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Effects of an acetylcholinesterase inhibitor and an N-methyl-D-aspartate receptor antagonist on inflammation and degeneration of the nucleus pulposus

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Abstract. – OBJECTIVE: The study aimed to examine the effects of two drugs, an acetylcholinesterase inhibitor (AChEI) and an N-methyl-D-aspartate receptor (NMDAR) antagonist, on degenerated annulus fibrosus (AF) and nucleus pulposus (NP) cells and the extracellular matrix (ECM) structure *in vitro*.

PATIENTS AND METHODS: Tissue samples were obtained from patients with intervertebral disc herniation (four males and four females; classified as Pfirmann stage IV) and used to prepare cell cultures. Untreated cell culture samples served as the control group. Study group samples were treated with donepezil, memantine or a combination of the two drugs. Cell viability, toxicity and proliferation were evaluated in all groups. Western blotting was used to examine changes in protein expression of signal transducer and activator of transcription 3 (STAT3), phospho-STAT3 (ser727), hypoxia-inducible factor (HIF)-1 alpha (HIF-1α) and nucleotide-binding oligomerisation domain (NOD) leucine-rich repeat (LRR)-containing proteins (NLR) family pyrin domain containing 3 (NLRP3) inflammasome. The alpha significance value was < 0.05.

RESULTS: Analysis of the microscopy and commercial kit results revealed that cell proliferation was suppressed, and no cell death was observed. The protein expression levels of NLRP3, STAT3, ser727 and HIF-1α were lower in the samples treated with donepezil and memantine at 72 h ($p < 0.05$). The protein expression levels of NLRP3, STAT3, ser727 and HIF-1α were higher in the samples treated with the combination of donepezil and memantine ($p < 0.05$).

CONCLUSIONS: The combined administration of memantine a NMDAR antagonist which can prevent neurodegeneration and donepezil an AChEI used for pain relief increased the protein expression levels in the anabolic pathway. However, it did not reduce the protein expression levels in the catabolic pathway. Therefore, further studies are needed to provide extensive insight into whether it may be among the potential targets for the therapy of intervertebral disc (IVD) diseases.

Key Words:
Donepezil, HIF-1α, Memantine, NLRP3, ser727, STAT3.

Abbreviations
AChEI: acetylcholinesterase inhibitor; AF: Annulus fibrosus; ECM: extracellular matrix; HIF-1α: hypoxia-inducible factor-1 alpha; IVDD: intervertebral disc degeneration; NMDAR: N-methyl-D-aspartate receptor; NLRP3: nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR)-containing proteins (NLR) family pyrin domain containing 3; STAT: signal transducer and activator of transcription protein.

Introduction

Intervertebral discs (IVDs) are anatomical structures that have crucial tasks, such as imparting mobility onto the spinal column and providing support for managing mechanical loads. Degradation of the biomechanical characteristics of the discs can lead to numerous functional losses and low back pain, which significantly impair quality of life[1].

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To date, many conservative medical and surgical treatment modalities have been used to arrest degeneration and initiate regeneration in IVD-related diseases. Despite all the treatment protocols applied, satisfactory outcomes cannot be achieved, leading to a significant loss in the labour force and economic burden on countries' healthcare systems[2]. Therefore, research on drugs that prevent inflammation of nucleus pulposus (NP) cells or IVDD has recently attracted interest[3-5].

Drugs, whether taken orally or parenterally, accumulate in the tissues, especially in the synovial fluid compartment, as soon as they enter the body[6]. Most drugs absorbed into the body diffuse into hyaluronic or synovial tissues. They then pass across the hyaline membranes into the IVD cavity through pores and reach IVD cells, such as annulus fibrosus (AF) and NP cells[7].

Potential drugs that can be used to relieve intense pain experienced during IVDD, currently prescribed to prevent neurodegeneration or for pain relief; Memantine (1-amino-3.5-dimethyladamantane), an N-methyl-D-aspartate receptor (NMDAR) antagonist, and donepezil, a potent and selective acetylcholinesterase inhibitor (AChEI), have both been approved by the United States Food and Drug Administration as pharmacological agents for the treatment of Alzheimer's disease (AD)[8] was evaluated in our study.

Increased NMDAR activity contributes to central sensitisation in certain types of neuropathic pain[9]. NMDAR antagonists can alleviate hyperalgesia and allodynia neuropathic pain caused by nerve damage and diabetic neuropathy in animal models[9]. Different NMDAR antagonists are used to treat neurodegeneration in mice with vanadium-induced neurotoxicity[10]. In addition, amantadine, an NMDAR antagonist administered to rats, can restore spinal cord damage by preventing oxidative stress[11]. The effects of NMDAR antagonists on retinal tissue in autoimmune optic neuritis[12] and on brain tissue in status epilepticus[13] have also been investigated.

Perioperative intrathecally administered AChEIs have a pain relief effect in the postoperative period and reduce the need for narcotic analgesics during lumbar disc surgery[14].

Acetylcholine is a well-known neurotransmitter. It is derived from acetyl coenzyme A, a product of cellular respiration in mitochondria, and choline, which plays an important role in lipid metabolism. The potential therapeutic effects of donepezil, which is a selective and reversible inhibitor of acetylcholinesterase (a predominant cholinesterase in the brain), are remarkable; it has been shown to efficiently treat osteoarthritis caused by cartilage degeneration[15].

No studies have yet investigated the effects of memantine on cartilage destruction. However, memantine has potential therapeutic effects in the treatment of osteoarthritis due to cartilage degeneration by weakening the activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription protein (STAT)-1 signalling pathway[16].

Donepezil[17] and memantine[18] can also accumulate in various tissues in the body. In addition, no studies have examined the effects of combinations of AChEIs and NMDAR antagonists on AF/NP cells extracted from IVDs and the extracellular matrix (ECM) structure.

In this study, the effects of donepezil, memantine and donepezil combined with memantine administered separately to human primary cell cultures on IVD cells and the ECM structure were investigated. Changes in the expression of the following proteins were examined: hypoxia-inducible factor (HIF)-1 alpha (HIF-1α)[1], an NP-specific marker; STAT-3 and phospho STAT-3 (ser727)[19], the inhibition of which reduce IVDD; and nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR)-containing proteins (NLR) family pyrin domain containing 3 (NLRP3) (previously known as NACHT, LRR and PYD domains-containing protein 3 [NALP3] and cryopyrin)[2].

Patients and Methods

Criteria for the Inclusion or Exclusion of Tissues Obtained from Patients

When donepezil or memantine are used with fluoxetine, paroxetine, ketoconazole or anti-dementia drugs, anticholinergic drugs, conventional antipsychotics and bradycardia-inducing drugs with major pharmacodynamic interaction potential, drug-drug interactions may occur at the pharmacokinetic level[20]. Therefore, patients who used these drugs were excluded from the study.

Lumbar microdiscectomy or lumbar sequestrectomy was performed to treat patients with lumbar disc herniation, progressive neurological deficits and sphincter defects who were unresponsive to medical and conservative treatments. The resected IVD tissues were included in the study.

Table I. Drug concentrations administered to the samples and the contents of the groups.

Groups	Donepezil (20 µM)	Memantine (10 µM)	Donepezil and Memantine Combination
Group 1 (Control)	-	-	-
Group 2	+	-	-
Group 3	-	+	-
Group 4	-	-	+

Surgical Resection of Tissues and Preparation of Primary Cell Cultures

The tissues obtained from the patients (four males and four females; mean age 44.98 ± 7.65 years; classified as Pfirmann stage IV) were transferred to Falcon tubes containing freshly prepared medium. The samples were then transferred to the laboratory at 4°C.

The tissues were moved into a flow cabinet, transferred to Petri dishes and washed three times consecutively with sterile phosphate-buffered saline (PBS). The tissues were minced mechanically and degraded enzymatically. After centrifugation, suspension and resuspension, the isolated cell samples were transferred to flasks and fed with a freshly prepared nutrient medium every two days for 21 days in an incubator with 5% CO_2 at 37.4°C[21]. The experiment was performed during the third passage.

Drug Treatment of the Cell Cultures

The selection of the donepezil and memantine doses was based on dose-response curves produced in previous studies. The concentrations used were 20 µM donepezil[15] and 10 µM memantine[16] per well in Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS; Table I).

Cell Viability, Toxicity and Proliferation Analyses

Cell viability was assessed using enzyme-linked immunosorbent assays (ELISAs) and a commercial 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) assay. The half-lives of donepezil and memantine were between 24 h and 60 h in the MTT assay[22]. Therefore, analyses were performed at 0, 24, 48 and 72 h.

Drug-treated culture medium was discarded from the wells and 100 µl of MTT solution (MTT dissolved in 1 ml of sterile PBS at pH 7.4 to prepare the 12 mM stock solution) was added to each well. After the primary cell culture samples had been incubated for 2 h at 37°C in the dark, dimethyl sulfoxide (DMSO) was added to the samples to stop the reaction. Primary cell cultures were then incubated for an additional 10 min at 37°C before photometric analysis, which was performed at a wavelength of 540 nm. The cell viability of the untreated control group was taken as 100%[6].

Acridine Orange (AO) and Propidium Iodide (PI) and Janus Green-B Staining

When stained with both AO and PI, cells with viable nuclei produce green fluorescence and cells with dead nuclei produce red fluorescence. To prepare the AO/PI dye, 4 mg AO (dissolved in 2 ml 99% ETOH), 10 g sodium–ethylenediaminetetraacetic acid, 4 mg PI and 50 ml fetal bovine serum (FBS) were mixed well. Sterile distilled water was added to this mixture to produce a volume of 200 ml[23].

Janus Green-B was used to supravitally stain mitochondria in cells. The Janus Green-B indicator changes colour according to the amount of oxygen. When oxygen is present, the indicator oxidises to a blue colour. In the absence of oxygen, the indicator decreases and turns pink[24].

Evaluation of Protein Levels by Western Blotting

Proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was used to determine protein expression levels. A polyvinylidene difluoride (PVDF) transfer membrane was used to transfer the proteins. Immunoblotting was performed using the WesternBreeze™ Chemiluminescent kit (Catalogue no: WB7104, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primary antibodies specific for HIF-1α (Catalogue no: MA1516, Thermo Fisher Scientific, Waltham, MA, USA), STAT 3 (Catalogue no: MA113042, Thermo Fisher Scientific, Waltham, MA, USA), ser727 (Catalogue no: MA515208, Thermo Fisher

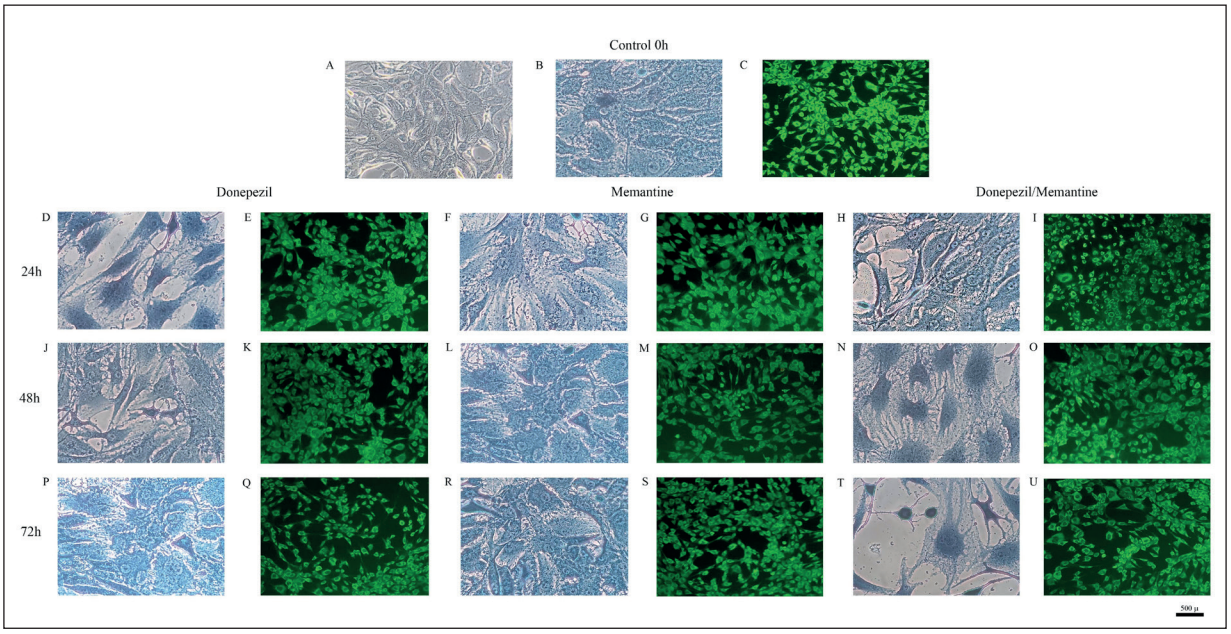

Figure 1. Images obtained via inverted microscopy. **A**, **B** and **C** represent the control group samples and show images of unstained, Janus Green-B stained and AO and PI stained cells. **D**, **E**, **J**, **K**, **P** and **Q** show donepezil-treated samples at 24, 48 and 72 h stained with Janus Green-B and AO and PI. **F**, **G**, **L**, **M**, **R** and **S** show memantine-treated samples at 24, 48 and 72 h stained with Janus Green-B and AO/PI. **H**, **I**, **N**, **O**, **T** and **U** show cells treated with the combination of donepezil and memantine at 24, 48 and 72 h stained with Janus Green-B and AO/PI (Janus Green-B stained, 40X magnification; AO and PI stained, 10X magnification).

Scientific, Waltham, MA, USA) and NLRP-3 inflammasome (Catalogue no: MA516274, Thermo Fisher Scientific, Waltham, MA, USA) were used. β-actin (Catalogue no: MA1-140, Thermo Fisher Scientific, Waltham, MA, USA) was used as an endogenous control. After treatment with a primary antibody, sequential washes were performed. The membranes were incubated with an alkaline phosphatase-conjugated secondary antibody. Then, the membranes were treated with substrate solution after three washes. The protein bands were transferred to an X-ray film (Catalogue no: 34090, Thermo Fisher Scientific, Waltham, MA, USA) and analysed using ImageJ software, and the specific amount of protein in each sample was determined[24,25].

Statistical Analysis

Statistical analyses were performed using Minitab version 22.0. Data are presented as a percentage (%), mean ± standard deviation, minimum (min) and maximum (max). Tukey honestly significant difference (HSD), a post hoc test, was performed after a one-way analysis of variance (ANOVA) to evaluate differences across the group means. The confidence level was 95%, and the alpha significance value was accepted as < 0.05.

Results

Analysis of the MTT assay results revealed that cell proliferation was suppressed after drug administration. However, AO/PI staining showed that there was no cell death (Figure 1).

Cell proliferation decreased by 25.10%, 22.66% and 35.13% at 24, 48 and 72 h, respectively, in the donepezil-treated samples compared to the control group samples. Cell proliferation decreased by 26.10%, 19.80% and 44% at 24, 48 and 72 h, respectively, in the memantine-treated samples compared to the control group samples. Cell proliferation decreased by 18.87%, 27.80% and 27.26% at 24, 48 and 72 h, respectively, in the samples treated with the combination of donepezil and memantine compared to the control group samples (Table II and Figure 2). The obtained results were statistically significant ($p < 0.05$) (Table III).

Western blot analyses were performed after SDS-PAGE, and the results are shown in Figure 3.

Illustrative Western blots for all proteins are given in the **Supplementary Data.**

The protein expression levels of NLRP3 expression (1%) remained at the same level as the control group, but STAT3, ser727 and HIF-1α in-

Table II. Tukey HSD grouping presentation of proliferation data after descriptive analysis and ANOVA.

Variable	Time (hours)	Mean±StDev	Tukey HSD Grouping*
Control absorbance (nm)	0	0.406±0.00089	F
	24	0.498±0.00121	C
	48	0.525±0.00422	B
	72	0.609±0.00894	A
Donepzil absorbance (nm)	0	0.406±0.00089	F
	24	0.373±0.00089	H I
	48	0.406±0.00089	F
	72	0.395±0.00089	G
Memantin absorbance (nm)	0	0.406±0.00089	F
	24	0.368±0.00089	I
	48	0.421±0.00089	E
	72	0.341±0.00089	J
Memantine in combination with donepezil absorbance (nm)	0	0.406±0.00089	F
	24	0.404±0.00089	F
	48	0.379±0.00767	H
	72	0.443±0.00105	D

*A: Highest rate of cell viability and proliferation. J: Lowest rate of cell viability and proliferation. Dev stands for standard deviation, and HSD stands for honestly significant difference.

creased by 82%, 13% and 106% at 24 h, respectively, in the donepezil-treated samples compared to the control group. NLRP3 decreased by 28%, while STAT3, ser727 and HIF-1α increased by 18%, 19% and 88% at 48 h, respectively. The protein expression levels of NLRP3, STAT3, ser727 and HIF-1α decreased by 46%, 17%, 27% and 12% at 72 h, respectively (Table IV).

The protein expression levels of NLRP3, STAT3, ser727 and HIF-1α decreased at 24 h (43%, 48%, 93% and 75%), 48 h (27%, 60%, 93% and 91%) and 72 h (54%, 43%, 82% and 66%) in the memantine-treated samples. The protein expression levels of NLRP3, STAT3, ser727 and HIF-1α increased by 97%, 83%, 11% and 13% at 72 h, respectively, and the changes in the protein expression levels were statistically significant ($p < 0.05$).

Discussion

IVDD is a pathology characterised by excessive apoptosis of NP cells and disruption of ECM microarchitecture and is a major cause of low back pain[26]. Degenerative changes can lead to a wide variety of symptoms, from simple axial pain to advanced myelopathic symptoms, depending on the location in the spine. Symptoms are more common in male patients and degeneration is observed more frequently in patients over 40 years of age. It is also seen in female patients, and it negatively affects their quality of life. The increase in IVDD-related pathologies in the ageing population means that there is an acute and urgent need to develop health care strategies to not only prevent, delay or stop disc degeneration, but also to regenerate affected discs to maintain or improve quality of life[27]. Therefore, many studies have investigated different pharmaceutical and biomolecular approaches that might have positive therapeutic effects on IVDD.

Ling et al[28], reported that a deeper understanding of the pharmaco-molecular mechanisms of disc degeneration and inflammation may provide a theoretical basis for the development of intervention strategies for lumbar disc tissue. The authors suggested that ECM proteolysis mediated by various proteases is involved in IVDD and that targeting such proteases may be an effective intervention for IVDD[28].

Kedong et al[29] reported that if disc inflammation (which involves several pathological events) can be downregulated in IVDD, then disc degeneration and low back pain can be eliminated.

The active ingredients of donepezil[15] and memantine[16] have positive therapeutic effects on cartilage degeneration and destruction. However, the effects of sole or combined administration of donepezil and memantine on IVD cells and the ECM structure have not been investigated to date. Therefore, in the present study, the effects of donepezil (an AChEI) and memantine (an NMDAR

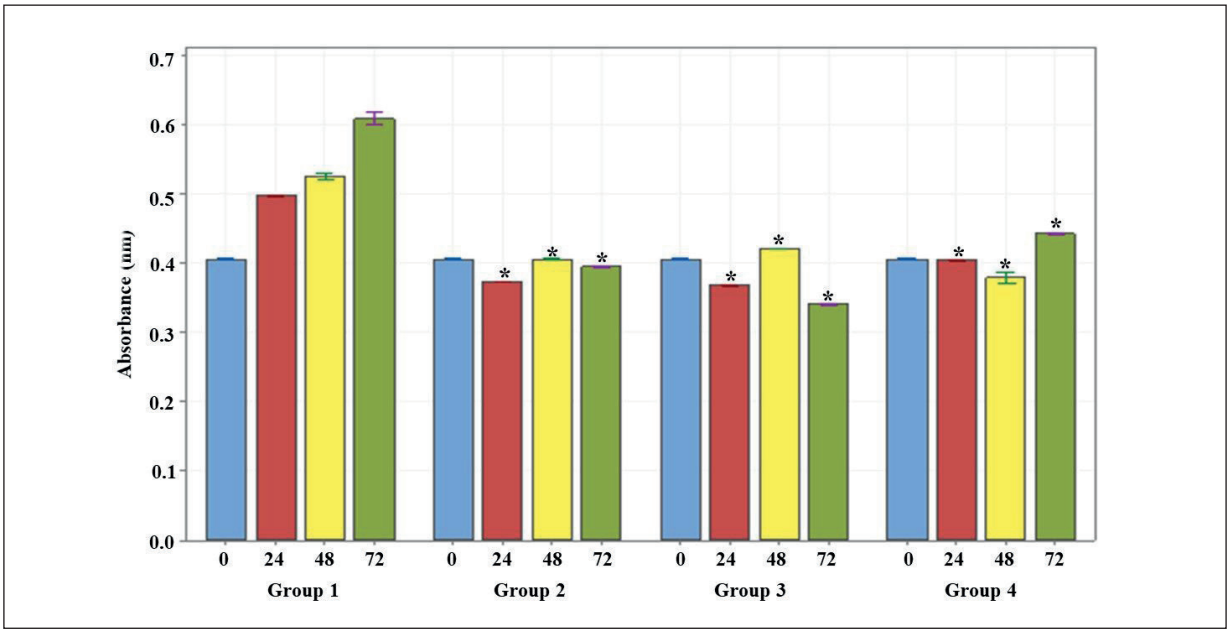

Figure 2. MTT-ELISA cell viability, toxicity and proliferation results. The MTT-ELISA is a colorimetric method for assessing cell metabolic activity (optical density, 540 nm absorbance).

antagonist) applied alone or in combination on human degenerated primary AF/NP cells were investigated by examining the HIF-1α, NLRP3 and STAT3 levels.

Some studies have reported that HIF-1α has an important role in both NP proliferation and the anabolic and catabolic reactions of ECM components[21,30,31]. Here, HIF-1α levels were found to increase at 24 and 48 h in the samples treated with donepezil and decrease at 72 h. The HIF-1α levels decreased in the samples treated with memantine. In addition, HIF-1α increased by 13% at 72 h in the samples treated with the combination of donepezil and memantine ($p < 0.05$).

The JAK-STAT pathway, a main intracellular signalling pathway, directly affects transcription factors. STAT proteins involved in transcription are inactive in the cytoplasm; they are phosphorylated after ligands bind to their receptors. Phosphorylated STAT proteins then dimerise, translocate to the cell nucleus and activate the transcription of target genes. The presence of different levels of negative feedback control of STAT signals in the intracellular signalling pathway has been reported[32,33]. The persistent activation of STAT3 also mediates tumour-promoting inflammation[33].

Wu et al[34] suggested that interleukin, which are inflammatory factors, and the downstream JAK/STAT3 pathway are involved in NP cell degeneration, and this idea has attracted interest. Suzuki et al[35] reported that the JAK/STAT3 pathway plays a crucial role in the pathogenesis of IVD degeneration and suppresses the catabolic effect.

Bai et al[36] indicated that the JAK2/STAT3 signalling pathway is involved in the regulation of autophagy. They reported that abnormal activation of the JAK2/STAT3 pathway is involved in a variety of pathophysiological processes, including apoptosis and autophagy[36]. The authors also reported that they could improve the function of NP cells in IVDD by negatively regulating the JAK/STAT3 signalling pathway, inhibiting cell apoptosis and ECM degradation[36].

It is noteworthy that many cytokines signal ***via*** the JAK/STAT pathway[37]. For example, STAT3 is involved in the differentiation of proinflammatory T helper cells, such as T helper-17 cells, which play a role in various autoimmune diseases[38]. Activation of STAT3 may occur *via* phosphorylation of Ser 727 by mitogen-activated protein kinases and through c-src, a non-receptor tyrosine kinase[39,40]. However, an increase in STAT can reduce degeneration[41].

Both donepezil and memantine have been reported to reduce the degeneration of cartilage cells. Zhang and Zhou[15] reported that donepezil may prevent the degeneration of cartilage cells by suppressing matrix metalloproteinase-13 (MMP-13)-induced tumour necrosis factor-alpha and by

Table III. Assessment of the cell viability of AF and NP cells following drug treatment.

Source	Adj SS	Adj MS	F-value	*p*-value*
Groups	0.239587	0.079862	7613.48	0.000
Time (hours)	0.026319	0.008773	836.35	0.000
Groups *vs.* Time	0.139484	0.015498	1477.49	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.

inhibiting collagen type 2 degradation ***via*** STAT1/interferon response factor-1. Whereas memantine mediates an anti-inflammatory effect against astroglia activation and neuroinflammation by blocking NMDA receptors[42, 43]. It also inhibits aggrecan and collagen-2 degradation induced by advanced glycation end products[16]. Memantine can also slow down articular cartilage degeneration by suppressing MMP-13 and affecting the JAK/STAT signalling pathway, which is important in chondrocyte pathophysiology[16].

The JAK2/STAT3 signalling pathway plays a crucial role in central nervous system inflammation, the immune response, synaptic plasticity, neurodegeneration and memory formation, and is related to IVDD[44]. Suppressing or inhibiting the STAT3 signalling pathway has a beneficial effect in IVDD treatment[44,45].

In the present study, the STAT3 and ser727 levels increased in the samples treated with donepezil compared to the untreated control group samples at 24 and 48 h; however, they decreased at 72 h. The STAT3 and ser727 levels decreased in the samples treated with memantine at 24, 48 and 72 h. The STAT3 and ser727 levels increased in the samples treated with the combination of donepezil and memantine at 24, 48 and 72 h ($p < 0.05$).

NLRP3 inflammasome signalling plays an important role in the treatment of IVDD, which causes the collapse or herniation of the NP or leads to radiculopathy[46]. As a pattern recognition receptor, NLRP3 is involved in the pathological processes of many diseases[47]. Furthermore, the NLRP3 inflammasome is associated with IVD inflammation, pyroptosis, ECM degradation and IVD cell apoptosis[48]. Activation of the NLRP3 inflammasome facilitates the induction of matrix metalloproteinases, causing damage to NP tissue and IVDD[48,49]. In the present study, the NLRP3 level decreased in the culture samples treated with donepezil and memantine alone compared to the untreated control group samples at 72 h. However,

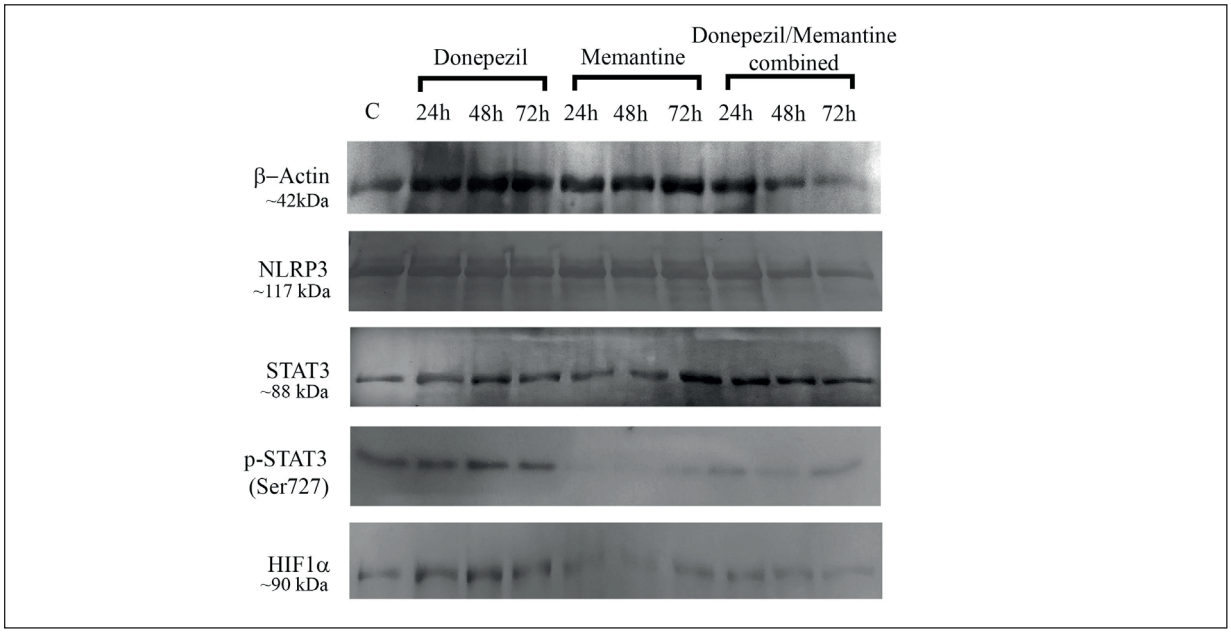

Figure 3. Demographic Western blot images of drug-treated samples (20 μM/well donepezil and 10 μM/well memantine, alone or in combination) and untreated samples. "C" represents control group samples.

Table IV. Presentation in folds of changes in protein expressions value (r) in drugs treated samples.

	βActin	NLRP3	STAT3	Ser727	Hif-1α
Control	1	1	1	1	1
Donepezil 24 h	1	1.01	1.82	1.13	2.06
Donepezil 48 h	1	0.72	1.18	1.19	1.88
Donepezil 72 h	1	0.54	0.83	0.73	0.88
Memantine 24 h	1	0.57	0.52	0.07	0.25
Memantine 48 h	1	0.73	0.40	0.07	0.09
Memantine 72 h	1	0.46	0.57	0.18	0.34
Donepezil/Memantine 24 h	1	0.78	0.89	0.30	0.50
Donepezil/Memantine 48 h	1	1.29	1.43	0.21	1.01
Donepezil/Memantine 72 h	1	1.97	1.83	1.11	1.13

the NLRP3 level increased in the culture samples treated with the combination of donepezil and memantine at 72 h.

Commercial cell lines[50] and animal tissue[51] are generally used in research. However, it is well known that the phenotype and genotype of commercial cell lines are often different from those of primary cells. Furthermore, results obtained in studies that use animal tissue may differ from results obtained in studies that use human tissues[6,24,25,52]. In the present study, primary cell cultures prepared from human degenerated IVDs were used. This may enhance the value of the study.

Cell proliferation decreased in the culture samples treated with donepezil (35.13%) and memantine (44.00%) alone, and the combination of donepezil and memantine (27.26%) at 72 h. Typically, the toxicity of a drug administered alone is potentiated when it is administered in combination. However, in this study, cell proliferation was less affected by the drug combination than the drugs administered alone.

The decrease in NLRP3 and STAT3 levels, as well as the significant decrease in STAT3 phosphorylation when memantine was administered alone showed that this drug may be beneficial in suppressing inflammation and pain caused by inflammation in IVDD. However, a decrease in the amount of HIF-1α was observed in memantine application. It is also an important fact that the response to decreased HIF-1α in IVD cells should be evaluated in more detail. In addition, the results of this study, which was consistent with a limited number of cases, need to be confirmed with a larger number of cases.

The increase in STAT3 and STAT3 phosphorylation in the first 48 hours in cultures treated with donepezil can be interpreted as an immediate response by the cells. Likewise, HIF-1α expression increased in the first 48 hours in this treatment group. For this reason, it can be said that donepezil does not have the potential to be used for pain relief in IVDD. In addition, the increase in NLRP3 and STAT3 in donepezil/memantine combined applications showed that these drugs would have a negative effect on the inflammation observed in IVDD.

All culture samples were prepared using tissues obtained from a small number of patients. This is the first limitation of this research. The experiments were repeated to eliminate this limitation. It is known that memantine is excreted mainly *via* the hepatic system, with a minor portion excreted *via* the renal system[53]. Donepezil is metabolised by hepatic enzymes and reduced to its active metabolite, 6-deoxy-donepezil[54]. Cytochrome P450 (CYP) 2D6, CYP3A4, CYP3A5 and CYP2C9 are involved in donepezil metabolism[54]. The present research was performed using an *in vitro* experimental set-up; therefore, clinically relevant results can only be predicted. Unlike *in vivo* experiments, no compensatory mechanism is present in an *in vitro* experimental set-up. Due to the lack of any *in vivo* experience with an inflammatory pain model, we cannot further support our *in vitro* findings. Further *in vivo* studies are required to inform the level of evidence that reduced pain signaling is observable with memantine alone. Therefore, the *in vivo* effects of memantine and donepezil cannot be predicted using the results of this study. This is a second limitation of the research.

Conclusions

HIF-1α, NLRP3, STAT3 and ser727, the phosphorylated form of STAT3, are involved in signalling pathways associated with IVD inflammation, pyroptosis, ECM degradation and apoptosis of

AF/NP cells. In the present study, both donepezil and memantine changed the expression of these proteins. The results indicate that if the pharmaceutical formulations memantine (an NMDAR antagonist) can be manipulated may have potential as treatment options for NP inflammation and IVDD but not donepezil (an AChEI) administered -alone or in combination- However, it should be kept in mind that the present research was conducted using an *in vitro* experimental set-up.

Conflict of Interest

The authors declare that they have no conflict of interests.

Availability of Data

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

Ethical Approval

Informed consent forms were obtained from patients whose tissues were used to prepare primary cell cultures. Approval was also obtained from the Local Ethics Committee of the School of Medicine of Haliç University (Date: 23.02.2022, Number: 46).

Authors' Contribution

I.Y., is the principal author of this study, and idea initiation, study design and data analysis, data collection, drafting of the article; IY and HA: Preparation of human primary cell cultures from tissues and application to cultures after preparation of drugs; IY, HA and DYS: Molecular analysis; NK and NeK: Inclusion criteria of the cases whose tissues were used and surgical resection of tissues; NK, NeK and HO: Revising the article for scientific and intellectual content; IY and HO: Statistical analysis and interpretation of the data; IY, DYS, NK and HO: Final approval of the version to be published. All authors read and approved the final manuscript.

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