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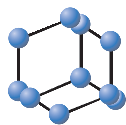

RESEARCH ARTICLE

Does Nimodipine, a Selective Calcium Channel Blocker, Impair Chondrocyte Proliferation or Damage Extracellular Matrix Structures?

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ARTICLE HISTORY

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Abstract: ***Background***: The study aimed to investigate the effects of the active ingredient, nimodipine, on chondrocyte proliferation and extracellular matrix (ECM) structures in cartilage tissue cells.

Methods: Chondrocyte cultures were prepared from tissues resected *via* surgical operations. Nimodipine was then applied to these cultures and molecular analysis was performed. The data obtained were statistically calculated.

Results: Both, the results of the (3-(4,5 dimethylthiazol2-yl)-2,5-diphenyltetrazolium (MTT) assay and the fluorescence microscope analysis [a membrane permeability test carried out with acridine orange/propidium iodide staining (AO/PI)] confirmed that the active ingredient, nimodipine, negatively affects the cell cultures.

Conclusion: Nimodipine was reported to suppress cellular proliferation; chondroadherin (CHAD) and hypoxia-inducible factor-1 alpha (HIF-1α) expression thus decreased by 2.4 and 1.7 times, respectively, at 24 hrs when compared to the control group ($p < 0.05$). Furthermore, type II collagen (COL2A1) expression was not detected ($p < 0.05$). The risk that a drug prescribed by a clinician in an innocuous manner to treat a patient by relieving the symptoms of a disease may affect the proliferation, differentiation, and viability of other cells and/or tissues at the molecular level, beyond its known side effects or adverse events, should not be forgotten.

Keywords: Chondroadherin, chondrocyte, hypoxia-inducible factor-1 alpha, nimodipine, type II collagen, matrix structures.

1. INTRODUCTION

Traumatic subarachnoid bleeding may present as complex, incomplete, and single or multi-traumatic ruptures of cortical tissue by means of diffusion caused by head traumas from car accidents and falls. Bleeding suppresses cerebrospinal fluid and increases intracranial pressure; thus, secondary brain lesions may develop [1].

Various studies have indicated that vasospasm is the most important factor in adverse outcomes among head trauma patients [2]. Nimodipine is frequently prescribed in clinics to prevent vasospasms; it suppresses the activity of L-type channels located in brain veins and presynaptic neurons susceptible to voltage change [3].

The active ingredient nimodipine is thought to block calcium intake in the veins of smooth muscle, thereby preventing vasospasms. As a result, it increases tolerance to ischemia by means of cerebral blood flow. Some research indicates that nimodipine decreases calcium entrance into nerve cells after ischemia or prevents cell proteolysis and lipolysis by antagonizing intracellular calcium. Therefore, the formation of fatty acids and oxygen free radicals prevents early morphological and functional damage to nerve cells [4].

In clinical and experimental studies, nimodipine, a calcium antagonist, a pharmacological agent of dihydropyridine group, has been shown to have neuroprotective effects by

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increasing cerebral blood flow. The chemical formula of nimodipine is isopropyl 2-methoxyethyl 1,4-dihydro-2, 6-dimethyl-4-(m-nitrophenyl)-3,5-pyridine dicarboxylate. Even though side effects such as local vascular irritation and phlebitis risk, headache, gastrointestinal symptoms, nausea, flushing, drop in the blood pressure, sweating, fatigue, nervousness, and a decrease or, rarely, an increase in heart rate have already been indicated [5-7].

Osteoblast/chondrocytes secrete vesicles containing calcium and phosphorus called matrix vesicles (MVs). These MVs bind to extracellular matrix proteins to initiate mineralization. Intracellular $Ca2^{+}$ is tightly regulated in especially smooth muscle cells and calcium channels are the major route by which $Ca2^{+}$ enters the smooth muscle cells. These channels are critical in regulating differentiation, function, and synthesis of matrix components. L-type calcium channel blockers are a widely used group of agents, also having effects beyond changes in the calcium influx [8].

As far as we know, no research concerning nimodipine's effects on cartilage cells and environmental structures has yet been carried out [5-7].

Articular cartilage does not contain nerves, veins or lymphatic tissues [9-11]. Therefore, cartilage cells, which are the articular surfaces of bones, are fed by a perichondrium layer in the vascular structure or by synovial fluid that washes the articular surface when there is no perichondrium [9]. Cartilage also has a double diffusion system [9]. Since the outer layer of the synovial fluid is thick, nutrients or drugs are diffused from the synovial tissue to the synovial fluid and thereafter pass from the synovial fluid through pores in the cartilage structure prior to reaching chondrocytes, where diffusion occurs again [9-11]. Indeed, planning a treatment to regain the functionality of tissues or organs is indicated in the literature nowadays, and a simple comparison of clinical observations may not be sufficient. Therefore, additional researches based on cellular response and pharmacomolecular approach are still needed before clinical applications of nimodipine. In this novel study, the aim was to evaluate the effects of nimodipine on both chondrocyte proliferation and changes in gene expressions effective in the formation of peripheral tissues such as the ECM structures.

2. MATERIALS AND METHODS

2.1. Patient Selection Criteria

Osteochondral tissues used for primary chondrocyte cultures were obtained from patients who were not oversensitive to nimodipine. Patients who received rasagiline, selegiline, moclobemide, or monoamine oxidase inhibitors in the previous 14 days were excluded from the study. Patients who were diagnosed with Parkinson's disease or taking antidepressants [9] were also excluded, as were those with malignancy. The mean age of patients meeting the inclusion criteria was 56.11 years [(standard deviation (SD) = 7.68)]. The study consisted of patients with stage-IV gonarthrosis according to the *Kellgren-Lawrence Grading Scale* who did not respond to the conservative treatment [10, 11]. The study was carried out with the approval of the local ethics committee (Namik Kemal University -2017/26.02.09).

2.2. Preparation of Primary Human Chondrocyte Cultures and Delivery of Nimodipine to Samples

Osteochondral tissues were resected from femurs as well as distal and proximal ends of tibias that were resected during total knee arthroplasty. Standard human primary chondrocyte culture protocols were conducted [9, 12]. During the prosthesis application, chondrocyte cultures were prepared from chondral tissues with undamaged articular surfaces. These undamaged, intact tissues contained not only cartilage cells but also their microenvironments, including ECMs. Primary chondrocytes cultures were separately prepared using six patients satisfying inclusion criteria. After surgery, the leftover osteochondral tissue was immediately transferred to cell culture laboratory in the transfer medium (100 mL DMEM contained 5 mL of penicillin-streptomycin (10,000 U/mL) and 2.50 mg/mL Amphotericin B) under sterile conditions at 4°C.

Resected tissues were transferred to a petri dish and smashed into small pieces, approximately 0.25 cm3, either with scalpel or rongeur. The tissues were transferred to 50 mL conical tubes and centrifuged at 1200 rpm for 10 min. The supernatant was discarded. To perform enzymatic digestion, tissues were incubated in a 200 units/mL collagenase type II enzyme mixture, dissolved in the complete medium (prepared with 100 mL DMEM containing 1 mL of penicillin-streptomycin (10,000 U/mL) and 2.50 mg/mL Amphotericin B, 1 mL L-glutamine (200 mM), 1 mL ITS, and 10% FBS) for 16 hrs in a CO2 incubator. Afterward, tissue samples were centrifuged at 4°C at 1200 rpm for 10 min to discard collagenase. Sedimented cartilage cell pellets were resuspended in a fresh complete medium, and transferred to flasks to obtain primary cultures. Confluent primary chondrocyte cultures were passed using trypsin–EDTA. Cells were counted and transferred to different culture plates or Petri dishes to carry out the various analyses. The cells were seeded at 1.6 x 10^{4} cells per well in 96 well plates for MTT analysis, at 4.3 x10^{4} cells per well in 24 well plates for inverted light microscopy and AO/PI assays, and at 5.1 x10^{6} cells per Petri dish for ribonucleic acid (RNA) isolation and qRT-PCR analyses. After additional overnight incubation, nimodipine solutions prepared with different concentrations were added to the samples that had over 95% confluency in the cell culture container and adhered to the surface.

Nimodipine (Nimotop®, Bayer Turkish Chemical Industry), the main stock solution, was freshly prepared using Dulbecco's Modified Eagle's medium in a flow cabin at 30 mg/ml. After being diluted in the medium, the solution was then transferred to the dark brown glass bottles and letter-coded to ensure that the researchers were blind during the analyses. In this respect, both the researchers administering drugs to the cell cultures and those performing cell culture analyses were blind to experimental specifics.

Before the experiments were initiated, diluent solutions of nimodipine at concentrations of 1, 50, 100, and 1000 μmolar were added to the cell cultures. It has previously been reported that cell proliferation cultures ceased when nimodipine was applied at concentrations greater than 100 μmolar [13]. This dose range was accepted as 1-1000 μmolar to represent the dose of nimodipine administered clinically.

Therefore, doses of nimodipine at the concentrations necessary to permit proliferation were applied to the cell classes found in the cultures. Human primary chondrocyte cultures were prepared according to effective concentrations to imitate clinical practices. The highest concentration applied was 100 μM of nimodipine. Chondrocyte cultures with no drug administration were used as control groups for all the tests and named Group I and cultures receiving nimodipine were named Group II. All the experiments were performed at 0, 24, and 48 hrs in both the groups. Group I (0 h) culture was accepted as a reference sample and used to compare differences between the groups.

2.3. Inverted/Fluorescence Microscopy

Cell morphology and confluency were analyzed using an inverted microscope (Olympus CKX41). Using the Olympus Cell Soft Imaging System software, microphotographs of cell organization were obtained and analyzed during the confocal/contrast phase, both before and after nimodipine applications. A fluorescent microscope (Leica DM 2500) was used for AO/PI analysis. Cell organization microphotographs were obtained before and during nimodipine applications, and the images were evaluated using the Cytovision Capture Station imaging software.

2.4. MTT-ELISA Viability, Toxicity, and Proliferation Analyses

This method is based on the spectrophotometric measurement of the color change, in the form of absorbance observed as the result of formazan (purple) dye production by cells undergoing proliferation that make use of tetrazolium (MTT: yellow) with increasing dehydrogenase enzyme activity [9-14].

In this way, MTT analyses of chondrocytes viability and proliferation were performed at 24 and 48 hrs according to the manufacturers' instructions, and nimodipine toxicity was detected. The culture media containing nimodipine discarded from the 100-mL MTT wells (5 mg MTT prepared by adding 1 mL sterile PBS) was added. Cultures were incubated at 37°C for 2 hrs without light exposure. Dimethylsulfoxide was added to these samples to stop the reaction and the samples were incubated at 37°C for an additional 10 min. 100 μL of the SDS-HCl solution was added to each well and mixed thoroughly using the pipette, followed by incubation at 37°C for 4 hours in a humidified chamber prior to photometric analysis of a 570 nm wavelength absorbance. The viability of the control group was assumed to be 100%. After proliferation and inhibition of proliferation were calculated using the "*Test OD/Control ODX100"* and "*1- Test OD/Control OD*" formulas, respectively, data were recorded for statistical analysis [9-16].

2.5. Gene-Specific RT-PCR Analysis

Total RNA was extracted from cultured primary human chondrocytes using a PureLink RNA Mini Kit (Ambion, Cat#12183018A) and 2-mercaptoethanol (Thermo Fisher Scientific, Cat#31350010). To obtain complementary DNA (cDNA), 50 ng RNA was reverse-transcribed *via* a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Cat#4368814) using a thermal cycler (ProFlex, Thermo Fisher Scientific) in accordance with the manufacturer's instructions. For qRT-PCR analysis, all the genes were amplified using TaqMan® Gene Expression Assays for CHAD (Cat#4331182, Hs00154382_m1), HIF1α (Cat#4331182, Hs00153153_m1), COL2A1 (Cat#4331182, Hs00264051_m1), and internal control genes (housekeeping genes: actin beta (ACTB, Cat#4331182, Hs01060665_g1) in accordance with the manufacturer's protocol [11, 15, 16]. Each gene was amplified using an RT-PCR reaction mix prepared with 1 μl TaqMan Gene Expression Assay, 10 ml of TaqMan Gene Expression Master Mix (Cat. #4369016), 4 ml of cDNA template, and UltraPure DNase/ RNase-Free distilled water (Cat. #10977035) on MicroAmp Fast Optical 96-well reaction plates (Cat. #4346906). The Applied Biosystems 7300/7500 RT-PCR system was used with the following reaction protocol: maintained at 50°C for 2 min, maintained at 95°C for 10 min, and alternating between 95°C for 15 s and at 60°C for 1 min and 40 cycles.

2.6. Statistical Analysis

The analyses were performed with the SPSS (version 20.0) software, while the data were evaluated at 95% confidence intervals (CI). Descriptive statistics from the evaluated data were presented as mean ± SD. Analysis of variance (ANOVA) was used to compare the experimental and control groups, and Tukey's honest significant difference (HSD) test was used for the evaluation of statistical significance. *P*, a statistical significance value, was accepted as < 0.05.

3. RESULTS

3.1. Cell Viability and Proliferation

MTT analyses were used to evaluate both cell vitality and proliferation.

Cell viability was the same in both the experimental groups at 0 h. Cell proliferation time-dependently decreased in the control and experimental groups at 24 and 48 hrs. (Table **1** and Fig. **1**). The decrease in cell proliferation in the control group was thought to originate in the cell confluency (Fig. **2**). AO/PI staining revealed that 50% of the cells in the nimodipine-administered group samples underwent apoptosis at 24 hrs (Fig. **3**). Cell death occurred due to nimodipine at 48 hrs was lower than that observed at 24 h. Resistant cells did not experience apoptotic cell death. However, MTT assay showed that cell proliferation was lower as compared to the control group, which might indicate the occurrence of cellular senescence.

Those results were evaluated statistically and revealed that nimodipine application did not have cytotoxic effects, but application time did affect cell proliferation ($p = 0.000$). As the application time increased, cell proliferation suppressed.

All the experimental groups had approximately the same number of cells. Cell density was the lowest in the nimodipine group after 48 h. The results of numerical proliferation obtained from the MTT assays were also visually supported by images obtained from the fluorescence microscope after AO/PI staining.

Table 1. Non-nimodipine-administered control group samples and nimodipine-administered samples were evaluated by Tukey's HSD test (a multiple comparison test) after one-way ANOVA.

Time (Hour) -Application	Absorbance (Nanometer- mean±SD)	Grouping[¥]	P-value*
Control 0h	0.3006±0.014	A	P<0.05
Nimodipine 0h	0.3006±0.014	A	
Control 24h	0.2725±0.032	A B	
Nimodipine 24h	0.2657±0.013	B C	
Control 48h	0.2359±0.026	C D	
Nimodipine 48h	0.2230±0.035	D	

¥ Information obtained using the Tukey HSD method and 95% CIs; From A to Z, A is best and Z is the worst, * one-way ANOVA.

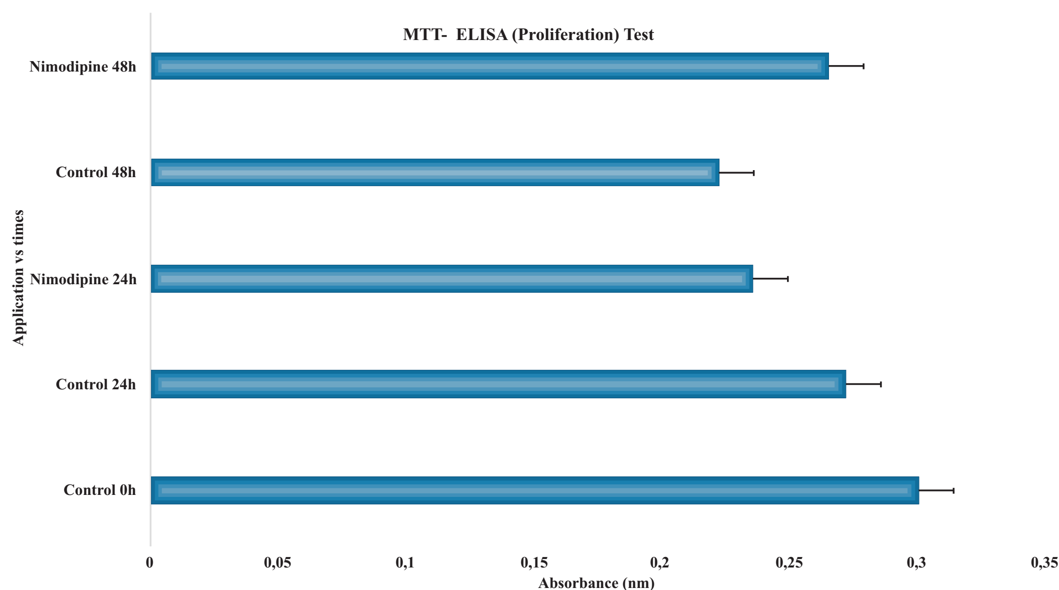
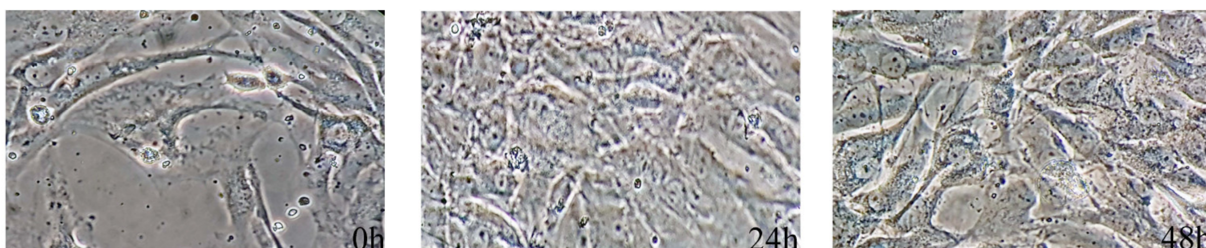


Fig. (1). Comparison of the proliferation against time among the groups at 570 nm absorbance.

Group 1



Group 2

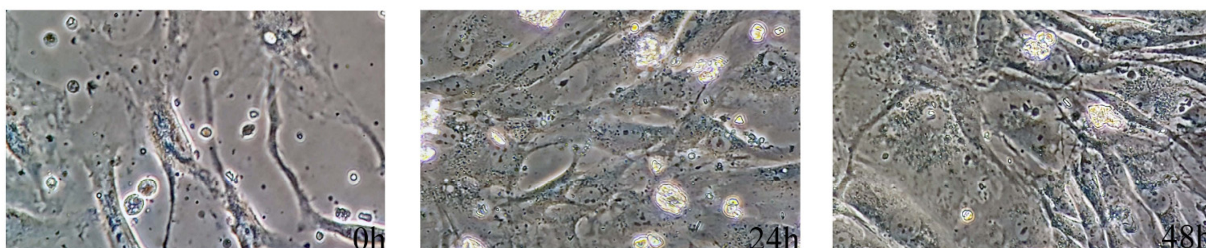
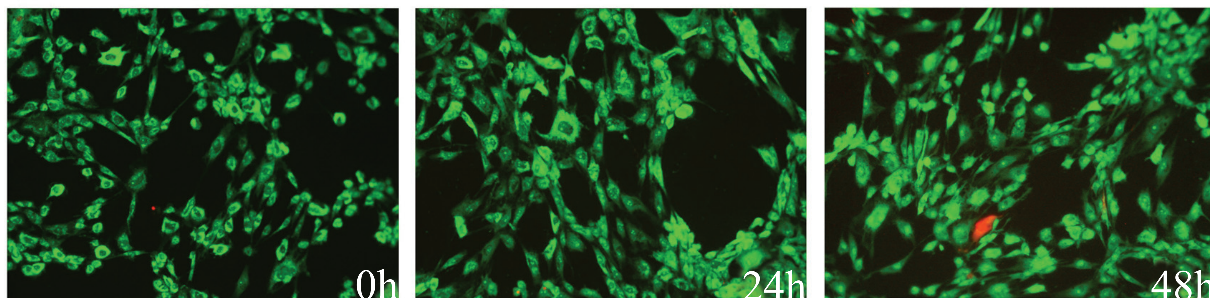


Fig. (2). Inverted microscopy of intervertebral disc tissue cells treated with or without nimodipine for 0h, 24h, and 48h.



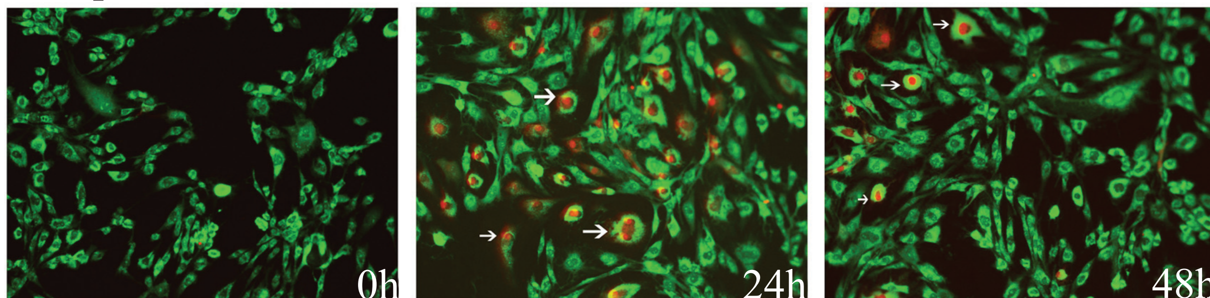


Fig. (3). The red cells highlighted by white arrows show dead cells. The cells in Group II (24 and 48 h) with red nuclei and green cytoplasm are dead and have damaged membrane integrity.

In the MTT proliferation assays, proliferation was observed in the non-nimodipine-administered control. However, it was observed in the MTT assays that proliferation was suppressed in the nimodipine-administered study group samples (Fig. **1**). In addition, in the images obtained *via* fluorescence microscopy, numerous dead cells were recorded, particularly those in nimodipine-applied samples at 24 hrs (Fig. **3**).

3.2. Expression of CHAD, HIF1α, and COL2A1 Genes in Chondrocyte Cultures

The expression levels of CHAD, COL2A1, and HIF1α genes were assessed after 24 and 48 hrs of nimodipine application on different molecular weights by gene-specific TaqMan Gene Expression Assays. Relative quantity (RQ) values were analyzed for each experimental group using the 7500 Fast-SDS software. ACTβ was used as an endogenous control, and the control (0 h) group was accepted as a reference sample. Gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method, meaning that the expression of the gene of interest is 100% and the RQ value is 1. The decrease and increase in gene expressions were calculated as RQ at 24 hrs and 48 hrs. Experiments were repeated at least three times and the RQ values obtained from all the experimental groups are presented in Fig. (**4**).

The CHAD expression increased about 90% (RQ = 1.9) at the 24th hrs in group 1, where nimodipine was not administered and which was included in the experiments as a control group. However, the expression of COL2A1 was found to be 3.3 (330%) times greater at 24 hrs. A decrease in the expression of all three analyzed genes was observed at 48 hrs.

CHAD expression (RQ=0.8) in the nimodipine-administered group samples decreased 2-fold and HIF1α expression decreased 1.7-fold as compared to the control group samples at 24 hrs. COL2A1 expression was not assessed as it was below the level that could be measured through the assay used. CHAD expression in nimodipine-administered group samples decreased 1.4-fold, and HIF1α expression decreased 2.3-fold as compared to the control group samples at 48 h.

4. DISCUSSION

During ischemia, which occurs due to subarachnoid bleeding, oxygen and glucose deprivation results in calcium overload, prevalent oxidative stress, neuroinflammation, and eventually, massive neuronal loss [17]. Treatment for this condition frequently includes pharmacological agents containing the active ingredient nimodipine [18].

Scientists are currently searching for novel generative treatment modalities while also paying attention to protect tissue regeneration and prevent drugs from causing damage to tissues. When any drug is prescribed, any damage it may cause to tissues should be considered. In the research literature, many side effects and adverse reactions have been reported for various drugs [5-7]. However, there has not been a well-designed study indicating the effects of pharmacological agents with nimodipine as the active ingredient on the cartilage tissue.

The present study is the first to examine the effects of nimodipine on chondrocyte proliferation and ECM structures. It also aimed to study the effects on CHAD, a cartilage matrix protein that mediates the adhesion of isolated chondrocyte

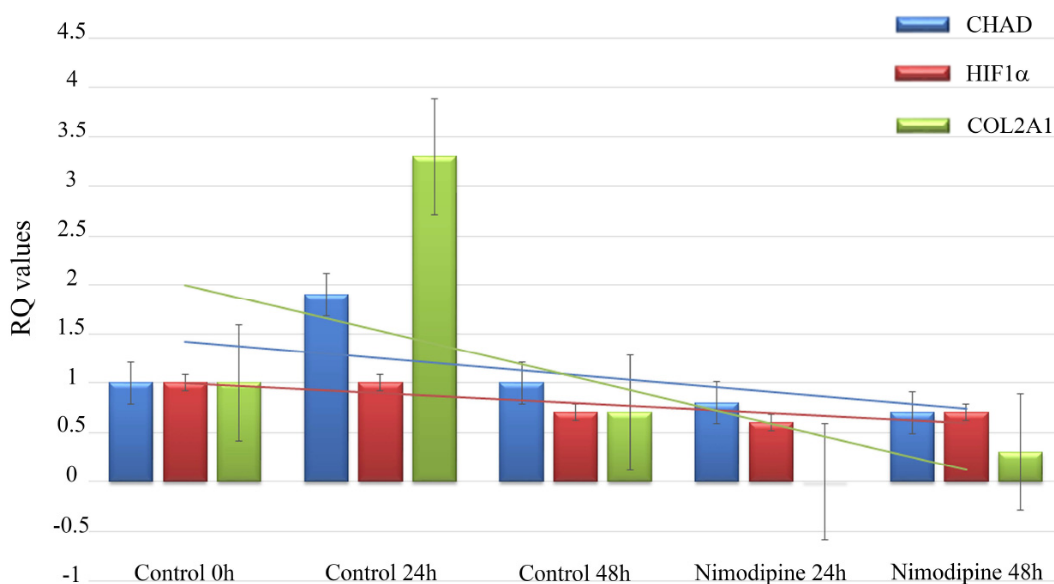


Fig. (4). RQ values obtained from experimental groups.

[19], COL2A1, which is responsible for ECM development [20], and HIF-1α expressions, which are components of a key pathway supporting chondrocyte survival during embryonic bone development [21] at the molecular level.

CHAD is a class IV small, leucine-rich proteoglycan/protein (SLRP) that mediates signaling between chondrocytes and the ECM [9, 12]. CHAD proteins bind to integrins, cell surface proteoglycans, and COL2A1; they play a role in regulating chondrocyte signaling and cartilage homeostasis. COL2A1 is an important cartilage-specific matrix protein, and the existence of COL2A1 in normal cartilage is a marker for anabolism [15, 16]. COL2A1 gene expression is regulated by transcription or growth factors, and an increased expression of this gene leads to the induction of ECM production [15, 16]. HIF1 is a transcription factor that regulates the transcription of a wide range of genes involved in cell survival [9, 12]. Hypoxic environment, hormones, and growth factors induce HIF1 translocation to the nucleus and regulate the expression of its target genes. Therefore, HIF-1α controls hypoxia-induced ECM synthesis in chondrocytes and is considered a positive regulator of cartilage regeneration [9, 12, 15, 16].

There are some contradictory results in the literature, indicating that nimodipine has a neuroprotective effect on one hand [17], and increases neurotoxicity on the other hand [22].

Erol *et al.* [22] reported that mitochondria played a crucial role in maintaining intracellular calcium homeostasis in combination with their buffering ability. Their study also indicated that the disruption of mitochondrial calcium homeostasis might be responsible for the neuropathy induced by cisplatin. More importantly, it indicated a significant increase in neurotoxicity in the group where 300 μM nimodipine was co-administered with 600 μM cisplatin [22]. Another study reported that nimodipine, which prevents cytosolic calcium increase, may have cardiotoxic effects apart from its effects on neurons [23]. Turner *et al.* [24] reported that nimodipine, as an L-type calcium channel antagonist, caused a widespread and significant age-related injury in the newborn rat brain.

In the present study, neither animal tissue nor commercial cell lines were used to prepare cell cultures; the human primary chondrocyte cultures were prepared using human osteochondral tissues. Therefore, it is believed that the results obtained in this research may be clinically valuable.

The main limitation of this study was the use of tissues obtained from actual cases. The small number of samples and the fact that the patients were all of the same race were also a limitation, although at least three cultures were prepared for each case, and tests were repeated at least three times. Given that the gene expression arises from individual differences, it would be better to perform the study with more cases. However, the changes in gene expressions in RT-PCR analyses detected in this study are informative.

CHAD and COL2A1 expressions, which are involved in the ECM formation, were higher in the control group at 24 hrs. CHAD and COL2A1 expressions did not continue at 48 hrs since the cells reached the confluence, and the contact inhibition occurred (Fig. **1**). MTT assay also revealed the discontinuation of proliferation in the control group at 48 h, thus, the cells protected their basal metabolism. HIF-1α expression remained stable in the control group, demonstrating that the culture medium was not hypoxic or that the cultured cells were not exposed to stress. Targeted gene expressions, especially CHAD and COL2A1 expressions, decreased in the nimodipine-administered cultures. Morphological analyses performed through changes in gene expressions were correlated with the results of the MTT assay and AO/PI staining. The latter revealed that 50% of the cells in the nimodipine-administered culture samples underwent apoptosis at 24 h. Resistant cells did not experience apoptotic cell death. However, the MTT assay revealed that cell proliferation was lower as compared to the control group samples. HIF1α expression remained stable in the nimodipine -administered

group samples, supporting the view that the culture medium was not hypoxic, and the cell deaths occurred due to the apoptosis rather than an acute toxic condition. In conclusion, AF/NPC cells exposed to nimodipine experienced apoptosis. The proliferation was suppressed in the cells which did not undergo apoptosis. Clinically, the administration of nimodipine to the operated patients may slow down the healing process.

CONCLUSION

Clinicians should now be aware of the risk that chondrotoxicity may be present when prescribing drugs with the active ingredient nimodipine while also paying attention to the posology and duration of treatment. Even though the data in this study were derived from an *in-vitro* assay system and even though only one dose of nimodipine was administered in the samples, the fact that the relevant gene expression and cell proliferation may be suppressed should not be disregarded. It should also be kept in mind that the ECM structure may deteriorate.

LIST OF ABBREVIATIONS

AO	=	Acridine orange
CHAD	=	Chondroadherin
COL2A1	=	Type II Collagen
ECM	=	Extracellular Matrix
HIF-1α	=	Hypoxia-Inducible Factor-1 Alpha
MTT	=	3-(4,5 Dimethylthiazol2-yl)-2,5-diphenyltetrazolium
PI	=	Propidium Iodide
RNA	=	Ribonucleic Acid
RQ	=	Relative Quantity
qRT-PCR	=	Quantitative Reverse Transcriptase Real-Time Polymerase Chain Reaction

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was carried out with the approval of the Local Ethics Board Namik Kemal University, Turkey (NKU-2017/26.02.09).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study all reported human were experimented in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the *Helsniki Declaration* of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written consent forms were obtained from all the patients undergoing surgery whose tissues were used in the preparation of the primary cell cultures.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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