European Review for Medical and Pharmacological Sciences 2022; 26: 2990-3000

Examining the effects of HMG-CoA reductase inhibitors on anabolic and catabolic signaling pathway proteins associated with degenerative disc disease

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Abstract. – OBJECTIVE: Recent evidence suggests that statins, among lipid-lowering drugs, can be used to lower serum cholesterol levels for reducing the risk of cardiovascular disease in individuals with high cholesterol, as well as reducing DNA damage and having anti-ageing and pleiotropic effects. Additionally, nuclear factor kappa B (NF-κB) is reported to be suppressed in statin-administered nucleus pulposus (NP) cells for the prevention of interleukin (IL) -1 beta (IL-1β)-induced apoptosis and extracellular matrix (ECM) degradation. The purpose of this study is the examine whether it is possible for pharmacological synthetic statin agents added into primary cell cultures obtained from human intervertebral disc tissue (IVD) to stop and eliminate tissue degeneration through the anabolic/catabolic signaling pathways associated with inflammation.

MATERIALS AND METHODS: Pitavastatin and rosuvastatin were added to monolayer grown human primary annulus fibrosus (AF)/NP cells. Cytotoxicity and proliferation analyses were carried out. AF/NP cells and ECM structure were also examined microscopically. In addition, changes in transcription factors and protein expressions of proinflammatory cytokines, which play important roles in anabolic and catabolic pathways associated with inflammation, were analyzed.

RESULTS: Decreased proliferation and cell necrosis were observed at the end of 72 hours in the samples, in which statins were added, compared to the samples in the control group to which no pharmacological agent was administered. In addition to this, changes were observed in the expressions of proteins. All results were statistically significant ($p<0.05$).

CONCLUSIONS: To better understand the regenerative effects of these two pharmacological agents on degenerated AF/NP cells, there is an urgent need for prospective studies in which different signaling pathways and receptors on these pathways are investigated, apart from IL-1β; NF-κB signaling pathway and SOX9.

Key Words:
IL-1β, NF-κB, Pleiotropic effect, Pitavastatin, Rosuvastatin, SOX9.

Abbreviations

ECM: extracellular matrix; ETOH: ethanol; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase; IDD: intervertebral disc degeneration; IL-1β: interleukin (IL) -1 beta; IVD: intervertebral disc; NF-κB: nuclear factor kappa B; NP: nucleus pulposus; SOX9: (sex-determination region Y [SRY] protein-related high-mobility group box genes)-9.

Introduction

Statins, which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are strong inhibitors of cholesterol biosynthesis[1]. This group of drugs is widely used for prevention of cardiovascular diseases and lowering serum cholesterol levels[2]. Statins have many beneficial effects on the vascular system functions in addition to lowering serum cholesterol levels[3].

Some of these beneficial effects, also called pleiotropic effects, include regulation of endothelial functions, antioxidant properties, plaque sta-

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bilization, inhibition of inflammatory response, and immunomodulatory effects[4-6]. Additionally, it is reported in the literature that statins have positive effects on osseous tissue and some systemic diseases, inhibit osteoclastic bone destruction[7], and stimulate the production of specific proteins involved in bone and cartilage development[8].

Intervertebral disc (IVD) degeneration (IDD) is a major contributor to chronic low back pain and is characterized by decreases in cellularity[9] and proteoglycan synthesis, upregulation of extracellular matrix (ECM) degradation, and increases in pro-inflammatory factors with neurovascular invasion[10].

The concept of disc degeneration, accepted as a physiological process, refers to the changes that occur due to ageing. Degenerative disc disease, on the other hand, is a clinical condition emerging early in life as a result of accelerated degenerative changes, which cause clinical symptoms due to radiculopathy and myelopathy in the spinal cord. Surgery is recommended for cases with progressive neurological deficits unresponsive to conservative and medical treatments. Despite available treatment modalities, upon obtaining satisfactory results in the treatment of disc degeneration, scientists have focused on both understanding the molecular mechanisms underlying pathogenesis and investigating the effect of different molecules in treatment, apart from the pharmacological agents used in existing degenerative disc treatment. In this respect, there is ongoing research on the positive and negative effects of various pharmaceuticals on the physiopathology of intact or degenerate IVD tissue at the molecular level. Statins are also among these pharmaceuticals[11].

In the past[9], SOX9 was reported to be a chondrogenic marker acting in the anabolic pathway of IVD and increasing ECM production. IDD is characterized by elevated levels of pro-inflammatory cytokines including and interleukin (IL)-1 beta (IL-1β), as well as down-regulation of expression of the important ECM molecules[12]. Nuclear factor-kappa B (NF-κB) is an ancient protein transcription factor and the NF-κB signaling pathway links pathogenic signals and cellular danger signals, thus organizing cellular resistance to invading pathogens, IVD cells[13,14]. IL-1β and NF-κB play an important role in autophagy, senescence, and apoptosis of IVDD[15,16].

There are studies in the literature reporting that osteoporosis and IDD occurring in the osseous tissue of the spinal column are common conditions that primarily affect the elderly and significantly affect the quality of life, and that there is a relationship between IDD and osteoporosis. A study on hypercholesterolemic postmenopausal women diagnosed with osteoporosis or osteopenia[17] evaluated bone mineral density in the lumbar vertebral corpus in cases given atorvastatin combined with risedronate. Compared to patients given risedronate alone, bone mineral density was reported to increase twice in the group to which atorvastatin was added[17]. A meta-analysis[18] reported that hip fractures decreased by up to 40% and hip bone mineral density improved in patients receiving statin.

A new function and mechanism were demonstrated for pitavastatin in bone remodeling, indicating its potential as a therapeutic candidate in treating osteoporosis by inhibiting osteoclastic resorption and promoting osteoblastic formation[19]. Rosuvastatin was reported to promote osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by the Wnt/β-catenin signal[20].

As with any pharmacological treatment, adverse effects of statin have been reported over the years and the clinical spectrum of statin has been reported to cause myalgia, myopathy, and rhabdomyolysis[21,22]. Today, as with any medication, the safety of statins is questioned, due to side effects/adverse events reported in various studies.

There are also *in vitro* studies in which NP cell cultures treated with lovastatin, a naturally occurring statin, were examined[23,24].

However, in the literature there is no experimental study in which synthetic statins pitavastatin and rosuvastatin were evaluated separately or in combination in human primary IVD cell cultures. Therefore, we believe that the findings of this research can contribute to the literature.

This study was aimed at examining the effects of pitavastatin and rosuvastatin pharmaceuticals, which are synthetic statins, on primary cell cultures obtained from human degenerative IVD tissue. In this way, it was aimed at evaluating the effects of these two drugs on the annulus fibrosus (AF), NP cells and ECM structure, as well as the protein expressions of SOX9, an anabolic factor, and IL-1β and NF-κB together, which are catabolic factors.

Materials and Methods

Inclusion/Exclusion Criteria of the Cases

It has been known that when statins are used in combination with cyclosporine, fibrates, mac-

rolide antibiotics, antifungal drugs, and niacin, they interact with drugs, increasing the incidence of side effects of statins[25,26]. Therefore, for the preparation of primary cell cultures, no tissue was collected from patients who were using cyclosporine, fibrates, macrolide antibiotics, antifungal drugs, and niacin, and they were excluded from the study. In addition, no tissue was collected from patients with any hepatobiliary system disease, and they were excluded from the study. For the preparation of primary IVD cell cultures, tissue was collected from patients undergoing lumbar microdiscectomy or lumbar sequestrectomy for lumbar disc herniation (4 men, 4 women; Pfirrmann Grade III-IV; mean age 44.12±4.87 years)[27].

Obtained tissues were transferred into Falcon tubes containing freshly prepared solutions. They were transferred to the laboratory at 4°C under sterile conditions.

Preparation of Human Primary Cell Cultures

The tissues transferred in Falcon tubes containing 5% penicillin-streptomycin and Dulbecco's Modified Eagle Medium (DMEM) were taken into the flow cabin. Then, the tissues were washed in sterile phosphate buffer saline (PBS) three times consecutively to remove any red blood cell. After the samples were mechanically disintegrated, they were transferred into Falcon tubes containing Clostridium histolyticum based collagenase type I (475 µg/mL) and type II (125 5 µg/mL) enzymes dissolved in Hank's Balanced Salt Solution (HBSS). They were kept overnight in an incubator at 37.4°C and 5% CO_2. Finally, the tissues were centrifuged twice consecutively at 4°C and 1,300 rpm for 10 minutes, thus ensuring that they were enzymatically broken down. Cell pellets at the bottom of the centrifuge were re-suspended using the culture medium prepared with DMEM. After the samples were transferred to the flasks, they were left in incubation for 72 hours. Human NPC/AFC samples were transferred into wells and fed for 21 days[28]. Cells were cultured in freshly prepared growth medium containing DMEM, 5% fetal bovine serum (FBS), and 1% penicillin-streptomycin every other day. For analysis, the cells were counted on a Thoma Slide in the presence of Trypan blue and were plated in 96-well plates at a concentration of 1.5×10^6 cells per well.

Application of Pitavastatin and Rosuvastatin to Cultures

In 96-well plates, 1 µM/L of pitavastatin[29] and 20 µmol/L of rosuvastatin[30] were added in culture media containing DMEM and 10% FBS per well. No statin class drugs were added in culture media for the control group. The control group was named as "group 1". The groups in which pitavastatin and rosuvastatin were added were named as "group 2" and "group 3", respectively.

Cell Viability, Toxicity, and Proliferation

Viability tests were performed using the enzyme-linked immunosorbent assay (ELISA) with the help of a commercial kit [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (MTT). With the help of this kit, MTT viability and proliferation in AF/NP cells were analyzed and the cytotoxicity of pitavastatin and rosuvastatin was examined.

Considering the half-lives of pitavastatin and rosuvastatin, MTT analyzes were performed at zero, 24, 48, and 72 hours (h).

For this purpose, the supernatant culture medium was discarded from the wells and 100 µl MTT solution [MTT dissolved in 1 ml sterile phosphate-buffered saline (PBS) at a pH of 7.4] to prepare stock solution (12 mM) was added per well. After primary cell cultures were incubated for two hours at 37°C in a dark environment, dimethyl sulfoxide was added to stop the reaction. Following this, the wells were incubated for 10 minutes at 37°C at 540 nm wavelength prior to photometric absorbance analysis. The viability of the control group was assumed to be 100% before the addition of pitavastatin and rosuvastatin[31].

AO/PI Staining and Janus Green B staining

Acridine orange (AO) and propidium iodide (PI), nucleic acid binding dyes, were used to determine cell viability and confirm MTT-ELISA data. AO produces green fluorescence by staining all nucleated cells, whether live or dead. PI only permeates through dead cells with poor membrane integrity and stains nucleated cells to form red fluorescence. When stained with both AO and PI, live nucleated cells only fluoresce green fluorescence while dead nucleated cells only fluoresce red. To prepare AO/PI stain, 4-mg AO (dissolved in 2-mL 99% ETOH), 10-g sodium-ethylenediaminetetraacetic acid, 4-mg PI, and 50 mL FBS were mixed into wells. Sterile distilled water was added to the mixture until a volume of 200 mL

was reached[32]. In addition, Janus Green B was used for supravital mitochondrial staining. The indicator Janus Green B changes color according to the amount of oxygen present. When oxygen is present, the indicator oxidizes to a blue color. In the absence of oxygen, the indicator is reduced and changes to a pink color[33].

Western Blotting for SOX9, NF-κB, and IL-1β

Total protein concentration in the protein lysates from each sample was determined spectrophotometrically with Bradford assay. Proteins were first isolated using the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the determination of expression levels by immunoblotting. Then, polyvinylidene difluoride transfer membrane was used, which provides high binding capacity for proteins and nucleic acids and is an ideal membrane for the transfer of proteins. Immunoblotting was performed for SOX9, phospho-NF-κB, and IL-1β target proteins using the WesternBreeze™ Chemiluminescent kit (Catalog no: WB7104, Thermo Fisher Scientific), in accordance with the manufacturer's instructions. Primary antibodies specific to IL-1β (Catalog no: M421B, Thermo Fisher Scientific, Waltham, MA, USA), SOX9 (Catalog no: 14-9765-82, Thermo Fisher Scientific, Waltham, MA, USA), phospho-NF-κB (SER536, catalog no: MA515181, Thermo Fisher Scientific, Waltham, MA, USA) proteins were used. In the Western-blot method, β-Actin (Catalog no: MA1-140, Thermo Fisher Scientific, Waltham, MA, USA) protein was used as the endogenous control.

After treatment with primary antibodies specific to these proteins, sequential washing was performed. Alkaline phosphatase was incubated with a conjugated secondary antibody. In the last stage, the membranes were washed three times and treated with substrate solution. The protein bands transferred to an X-ray film (Thermo Fisher Scientific, Cat# 34090, Waltham, MA, USA) were analyzed using the ImageJ software, where the specific amount of protein in each sample was determined[34].

Statistical Analysis

Statistical analyses were carried out on Minitab version 19.0. Data were given as percentage (%), mean±standard deviation. The post hoc Tukey's honestly significant difference (HSD) was used for intergroup comparisons after one-way analysis of variance was carried out (ANOVA). The results were interpreted with a 95% confidence interval and an alpha significance of <0.05.

Results

In the microscopic evaluation, although not prominent, proliferation was partially suppressed in the pitavastatin group, while a complete suppression was observed in the rosuvastatin group (Figure 1, Figure 2).

While the first column displays images of normal inverted light microscopy, the second column displays images of Janus Green-B staining, and the third column displays images of AO/PI staining. A, D, G, and J display AF/NP samples belonging to the control group where no drugs were administered. B, E, H, and K display the groups treated with pitavastatin. C, F, I, and L display the cells treated with rosuvastatin. In the pitavastatin group, blue color indicator gradually changed into pink at the end of 72 hours in the absence of oxygen (Figure 1K).

Cell necrosis was reported in addition to suppressed proliferation, especially in the rosuvastatin group (Figure 2 F, I, and L).

In the pitavastatin groups, proliferation decreased by 20.35%, 31.32% and 49.94% at 24, 48, and 72 hours, respectively, compared to the control group. In the rosuvastatin groups, proliferation decreased by 39.53%, 42.92%, and 47.42% at 24, 48, and 72 hours, respectively, compared to the control group. This decrease in proliferation was also statistically significant ($p<0.05$) (Table I, Table II, Figure 3).

While presenting averages of the data obtained from Western blotting in the ImageJ program, the protein expressions of IL-1β, NF-κB and Sox9 signaling pathway were accepted as 100% (1-fold) in the control group (Table III).

The protein expression of SOX9 increased by 59%, 7%, and 47% at 24, 48, and 72 hours, respectively, in the pitavastatin group compared to the control group in which no statin was administered ($p<0.05$). It was observed that IL-1β expression increased by 105%, 4%, and 79%, respectively ($p<0.05$). In the same time periods, NF-κB increased by 60%, 12%, and 84%, respectively ($p<0.05$).

In the rosuvastatin groups, the protein expression of SOX9 increased by 186% and 255% at 24 and 48 hours, respectively, compared to the control group ($p<0.05$). However, elevated SOX9

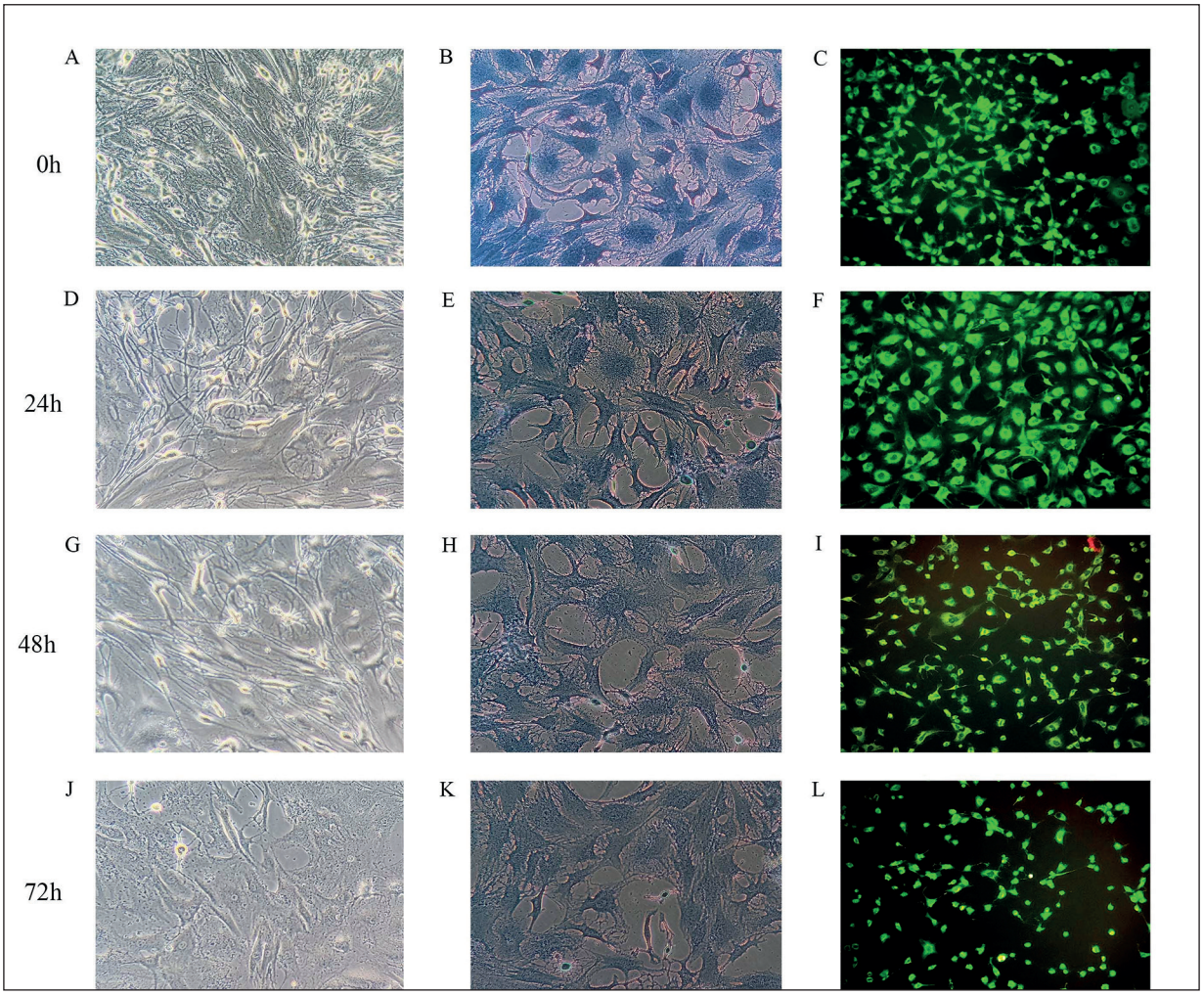

Figure 1. Stained and unstained images of inverted microscopy after the administration of pitavastatin (Janus Green-B stained, 20× magnification; AO and PI stained, 10× magnification).

expression was reported to decrease by 4% by the end of 72 hours ($p<0.05$). It was observed that IL-1β expression increased by 107%, 185%, and 113%, respectively ($p<0.05$). Concurrently, NF-κB increased by 37%, 65%, and 48%, respectively ($p<0.05$) (Figure 4).

Discussion

Pitavastatin has a long half-life (up to 12 hours)[35]. Rosuvastatin is a hepatoselective synthetic enantiomer that is relatively hydrophilic and has minimal metabolism via the cytochrome P450 3A4 system. Rosuvastatin, like atorvastatin, has a plasma half-life of approximately 20 hours and is a potent HMG-CoA reductase inhibitor[36]. Both pitavastatin and rosuvastatin[37] are frequently prescribed in clinics as they significantly lower serum low-density lipoprotein-cholesterol levels. In addition to beneficial cardiovascular effects, this group of drugs has been investigated quite extensively due to their pleiotropic effects.

Today, IVD is known to be a lymphatic tissue[28,31,32]. Another well-known scientific fact is that many of the drugs administered orally or parenterally to the body in any way accumulate in the synovial fluid compartment[28,31,32]. Drugs and/or nutrients are diffused from hyaluronic or synovial tissues and then into body fluids in significant amounts, and then pass through the pores in the hyaline membrane in the cartilage structure, where they diffuse into the cells[28,31,32]. In the lit-

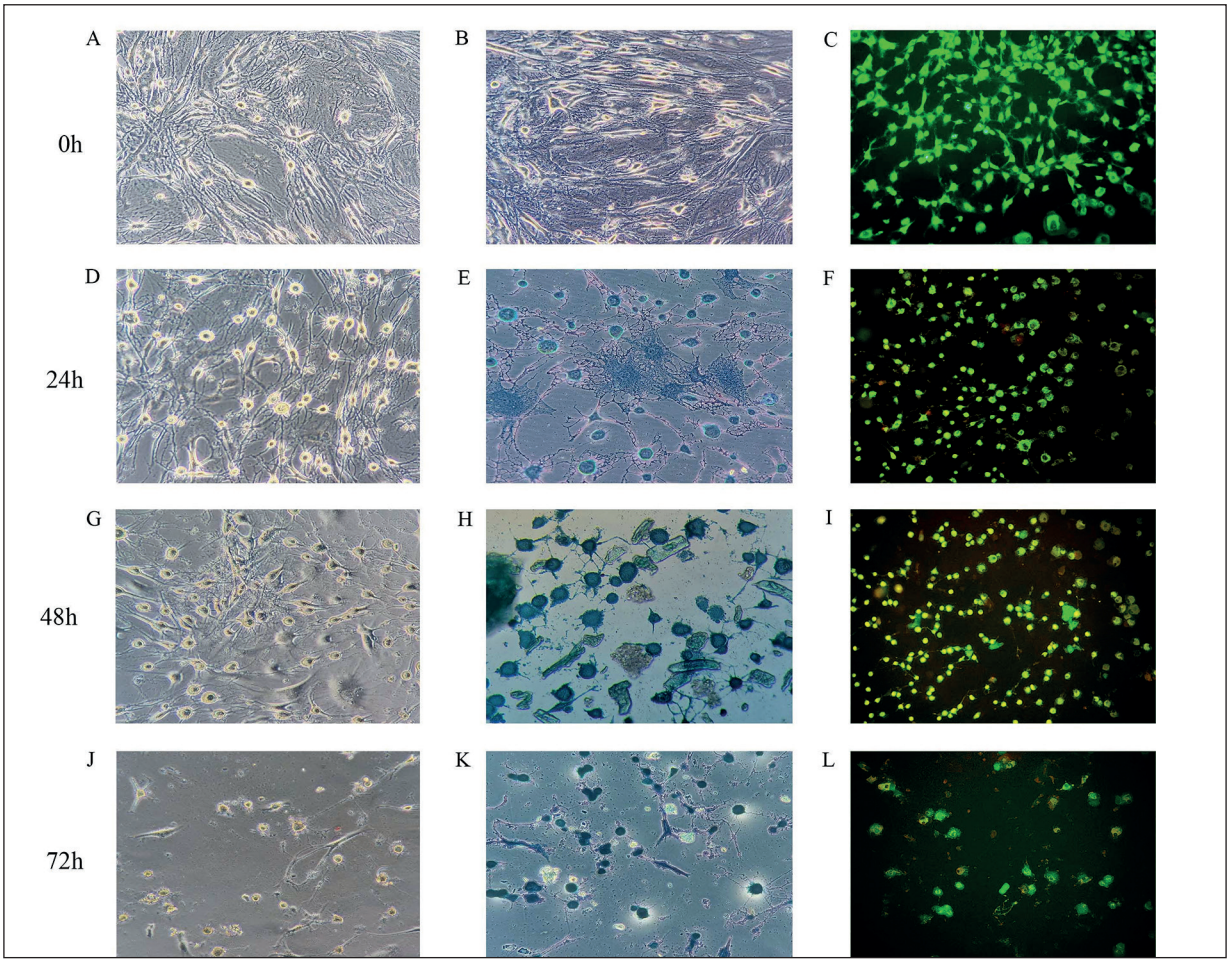

Figure 2. Stained and unstained images of inverted microscopy after the administration of rosuvastatin (Janus Green-B stained, 20× magnification; AO and PI stained, 10× magnification).

erature, statins are also reported to accumulate in IVD[38,39]. Various studies report that statins cause cell differentiation, cell apoptosis, cell proliferation, cell migration, and angiogenesis[40].

Table I. Presentation of the findings of Tukey HSD analysis and cell proliferation data after one-way analysis of variance.

Variable	Time (hours)	Mean ± StDev	Tukey HSD Grouping*
Control absorbance (nm)	0	0.425 ± 0.001	D
	24	0.511 ± 0.001	C
	48	0.565 ± 0.001	B
	72	0.614 ± 0.003	A
Pitavastatin absorbance (nm)	0	0.425 ± 0.001	D
	24	0.407 ± 0.001	E
	48	0.388 ± 0.001	F
	72	0.308 ± 0.001	H
Rosuvastatin absorbance (nm)	0	0.425 ± 0.001	D
	24	0.309 ± 0.001	H
	48	0.323 ± 0.001	G
	72	0.323 ± 0.001	G

**p <0.05 and Data were analyzed using a one-way analysis of variance followed by a post-hoc Turkey Pairwise Comparison test. A: Highest rate of cell viability and proliferation. J: Lowest rate of cell viability and proliferation.

Table II. Assessment of the cell viability of AF/NP cells following drugs treatment.

Source	Adj SS	Adj MS	F-Value	*p*-value*
Groups	0.454451	0.227226	211920.32	0.000
Time (hours)	0.003412	0.001137	1060.88	0.000
Groups* Time (hours)	0.215372	0.035895	33477.47	0.000

*p <0 .05 and data were analyzed using a one way analysis of variance. Adj SS, adjusted sum of squares; Adj MS, adjusted mean square.

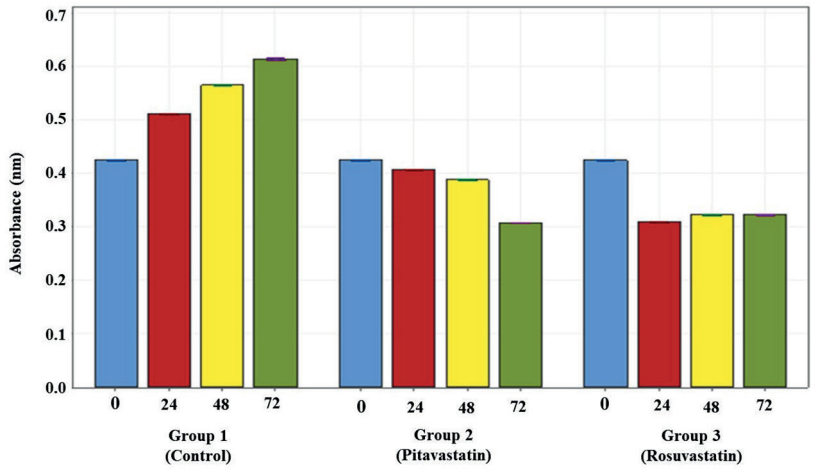

Figure 3. Interval plot of absorbance (540 nm, O.D.) in the experimental groups treated with pitavastatin and rosuvastatin compared to the control group.

Many experimental studies examining the effects of statins have used commercial cell lines[41,42] or animal tissues[43,44].

However, it is well known that commercial cell lines contain only a single cell type. These cells do not possess complex coordination mechanisms. The results of experimental studies using cell lines may also be misleading as they do not have the same genotypic and/or phenotypic characteristics in the human body[45]. The sensitivity of animal tissue is known to differ from that of human tissue[45]. The results obtained from the analyses conducted using animal tissues may differ from those using human tissues, which may lead to misleading results[34,46]. For such reasons, commercial cell lines or animal tissues were not used in this study.

Terabe et al[47] reported that simvastatin may promote restoration of chondrocyte morphology and phenotype. In their study, it is emphasized that treatments with simvastatin stimulate the expression of SOX9 and type-2 collagen in human osteoarthritic chondrocytes and strengthen the SOX9 protein, and that simvastatin has positive effects on the re-differentiation of chondrocytes[47]. Simvastatin promotes osteogenic differentiation of mesenchymal stem cells in osteoporosi[48].

Table III. Presentation in folds of changes in protein expressions value (r) in pitavastatin and rosuvastatin treated samples.

Groups	SOX9	IL-1β	NF-κB
Control (no drug administration)	1	1	1
Pitavastatin 24h	1.59	2.05	1.60
Pitavastatin 48h	1.07	1.04	1.12
Pitavastatin 72h	1.47	1.79	1.84
Rosuvastatin 24h	2.86	2.07	1.37
Rosuvastatin 48h	3.55	2.85	1.65
Rosuvastatin 72h	0.96	2.13	1.48

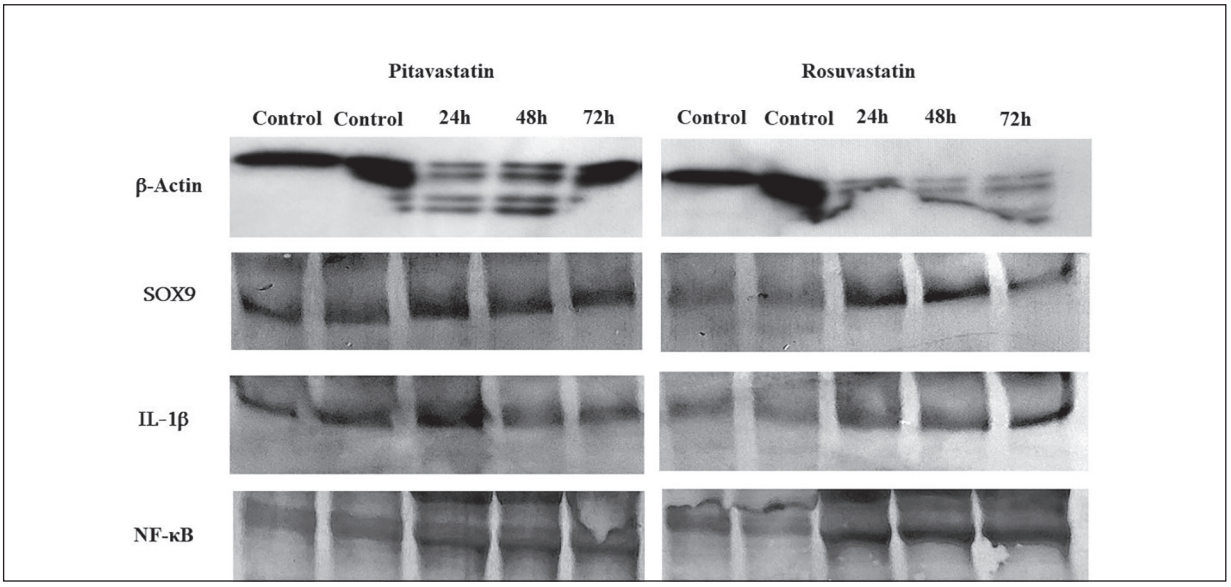

Figure 4. The expression levels of β-actin, SOX9, IL-1β, and NF-κB were detected by Western blotting. Three independent experiments were performed for each study. Data were presented as frequency.

On the other hand, Chen et al[49] reported that 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) inhibition expanded the number of craniofacial and pectoral fin tendon progenitors in the zebrafish embryo using chemical and genetic approaches.

In this study, the protein expression of SOX9 increased by 59%, 7%, and 47% at 24, 48, and 72 hours, respectively, in the pitavastatin-combined group compared to the control group. Compared to the control group, SOX9 protein expression was observed to increase in the rosuvastatin groups by 186% and 255% at 24 and 48 hours, respectively; however, this increase of SOX9 decreased by 4% by the end of 72 hours.

In an experimental study[50] suggesting that statins play a potential role in chondrocyte cell proliferation, the authors reported proliferation-regulatory effects of lovastatin in rabbit chondrocytes, as well as the underlying signaling mechanisms, thereby exploring its potential benefits in chondrocyte-related disorders, such as cartilage damage and osteoarthritis[50].

In this study, we observed that cell proliferation was suppressed and reduced in the pitavastatin and rosuvastatin groups. In addition to the suppression of proliferation, cell necrosis was detected in AF/NP cells in the rosuvastatin group compared to the pitavastatin group. Moreover, in the group to which pitavastatin was added, it was observed that the blue color indicator changed into pink in the absence of oxygen at the end of 72 hours.

There is also a study[42] in the literature in which statins were found to have anti-inflammatory effects in chronic degenerative joint diseases characterized by cartilage degradation related to proinflammatory cytokines. It was underlined that pravastatin alleviated interleukin 1β-induced cartilage degradation by restoring impaired autophagy associated with mitogen-activated protein kinase signaling pathway inhibition[42].

In this study, it was observed that IL-1β expression, one of the proinflammatory cytokines inducing IDD, increased in all time periods, including 72 hours, in both the pitavastatin and rosuvastatin groups compared to the control group in which no statins were administered.

Atorvastatin is reported to stimulate angiogenesis in experimental disc degeneration in rats, but to exert no biphasic effect[11]. It has been reported that Atorvastatin inhibits tumor necrosis factor-α (TNF-α) induced matrix degradation in rats' nucleus pulposus cells by suppressing NLRP3 inflammasome activity and inducing autophagy through NF-κB signaling[51]. An available study reported that intradiscal injections of simvastatin set back the progression of intervertebral disc degeneration induced by stab injuries[52].

In their study, Tian et al[53] emphasized that orally administered simvastatin partially preserved lumbar vertebral bone mass but not the integrity of IVD in ovariectomized rats. In the literature, there are studies reporting that simvastatin can prevent

IL-1β-induced apoptosis and ECM degradation by suppressing NF- κB and mitogen-activated protein kinase (MAPK) pathways in NP cells[54].

We observed that NF-κB protein expression increased in both the pitavastatin-combined group (60%, 12%, and 84%) and the rosuvastatin-combined group (37%, 65%, and 48%), compared to the control group.

In this study, primary cell cultures were prepared from human degenerate IVD tissues. These cultures were prepared using samples from the same race, which was one of the limitations in our study. However, a more important limitation was that the experiments were carried out *in vitro* without the compensatory mechanisms occurring in the body, since all statins, with the exception of pitavastatin, showed a very low systemic bioavailability, due to an extensive first-pass effect at the intestinal and/or hepatic level[55,56]. It is important that the results of our study are supported by prospective studies in which primary cell cultures are prepared using degenerate and/or intact IVD tissues of different races.

Conclusions

Although less prominent in cells treated with pitavastatin, suppression of proliferation and necrosis of AF/NP cells were clearly demonstrated in the rosuvastatin group both numerically and visually by MTT data and inverted light microscopy. Our findings indicate that pitavastatin and rosuvastatin considerably increased IL-1β/NF-κB-induced apoptosis in NP cells. Although SOX9 expression increased in the pitavastatin group, it was noted that SOX9 protein expression decreased by 4% in the rosuvastatin group compared to the control group. If protein expressions of IL-1β/ NF-κB signaling pathways had been suppressed in the pitavastatin and rosuvastatin treated groups or SOX9 protein expression increased in the rosuvastatin group, as in the pitavastatin group, we could have reported both these synthetic statin drugs as potential agents in the prevention and treatment of IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We, as the authors, would like to express our gratitude and respect to Assoc. Prof. Ph.D., Duygu YASAR SIRIN, Faculty Member at Namik Kemal University, Department of Molecular Biology and Genetics, for her support and blind review in the interpretation of Western Blotting analyses after SDS-PAGE.

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Availability of Data

The data and materials generated/analyzed in the present study are available from the corresponding author upon request.

Informed Consent

Informed consent was obtained from the participants prior to the collection of primary cell cultures.

Ethical Approval

Approval was obtained for the study from Local Ethics Committee of Halic University, Faculty of Medicine (Date: 23.02.2022 Number: 44).

Authors' Contribution

I.Y., designed the study and the experiments. N.K., identified the cases to be included in the study and surgically resected the tissues from the cases to be used in the preparation of cell cultures. I.Y., provided the dissolution of the drugs in suitable solvents and applied these drugs to the cell cultures he prepared from the tissues. I.Y., and N.K. worked on the experiments and the pharmaco-molecular analysis of the samples. I.Y. collected the data. I.Y., and N.K. carried out the statistical analysis. I.Y., and N.K. read and approved the final manuscript.

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