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Original Article

Evaluation of the effect of apixaban on the primary intact intervertebral disc cell cultures

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Abstract

Aim: Apixaban is a frequently preferred pharmacological agent in clinics to prevent deep vein thrombosis and pulmonary embolism. Such new oral anticoagulants may cause hemorrhage's in tissues and/or organs or may cause gastrointestinal symptoms without bleeding. It is also reported in the literature that it may lead to mental disorders, unwanted disorders in the urinary tract and skeletal-muscle system. However, when the literature is examined, there are no studies, which are of high-evidential value, evaluating the efficacy of apixaban on healthy, intact intervertebral disc tissue, and matrix-like structures. In this pharmaco-molecular study, it was aimed to investigate the effects of a new oral anticoagulant agent containing the active ingredient apixaban on the intact intervertebral disc tissue cells, extracellular matrix (ECM) structure and to evaluate its positive and / or negative effects on gene expressions of cartilage oligo matrix protein (COMP), chondroadherin (CHAD), and Matrix Metalloproteinase (MMP)s.

Material and Methods: The primary cell cultures were prepared from the intact tissues of the patients with the traumatic intervertebral disc herniation. Apixaban was administered to the cultures and molecular analyses were performed for 21 days. The data obtained from the apixaban-administered and non-apixaban-administered samples were evaluated statistically and the significance value was accepted as $P < 0.05$.

Results: The changes were observed in the cell proliferation and the expressions of the mentioned genes in the apixaban-administered group. The suppression of COMP value and the increase in MMP-13 value may be indicative of the development of matrix degeneration in the apixaban-administered group, compared to the non-drug-administered control group.

Conclusion: The selectivity is one of the most important features of the drugs. However, it should not be forgotten that no drug will only produce the desired effect.

Keywords: Apixaban; cartilage oligo matrix protein; chondroadherin; intact intervertebral disc; matrix metalloproteinase.

INTRODUCTION

Anticoagulant therapy is an indispensable and vital treatment method in the prophylaxis and treatment of thromboembolic diseases manifesting itself in different localizations and on various clinical pictures. Amongst these treatment modalities, acetylsalicylic acid, clopidogrel combined with acetylsalicylic acid, and warfarin, one of the vitamin K antagonists, are frequently used pharmacological agents. Warfarin is an oral anticoagulant capable of interacting with different nutrients and a multitude of pharmacological agents (1). Its anticoagulation effect cannot be fully predicted and, this may lead to a bleeding risk in different tissues (2).

Thereby, the international normalized ratio (INR) monitoring is routinely required in a narrow therapeutic range. In addition, the reasons such as the difficulty of

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the training given to patients using this pharmaceutical by the clinicians regarding the usage range and dosage of the drug and the lack of awareness regarding side effects/ adverse events of the drug may raise problems (3).

New oral anticoagulants have begun to take their place in the treatment of the diseases to overcome these problems. These new anticoagulants are a direct thrombin inhibitor, dabigatran, and direct factor Xa (FXa) inhibitors, rivaroxaban, edoxaban, apixaban, and their clinical usages have gained popularity (4).

The apixaban, is a frequently preferred pharmacological agent in clinics for the prevention of deep vein thrombosis and pulmonary embolism (5). Such new oral anticoagulants may cause tissue and/or organ haemorrhage or may cause gastrointestinal symptoms without bleeding. It is also reported in the literature that it may lead to mental disorders, unwanted disorders in the urinary tract and skeletal-muscle system (6).

However, when the literature is examined, there are no studies with high-evidential value, evaluating the efficacy of apixaban on healthy, intact intervertebral disc tissue, and matrix-like structures.

In this research, cell viability, toxicity, and proliferation analysis were, thus, performed spectrophotometrically and microscopically in both the apixaban-administered samples and the non-apixaban-administered samples in the primary cell cultures established from the tissues obtained surgically from the patients with traumatic intervertebral disc herniation. In addition, the nucleus pulposus-specific chondroadherin gene (CHAD) (7-11) expression was tested. Simultaneously, the changes in both the expression of extracellular matrix (ECM) protein (12) in cartilage, encoded by the cartilage oligo matrix protein (COMP) gene in humans, and the matrix metalloproteinases (MMP) -13 and MMP-19 expressions, involved in the anabolic and catabolic reactions in degenerative disc diseases, and playing a significant role in reconstituting and repairing of the damaged tissues (13), were also tested.

MATERIAL and METHODS

The required approval was obtained to use the intervertebral disc tissue in the preparation of the human primary cell cultures (Namik Kemal University School of Medicine, Local Ethics Committee; 10840098-604.01.01-E.44192-29.11.2017). In addition, a written informed consent was obtained from the patients whose tissues were used.

To minimize experimental errors, the surgically obtained tissues were resected by the same surgeons and transferred to the laboratory. Similar analyses were performed by the same researchers during the assays. The assays were repeated at least three.

The primary cell cultures were fed with freshly prepared medium every two days. Confluent cells were trypsinized with 0.25% EDTA-trypsin and passaged. It was waited for third passage to start performing the assay with the cells in the flasks. Following the third passages, the cells were counted using the Trypan blue in Neubauer chamber with the help of an inverted light microscope at a magnification of x10 and transferred to the culture dishes for further tests.

The samples were then incubated overnight in an incubator which was set at a temperature of 37°C and 5% CO2, and the assays were started after incubation.

Eligibility criteria, surgical resection of the tissues and preparation of the cultures.

The patients having normal blood coagulation test values were included in the study (n=15). However, since a pharmacological agent containing the active ingredient apixaban may interact with drugs (14) such as ketoconazole, enoxaparin, aspirin, non-steroidal anti-inflammatory drugs, rifampicin, diltiazem, the patients using these, or similar drugs were excluded from the study. The resected tissues of the patients using ketoconazole (n = 1), enoxaparin (n = 1), acetylsalicylic acid (n = 1) naproxen (n = 2), diltiazem (n = 1), rifampicin (n=1), and St John's Wort plant extract (n = 1) were not used in the cell cultures and were excluded from the study. The remaining six patients' tissues were used for the primary cell culture preparation.

The evaluation of the patients, referred to the emergency department following general body trauma and suspected of the development of the spinal trauma, was performed. Following neurological and imaging examinations, the patients diagnosed with traumatic intervertebral disc herniation, neural tissue compression and instability in the traumatic spinal localization were operated. A midline incision was made to reach the operation site. Total laminectomy was then performed, and the dura was observed to be significantly suppressed. The spinal fusion was performed through neural decompression, herniated intervertebral disc excision and transpedicular instrumentation. Intact intervertebral disc tissues resected during surgery were placed in a DMEM solution containing 5% penicillin-streptomycin (PS). The obtained tissues were taken in the sterile and capped Falcon tubes and transferred to the laboratory at a temperature of 4^0C.

After mechanically degraded, the tissues taken in a laminar flow cabinet (Air Flow-NUVE/NF-800 R, Ankara, Turkey) were treated with the collagenase type II enzyme (1 mg / mL; Invitrogen Corp., Sigma Chemical, St. Louis, Mo.) and Hank's Balanced Salt Solution (HBSS-1X, 14025, Gibco) to ensure enzymatic degradation. The samples were then incubated overnight in an incubator (NUVE, 06750, Ankara, Turkey) which was set at a temperature of 37°C and 5% CO^2. The samples were centrifuged at 1.200 rpm consecutively for five minutes in a centrifuge which

was set at a temperature of 4°C. The supernatants in the tubes were taken out. The cell pellets were re-suspended with the freshly prepared culture medium.

Analyses and statistical calculations after application of the drugs to culture media Apixaban (Eliquis®, Pfizer) was not added to the control group samples. Human primary intact intervertebral disc tissue cell cultures were prepared according to concentrations that could be effective to mimic clinical applications. To determine the apixaban dose to be applied to the samples in the study groups, dose-response curves were generated for the calculation of the apixaban dose which would not be toxic before the assays started. In doing so, the apixaban solutions having the concentrations of 1, 25, 50, 100 and 1000 µmol were added respectively to the culture samples. The cell proliferation ended at doses above the concentration of 0.001 mg / mL. For this reason, apixaban solution having a concentration of 0.001 mg / mL / well, which allowed cell proliferation, was administered.

Analyses were performed simultaneously at baseline and on the 10th and 21st days for the non-drug-treated samples in the control group and the apixaban- treated samples in the study group.

Different magnifications such as x4, x10, x20 and x40 were used to evaluate cell surface morphology with the aid of an inverted light microscope (Olympus CKX41). Membrane permeability tests were performed via Acridine orange / Propidium iodide staining (AO and PI). To prepare the AO/PI stain, 4mg AO (dissolved in 2ml 99% ETOH), 10g sodium-ethylenediaminetetraacetic acid (Na-EDTA), 4mg PI, and 50 ml fetal bovine serum (Cat#10082147, Sigma Chemical, St. Louis, MO) were mixed well and sterile distilled water was added to reach the 200 ml final volume. This analysis, based on the principle (7-11) that live cells produce green fluorescent and that dead cells produce red fluorescent, was performed through a fluorescent microscope (Leica; DM 2500). Microphotographs were recorded and evaluated with the Cytovision Capture Station imaging software program. Proliferation assays were performed at 570 nanometers (nm) wavelength absorbance using MTT [3 (4,5 dimethylthiazol 2 yl) 2,5-diphenyltetrazolium bromide (Vybrant MTT Cell Proliferation Assay Cat # V13154, Thermo Fisher Scientific, USA)] commercial kit. The enzyme-linked immunosorbent assay (ELISA) /Optical density-OD) microplate reader, with which spectrophotometric analysis was carried out, was Mindray (MR 96A, PRC). During the evaluation of viability, toxicity and proliferation, the measurements were performed both in the non-apixaban-administered control group samples and in the apixaban-administered study group samples. The culture media containing apixaban discarded from the wells and 100mL MTT (12 mM MTT prepared by adding 1 mL sterile phosphate buffer saline) was added. The cultures were incubated at 37°C for 3 h without light exposure. Dimethylsulfoxide was added to these samples to stop the reaction and incubated at 37°C for an additional 10 min, prior to photometric analysis of a 570nm wavelength absorbance.

After the proliferation and the inhibition of the proliferation were calculated using following formulas respectively: “Test OD/ Control ODX100” and “1- Test OD/ Control OD”, data were recorded for statistical analysis (15-19).

PureLink RNA mini kit was used to extract total RNA from primary human intact intervertebral disc cell cultures. The quantity of RNA was measured using an UV spectrophotometer. RNA (50 ng) was reverse tran¬scribed using a high capacity cDNA reverse transcription kit and using a thermal cycler to obtain cDNA. All genes were amplified using TaqMan® Gene Expression assays for CHAD, actin beta (ACTβ: Housekeeping gene), COMP, MMP-13, and MMP-19. qPCR was performed on an Applied Biosystems 7300/7500 real-time PCR system (Thermo Fisher Scientific, Inc.). After appropriate thermocycling conditions were provided, ACTβ, an endogenous control for the normalization of target gene expression, was used. A calibrator sample was established for the evaluations. The relative quantity (RQ) value was accepted as 1 at the 0th hour when no drug was applied in cell culture samples for this calibrator reference. RQ values were calculated using the $2^{\Delta\Delta Cq}$ method during the comparison of the results obtained after analyses (7-11).

Prior to monitoring, the fixation solution prepared with 8% glutaraldehyde and 25% cacodylate buffer was used. After preserving for two hours at the room temperature for fixation, the samples were washed three consecutively with phosphate buffered saline. Images of the samples evaluated at a magnification of 4x, 10x, 20x and 40x with the aid of an inverted light microscope were recorded.

The statistical analyses were performed using Minitab software (version 18.0) and the data were evaluated at a 95% confidence interval. Descriptive statistics were presented as a mean ± standard deviation (SD). Variance Analysis (ANOVA) and Tukey's Honestly Significant Difference (HSD) post-hoc test were performed respectively. The alpha significance value was accepted as $P < 0.05$.

RESULTS

It was observed that the proliferation of the cells in the apixaban-administered samples decreased from 0.494 nanometers (optical density = OD) to 0.489 on day 10 compared to the non-apixaban-administered samples. However, it was observed that this suppression increased to 0.567 compared to the control group on day 20. This result was reported to be statistically significant (Table 1, Figure 1).

CHAD gene expression increased in the control group compared to the reference sample (RQ = 3.06 on day 10, and RQ = 3.37 on day 20). It also increased in the apixaban-administered experimental group on day 10, compared

to the control group (RQ=2.61). Interestingly, CHAD was expressed 1.9 times more in the apixaban-administered cultures on day 20 than the control group of the same day. The COMP gene expression remained unchanged in the control group. The COMP expression decreased by 63% in the apixaban-administered cultures on day 10 (P<0.005).

Table 1. Analysis of Variance results of cell toxicity and proliferation

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Apixaban	1	0,01834	0,018336	16,85	0,000
Times	2	0,17123	0,085616	78,69	0,000
Model summary					
S		R-sq	R-sq(adj)		R-sq(pred)
0,0329842		87,75%	86,73%		85,00%

DF, degrees of freedom; Adj, adjusted; SS, Sum of Square; MS, mean square.

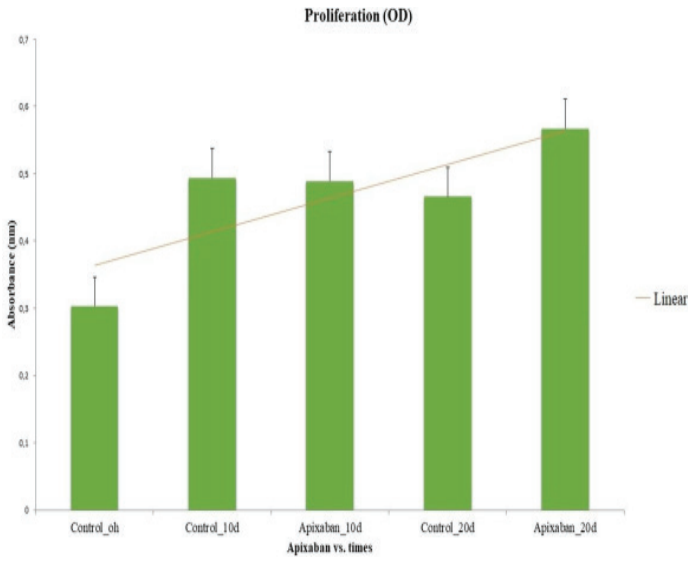

Figure 1. The graph reveals the results of the analysis performed through the spectrophotometric methodology at 570 nm concerning the suppression of cell proliferation in the drug-administered group compared to the control group on day 10.

However, it was normalized on day 20 reaching the same level as the reference sample. IL-1B expression increased in both the control group and apixaban-administered cultures on days 10 and 20. The highest increase was observed on day 20 of the apixaban administration (RQ = 5.99). MMP-13 gene expression decreased in both control and apixaban-administered cultures on days 10 and 20 (P<0.005).

However, MMP-13 was approximately 5.2-fold lesser in apixaban-administered cultures on day 10. MMP-13 expression increased by 6.8-fold in the apixaban-administered cultures, compared to the control group on day 20 (P<0.005).

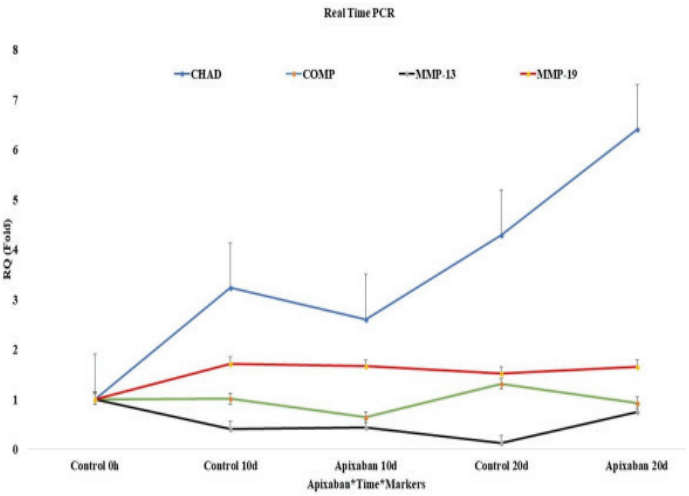

Figure 2. The figure shows that the expression of the other three genes except MMP-13 continue to decrease and suppress in the apixaban-administered samples.

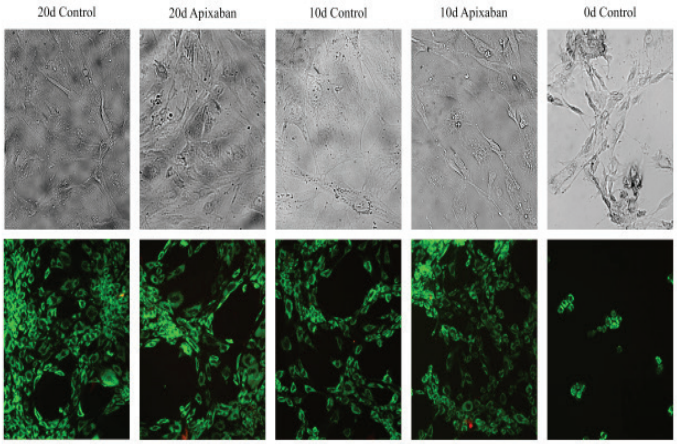

Figure 3. The line 1: the micrographs obtained from cell cultures using an inverted light microscope. The cell proliferation is observed to time-dependently continue in both the apixaban group and the control group as from day 0. AO/PI staining is used to determine apoptotic cell death that may occur due to aging or cytotoxicity. Line 2: AO/PI staining images of the same cultures.

Although MMP-19 expression increased slightly in both the control and the experimental groups on days 10 and 20 compared to the reference sample, this increase was not significant. MMP-7 gene expression significantly decreased in both control and apixaban groups on days 10 and 20 (P<0.005) (Figure 2).

When the apixaban-administered and non-administered control group culture samples were evaluated morphologically, it was observed that cell proliferation continued in both the experimental groups and the cells secured their specific morphology in the culture medium (Figure 3).

The drug administration or the effect of the direct culture medium on cell viability was confirmed by MTT analysis as well as AO / PI staining. No apoptotic cell death was observed in cell cultures.

DISCUSSION

Coumarin-derived drugs have become indispensable in the thromboembolic diseases' treatment since almost half a century. Due to the disadvantages (2,20) of using conventional anticoagulant drugs, the pharmaceutical industry has produced alternative oral anticoagulants that can be used by clinicians in their treatment protocols. Side effects such as brain hemorrhage which may be morbid or non-bleeding gastro intestinal symptoms which may be less morbid are mentioned in the literature (17,18).

However, there is no research handling its positive or negative effects on the intervertebral disc tissue. Therefore, in this research, it was aimed to microscopically evaluate the effects of apixaban on the healthy, intact, human primary cell cultures at pharmaco-molecular level.

Dhar et al. investigated the role of FXa in hepatic fibrosis both in vivo and in vitro (16). In this research, commercial HSC-LX2 cell line was used during in vivo experiments. They analyzed the gene expression of αSMA through these cell lines in the presence of FXa and/or thrombin. In in vivo experiments, the mice, which are used to induce fibrosis in the liver, were treated with thioacetamide, for eight weeks combined with Rivaroxaban or Dabigatran. The thioacetamide was administered alone to the control group. The hepatic tissue hydroxyproline was analyzed in addition to visual evaluations when evaluating fibrosis (16). FXa and thrombin- administered stellate cells were reported to demonstrate upregulation of procollagen, transforming growth factor beta and αSMA compared to culturing with FXa or thrombin alone. They underlined that FXa inhibition alone was more effective in reducing the percentage area of fibrosis and hepatic hydroxyproline content than inhibition of thrombin alone. They suggested that early inhibition of coagulation using an FXa inhibitor significantly decreased TAA-induced murine liver fibrosis (16).

In their study, Bastiaans et al. (21) reported that the vitreous of patients with proliferative vitreoretinopathy included elevated thrombin activity which induced pro-inflammatory and proinflammatory programs in retinal pigment epithelial cells. Therefore, the hypothesis that the inhibition of intravitreal thrombin activity might be a therapeutic option for proliferative vitreoretinopathy was tested. Based on this hypothesis, dabigatran was used with the aim of inhibiting thrombin activity in vitreous fluids (21). Thrombin, vitreous without thrombin activity, and vitreous with elevated thrombin activity were administered, either in the presence or absence of dabigatran, to the ARPE-19 cells. Subsequently, CCL2, CXCL8, GM-CSF, IL6 and PDGFB mRNA expression levels were determined with RQ-PCR. The protein levels of 27 cytokines, chemokines, and growth factors were detected in the culture supernatants (21).

Thrombin and vitreous fluids containing thrombin activity were reported to induce CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB expression by ARPE-19 cells inhibited by dabigatran. They suggested that this new oral anticoagulant agent reaching the vitreous after repeated oral ingestion did not inhibit thrombin activity in an in-vitro activity assay and emphasized that the pathway of proliferative vitreoretinopathy activation might be inhibited by dabigatran (21).

Vianello et al. (22) investigated the efficacy of the appropriate antithrombin agent for adjuvant treatment of the patients with cancer. They tested whether dabigatran might affect the mechanisms favoring tumor growth by interfering with thrombin-induced PAR-1 activation. The exposure of tumor cells to thrombin was reported to significantly increase cell proliferation and it was correlated with the downregulation of p27 and concomitant induction of cyclin D1 (22). The dabigatran was found to be effective in provoking thrombin-induced proliferation and that it restored the initial pattern of cell cycle protein expression (22).

Castle et al. (23) reported in their study that cancer stem-like cells (CSCs) were the cause of tumor development, resistant to chemotherapy and play a role in metastasis. They hypothesized that new oral anticoagulants might inhibit CSC activity by targeting specific factors in the thrombin pathway (23).

In this research, thrombin and dabigatran were applied to breast cancer commercial cell lines (23). During the analyses, MDA-MB-231, MCF-7, SKBR3 and MDA-MB-157 cell lines representing the spectrum of breast cancer subtypes were cultured with 0.1 NIH Units/ml human thrombin, and 0.5 μM dabigatran. They emphasized that dabigatran removed the stimulatory effect of thrombin on MFE in MDA-MB-231 and MDA-MB-157 cells treated with thrombin (23).

In a study in the literature (24), human-derived sinus rhythm and AFB hemodynamic shear stress patterns were applied to primary human endothelial cells in culture. Subsequently, it was found that endothelial cells exposed to atrial fibrillation hemodynamics increased thrombotic potential as calculated by increased expression of pro-thrombotic gene markers and fibrin deposition on the endothelium. It was emphasized that treatment with the FXa inhibitor, apixaban, increased fibrin density on the endothelial cell surface but decreased fibrous accumulation (24).

When the literature is examined, no such research has been found in the field of neurosurgery at the pharmaco-molecular level. Moreover, when the literature is searched with the keywords such as “cell culture” and/or “apixaban” and similar words in the Pubmed electronic database, it is observed that the effects of the drugs have been investigated using experimental setup established with commercial cell lines, or using the animal tissues obtained from live mammal subjects in the studies with high-evidential value (16,21-24).

However, it is well known (7-11) that commercial cell lines contain single cell type and their genetic structure has

been modified. Thereby, the results of the studies where the commercial cell lines are used may be misleading. It has also been indicated in the literature that the sensitivity of human and animal tissues is different; therefore, the results obtained may be different and misleading as well (7-11).

The commercial cell lines or animal tissues were not used in this study. Instead, primary human cell cultures, derived from intact and healthy tissues obtained from intervertebral disc herniation operations performed due to the spinal trauma were prepared. The evaluations of the apixaban were performed on these prepared cultures. Hence, we believe that the results obtained from this research may contribute positively to the literature.

The disc degeneration is a part of the natural aging that occurs due to multifactorial causes. It is a progressive change affecting the histopathology and mechanism of the spinal cord. Even though it is not considered as a disease, it dramatically accelerates degenerative changes with the effect of some etiological factors causing impairment of spinal biomechanics and compression of neural tissue. It may also cause clinical conditions that adversely affect the quality of life due to the pain that develops afterward (25). Despite the intense interest in the clinical significance of the degenerative disc disease, there is still no consensus on the factors that may initiate physiopathological mechanisms. The inferior and superior faces of the disc are attached to the corpus vertebra with an end-plate consisting of hyaline cartilage containing chondrocytic cells. The collagen fibers of AF hold directly on to the cartilaginous end-plate and the corpus vertebra in an embedded manner. The end-plate prevents NP from protruding into the vertebral corpus and plays a very important role in feeding the disc and removing waste materials (26,27). ECM catabolism through the lytic enzymes and deterioration in the regulation of matrix synthesis cause progressive degenerative changes.

Unlike other tissues in the body, degeneration that occurs in the intervertebral disc begins due to the transformation of the nucleus pulposus cells from notochord-like cells into chondrocytic cells and consequently changes in the matrix proteoglycan synthesis. The basic components of the ECM are collagen, adipic glycoproteins, and proteoglycans (28).

ECM helps to keep cells and tissues together, fills spaces between cells, acts as a support, functions in the cell structure and movement. ECM also plays a role in the cell development and differentiation, in the exchange of nutrients and substances, in the continuity of cell viability, in the migration of cells and in the regulation of their relations with each other (28).

Cell-matrix interactions are regulated by the proteolytic enzymes responsible for the hydrolysis of ECM components. These enzymes regulate the composition and integrity of the ECM structure and play an essential role in the control of signals generated by matrix molecules, cell proliferation, differentiation and cell death. MMPs have a prominent place in these enzyme systems. MMPs are Zn(2+) dependent and Ca(2+) containing endopeptidases that degrade ECM components (29).

In this degeneration cycle type II collagen synthesis decreases and type I collagen synthesis increases in the matrix. As a result, the activation of MMPs that break down the matrix tissue and provide more fragile collagen can play a significant role. It may histologically cause vascularization, annulus tears, and cluster in nucleus pulposus cells, apoptosis, myxoid degeneration, microcystic changes and calcification and/or osteophytes in the vertebral body (30). MMP-9 from the gelatinase B enzyme group contains three fibronectin type II inserted in the collagen, laminin and gelatin-binding catalytic domain. This region enables the gelatinases to bind with high affinity to gelatin and collagen, thereby increasing their proteolytic activity. This feature is also the basis for elastolytic activity (31,32).

MMP-19 hydrolyzes some basal membrane proteins (33). It should be remembered that the loss of control of MMP activity may lead to an increase in developing diseases such as arthritis, cancer, atherosclerosis, ulcers, fibrosis, and vascular aneurysms (31).

The increase in fibronectin fragments as well as fibronectin during disc degeneration stimulates MMP production. Due to this stimulation, it has been reported that the proteoglycan production is suppressed, and the degeneration process of the intervertebral disc is accelerated (15,34).

To date, many studies have been carried out on catabolic enzymes that cause cartilage destruction. These enzymes are the family of MMPs and ADAMTS family members, but the ADAMTS5 enzyme is more specific for cartilage. COMP is an ADAMTS-5 gene (35).

The studies on the regulation of this gene in cartilage tissue have become the new focus of studies on the pathogenesis of OA. The studies, performed up to now, have shown that inflammatory cytokines increase cartilage damage by stimulating the ADAMTS5 gene (36).

Proteolytic enzymes also play a significant role in the pathophysiology of disc degeneration. Amongst these enzymes, the MMP family, which causes degradation by decomposing the constituents of ECM, is very important (37).

Another important proteolytic enzyme is a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). The ADAMTS family enzymes have a different effect from the MMP family enzymes and cause much stronger proteoglycan degradation than MMP (38-40). It has been reported that these proteolytic enzymes increase in the aged and diseased disc, and their amounts increase as the degree of the degeneration increases (41,42).

Degenerative disc disease is a complex and multifactorial process. The cells and ECM structures in the disc tissue

play an essential role in the degeneration process. It should be kept in mind that the loss of balance of COMP enzymes from ADAMTS family members, and MMP-13, MMP-19 from MMP family members is responsible for degradation of the ECM and that the loss of proteoglycan in the ECM may occur during degeneration (43).

It is asserted that the water content of the disc tissue decreases due to the loss of proteoglycan, the amount of collagen towards type 1 collagen increases, and the fibrous tissue develops. The resistance to the axial loading increases due to the deterioration in the diffusional feeding system of the intervertebral disc tissue. Furthermore, the deterioration in the feeding system causes a hypoxia which decreases pH value in the disc tissue by increasing the lactate production (44).

It should be also kept in mind that cell deaths dependent on the morphological, biological and biochemical changes in the disc tissue may develop due to the decrease in the matrix synthesis.

As a result, the proliferation of the cells in the apixaban-administered samples was observed to decrease on day 10 compared to the non-apixaban-administered samples, but this suppression ended on day 20, and the proliferation of the cells in the apixaban-administered samples increased compared to the control group.

This result was attributed to the fact that the drug was administered at a single and very low dose only at the beginning of the assays in the laboratory. Since the media in the cell culture medium is changed every two days, it is thought that there may be a result occurred due to the removal of the drug from the medium. However, it is important to remember that this drug is taken orally into the human body every day.

When the changes in gene expressions of CHAD, COMP, MMP-13 and MMP-19, the initial RQ value of which was accepted as 1-fold, were examined, it was seen that the CHAD and MMP-19 expressions increased in the control group on day 10 while the COMP and MMP-13 expressions decreased.

However, an increase in CHAD gene expression was seen on day 20 and the increase in the apixaban-administered group was interestingly more than that in the control group. The MMP-19 expression did not change in both the control and apixaban-administered groups.

It was also reported that COMP value was suppressed, and that MMP-13 value increased on day 20 in the apixaban-administered groups compared to the control group. All these results were found to be statistically significant.

In addition to the changes in the proliferation, the changes were observed to occur in the gene expressions of CHAD, COMP, MMP-13, and MMP-19. The CHAD and MMP-19 expressions in the control group samples were seen to increase, while the COMP and MMP-13 expressions decreased on day 10. The expressions of the other three genes except MMP-13 were also observed to decrease and suppress in the apixaban- administered samples. Inhibition of the COMP expression and the increase in the MMP-13 expression in the drug-administered group, compared to the non-drug-administered control group, revealed that the apixaban might cause a degeneration in the ECM structures. All these results were found to be statistically significant ($P < 0.05$).

Although no morphological change or toxicity-related death were observed in the apixaban- administered cell cultures, the changes in the level of gene expression are remarkable. The fact that the number of our sample was six is one of the limitations of our study. The obtained real-time PCR results should be confirmed in larger experimental groups.

The drug effect in the body can be changed by a variety of factors. Some of these factors alter its concentration at the site of effect by differentiating the pharmacokinetics of the drug. The change in its effect is dependent on this situation. Some other factors may alter the response of the target organ or cells to the drug without altering the pharmacokinetics of the drug.

However, since this research has been carried out using an in-vitro experimental setup, it may not provide clear information on what kind of effect the apixaban-active pharmacological agent would have on the components of the intervertebral disc tissue in the living organism, either positively or negatively. This is the first limitation of our research.

Genetic differences in the carrier enzymes involved in the drug metabolism are amongst the most crucial factors affecting the drug response. These genetic factors alter the drug metabolism, the elimination rate of the drugs in individuals, the quality and quantity of the drug receptors and other structures in the target cell. Thus, these factors play a significant role in varying the effect of the drug between individuals and between races (9).

The fact that the tissues used for the preparation of cell cultures were obtained from the patients having the same race, and the small number of the patients may be considered as the second limitation of our study. However, the fact that the experiments were repeated at least three, and the cell cultures were prepared from intact intervertebral human disc tissues may weaken the effect of this limitation.

CONCLUSION

The purpose of the treatment with drugs is to prevent various diseases, to reduce or control disease effects. To achieve this aim, it is necessary to deliver sufficient drug doses that do not reach toxic levels to the target tissues. Indications and positive effects of the apixaban have been explained in different studies in the literature. However, clinicians should prefer such new pharmaceuticals in the clinical use, considering the possibility that this new oral anticoagulant may damage intervertebral disc tissue cells and/or lead to alterations in the gene expression

of important components involved in the anabolic and catabolic cascades of the disc tissue.

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