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Investigation of the Potential Effects of Alpha-Lipoic Acid on Human Degenerated Intervertebral Disc Tissue Primary Cell Cultures

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ABSTRACT

AIM: To investigate the supplementation of alpha-lipoic acid (ALA) at the molecular level to determine its effect on primary cell cultures prepared from human intervertebral disc (IVD) tissue in an in vitro environment.

MATERIAL and METHODS: Human primary cell cultures were prepared from IVD tissue resected during surgery. While cell cultures without ALA supplementation formed the control group, those with ALA supplementation formed the study group. All cell groups were stained using acridine orange/propidium iodide (AO/PI), and the incidence of apoptotic cell death was determined under a fluorescent microscope. Cell surface morphology and extracellular matrix (ECM) structures were evaluated under an invert light microscope. Simultaneously, cell proliferation was evaluated by MTT–ELISA analysis, and the expressions of chondroadherin (CHAD), cartilage oligomeric protein (COMP), interleukin-1 beta (IL-1β), and matrix metalloproteinase (MMP)-7 and-19, which are genes associated with ECM regulation, were tested using qRT–PCR. The data obtained were evaluated statistically using Tukey's honestly significant difference (HSD) test after analysis of variance (ANOVA) was performed. The alpha significance value was accepted as $< .05$.

RESULTS: Compared to the cells in the control group, it was observed that both proliferation was suppressed and ECM structures deteriorated in the cells in the study group.

CONCLUSION: Also, it was reported that the all-gene expression levels changed. ALA supplementation can negatively affect human IVD primary cell cultures in an in vitro environment.

KEYWORDS: Alpha-lipoic acid, Interleukin-1 beta, Intervertebral disc, Cartilage oligomeric matrix protein, Chondroadherin gene, Matrix metalloproteinase

INTRODUCTION

In recent years, although vitamin and mineral supplements have been prescribed frequently, they can also be obtained from pharmacies or online without a prescription (13). These supplements are intended to provide the molecules that the body cannot produce or that the body produces in insufficient amounts (34).

Alpha-lipoic acid (ALA), a water-and fat-soluble coenzyme, has become one of the most popular nutritional supplements today. Lipoic acid is an organosulfur compound derived from caprylic acid, and it is also known as α-lipoic acid or thioctic acid. ALA is normally produced in animals, as it is required for aerobic metabolism (3). However, taking such supplements as reinforcements outside the body's need can cause molecular-level damage to tissues, cells, and cellular structures instead of being beneficial (13).

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Research suggests that drugs and dietary supplements can accumulate in various tissues, including the synovial fluid compartments, after oral or parenteral application (20-23,38).

Following this scientific reality, drugs diffuse significant amounts first into the hyaluron or synovial tissues and then into the body fluids. After this diffusion, drugs pass through pores in the hyaline membrane located at the boundaries of the intervertebral discs (IVDs), reaching IVD tissue cells, such as anulus fibrosus (AF) or nucleus pulposus (NP) cells. IVD tissue is fed with arterial blood tissue until the age of 18, but as an individual reaches their late 20s or early 30s, the vascular nutrition of the entire NP tissue and many parts of the peripheral part of the AF tissue are subject to obliteration. After 30 years of age, AF/NP continues to be fed by diffusion from surrounding tissues (20-23,38).

Particularly in the last decade, scientists have been working intensively to determine whether prescribed or nonprescription drugs and similar food supplements cause harm at the cellular level (20,38). However, there is no research in the literature that reports the effects of ALA on IVD cells, such as human primary AF and NP cells. More importantly, no research in the literature has evaluated the effect of ALA on the surface morphology and extracellular matrix (ECM) structures of NP and AF cells.

Further, it is understood that investigations into how ALA can cause changes in the proliferation of degenerated IVD tissue cells and the gene expression of chondroadherin (CHAD), cartilage oligomeric protein (COMP), interleukin-1 beta (IL-1β), and matrix metalloproteinase (MMP)-7 and-19 remain unexplored.

Therefore, this study primarily evaluates the molecular-level effects of ALA on the cells and cellular structures of primary human AF and NP cell cultures.

MATERIAL and METHODS

This study was conducted with the permission of the Non-Invasive Clinical Research Ethics Committee of Istanbul Medipol University School of Medicine, dated 27/02/2019, numbered 10840098-604.01.01-E8223. For the tissues used in the primary cell cultures in the study, approved informed volunteer consent forms were obtained from the patients. Analyses were repeated at least three times to minimize experimental errors. The medium of the primary cell cultures was replaced with fresh medium every two days.

Tissue Inclusion and Exclusion Criteria

Additional examinations were performed on patients who did not respond positively to surgical intervention or medical and conservative treatment. Resected tissues were collected from patients diagnosed with lumbar IVD herniation after preoperative neurological examination, lumbar spinal magnetic resonance imaging (MRI), lumbar spinal computed tomography, or lower extremity electromyography examinations (n=15). The rating of IVD degeneration was conducted using T2-weighted MRI assisted by the Pfirrmann classification (21-23).

Tissues belonging to patients with any rheumatological, inflammatory, or infectious disease were excluded from the study (n=3). Tissues belonging to those patients using nonsteroidal anti-inflammatory drugs (3 females, 3 males; n=6) in the last three weeks were also excluded from the study.

There was no history of type-2 diabetes mellitus and/or smoking in the history of the cases in which the tissues used in the establishment of primary cell cultures were taken.

Resection and Preparation of Human Primary Cell Cultures from Tissues

Primary cell cultures were prepared from tissues obtained from patients who underwent lumbar microdiscectomy or lumbar microsequestrectomy operations due to lumbar IVD herniation. The resected tissues were placed in Falcon tubes containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, CatNo# 11965092; Thermo Fisher Scientific, Waltham, MA, USA) solution with 1% Penicillin-Streptomycin (CatNo#15070063; Thermo Fisher Scientific, Waltham, MA, USA). The tissues were stored at 4°C when transferred to the laboratory within a maximum of 2 h.

The mean age of the patients from which the tissues included in the study were obtained was 38.43 ± 9.74 years. The tissues were transferred to petri dishes in the flow cabinet and irrigated three consecutive times with 0.9% isotonic sodium chloride solution. Then, the mechanically disrupted tissues were transferred to Falcon tubes for enzymatic digestion (21-23).

Collagenase type-II (CatNo#17101015; Thermo Fisher Scientific, Waltham, MA, USA) dissolved Hanks balanced salt solution (HBSS, CatNo#88284; Thermo Fisher Scientific, Waltham, MA, USA) was added to these tubes. The tubes, whose lids were left slightly open, were placed in a 37°C 5% carbon dioxide incubator overnight (21-23).

At the end of the overnight period, according to the protocol obtained from publications with a high level of evidence (21-23), the Falcon tubes were centrifuged twice consecutively for 5 minutes at 1200 rpm. The resulting cell suspensions were transferred to T-25 flasks. DMEM medium containing fetal bovine serum, L-glutamine (CatNo# 25030081; Thermo Fisher Scientific, Waltham, MA, USA), penicillin, streptomycin, and amphotericin B (CatNo# 15290026; Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. The cells were placed in a 37°C 5% CO_2 incubator, where they were attached to the flask surface and proliferated (21-23).

Primary cell cultures that gained 70% to 80% confluency were passaged using the trypsinization process. Cells from cultures that became approximately 90% confluent after the third passage were removed and stained with Trypan blue. Then, a cell count was performed using a Thoma slide. The counted cells were planted in various cell dishes according to which analysis would be performed: 1.5×10^4 cells/well for 96-well plates and 4.4×10^6 cells/petri dishes and 10-mm petri dishes were cultivated. Subsequently, all samples were incubated overnight in a 37°C 5% carbon dioxide incubator.

Application of ALA to Primary Cell Cultures

To determine the dose of ALA, the studies of Tibullo et al. (39),

Gomes et al. (10), Dinicola et al. (5), and Jeon et al. (18) were considered. ALA was dissolved in 50 mg ethanol per milliliter and was then aliquoted. According to previous studies, dose-response curve trials were calculated based on doses of 50, 100, and 250 µm (5,10,18,39), from freshly prepared ALA solutions applied to the cell culture samples with a final concentration of 250 µm.

Molecular Analyses Performed on Primary IVD Cell Cultures Given ALA

Evaluations of AF and NP cell surface morphology and ECM structures were performed under 4x, 10x, 20x, and 40x magnifications using an inverted light microscope (Olympus CKX41; Olympus, Tokyo, Japan). For this, microphotographs of the cell organization in the control samples and the samples with ALA supplementation were examined using the Olympus Cell Soft Imaging (Olympus Corporation; version, Cell^A5.1) program.

MTT-ELISA Cell Viability, Toxicity, and Proliferation Assays

A methyl thiazole diphenyl tetrazolium (MTT; 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) kit (Vybrant MTT Cell Proliferation Assay, Cat#V13154; Thermo Fisher Scientific, Waltham, MA, USA) was used during spectrophotometric analysis.

Using the MTT kit, enzyme-linked immunosorbent analysis (ELISA/optical density [OD]) was performed at 24, 48, and 72 h on both the control group without ALA supplementation and the samples with ALA supplementation. Measurements were performed with a Mindray microplate reader (MR 96 A, China) at an absorbance of 570 nanometers (nm). The survival rate of the cells in the control group was accepted as 100%. Proliferation was calculated with the formula: Test OD / (Control OD × 100), and proliferation inhibition was calculated with the formula: (1 – Test OD) / Control OD. The data were recorded for statistical analysis (1, 20-23, 38).

Fluorescence Microscopy Analysis

To determine cell viability and confirm the results of the MTT test, nucleic acid binding was performed using acridine orange/propidium iodine (AO/PI) staining, and microphotographs were obtained using fluorescence microscopy (DM 2500; Leica Microsystems, Wetzlar, Germany). Images were evaluated using Cytovision Capture Station imaging software (version 7.0; Genetix, Leica Microsystems, Wetzlar, Germany).

All nucleated cells were stained with AO because all cells, whether alive or dead, will generate green fluorescence (1), whereas PI penetrated only dead cells with poor membrane integrity and stained nucleated cells to generate red fluorescence (1). When stained with AO and PI, all live nucleated cells exhibited green fluorescence and all dead nucleated cells displayed red fluorescence (1).

Gene Expression Measurements Using qRT–PCR

The gene expression results were obtained using 7500 Fast-SDS program V.2.3 (Thermo Fisher Scientific, Waltham, MA, USA) and presented as relative quantification (RQ). During the analyses, endogenous control actin beta (ACTβ) was used to normalize the target gene expression.

Before the analysis, a PureLink RNA mini kit (cat. no. 12183025; Thermo Fisher Scientific, Waltham, MA, USA) was used to obtain total RNA from the primary cell cultures, and then a high-capacity complementary DNA (cDNA) real-time kit (cat. no. 4368814; Thermo Fisher Scientific, Waltham, MA, USA) was used to obtain cDNA.

The amount of RNA obtained from each sample was measured using an ultraviolet spectrophotometer. To obtain total cDNA, 50 ng RNA, a high-capacity cDNA reverse transcription kit, and a thermal cycler were used.

All genes were amplified using TaqMan® Gene Expression assays for ACTβ (Cat#4331182, Hs01060665_g1 Thermo Fisher Scientific, Waltham, MA, USA), CHAD (Cat#4331182, Hs00154382_m1 Thermo Fisher Scientific, Waltham, MA, USA), COMP (Cat#4331182, Hs00164359_m1 Thermo Fisher Scientific, Waltham, MA, USA), IL-1β (Cat#4331182, Hs01555410_m1 Thermo Fisher Scientific, Waltham, MA, USA), MMP-7 (Cat#4331182, Hs01042796_m1 Thermo Fisher Scientific, Waltham, MA, USA), and MMP-19 (Cat#4331182, Hs00418247_g1 Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time polymerase chain reaction (qRT–PCR) was performed using an Applied Biosystems 7300/7500 qRT–PCR system (Thermo Fisher Scientific, Waltham, MA, USA).

Heat cycle conditions for the 40 cycles were two minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, and one minute at 60°C, consecutively. To obtain the results, a reference (calibrator) sample (Control group, 0h-baseline) was used, and the RQ values were calculated with the $2^{\Delta\Delta Cq}$ methodology (1, 21-23, 38).

Statistical Analysis

To evaluate the data, the Minitab® (version 18) package program was used. While the data were evaluated at a 95% confidence interval, an alpha significance value was accepted as $< .05$. After analysis of variance (ANOVA), Tukey's honestly significant difference (HSD) test was used to test between which groups the observed significant difference was more important.

RESULTS

Cell viability, toxicity, and cell proliferation analyses were performed using the MTT-ELISA test. It was observed that healthy proliferation of the cells in the control group continued, and these results were statistically significant ($p<.05$). However, it was noted that in the study group, proliferation was suppressed from 24 to 72 h, and there was a lower number of total cells compared to the control group. These results were also statistically significant ($p<.05$; Table I, Figure 1).

Cell morphology and proliferation were monitored during the experiment using an inverted microscope (Figure 2, Lane1 A, D, G, J, M, P, and S). Also, cells were stained with gieamsa at the end of the experiment and examined regarding morphology and extracellular matrix development (Figure 2, Lane 2 B, E, H, K, N, Q, and T). To determine the cell viability and to prove the accuracy of the results obtained from the

Table I: Cell Viability, Toxicity and Proliferation Analysis Results Performed by the ANOVA Test

	DF	Adj SS	Adj MS	F-value	p*
ALA	1	0.93954	0.939542	1.17443	**0.00**
Times	2	0.00004	0.000020	2551.25	**0.00**
ALA *vs.* Times	2	0.11062	0.055310	6913691.25	**0.00**

One-way ANOVA, analysis of variance; **DF: degrees of freedom, **Adj:** adjusted, **SS:** Sum of square, **MS:** Meansquare*

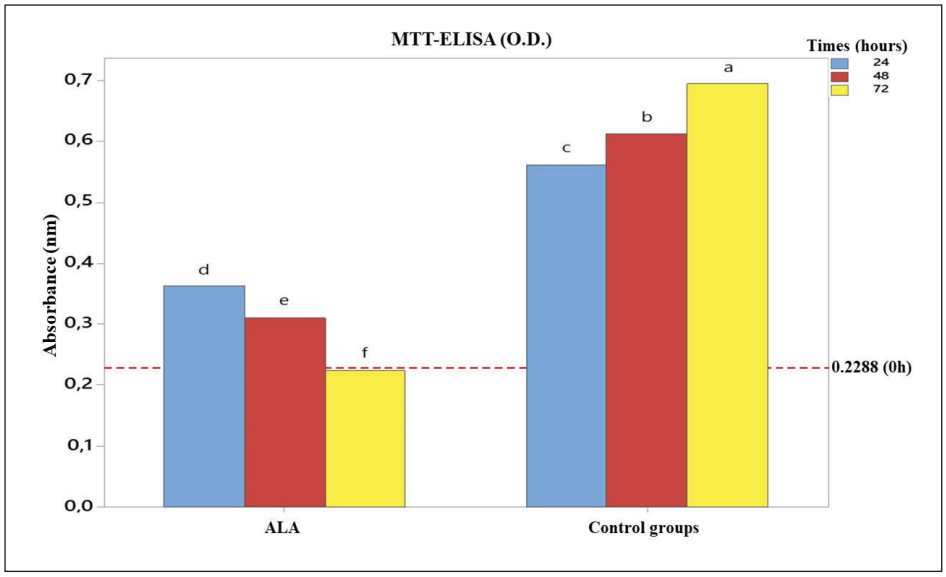

Figure 1: Time dependent proliferation indicator of the control group without ALA and the study group treated with ALA. As we go from A to Z, the order of importance of the difference between groups worsens. The 0.2288 nm absorbance value belonged to the control group that did not receive ALA at 0 h.

MTT-ELISA proliferation and toxicity tests, the fluorescence microscopy images of the AO/PI staining also exhibited decreased proliferation (Figure 2, Lane 3 C, F, I, L, O, R, and U). In microscopic evaluations, it was observed that ALA application negatively affected cell proliferation and ECM organization. However, as observed in AO/PI staining, no apoptotic cell death due to cytotoxicity was observed at the doses and durations where ALA was applied.

In addition to these analyses performed at both the microscopic and molecular levels, it was observed that gene expression also changed.

The RQ values of the CHAD, COMP, IL-1β, MMP-7, and MMP-19 genes were 0.71, 0.62, 0.75, 0.94, and 0.87-fold, respectively, in the control group based on the RNA reference at 0 h. In the control group samples, it was noted that CHAD expression peaked at 24 h and increased to 1.90-fold, but at the same period, this expression decreased to 0.39-fold in the study group. CHAD gene expression was unobserved as time progressed toward 72 h in both groups ($p<.05$).

COMP gene expression increased in the control group from 24 to 72 h compared to the reference RQ value. However, in the study group, the COMP value decreased as 72 h approached, and had decreased by 0.34-fold at the 72 h measurement ($p<.05$).

While the RQ value of IL-1β gene expression decreased in both the control and study groups in the 24-hour analysis, it peaked at 48 h in the control group samples. No gene expression was observed at 48 or 72 h in either the control group or study group ($p<.05$).

MMP-7 gene expression decreased to 0.79-fold in the control group samples at 24 h and to 0.50-fold in the study group samples. Gene expression was observed in either the control group or the study group at 48 or 72 h ($p<.05$).

In the control group, MMP-19 gene expression increased at 24 and 48 h and decreased over time from the initial RQ of 0.87-fold to 0.10-fold at 72 h ($p<.05$). It was noted that the level of MMP-19 expression in the study group samples decreased in the other periods, except for 48 h. These results were all statistically significant ($p<.05$; Figure 3).

DISCUSSION

It has been reported that low back pain affects millions of people worldwide, and the average lifetime incidence is approximately 85% (9,14). Low back pain is a widespread condition that negatively affects quality of life and induces economic burden, especially regarding the health economy (28). IVD degeneration is among the prevalent causes of low back pain (9,14).

Several genomic loci have been previously found to be associated with intervertebral disc degeneration. Single nucleotide polymorphisms in COL1a1, COL9a3, and vitamin D receptor genes have been associated with the development of lumbar disc degeneration. In fact, it is suggested that this relationship is stronger when more than one polymorphism is found in the same individual (40). It was determined that plasma VDR levels and VDR expression were significantly lower in patients with LDD, and TT genotype, a TaqI polymorphism, was significantly associated with lower plasma Vitamin D Receptor levels in patients with lumbar disc degeneration (42).

Depending on the degeneration level of the IVDs, the treatment is either conservative or invasive. However, all treatment methods involve symptomatic treatment, so the pathological process continues (26). Hence, scientists have

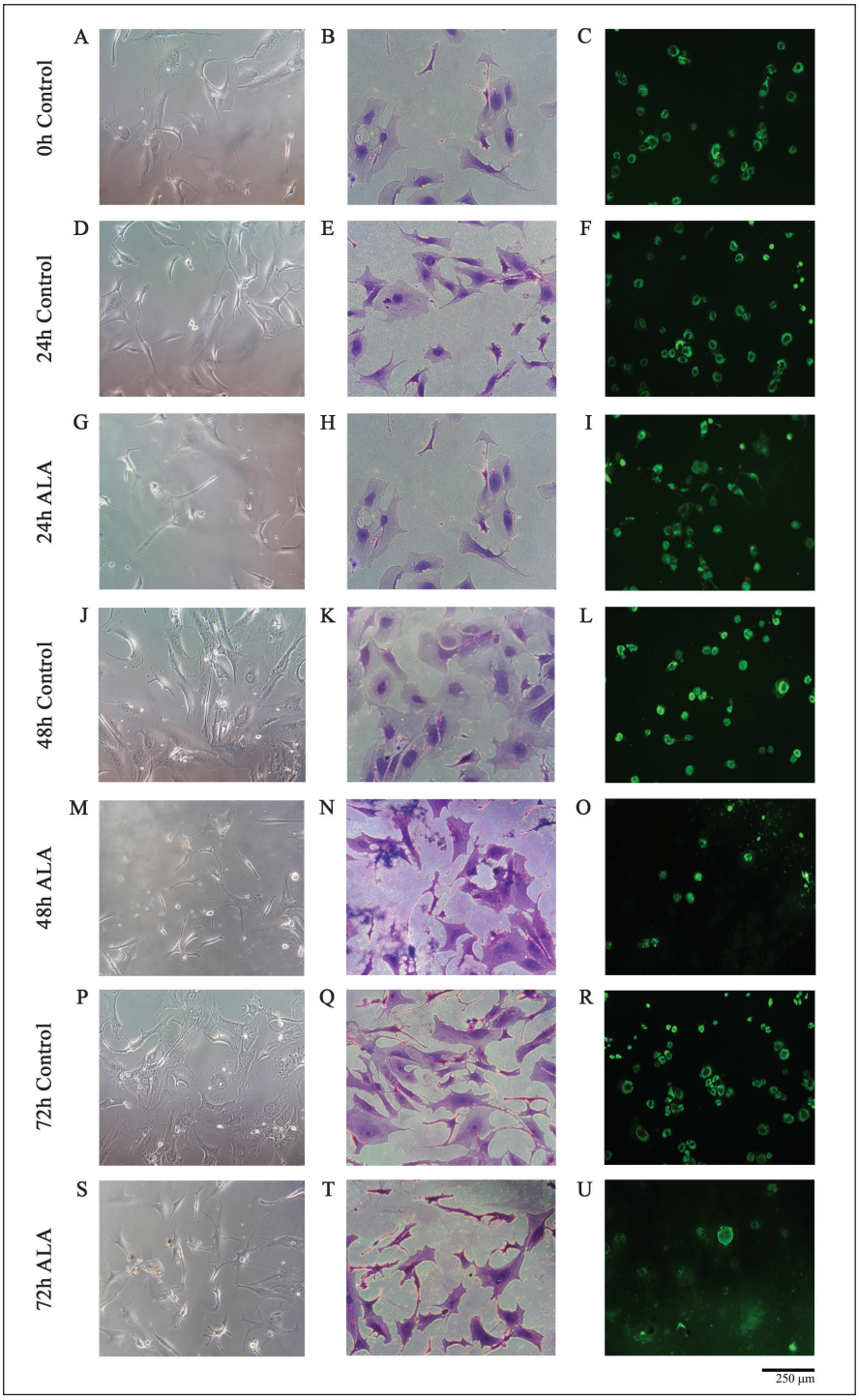

Figure 2: Evaluation of the cell morphology of NP and AF cells and fluorescence microscopy after AO/PI staining. Microphotos of inverted microscopy (Lane1: A, D, G, J, M, P, and S), Giemsa staining regarding morphology and extracellular matrix development (Lane 2: B, E, H, K, N, Q, and T) and the fluorescence microscopy images of the AO/PI staining (Lane 3: C, F, I, L, O, R, and U). A, B, and C; 0 h, D, E, and F; 24 h, J, K, and L; 48 and P, Q and R show the 72 h control group analysis. Simultaneously, G, H and I; 24 h, M, N and O; 48 and S, T and U; 72 h are demonstrative views of the microphotos of ALA-applied samples.

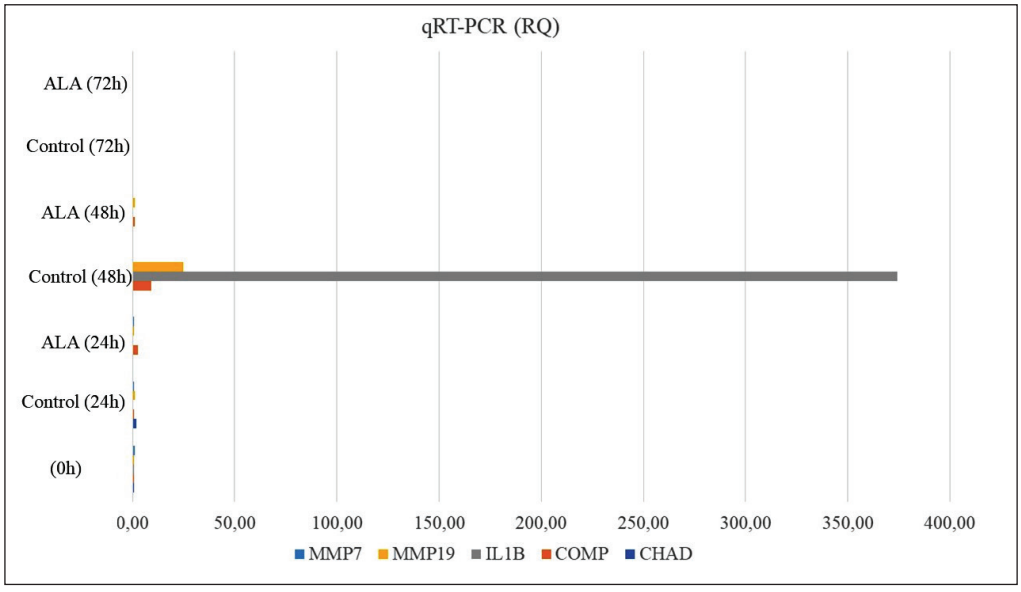

Figure 3: qRT–PCR change indicator of CHAD, COMP, IL-1β, MMP-7, and MMP-19 gene expressions.

turned to research aimed at stopping IVD degeneration or converting degeneration into regeneration. This research includes the implantation of active IVD cells (27), biomaterial (33), and mesenchymal stem cells (37); the injection of growth factors into existing cells (41); gene therapies for inhibiting inflammatory cytokines or intradiscal molecule injection (2); intact or artificial disc transplantation (16); and the testing of pharmacological molecules. However, no commercial product has emerged that provides the desired success in treatment from these studies, and research is still ongoing.

ALA is an organosulfur compound produced from octanoic acid during plant, animal, and human metabolism and has been reported to regeneratively affect many different tissues. Because ALA is both a water-and oil-soluble compound, it is valuable, and many publications have contributed to its clinical effects (36).

In addition to being known as a powerful antioxidant, ALA is a metal chelator and free radical cleaner (32). It also contributes to reproducing endogenous antioxidants (such as glutathione (7), vitamin C, and vitamin E) and repairing damage caused by oxidation (32). Also, many studies have shown that ALA contributes in treating common diseases, such as diabetes, Alzheimer's, schizophrenia, cancer, multiple sclerosis, and obesity. The effects of ALA on apoptosis and cell proliferation are frequently investigated in cancer cells (8).

However, no studies investigating the effects of ALA on IVD degeneration have been found in the literature. A single randomized, double-blinded comparative clinical study by Memeo et al. investigated the effect of ALA on sciatic nerve pain caused by IVD herniation (30). Since there are no similar studies that we can refer to, our research is critical, as it is unprecedented in this field.

Commercial cell lines contain only one type of cell, and their genotype/phenotype has been changed in these cells. Therefore, the reliability of the results determined from studies using only a single type of cell line is controversial (21,23,38). Also, the sensitivity of animal tissues differs from that of human tissues, so the responses from animal tissues during analyses can greatly differ (12,13). It is also known that experimental IVD tissues from animals have differences in cell density, IVD size, and metabolism compared to human IVD tissue.

Therefore, commercial cell lines or animal tissues were not used in this study. Instead, human primary IVD cell cultures prepared from tissues obtained from IVD herniation operations due to lumbar IVD herniation were used. The effects of ALA on these prepared cultures were evaluated.

Also, in the literature, it has been reported that the presence of type-2 diabetes mellitus or smoking causes disc and end-plate degeneration, which may lead to disc degeneration (4,6). However, in the patients where the tissues used in the preparation of primary cultures were taken in this study, type-2 diabetes mellitus and/or smoking were absent in the histories of the patients.

CHAD and COMP are proteins secreted from AF and NP cells that form the ECM and contribute to forming a healthy microenvironment in the IVD tissue (1,23,38). Decreased CHAD and COMP gene expression has been thought to negatively affect cell morphology, and disruptions in ECM structure are believed to induce IL-1β secretion from AF and NP cells (20).

The COMP gene encodes the cartilage oligomeric matrix protein, which is found in the ECM of cells that make up the ligaments, tendons, and cartilage (24). Ishii et al. examined the expression and distribution characteristics of COMP using rat IVDs by demonstrating that COMP is expressed at the protein and mRNA levels in both AF and NP cells in IVD tissue in the lumbar spine and tail (17). Another study also stated that COMP gene expression is higher in the AF region (17,35).

COMP preserves the structural integrity of the ECM. It binds other matrix proteins and catalyzes the polymerization of type II collagen fibrils while also preventing the vascularization

of cartilage. COMP has been said to play an important role in regulating cell movement and attachment alongside cell growth and proliferation. It may also play a role in the pathogenesis of osteoarthritis, a clinical syndrome that results from cartilage degeneration and acts as a potent apoptosis suppressor in primary chondrocytes, IVD cells, and transformed cells (17,35). COMP is believed to be present in cartilage during joint degeneration or osteoarthritis. However, studies have not yet fully explained COMP participation in IVD degeneration (24).

Kaplan et al. suggested that COMP is found as an ECM protein in cartilage and is a new biomarker involved in spinal canal stenosis, along with osteophyte, scoliosis, and joint metabolism (19). Also, it has been reported that MMP-19 is an enzyme that breaks down COMP (41).

CHAD, one of the main ECM components of a healthy IVD, binds to integrin and collagen and regulates cell metabolism, ECM structure, and matrix homeostasis. CHAD, which is also a continuously expressed NP-specific marker, is known to be associated with the development of the spinal cord and dorsal column (1,23,38). In some studies, it has been shown that CHAD expression is associated with an underdeveloped microenvironment and that significant changes in CHAD gene expression have been observed in degenerated IVD tissue (23).

MMPs have basic functions in the maintenance of growth, normal tissue regeneration and physiological events in the organism. These enzymes are also responsible for the degradation of ECM proteins and organogenesis. The continuation of these functions depends on the balance between MMP activity and MMP-specific endogenous tissue inhibitors. When this balance is disturbed in the direction of increased MMP activity, it causes matrix degradation and pathophysiological events (43). Under normal conditions, MMP synthesis and activity are quite low in healthy tissues, however, MMP levels increase in pathological or adverse conditions such as inflammatory disease, tumor progression and metastasis, and this increase may cause tissue destruction (44).

Increased expression of MMPs are known to cause the degeneration of healthy IVD tissue. The structure and interrelationships of collagen types found in the NP and AF regions play a vital role in the mechanical function of IVDs. Collagen molecules can only be degredated by collagenase enzymes specific to them and by MMPs, which are the main enzymes in collagen catabolism and can degrade all known matrix components. Degenerate IVDs are known to be more vascular than normal IVDs, and this vascularization results in an increase in MMP. When the ratio of IVD cells to vascular cells considered in the degenerate IVDs, based on limited immunohistochemical data, it can be said that IVD cells are mostly responsible for MMP enzyme production. High MMP levels in herniated IVDs also suggest that MMPs are responsible for repairing IVD herniation (1).

MMP-7 and MMP-19 are members of the MMP family and structurally comprise zinc-dependent endopeptidases. The primary role of activated MMP is to break down the ECM by breaking down macromolecules such as casein, gelatin, fibronectin, and proteoglycan (25).

Gruber et al. reported that MMPs disrupt the ECM of the IVD (11). They also reported that MMP-19 inhibits capillary formation, thus playing a role in the avascular nature of the IVD.

Yrube et al. reported that MMP-7 is the catabolic gene associated with the degeneration of IVD tissue in rats (45). MMP-7, also known as matrilin, can degrade aggrecan and collagen type II in the matrix, along with noncollagenase proteins, such as proteoglycan and MMP-3. MMP-7 can also break down denatured collagen molecules and the intermolecular network between collagen fibrils (1).

Kaya et al. compared the gene expressions of CHAD, IL-1β, MMP-7, MMP-19, and COMP in the primary cell culture of degenerated and non-degenerated IVD tissues (24). According to their research, they found that CHAD, IL-1β, MMP-7, and MMP-19 gene expressions were significantly changed in degenerated IVD tissue, while COMP gene expression did not change.

In the last two decades, it has been reported that cytokines—mostly chondrocytes, histiocytes, and fibroblasts—are produced by NP cells in the IVD structure (1). IL-1β, a proinflammatory cytokine, is associated with functions such as prostaglandin synthesis, T and B cell activation, as well as fibroblast proliferation and collagen production in the ECM. If tissue degenerates due to ECM degradation, low-molecular-weight degradation products are formed. These products induce inflammation and may stimulate macrophage-mediated IL-1β production (20-23,38). Research supports those disruptions in the ECM structure induce IL-1β secretion from AF and NP cells (19-23,38).

IL-1β, one of the predominant cytokines involved in IVD tissue metabolism; it can induce catabolic activity and angiogenesis in chondrocytes, and stimulate the release of pain mediators from IVD cells by increasing the release of molecules such as proteoglycan and prostaglandin-E2 (31). It has been reported that IL-1β negatively affects IVD tissue cells by causing apoptosis of AF cells (15), and that an increase in IL-1β expression causes an increase in MMP-7 (1).

It has been reported that ALA downregulates β1 and β3 integrin expression, consequently inhibiting focal adhesion kinase activation and preventing extracellular signal-regulated kinases activation. Because of this effect induced by ALA, the mRNA levels of MMP-9 and MMP-2 have been observed to decrease (29). In our study, it was observed that MMP-7 and MMP-19 expression decreased in the ALA group. In IVD degeneration, it has been reported that the number of terminal dUTP nick-end labeling-positive cells is higher in degenerated IVD cells compared to healthy IVD cells, and clear apoptosis is observed (46). It has been reported that NP and AF cells undergo apoptosis through complex mechanisms in degenerated IVDs (46), and apoptosis is clearly observed in cartilage plaque cells (47).

It is critical to replace the amount of cells lost in the biological treatment of IVD degeneration, so this study also investigated the effect of ALA on cell proliferation.

In our study, it was understood that the proliferation of AF and NP cells decreased, and the ECM structure was damaged in the group with ALA added. In microscopic evaluations, it was observed that ALA application reduces cell proliferation. However, as observed in AO/PI staining, no apoptotic cell death due to cytotoxicity was observed at the doses and durations where ALA was applied. Therefore, it was thought that the regression recorded in proliferation and extracellular matrix development may be due to changes in gene expression, not direct cytotoxicity.

Also, it was found that CHAD, COMP, IL-1β, MMP-7, and MMP-19 gene expression exhibited quite different changes compared to the control group. All these results were statistically significant ($p<.05$).

ALA produced surprising, unexpected results on IVD cells. It was observed that ALA suppressed CHAD and COMP gene expressions, which are important markers in the reconstruction of the matrix structure lost during degeneration compared with the control group. Consequently, the matrix formation was negatively affected. However, ALA decreased IL1-β, MMP-7, and MMP-9 gene expressions, which are markers indicating matrix degradation, and decreased matrix degradation was observed compared to the control group.

To summarize, in degenerated IVD cells, compared to the control group, ALA prevented not only the repair of the degenerated ECM structure but also prevented further progress of the degeneration of the ECM structure. Also, ALA suppressed the proliferation of IVD cells in primary cell cultures made from degenerated human IVD tissue.

These results reveal the importance of consuming only ALA very carefully, especially due to its easy availability as a food supplement.

In IVD degeneration, it should be noted that ALA usage is not beneficial or may even advance degeneration. Although our study showed that ALA negatively affects human primary IVD cell cultures, this research was conducted in an *in vitro* laboratory environment.

More importantly, the positive and negative effects of ALA administration outside the doses and durations specified in this study on the cell population and gene expression cannot be determined with the results obtained from this study. Therefore, the results obtained did not fully reflect the clinic. This is the most important limitation of this study.

Our aim was to explain the molecular physiopathology of IVD degeneration, which creates a significant decrease in quality of life and a large economic burden. We also aimed to provide effective information for future treatment strategies. Therefore, it is believed that the results obtained from this study could contribute to the literature.

CONCLUSION

Although it is stated that ALA has a low side effect profile in clinical studies in the literature, its effects on IVD, AF, and NP cells should be considered when prescribing such supplemental pharmaceuticals, even if the evaluation is at a cellular level and in an *in vitro* laboratory environment.

AUTHORSHIP CONTRIBUTION

Study conception and design: IA, HO, DYS, NK

Data collection: IA, DYS, NK

Analysis and interpretation of results: IA, DYS, NK, HO

Draft manuscript preparation: IA, HO

Statistical analysis: IA, HO

Critical revision of the article: DYS, NK, HO

Study supervision: HO

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