IV than in Pfirrmann grades I and II discs. As IVD degeneration progresses, MFN1 expression increases, indicating a correlation between the progression of disc degeneration and elevated MFN1 levels (Figure 5).



<Figure 4> ELISA of NPCs (grade III disc cells)

NPC: Nucleus pulposus cell, ELISA: Enzyme-linked immunosorbent assay, IVD: Intravertebral disc, PAX1: Paired box-1, OVOL2: Ovo-like zinc finger 2, AF: Annular fibrosus, LF: Ligament flavum, MRC5: Medical research council cell strain 5



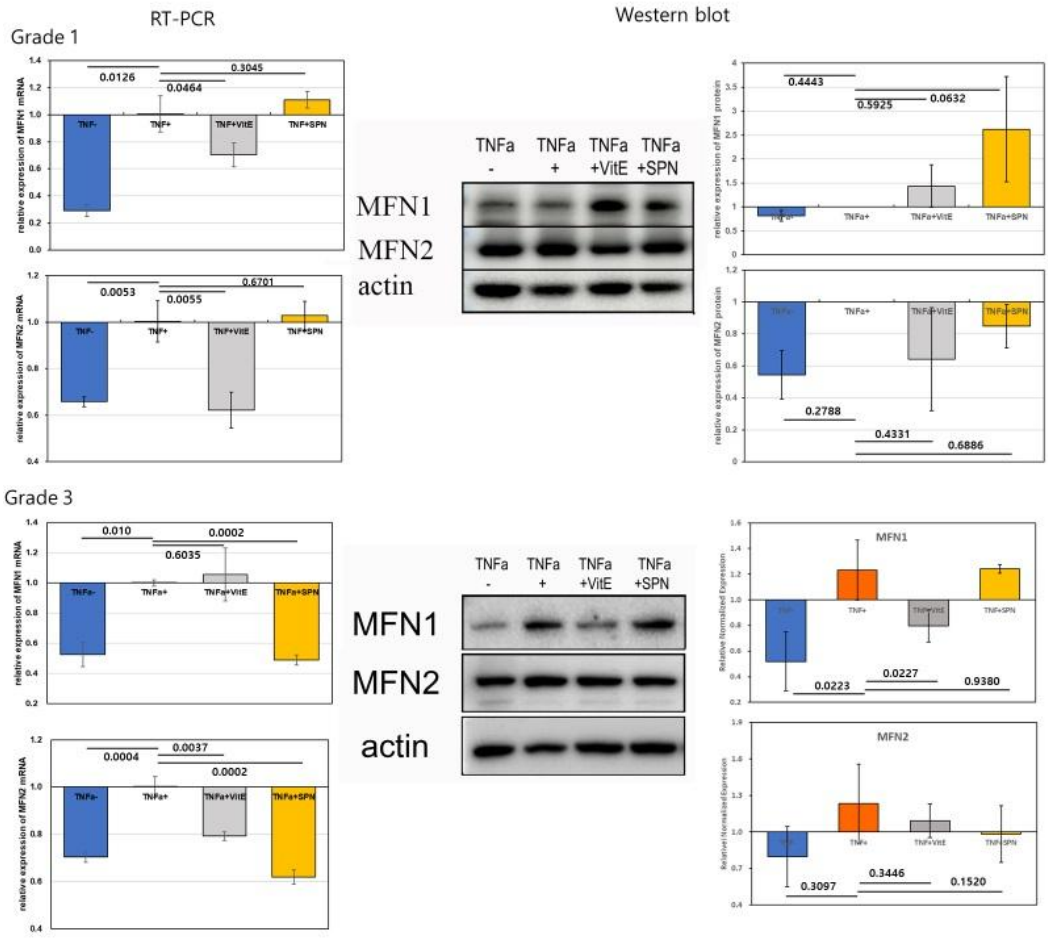
<Figure 5> *MFN1* expression in relation to disc degeneration

MFN1: Mitofusin 1

3.2 Effects of TNF-α on MFN proteins and their impact on antioxidants

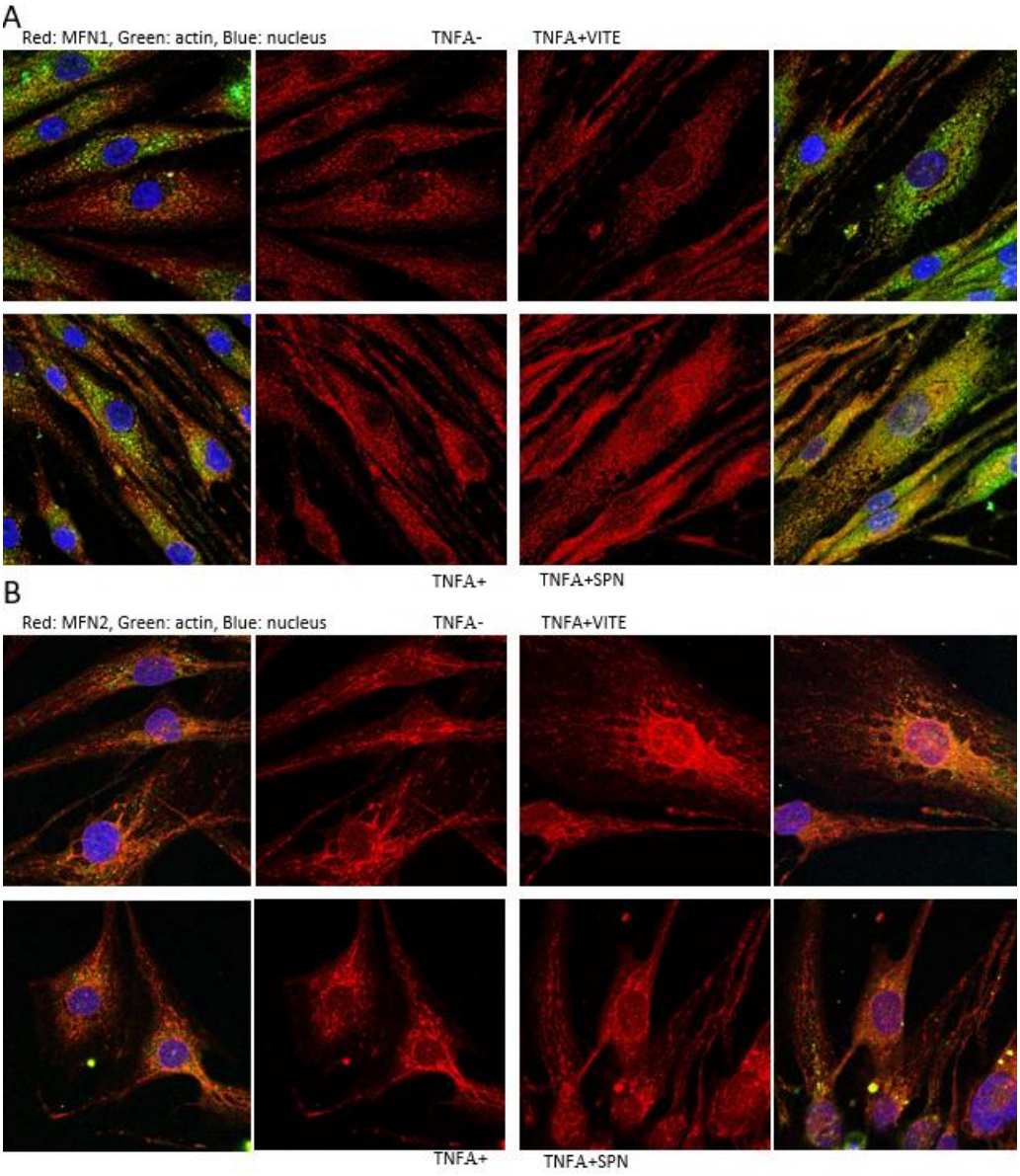
MFN1 and MFN2 exhibited similar trends despite some variations in magnitude. Quantitative PCR, including RNA extraction and real-time PCR analysis, revealed that TNF-α- induced inflammation resulted in an increase in the expression of MFN proteins, including MFN1, compared with the control. Subsequent treatment with Vit E or SPN demonstrated a trend toward decreased expression of these proteins. Western blot analysis revealed results similar to those of qPCR, showing an increase in MFN proteins, including MFN1, post-TNF-α treatment. Upon the administration of Vit E, a decrease in the expression of MFN proteins was observed, except for MFN1 in grade I disc NPCs. Notably, MFN1 showed a significant increase in response to TNF-α, with a more substantial decrease observed with Vit E compared with SPN, particularly when

compared with MFN2 in grade III disc NPCs (Figure 6). Immunocytochemistry indicated a greater increase in MFN1 expression than in MFN2 expression post-TNF-α treatment, followed by a more pronounced sequential decrease in Vit E expression (Figure 7).



<Figure 6> Expression levels of MFN1 and MFN2 in grades I and III disc NPCs

A. The expression levels of MFN1 and MFN2 in grade I and III disc NPCs were analyzed using qPCR and western blotting. In all experiments, the control, TNF-α-induced inflammation, anti-inflammation by vitamin E, and anti-inflammation by SPN groups were compared. MFN: Mitofusin, TNF-α: Tumor necrosis factor-α, Vit E: Vitamin E, SPN: Saponin, qPCR: Quantitative polymerase chain reaction, NPC: Nucleus pulposus cell, RT-PCR, real-time polymerase chain reaction



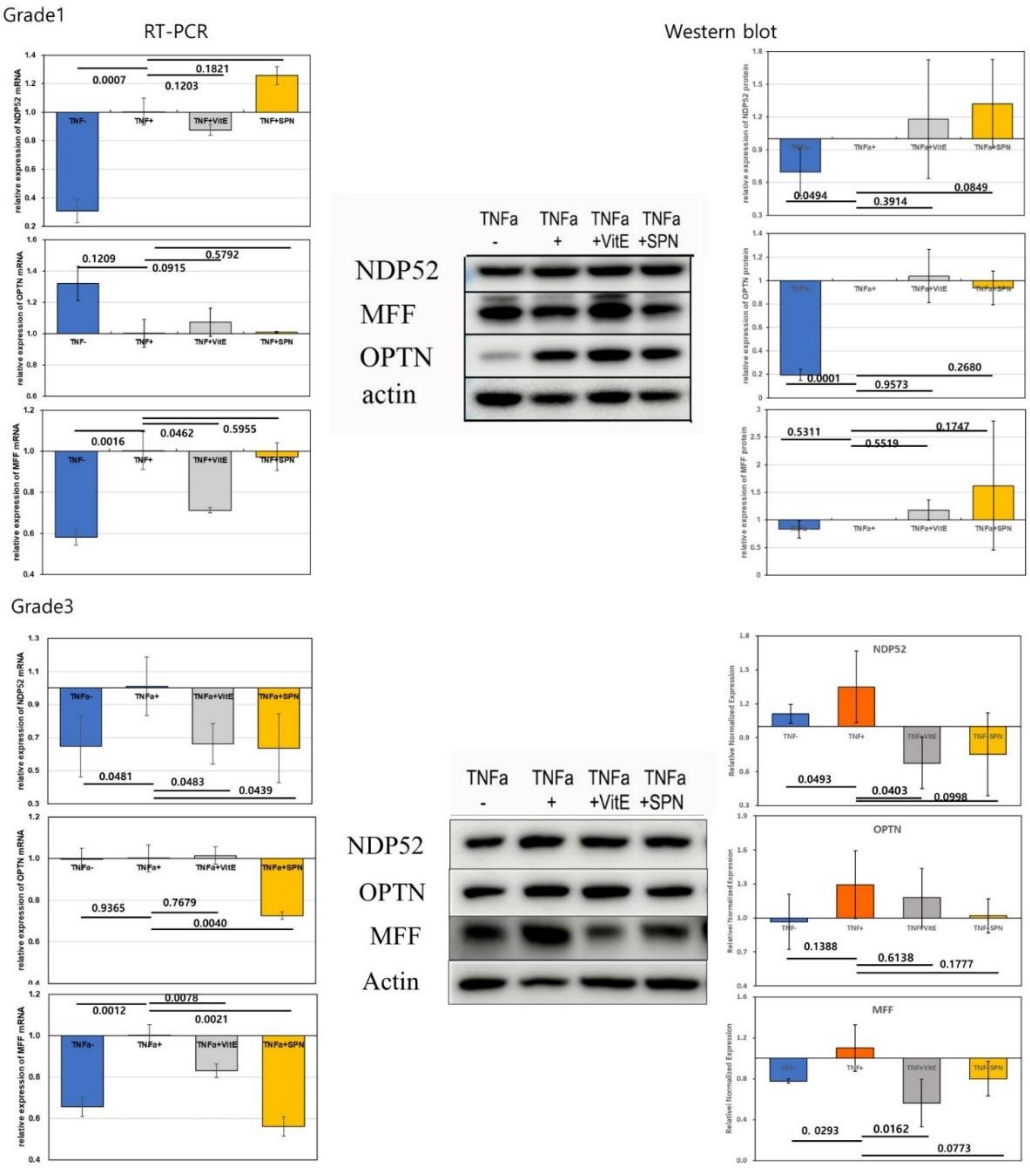
<Figure 7> MFN1 and MFN2 expressions observed through immunocytochemistry in grade III disc NPCs.

In all experiments, control, TNF-α-induced inflammation, anti-inflammation by vitamin E, and anti-inflammation by SPN groups were compared. MFN: Mitofusin, TNF-α: Tumor necrosis factor-α, Vit E: Vitamin E, SPN: Saponin, NPC: Nucleus pulposus cell

3.3 Effects of TNF-α on mitofission and autophagy receptor-related proteins and the role of antioxidants in mitigating these effects

Quantitative messenger RNA (mRNA) expression analysis revealed distinct patterns of NDP52, OPTN, and MFF in grades I and III disc NPCs under different experimental conditions, suggesting differential responses to inflammation and antioxidant treatments.

In grade I disc NPCs, NDP52 mRNA expression increased significantly post-TNF-α treatment compared to the control condition. The administration of Vit E and SPN did not result in a decrease in NDP52 mRNA expression. Conversely, in grade III disc NPCs, TNF-α treatment led to an increase in NDP52 mRNA expression, while Vit E and SPN significantly reduced its expression. These trends were consistent with the findings of the western blot analysis, which mirrored the mRNA results. For OPTN, in grade I disc NPCs, TNF-α treatment did not substantially alter its mRNA expression, nor did the administration of Vit E and SPN. However, SPN treatment alone led to a decrease in OPTN mRNA expression in grade III disc NPCs. Western blot analysis showed a distinct pattern: OPTN protein expression increased in response to TNF-α treatment in grade I disc NPCs, while no significant decrease in its expression was observed with Vit E or SPN in grade I or III disc NPCs. MFF exhibited an increase in mRNA expression in both grades I and III disc NPCs post-TNF-α treatment, indicating enhanced mitochondrial fission under inflammatory conditions. When treated with Vit E or SPN, MFF mRNA expression significantly decreased in both grades of disc NPCs. However, western blot results revealed some variation: no significant change was observed in MFF protein expression across TNF-α, Vit E, or SPN treatments in grade I disc NPCs. In grade III disc NPCs, MFF protein expression significantly increased under TNF-α treatment and decreased significantly with Vit E treatment. While SPN reduced MFF expression in grade III disc NPCs, the change was not statistically significant (Figure 8).



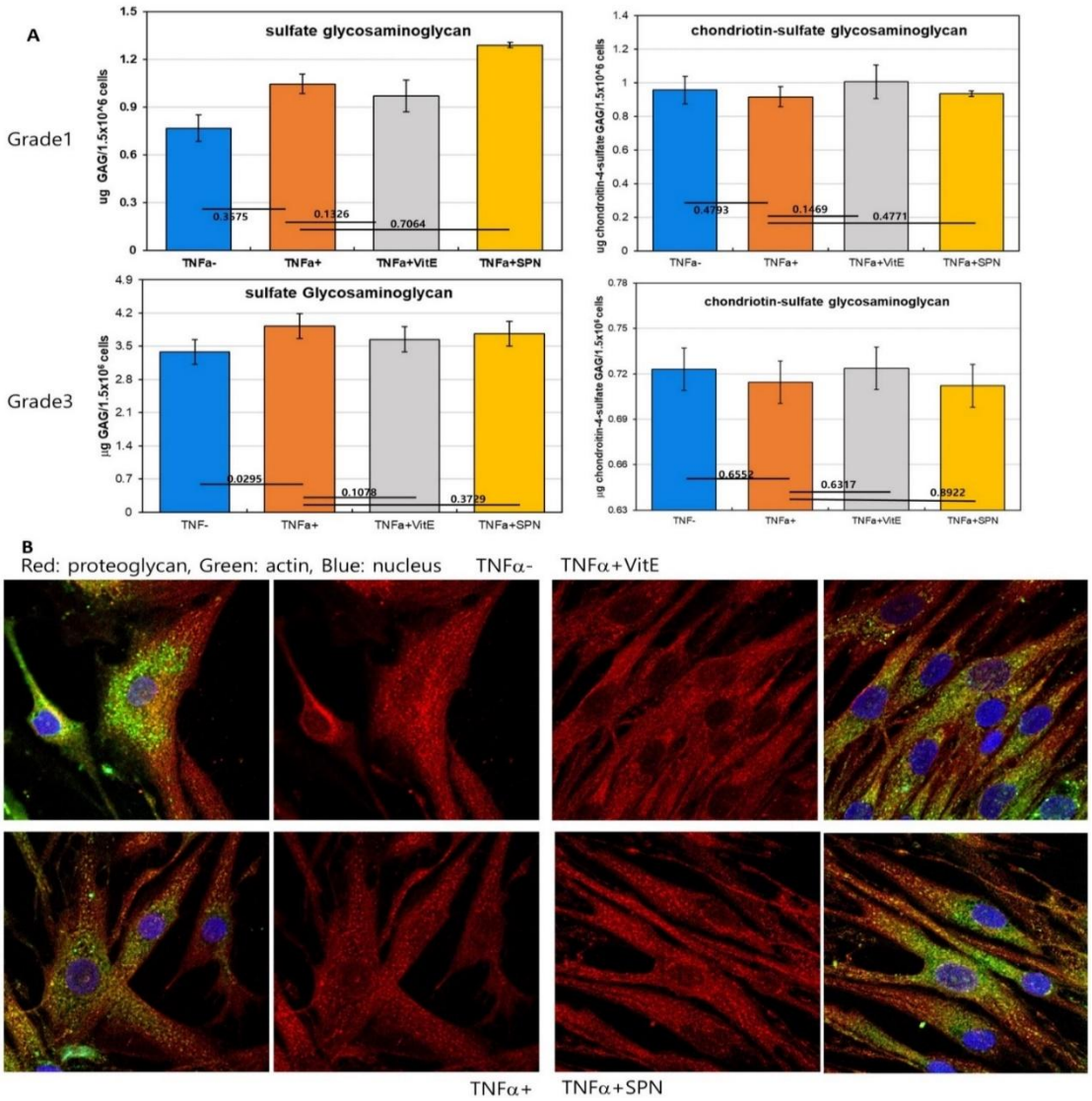
<Figure 8> Analysis of NDP52, MFF, and OPTN expressions in grades I and III disc NPCs

A. The expression levels of NDP52, MFF, and OPTN in grade I disc NPCs were analyzed using qPCR and western blotting. In grade 3 disc NPCs, the expression levels of NDP52, MFF, and OPTN were analyzed using qPCR and western blotting. In all experiments, control, TNF-α-induced inflammation, anti-inflammatory by vitamin E, and anti-inflammatory by SPN groups were compared. *NDP52*: Calcium binding and coiled-coil domain 2, *OPTN*: Optineurim, *MFF:*

Mitochondrial fission factor, qPCR: Quantitative polymerase chain reaction, TNF-α: Tumor necrosis factor-α, *MFF*: Mitochondrial fission factor, NPC: Nucleus pulposus cell

3.4 Effects of TNF-α on changes in the ECM and the effects of antioxidants

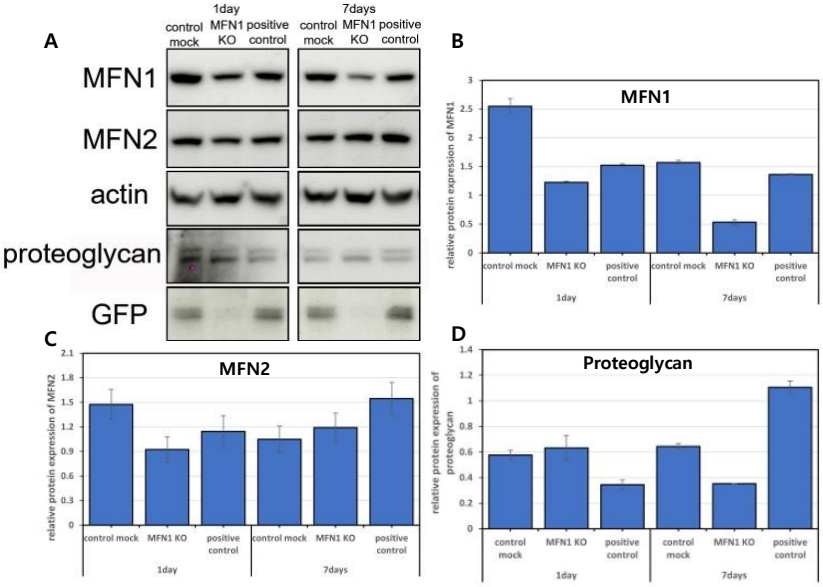
Sulfate GAG levels tended to increase in response to TNF-α treatment in grade III disc NPCs, with no significant changes observed post-treatment with Vit E and SPN. Additionally, immunocytochemistry analysis revealed a tendency for proteoglycan expression to slightly increase post-TNF-α treatment, whereas Vit E and SPN treatment did not affect its expression (Figure 9).



<Figure 9> Analysis of GAG and proteoglycan expression levels in grades I and III disc NPCs.
A. Sulfate and chondroitin-sulfate GAG expression levels in grade I and III disc NPCs as analyzed by ELISA. Proteoglycan expression levels as analyzed by immunocytochemistry. In all experiments, control, TNF-α-induced inflammation, anti-inflammation by vitamin E, and anti-inflammation by SPN groups were compared. GAG: Glycosaminoglycan, ELISA: Enzyme-linked immunosorbent assay, Vit E: Vitamin E, SPN: Saponin, TNF-α: Tumor necrosis factor-α, NPC: Nucleus pulposus cell

3.5 Expression levels of MFN1, MFN2, and proteoglycan in grade III NPCs from *in vivo e*xperiments

The expression levels of MFN1, MFN2, and proteoglycan in grade III disc NPCs from in vivo experiments were analyzed using western blot, dividing the samples into control (mock), MFN1 knockout, and positive control groups. Evaluations were performed on days 1 and 7 post-transduction. On day 1, MFN1 expression was significantly reduced in the MFN1 knockout group compared to the mock group, while the positive control group maintained relatively higher expression levels of MFN1. By day 7, MFN1 expression further decreased in the MFN1 knockout group, and even the mock group showed a decline in MFN1 levels compared to day 1. MFN2 expression level was higher in the MFN1 knockout group than in the mock group on both days 1 and 7. Notably, on day 7, the MFN1 knockout group exhibited a compensatory increase in MFN2 expression when compared to the mock group, indicating a potential adaptive response to MFN1 absence. The proteoglycan expression level was significantly reduced in the MFN1 knockout group compared to the mock group on both days 1 and 7, highlighting the essential role of MFN1 in maintaining proteoglycan levels (Figure 10).

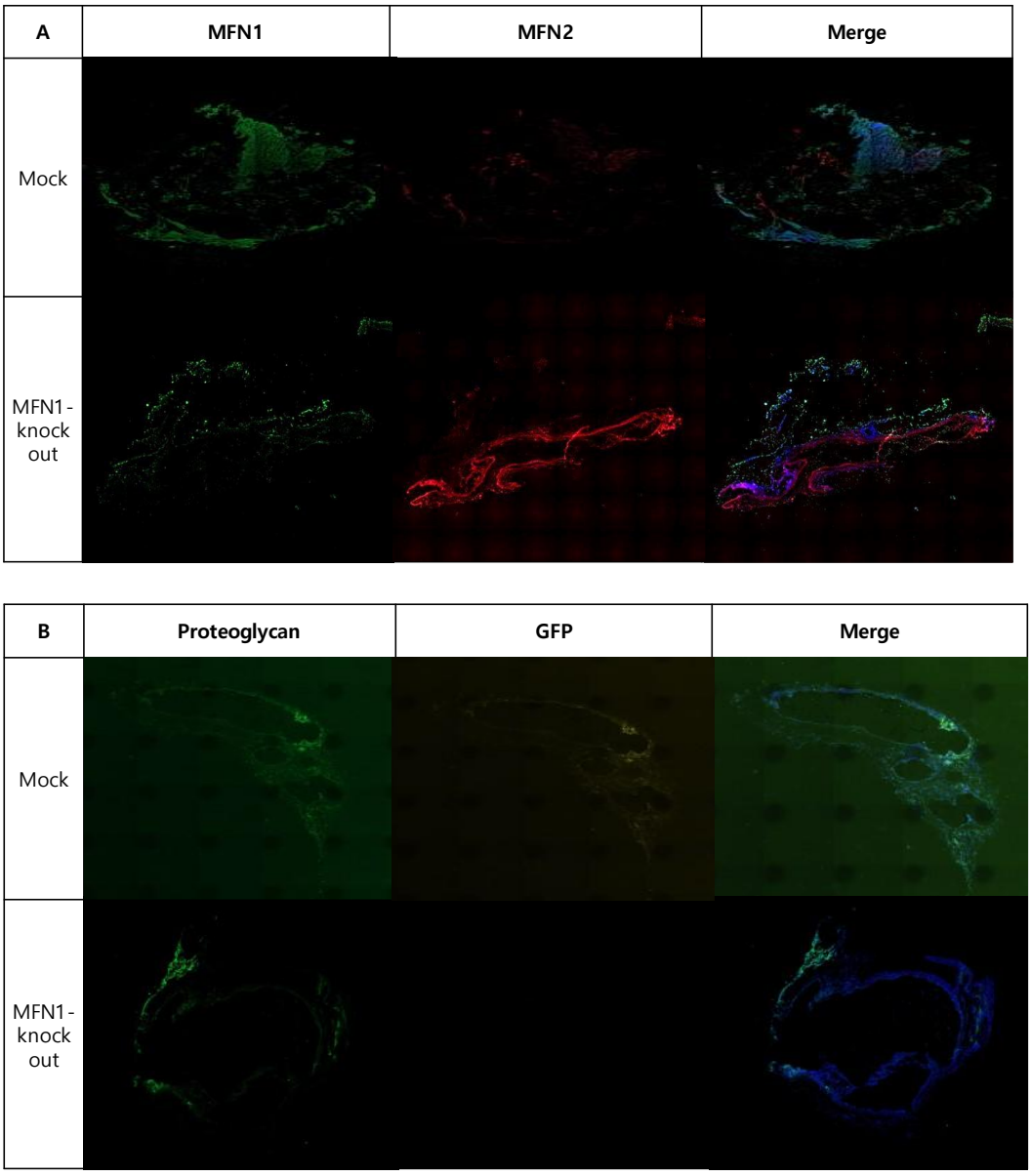


<Figure 10> Expression levels of MFN1, MFN2 and proteoglycan in grade III disc NPCs evaluated *in vivo* using western blot analysis. Samples were categorized into negative control (mock), MFN1 knockout, and positive control groups.

A. The expression levels of MFN1, MFN2, and proteoglycan in grade II disc NPCs were analyzed using western blot in *in vivo* experiments. The expression levels of MFN1 (B), MFN2 (C), and

proteoglycan (D) in grade III disc NPCs were analyzed using western blotting, and the results are presented as graphs. In all experiments, the samples were categorized into negative control (mock), MFN1 knockout, and positive control groups. Expression levels were evaluated at both days 1 and 7 post-transplantation to assess temporal changes among the groups. MFN: Mitofusin, KO: Knockout mice, GFP: Green Fluorescent Protein, NPC: Nucleus pulposus cell

Immunofluorescence analysis performed at day 7 post-transduction showed that MFN1 expression was absent in MFN1-knockout cells, confirming the successful knockout. In contrast, MFN2 expression was more prominently expressed in the knockout group than in the mock group. Proteoglycan expression was reduced in MFN1-knockout cells compared to the mock group (Figure 11).



<Figure 11> Immunofluorescence analysis of MFN1, MFN2, proteoglycan, and GFP in the mock and MFN1-knockout cells.

(A) Expression and co-localization of MFN1 (green) and MFN2 (red) in the mock and MFN1-knockout cells. MFN1 is absent in the knockout group; however, MFN2 is more prominently expressed in the knockout group compared to the mock group.

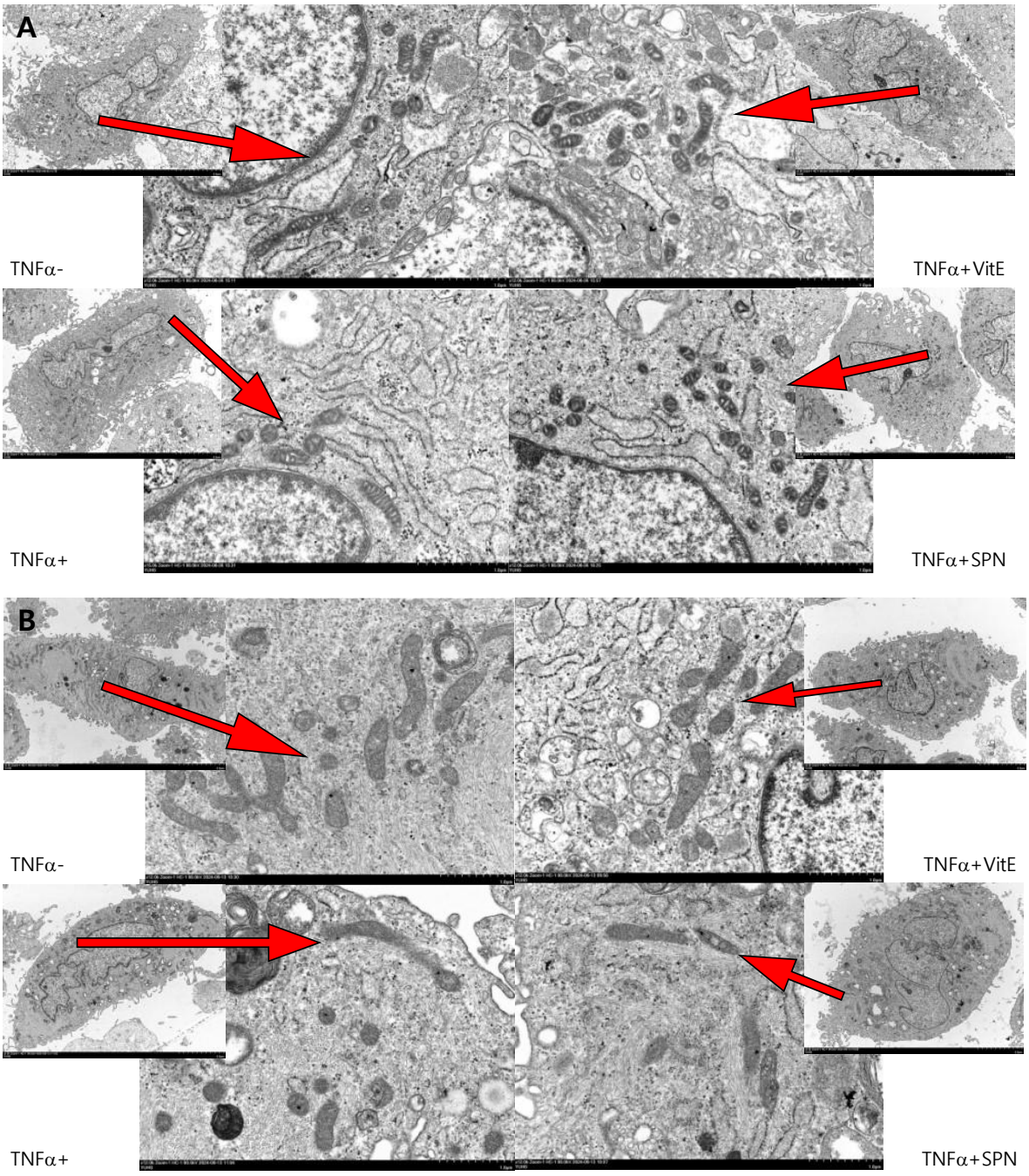
(B) Distribution of proteoglycan (green) and GFP (yellow) in Mock and MFN1-knockout cells. Proteoglycan expression is reduced in the MFN1-knockout group compared to the mock group.

4. Discussion

In IVD NPCs, MQC mechanisms are crucial for responding to inflammation by maintaining mitochondrial homeostasis through processes such as fission, fusion, and mitophagy. [13] These processes segregate and remove damaged mitochondria, preserving cellular function. However, persistent inflammation and accumulated stress can overwhelm these protective mechanisms, leading to mitochondrial dysfunction, which is characterized by impaired membrane potential and increased ROS levels. These factors contribute to the progression of IVD degeneration and result in structural and functional deterioration. [14,15] During inflammation, the activation of mitophagy and autophagy receptors plays a vital role in managing mitochondrial stress and damage. Mitofission helps isolate damaged mitochondrial fragments, whereas mitophagy clears them, preventing excessive cellular damage and maintaining energy balance. This coordinated response is essential for the survival of disc NPCs under inflammatory conditions. [13,16,17]

NPCs exhibit a complex cellular response aimed at managing inflammation, cellular stress, and tissue remodeling in response to inflammation. Mitophagy activation helps maintain mitochondrial health by removing damaged components and optimizing mitochondrial function. Concurrently, the increase in ECM suggests that NPCs actively stabilize and repair tissue structures, which is a crucial step in maintaining IVD integrity. Collectively, these processes reflect the mechanisms by which NPCs are activated to preserve homeostasis and protect against cellular damage under inflammatory conditions. This balance between cellular repair and tissue remodeling indicates the adaptive response of NPCs to maintain functionality and prevent degeneration. [13]

Based on TEM images, the morphology of mitochondria can vary depending on the cells' condition. In grade I disc NPCs, mitochondria tend to be smaller and more numerous, which is indicative of a metabolically active and healthy state. However, they tend to be larger and fewer in grade III disc NPCs, which may be associated with degenerative changes or a damaged cellular state. These changes may reflect mitochondrial dysfunction or impaired cellular energy metabolism. In grades I and III disc NPCs, the number of mitochondria decreased during inflammation, while it increased upon the introduction of anti-inflammatory agents, which also appeared more pronounced or dense. During inflammation, cellular energy metabolism and function are impaired, leading to a decreased number of mitochondria. Inflammatory conditions increase oxidative stress and decrease mitochondrial function, thereby affecting the number and structural integrity of mitochondria (Figure 12).



<Figure 12> Mitochondrial morphology of grades I and III disc NPCs using TEM.

(A) In grade I NPCs, the mitochondria are small, numerous, and structurally intact, reflecting a healthy state with active metabolism. In all experiments, control, TNF-α-induced inflammation, anti-inflammation by Vit E, and SPN-induced inflammation groups were compared.

(B) Grade III disc NPCs exhibit larger and fewer mitochondria with structural damage and reduced density, indicative of degenerative changes and impaired mitochondrial function. In all experiments, control, TNF-α-induced inflammation, anti-inflammation by Vit E, and SPN-induced inflammation groups were compared. Vit E: Vitamin E, SPN: Saponin, TNF-α: Tumor necrosis factor-α, TEM: transmission electron microscopy, NPC: Nucleus pulposus cell

The PCR results demonstrated that MFN1 and MFN2 expression levels increased in response to inflammation in disc NPCs, highlighting their roles in managing mitochondrial dynamics under inflammatory stress. While MFN1 showed a slightly more pronounced initial response, MFN2 exhibited comparable regulatory effects on mitochondrial fusion and protection of mitochondrial function. Following inflammation, MFN1 and MFN2 expressions decreased, even after Vit E treatment, with MFN1 showing a slightly greater reduction in grade III disc NPCs. This decrease in expression correlated with reduced ECM levels, suggesting that MFN1 and MFN2 jointly contribute to mitochondrial dynamics and ECM regulation under inflammatory conditions, albeit with minor differences in sensitivity to inflammatory signals. [13,18,19]

The in vivo results highlight the critical role of MFN1 in maintaining ECM integrity and regulating proteoglycan levels under physiological conditions. Additionally, the significant reduction in proteoglycan levels in the MFN1 knockout group at days 1 and 7 underscores MFN1's essential function in preserving ECM stability. Notably, the compensatory increase in MFN2 expression observed in the MFN1 knockout group suggests an adaptive mechanism to mitigate mitochondrial dysfunction and maintain cellular homeostasis in the absence of MFN1. Despite this compensatory response, MFN2 alone was insufficient to fully preserve proteoglycan levels, indicating that MFN1 has a unique and indispensable role in ECM maintenance. These findings emphasize the interplay between MFN1 and MFN2 in regulating mitochondrial dynamics and ECM stability, and they provide a foundation for exploring therapeutic strategies targeting mitochondrial function in degenerative disc diseases.

Vit E, SPN, curcumin, omega-3, glucosamine, and chondroitin are well-known anti-inflammatory agents recognized for their ability to reduce pro-inflammatory cytokines. In this study, we conducted experiments using Vit E and SPN to modulate the inflammatory pathway in disc NPCs. We observed that Vit E exhibited a more pronounced effect on expression changes in disc NPCs than SPN, with significant differences in the expression of MFN1, MFF, and NDP52 proteins in grade III disc NPCs. Specifically, Vit E demonstrated a greater ability to modulate the expression of genes associated with inflammation than SPN, leading to significant reductions in inflammatory markers and enhanced anti-inflammatory responses. Moreover, this suggests that Vit E plays an important role in regulating the inflammatory environment in disc NPCs, thereby providing better protection against inflammation-induced damage. [20,21]

The inflammation and anti-inflammatory effects on MFN1, MFN2, NDP52, OPTN, and MFF expression observed via western blotting were more pronounced in grade III disc NPCs than in grade I disc NPCs. This finding could be because more advanced disc degeneration (as observed in grade III) leads to greater cellular stress and mitochondrial dysfunction. As disc degeneration progresses, cells may become more sensitive to inflammatory stimuli, causing a heightened response in mitochondrial dynamics. Consequently, the anti-inflammatory effects of agents, such as Vit E, are also more pronounced in mitigating inflammation-induced stress, particularly in more severely degenerated discs.

4.1 Comparison with previous studies

Although prior research has established that mitochondrial dysfunction plays a central role in IVD degeneration, particularly through oxidative stress and disrupted mitochondrial dynamics, our study adds significant new dimensions. Previous studies have primarily focused on mitochondrial fission and autophagy as key mechanisms underlying degenerative processes. [2,4,7] The current study highlights the distinct and critical roles of MFN1 and MFN2 in regulating mitochondrial fusion under inflammatory conditions. In grade III disc NPCs, MFN1 demonstrated effects comparable to those of MFN2, suggesting that both proteins play significant roles in responding to inflammatory stimuli and maintaining mitochondrial dynamics. This is an important distinction, as the heightened sensitivity of MFN1 may make it a more relevant therapeutic target in conditions characterized by chronic inflammation, such as degenerative disc disease. Contrary to the earlier focus on general mitochondrial dysfunction, our results indicate that selective modulation of MFN1 enhances mitochondrial fusion and protects cellular function. While the use of antioxidants to combat inflammation is well established, our data provide detailed mechanistic insights into the modulation of proteins related to mitochondrial dynamics, including MFN1 and its associated pathways, a connection that has been previously underexplored. [21]

4.2 Clinical implications

The clinical implications of these findings are significant. As degenerative disc disease continues to be a major contributor to chronic pain, developing therapies that specifically target mitochondrial dynamics may improve patient outcomes. The ability of Vit E to downregulate MFN1 in response to inflammation suggests that antioxidant therapies serve as a viable, non-invasive treatment option for slowing or reversing disc degeneration. This finding holds particular relevance in the context of aging populations, where the prevalence of degenerative conditions is increasing.

Moreover, targeting MFN1 to modulate mitochondrial fusion and fission may represent a novel therapeutic strategy beyond traditional anti-inflammatory and surgical interventions. This could delay the need for surgical treatments, such as spinal fusion and disc replacement, thereby reducing the risk of surgical complications and improving the long-term quality of life of patients.

From a policy perspective, our findings suggest that early intervention with antioxidant therapies could be incorporated into clinical guidelines for managing early-stage disc degeneration. Such an approach could help reduce healthcare costs by delaying disease progression and reducing the need for invasive procedures. In clinical practice, these findings could support the development of personalized treatment protocols where patients with high inflammatory profiles may benefit from antioxidant therapies specifically targeting mitochondrial dysfunction. However, further clinical trials should explore the optimal dosing and timing of Vit E and other antioxidants to maximize their protective effects on disc NPCs.

4.3 Future research directions

In the future, therapeutic strategies that combine antioxidants with agents that specifically modulate mitochondrial fusion and fission should be explored. Clinical trials that evaluate the efficacy of antioxidant therapies in preventing or delaying disc degeneration would also provide direct evidence for integrating these treatments into clinical practice. MQC is involved in various pathways beyond those discussed, and its roles in inflammation and anti-inflammation should be considered in future research.

5. Conclusions

In disc NPCs, inflammation activates mitofusion, mitofission, and autophagy, which help maintain cellular homeostasis. Vit E has anti-inflammatory effects on NPCs. Notably, MFN1 tended to be activated as the pathogenesis of NPCs progressed, with significant differences observed between inflammatory and anti-inflammatory conditions. These findings highlight the critical roles of mitochondrial dysfunction and inflammation in IVD degeneration. Furthermore, the modulation of mitochondrial dynamics by antioxidants, such as Vit E, may provide a potential therapeutic strategy for treating and preventing degenerative disc disease.

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Abstract (in Korean)

염증 유발에 따른 추간판 수핵 세포에서의 미토콘드리아 품질 관리에 있어 Mitofusin 1의 역할 및 항염증제의 효과

서론

미토콘드리아 기능 장애는 산화 스트레스와 같은 외부 자극에 의해 유발되며, 이는 추간판(IVD) 퇴행의 주요 원인 중 하나입니다. 미토콘드리아 융합과 분열은 Mitofusin 1(MFN1), Mitofusin 2(MFN2), Dynamin-related protein 1(DRP1)등 과 같은 단백질에 의해 조절되며, 미토콘드리아의 구조적 안정성을 유지하는 데 필수적인 역할을 합니다. 항산화제는 염증을 억제하고 IVD 퇴행을 예방하는 효과가 있다고 알려져 있으나, 이들이 추간판 수핵 세포(NP) 내 미토콘드리아 기능에 미치는 구체적인 기전은 아직 명확하게 밝혀진 바가 없습니다.

목적

본 연구의 목적은 염증이 추간판 수핵 세포(NP)의 미토콘드리아 역동성에 미치는 영향을 조사하고, 특히 MFN1의 역할을 중심으로 비타민 E와 saponin이 염증 상황에서 항염증 효과를 나타내는지 평가하는 것입니다.

방법

퇴행성 디스크 질환으로 수술을 받은 환자 15명으로부터 추간판 조직을 채취하여 추간판 수핵 세포(NP)를 분리하고 배양하였습니다. 실험에 사용된 디스크는 피르만 그레이드 1과 3을 주로 사용하였습니다. 배양된 세포에 TNF-α를 처리하여 염증을 유도한 뒤, 비타민 E 및 사포닌을 투여하여 항염증 효과를 평가하였습니다. RNA 추출 후 실시간 PCR을 통해 유전자 발현을 분석하였고, 단백질 발현은 웨스턴 블롯, 면역세포화학염색, 그리고 전자현미경을 통해 확인하였습니다. 또한, in vivo 분석을 위해 MFN1 억제 수핵 세포를 BALB/c 누드 마우스에 이식하여 생리적 조건에서 MFN1 억제가 ECM 유지에 미치는 영향을 평가하였습니다.

결과

TNF-α에 의해 유도된 염증은 MFN1 및 MFN2와 같은 미토콘드리아 융합 단백질의 발현을 증가시켰으며, 비타민 E와 사포닌 처리 후 이들 단백질의 발현이 감소하는 경향을 보였습니다. 특히, MFN1은 염증 조건에서 세포외기질(ECM) 유지에 중요한 역할을 하는 것으로 나타났습니다. 염증은 미토콘드리아 분열 및 자가포식 관련 단백질의 발현을 증가시켰으나, 항산화제 처리 후 이들 단백질의 발현이 감소하는

양상을 보였습니다. ECM의 변화는 통계적으로 유의미하지 않았지만, 염증 및 항염증 반응에서 미토콘드리아 융합 및 분열 관련 단백질의 변화는 그레이드 1 디스크 NP 세포보다 그레이드 3 디스크 NP 세포에서 더 두드러진 반응을 보였습니다. 전반적으로, MFN1은 ECM 유지에 핵심적인 역할을 하며, 비타민 E는 사포닌보다 더 큰 항염증 효과를 디스크 NP 세포에서 나타냈습니다.

결론

염증 상태에서 NP 세포는 미토콘드리아 융합 및 분열 과정을 활성화하여 세포의 항상성을 유지하려는 기전을 나타내며, 비타민 E는 이러한 세포에서 항염증 효과를 발휘합니다. 특히 MFN1은 염증이 진행됨에 따라 더 크게 활성화되며, 항염증제 처리 시 염증과 항염증 간의 유의미한 차이를 보였습니다. 본 연구 결과는 추간판 퇴행성 질환에서 미토콘드리아 기능 장애와 염증 반응이 중요한 역할을 한다는 점을 시사합니다. 비타민 E와 같은 항산화제가 미토콘드리아 역동성을 조절하고 염증을 완화함으로써, 추간판 퇴행성 질환의 예방 및 치료에 잠재적 치료 전략으로 활용될 수 있음을 보여줍니다.

핵심되는 말 : 미토콘드리아 질 관리, 수핵 세포, 디스크 퇴행, 미토퓨진