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Is Favipiravir a Potential Therapeutic Agent in the Treatment of Intervertebral Disc Degeneration by Suppressing Autophagy and Apoptosis?

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ABSTRACT

AIM: To evaluate the effects of favipiravir (FVP) on cell viability and cytotoxicity in human degenerated primary intervertebral disc (IVD) tissue cell cultures. Furthermore, the protein expressions of hypoxia-inducible factor 1 alpha (HIF-1α), nuclear factor-kappa-b (NF-κB), and interleukin-1 beta (IL-1β) were also examined.

MATERIAL and METHODS: Untreated cell cultures served as the control group, named group 1. Cell cultures treated with FVP served as the study group, named group 2. Pharmacomolecular analyses were performed in all groups at 0, 24, 48, and 72 hours (h). Obtained data were evaluated statistically.

RESULTS: Cell proliferation was suppressed in the FVP-treated samples compared to the control group samples at 24 and 72 h, and this was statistically significant ($p<0.05$). Decreased or increased protein expression levels of HIF-1α, NF-κB, and IL-1β in FVP-treated samples may be an indication of suppression in anabolic events as well as proliferation in IVD cultures. FVP administration showed that AF/NP cells in a culture medium may induce a strong inflammatory response to FVP. This strong inflammatory response is likely to cause slowed proliferation. It may also be a trigger for many catabolic events. NF-κB expression increased within the first 24 h and then decreased rapidly. Based on the data obtained, it may be suggested that the rapidly increasing NF-kB may have stimulated the expression of many antiproliferative genes.

CONCLUSION: The suppression of IL-1β and NF-kB protein expressions in IVD cells treated with FVP is important in the treatment of IVD degeneration (IDD). If the protein expression of HIF-1α could be increased along with the suppression of IL-1β and NF-kB, FVP would perhaps be a promising pharmacological agent in the treatment of IDD.

KEYWORDS: Favipiravir, HIF-1α, IL-1β, NF-κB

ABBREVIATIONS: ECM: Extracellular matrix, **FVP:** Favipiravir, **HIF-1α:** Hypoxia-inducible factor-1 alpha, **IVD:** Intervertebral disc, **IDD:** Intervertebral disc degeneration, **NF-Kb:** Nuclear factor-kappa-b, **IL-1β:** Interleukin-1 beta, **MTT:** 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium-bromide.

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INTRODUCTION

Several pharmacomolecular studies on drugs conducted in recent years have suggested that many different signaling pathways are associated with the degeneration of the intervertebral disc (IVD) and/or inflammatory diseases of IVD (4). The nuclear factor kappa-B (NF-κB) signaling pathway is widely activated in immune reactions, the tumor microenvironment, senescence, and cell stress responses and plays an important role in the development of IVD degeneration (IDD) (4,32). NF-κB signaling is also crucial for the maintenance of IVD homeostasis (31).

NF-κB signaling is associated with the Wnt pathway, which plays an important role in IVD cell senescence, apoptosis, and extracellular matrix (ECM) degradation (5). Hypoxia-inducible factor-1 alpha (HIF-1α) is vital in the survival of nucleus pulposus (NP) cells (30). HIF-1α plays a key role in many pathophysiological events, including chondrogenesis (12). NF-kB is the central transcription factor of inflammatory mediators that plays a significant role in microglial activation (2). The acetylation of the NF-κB signaling pathway attenuates the inflammatory response by modulating microglia polarization, and this leads to neuroprotective effects (2). Interleukin-1 beta (IL-1β) promotes NF-κB signaling in NP cells (16,25). Some studies have reported that the inhibition of the NF-κB signaling pathway may suppress apoptosis in NP cells (23) and that IL-1β can activate NP cells with or without NF-κB inhibition (34).

Frequently prescribed drugs, administered orally or parenterally, accumulate in many different tissues, particularly in the synovial fluid compartment. Drugs taken into the body first diffuse into the hyaluronan or synovial tissues and then reach the IVD cells by passing through the pores in the hyaline membranes in the IVD cavity.

Used against the Ebola virus, favipiravir (T-705, FVP) is an inhibitor of CYP2C8. Many antiretroviral drugs have a narrow therapeutic index, including FVP (8), a purine nucleotide that inhibits viral replication via control of RNA polymerase (10). However, since 2019, it has been frequently prescribed as a treatment for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known as COVID-19 (11). Some studies have reported adverse events/side effects related to FVP (22). However, no studies have focused on the effects of FVP on IVD tissue, annulus fibrosus (AF)/NP cells, and the ECM structure. The present in vitro study aimed to evaluate the effects of FVP on human primary IVD tissue cell cultures. Not only cytotoxicity but also senescence/cytotoxicity/proliferation changes in AF/NP cells and whether there was a deterioration in the ECM structure were investigated. In this respect, the protein expressions in the signaling pathways of IL-1β, HIF-1α, and NF-KB, which are important in proinflammatory and inflammatory pathways, were evaluated.

MATERIAL and METHODS

Approval of the Local Ethics Committee

Approval has been obtained from the local ethics committee of the School of Medicine of Izmir Bakircay University (date: 12/01/2022 no. 464/483) to conduct the study.

Criteria for the Inclusion or Exclusion of Tissues Obtained From Patients

Tissues obtained from patients with liver/kidney dysfunction and those with an FVP allergy were not used in the preparation of cell cultures. Tissues of patients with lumbar disc herniation, Pfirrmann grade IV, and who were unresponsive to conservative management and medical treatment were included in the study.

Surgical Resection of Tissues and Preparation of Primary Cell Cultures

Patients diagnosed with lumbar IDD were taken to the operating table following preoperative preparations. The patients were operated on in the prone position under endotracheal general anesthesia. The surgical level was determined using a C-arm scopy (X-ray). Surgical site antisepsis was provided, and the site was covered in a sterile manner. A skin incision was made with a mid-lumbar approach, and a lumbar fascia incision and subperiosteal paravertebral muscle dissections were performed. Subsequently, hemilaminectomy, flavectomy, and microdiscectomy procedures were performed under the surgical microscope. Following the bleeding controls, the folds were sutured following their anatomical originals. The IVD tissues obtained through microdiscectomy were transferred into Falcon tubes containing 5% penicillin-streptomycin (PS) together with a freshly prepared cell culture medium. The samples were then transferred to the laboratory at 4 °C.

The tissues were first mechanically minced into small fragments using a rongeur in separate Petri dishes. They were then enzymatically degraded using a mixture of 200 units/mL collagenase type II enzyme/collagenase type I enzyme dissolved in Hank's Balanced Salt Solution (HBSS). After overnight incubation at 37 °C, 5% CO_2, the samples were centrifuged at 4 °C and 1200 rpm consecutively twice for 10 min. The supernatant on the tubes was discarded. Pellets precipitated at the bottom of the tubes were resuspended with a freshly prepared cell culture medium. The samples were then transferred to flasks. Cells that were viable and adhered to the surface of the flasks were counted by trypan blue exclusion using a Neubauer counting chamber. For enzyme-linked immunosorbent assay (ELISA) 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium-bromide (MTT) viability, proliferation, and toxicity assays, and acridine orange/propidium iodide (AO/PI) staining, cells were cultivated with 15,000 cells/well in 96-well plates. To evaluate the protein expression, change of IL-1β, NF-KB, and HIF-1α by western blotting, cells were transferred to petri dishes (29,000 cells/10-mm).

Drug Treatment of the Cell Cultures

The plasma half-life of FVP is approximately 4 h, and drugs are administered orally for a total of five days with 3×900 mg/1 day loading dose, followed by 2×300 mg/4 days maintenance doses in the treatment of COVID-19 patients in Turkey. In in vitro cell culture studies, the replicated doses have been administered, and the final concentration of FVP has been taken as 61.88 μM (3,17). Therefore, the doses applied to cell cultures were 61.88 μM FVP (Favicovir®, Atabay Kimya, Istanbul, Turkey) per well in 1 ml of 1%-FBS medium. No drug

was administered to the samples in the control group (Group 1). FVP at 61.88 μM was applied to the samples in the study group (Group 2).

Inverted Light/Fluorescence Microscopy and Acridine Orange and Propidium Iodide Staining

Cell and ECM surface morphologies were evaluated various magnifications using an inverted light microscope. In membrane permeability assays, the live and dead cells were determined by interpreting the images obtained after AO/PI staining, which is based on the principle that living cells generate green fluorescence, while dead cells generate red fluorescence. The cell morphology was determined using the supravital staining through Janus green B, which specifically stains mitochondria and is retained by living cells.

Cell Viability, Toxicity, and Proliferation Analyses Using MTT and Enzyme-Linked Immunosorbent Assays

MTT commercial kit (3 [4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide [Vybrant MTT Cell Proliferation Assay, Cat #V13154, Thermo Fisher Scientific, USA]), was used for this. As is known, the working principle of this kit is based on the blue color reaction that occurs due to the transformation of the thiazole ring into formazan crystals (13). This method is predicated on the measurement of color changes, which occur via formazan (purple) production as cells undergo proliferation using tetrazolium through increasing dehydrogenase enzyme activity by spectrophotometry as absorbance (13). The assays were performed at a wavelength of 570 nm, and the untreated control group cell viability was taken as 100%. The proliferation and the inhibition of the proliferation were calculated using the following formulas: "Test optical density (OD) / Control ODX100" and "1- Test OD / Control OD," respectively, and the data were recorded for statistical analysis (13).

Evaluation of the Expression Levels of HIF-1α, IL-1β, and NF-κB Proteins by Western Blotting

The total protein concentration in the samples was determined using the Bradford method (29). To determine the expression levels of the proteins by western blotting, the proteins were separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method (29). Then, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which provides high binding capacity for proteins and nucleic acids and is an ideal membrane for this purpose (29). Immunoblotting for the HIF-1α, IL-1β, and NF-κB target proteins was performed using the WesternBreeze™ Chemiluminescent kit (Catalog no: WB7104, Thermo Fisher Scientific) according to the manufacturer's instructions. Primary antibodies specific to the HIF-1α (Catalog no: MA1516, ThermoFisher Scientific, USA), IL-1β (Catalog no: M421B, ThermoFisher Scientific, USA), and phospho-NF-κB-P65 (Catalog no: MA515181, ThermoFisher Scientific, USA) proteins were used. The βActin (Catalog no: MA1-140, ThermoFisher Scientific, USA) protein was used as an endogenous control in the western blot method. After treatment with the primary antibody specific to the HIF-1α, IL-1β, and NF-κB proteins, sequential washings were performed. This was followed by incubation with an alkaline phosphatase-conjugated secondary antibody. The membranes were treated with substrate solution, the protein bands transferred to an X-ray film (Thermo Fisher Scientific, Cat#34090, Waltham, MA, USA) were analyzed using ImageJ software, and the specific amount of protein in each sample was determined (29).

Statistical Analyses

The statistical analyses were performed using Minitab (version 20.0) software. Descriptive statistics were presented as the mean ± standard deviation or frequency (%). An analysis of variance (ANOVA) was used to evaluate differences across the group means. When differences across groups were observed, Tukey's honestly significant difference (HSD) post-hoc test was used for multiple pairwise comparisons. The alpha significance value was accepted as < 0.05.

RESULTS

Demographic Data

The mean age of eight (4 male, 4 female) patients whose tissues were used in the preparation of the human primary IVD tissue cell cultures was 52.16 ± 2.18 years.

Microscopic Evaluations

All cell cultures were followed with an inverted microscope throughout the experiments. Figure 1 lane 1: A, D, G, and J are inverted microscopy images of cell cultures at 0, 24, 48, and 72 h, respectively. Cell proliferation decreased time-dependently. The cultures were stained with Janus green B for further morphological examination (Figure 1).

Figure 1 lane 2: B, E, H, and K are images of cell cultures at 0, 24, 48, and 72 h, respectively. No change or deterioration in cell morphology was observed due to FVP application. The decrease in the number of cells as the application time increases indicated decreased proliferation. The cultures were stained with AO/PI to evaluate whether there was cell death, and the images obtained are given in Figure 1 lane 3. AO/PI staining revealed no cell death in cell cultures at 0, 24, 48, and 72 h, respectively (Figure 1 lane 3: C, F, I, and L).

MTT ELISA Viability, Toxicity, and Proliferation Analysis

The cell viability of the untreated samples decreased by 17.31% at 24 h, by 22.09% at 48 h, and by 44.65% at 72 h compared to the FVP-treated samples (Figure 2, Table I, Table II).

Western Blotting of HIF-1α, IL-1β, and NF-κB

The HIF-1α, IL-1β, and NF-κB protein expression levels of the untreated and treated samples were evaluated by western blot at 24, 48, and 72 h (Figure 3).

The HIF-1a expression level of the study group samples decreased by 12%, 29%, and 27% at 24, 48, and 72 h, respectively, compared to the control group samples. The IL-1β expression level of the study group samples increased by 56% at 24 h but decreased by 36% and 11% at 48 and 72 h, respectively, compared to the control group samples. The NF-kB expression level of the study group samples increased

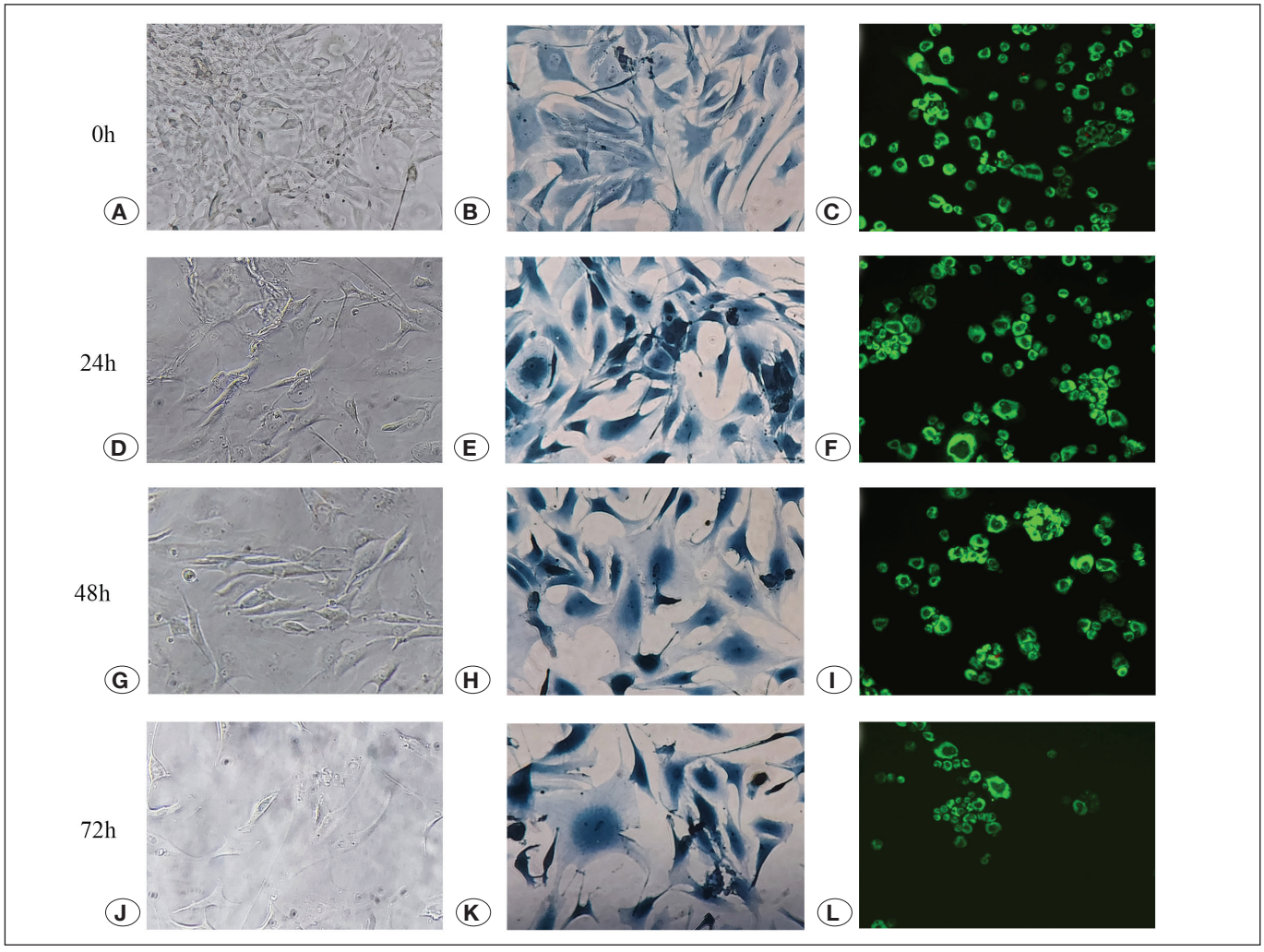

Figure 1: Inverted microscopy of cell cultures: **lane 1: A, D, G, J** are inverted microscopy images of cell cultures at 0, 24, 48, and 72 h, respectively. **Lane 2: B, E, H, and K** are images of Janus green B-stained samples and **lane 3: C, F, I, and L** are AO/PI staining of same cultures.

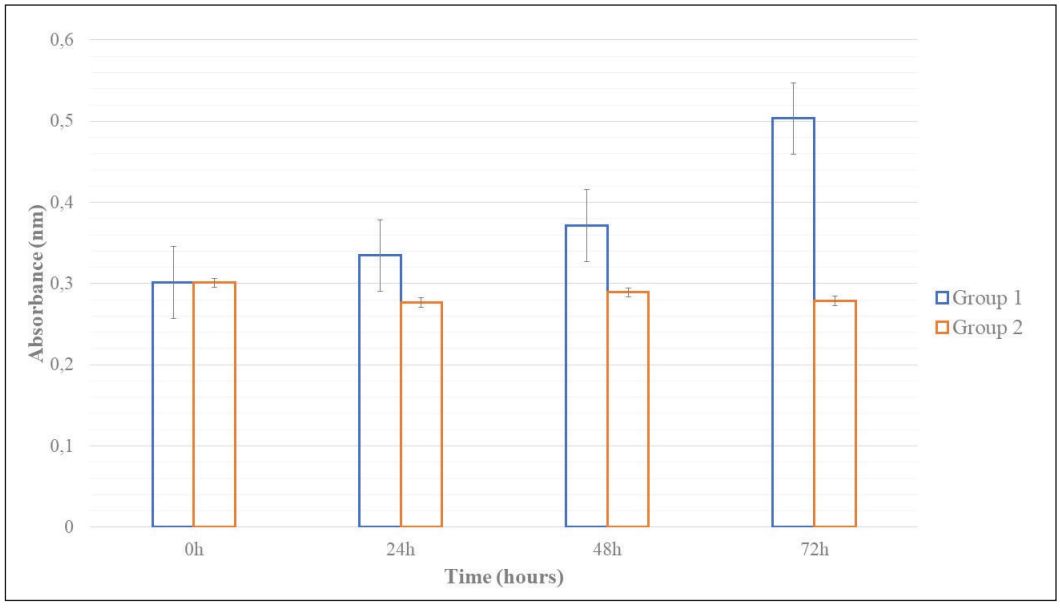

Figure 2: Comparison of cell viability, toxicity, and proliferation between groups at a wavelength of 570 nm (optical density) through ELISA.

Table I: Descriptive Statistics and Results of the Anova and Tukey HSD Tests

Variable	Time (hours)	Mean ± StDev	SE Mean	Minimum	Maximum	Tukey HSD Grouping*
Group 1 Absorbance (nm)	0	0.30100 ± 0.000000	0.000000	0.30100	0.30100	D
	24	0.33450 ± 0.000548	0.000224	0.33400	0.33500	C
	48	0.37133 ± 0.00532	0.00217	0.36800	0.38200	B
	72	0.50333 ± 0.00747	0.00305	0.49700	0.51500	A
Group 2 Absorbance (nm)	0	0.30100 ± 0.00126	0.000000	0.29900	0.30300	D
	24	0.2766 ± 0.0250	0.000224	0.2270	0.2960	D
	48	0.28925 ± 0.01016	0.00217	0.27800	0.30500	D
	72	0.2786 ± 0.0340	0.00305	0.2492	0.3250	D

***A:** Highest rate of cell viability and proliferation. **D:** The lowest rate of cell viability and proliferation.*

Table II: Assessment of the Cell Viability of AF/NP Cells Following FVP Treatment

Source	Adj SS	Adj MS	F-Value	p*
Groups	0.099773	0.099773	405.52	0.000
Time (hours)	0.061692	0.020564	83.58	0.000
Groups vs. Time	0.082018	0.027339	111.12	0.000

**p<0.05 vs. group 1 and p<0.05 vs. group 2. Data were analyzed using a one-way analysis of variance followed by a post-hoc Turkey Pairwise Comparison test.*

Table III: Protein Expression Values (r)

Protein	0h	24h	48h	72h
b-actin	1	1	1	1
HIF-1a	1	0.88	0.71	0.73
IL-1b	1	1.56	0.64	0.89
NF-kB	1	1.18	1.21	0.8

at 24 and 48 h (18% and 21%, respectively) but decreased by 20% at 72 h compared to the control group samples. The bActin protein expression level in all groups at the same time was accepted as 1 (100%), and the data were normalized (Table III, Figure 3).

DISCUSSION

Although there are many studies on FVP in the literature, no studies have investigated the effects of this drug on human primary IVD tissue cell cultures. In addition, no research has yet examined the protein expressions of the HIF-1α, IL-1β, and NF-κB signaling pathways, which play a key role in the degeneration mechanisms of IVD tissue cells. Therefore, we believe that the data obtained from the present study may contribute significantly to the literature on IDD.

HIF-1α and NF-κB play a critical role in AF/NP cells in IVD tissue (1,9,18,19,30). The NF-κB signaling pathway is responsible for

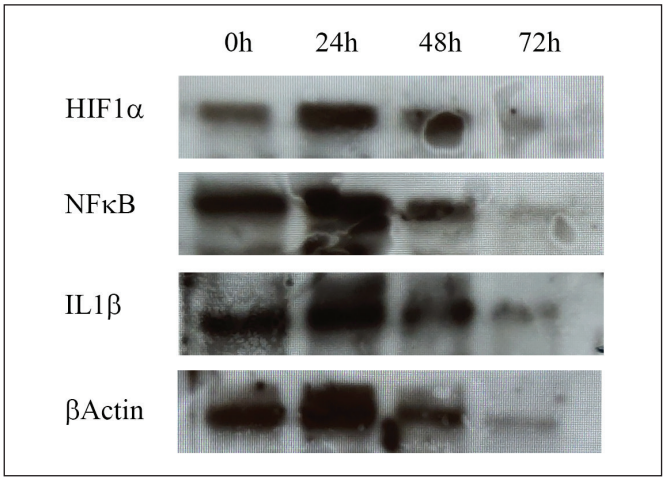

Figure 3: Image of western blot: HIF-1α, NF-kB, and IL-1β protein expressions at control group 0h and FVP administered experimental group (24, 48, and 72 h respectively).

the regulation of inflammatory cytokine response signaling, catabolic ECM enzyme signaling, integrin signaling, and AF/NP cells inflammatory signaling (21). Some experimental studies have suggested that if certain pro-inflammatory cytokines, such as IL-1β, can be neutralized, advances can be made in the potential medical treatment of many degenerative and inflammatory diseases (6).

To date, the complex and important roles of the HIF-1α and NF-kB signaling pathways triggered by some stress-related stimuli, primarily pro-inflammatory cytokines, through different signaling pathways in the pathogenesis of IDD have been described in many studies (7,26).

NF-κB plays a significant role in mediating cellular response to damage, stress, and inflammation (34). Many studies have reported that the chronic activation of NF-κB may be seen in many degenerative diseases, but its role in IDD has not been elucidated (34). NF-kB activation on chondrocytes causes osteoarthritis, a degenerative joint disease characterized by cartilage degradation and inflammation (33). The inhibition of NF-kB increases proteoglycan and collagen synthesis in aging tissues (21,27). In recent studies on IVD tissue, the NF-κB signaling pathway has become the central signaling pathway and has gained popularity in target drug therapy research (21,27). DNA damage may cause cell damage by activating the NF-κB signaling pathway to elevate the release of pro-inflammatory cytokines, thereby speeding up cellular senescence (31).

Hypoxia and a marked increase in inflammatory cytokines are common hallmarks of cartilage-like tissue degeneration, and degeneration is known to disrupt the ECM and the normal balance (24). The regulation of glycolysis is mediated by HIF-1α, a transcription factor, and is expressed by NP cells (14). HIF-1α promotes the differentiation and apoptosis of chondrocyte cells, and it is activated together with the NF-kB signaling pathway (20). A recent study (14) suggested that the activation of the HIF-1α signaling pathway may play a preventive role against IDD and be a future therapeutic agent.

IL-1β can be produced in both chondrocyte-like NP cells and AF cells, whether degenerated or not (15). It is known to increase the MMPs that degrade the ECM. IL-1β increases the production of aggrecanase in local cells, leading to dehydration and loss of disc height, which is characteristic of IDD (15).

Expressed in degenerative AF/NP cells, IL-1β plays a role in multiple pathological processes, inflammatory responses, ECM destruction, angiogenesis and innervation, apoptosis, oxidative stress, and cellular senescence (28). IL-1β also has a protective role against ECM destruction and disc degeneration (28).

The present study evaluated the cytotoxicity of FVP to IVD cells. Hif-1α, which plays a crucial role in the anabolic pathway, and IL-1β and NF-kB, which are important in the catabolic pathways, were evaluated. The effects of changes in expression levels on IDD were also examined.

FVP suppressed the proliferation of AF/NP cells in human primary IVD cultures ($p<0.05$) but did not adversely affect ECM or cell morphology. The HIF-1α expression level in the FVP-treated samples decreased by 12%, 29%, and 27% at 24, 48, and 72 h, respectively, compared to the control group samples. The IL-1β expression level in the FVP- treated samples increased by 56% at 24 h but decreased by 36% and 11% at 48 and 72 h, respectively, compared to the control group samples. The NF-kB expression level in the FVP-treated samples increased at 24 and 48 h (18% and 21%, respectively) but decreased by 20% at 72 h compared to the control group samples. The decreased expression of HIF-1α may be an indication of suppression in anabolic events as well as proliferation in IVD cultures. The increased IL-1β within the first 24 h revealed that AF/NP cells in the culture medium may induce a strong inflammatory response to FVP. This strong inflammatory response is likely to cause slowed proliferation. It may also be a trigger for many catabolic events. Similarly, NF-kB expression increased within the first 24 h and then decreased rapidly. Based on the data obtained, it can be suggested that the rapidly increasing NF-kB may have stimulated the expression of many anti-proliferative genes.

The fact that the tissues used in the preparation of primary cell cultures were obtained from patients who were of the same race and that the cultures were prepared from the tissues of only eight subjects may seem to be a limitation. However, the fact that tissues were obtained from a small number of cases can be compensated significantly by repeating the experiments. As is known, the fraction of any drug administered to the body that passes into the systemic circulation without undergoing chemical changes is defined as bioavailability. Orally administered drugs first pass through the liver and are extensively metabolized. Drug molecules absorbed from the gastrointestinal mucosa get into the portal circulation through the capillaries. Some of the absorbed drugs may reach the systemic circulation via the inferior vena cava or be converted to their inactive metabolites by enzymes in the mucous epithelium or liver cells. Moreover, the unchanged form or metabolic products of drugs getting into the enterohepatic cycle are excreted into the bile. However, the present study entails in vitro experimental research. That is, there is no in vivo experimental setup with compensatory mechanisms occurring in the body from the moment the drugs are administered. Therefore, the accurate effects of the metabolites that emerged after the hepatic first-pass effect of FVP on IVD cells or protein expression levels cannot be known.

CONCLUSION

This research is an in vitro experimental study; therefore, it is not possible to replicate the clinical research and clinical responses exactly. The findings obtained point to the need to perform further multicenter research on this drug using primary cultures prepared from tissues obtained from individuals of different and many more races. The data obtained herein should be confirmed by further research. FVP has changed the protein expression level of Hif-1α, IL-1β, and NF-κB. However,

not only was the expression of IL-1β and NF-κB, which play a vital role in the catabolic pathways, but also the expression of HIF-1α, which plays a significant role in the anabolic pathways, was also suppressed in the culture samples treated with FVP. If the protein expression of HIF-1α could be increased along with the suppression of IL-1β and NF-kB, FVP would perhaps be a promising pharmacological agent in the treatment of IDD. The pharmaceutical industry may manipulate these signaling pathways to produce anabolic effects, not catabolic effects, in the preparation of novel drugs, thereby providing a potential therapeutic option for the treatment of IDD.

AUTHORSHIP CONTRIBUTION

Study conception and design: IY, NK, HO

Data collection: IY, HA, NK, DYS

Analysis and interpretation of results: IY, HA, DYS, NK, HO, OA

Draft manuscript preparation: IY, HA, DYS, NK, HO

Critical revision of the article: DYS, NK, HO, OA

Other (study supervision, fundings, materials, etc...): IY, NK, HO

All authors (IY, HA, DYS, NK, HO, OA) reviewed the results and approved the final version of the manuscript.

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