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Original Article

In-vitro evaluation of the effects of tigecycline on annulus fibrosus and nucleus pulposus

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Abstract

Aim: This study aimed to examine the effects of tigecycline on primary cell cultures established using intervertebral disc tissues.
Material and Methods: Primary Annulus Fibrosus and Nucleus Pulposus cultures were obtained from human intervertebral disc tissue. Untreated samples served as the control group, and treated samples served as the study group. Treated and untreated samples were statistically evaluated using an alpha significance value of < 0.05.
Results: Proliferation and gene expression decreased in the tigecycline administered cultures when compared to the control group samples (P < 0.05).
Conclusion: Drugs used in clinics may have side effects other than those indicated in their package insert.

Keywords: Chondroadherin gene; cartilage oligomatrix protein; interleukin-1beta; intervertebral disc cell culture; matrix metalloproteinase; tigecycline

INTRODUCTION

Excessive antibiotic use is common and may cause damage to the liver and kidneys, as well as lead to antibiotic resistance, the formation of fungal infections, and the significant loss of probiotics in the intestine (1-4). In the past two decades, the marked increase in multi-drug resistant bacteria and antibiotic resistance has become a serious problem for many medical branches, especially infectious diseases (5). Furthermore, the rapid proliferation of Enterobacteriaceae that produce antimicrobial-resistant extended-spectrum β-lactamases (ESBLs) has become a serious global health problem. Although carbapenems are the preferred treatment for serious infections caused by ESBL-producing Enterobacteriaceae, reports of carbapenem-resistant Enterobacteriaceae infections have become increasingly more common worldwide, including in Turkey. Thus, older drugs, such as tigecycline, have regained popularity.

Tigecycline, a minocycline derivative, is known to have broad-spectrum effects on many gram-positive and negative microorganisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and ESBLs expressed by *Escherichia coli* and *Klebsiella pneumoniae* isolates. Tigecycline is a potent drug for atypical agents, including anaerobic bacteria and non-tuberculosis mycobacteria that grow rapidly, and is widely preferred for patients with renal insufficiency because it is excreted via the biliary tract and does not require dosage adjustments (6). For the mentioned reasons, tigecycline is commonly used for complicated intraabdominal and skin-soft tissue infections, as well as the washing of surgical instruments and the surgical field (1).

Studies that compared tigecycline with other drugs used for similar indications have reported the following common adverse events: nausea; vomiting; an increase in some enzymes, such as alanine aminotransferase; pancreatitis; and hepatic insufficiency (7,8). The less commonly reported adverse events include cholestasis, jaundice, and Stevens-Johnson syndrome (7,8). However, no studies have investigated the effects of tigecycline on

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intervertebral disc tissue elements, such as the annulus fibrosus (AF), nucleus pulposus (NP), and extracellular matrix (ECM) structure. This randomized, double-blind, *in vitro* study aimed to evaluate the effects of tigecycline on human primary intervertebral disc tissue cell cultures.

MATERIAL and METHODS

Patients who were not allergic to tetracycline-class antibiotics, such as minocycline, doxycycline, and tigecycline, were included in the study. Additionally, patients who had used ketoconazole for the treatment of fungal diseases, rifampicin for the treatment of tuberculosis, or cyclosporine were excluded from the study. Tissues from eight patients (four males and four females) were used to prepare primary cell cultures.

The patients (n=8), whose mean age was 33±10.69 years (age range: 22–44 years), underwent lumbar microdiscectomy and sequestrectomy due to disc herniation (9,10). Resected tissues were transferred to the laboratory in cell culture medium containing penicillin-streptomycin on ice. Using methods presented in previous studies by Karaarslan et al. primary cell cultures of intervertebral disc tissue were prepared (11-13).

Experiments were performed on AF and NF cell cultures after the third passage. Cells were stained with trypan blue, counted with an inverted light microscope (magnification ×10). Viable $6x10^4$ cells/well were cultivated in 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO)/ propidium iodide (PI) assays. Cells were also seeded at a density of $6x10^4$ cells per dish in Petri dishes (100 mm) to monitor ECM structure. All the cells were then incubated for 24 h. At the end of the incubation period, cells that had become confluent and adhered to the basement were treated with tigecycline. The researchers who conducted the experiments were blind to the components of cell cultures.

To prepare the main stock solution, 50 mg of tigecycline lyophilized powder (TYGACIL®, Pfizer) was used. A 10 mg/ml stock solution was procured by dissolving 50 mg of tigecycline powder with a half-life of 36 h (14) in Dulbecco's Modified Eagle Medium (DMEM) with 5% dimethyl sulfoxide (DMSO). This stock solution was diluted to reach a final concentration of 20 µg/ml. A 20 µg/ml tigecycline solution was added to the study group samples. Untreated samples served as the control group. Treated and untreated samples were analyzed at 0h on days 7 and 21. Using an inverted light microscope, cell surface morphologies and viability were examined under ×4, ×10, ×20, and × 40 magnifications. Membrane permeability was evaluated with fluorescence microscopy. The results of assays performed using the nucleic acid binding dyes AO and PI were evaluated in accordance with the principle that viable cells produce green light, and apoptotic or dead cells produce red light (15).

A commercial MTT kit (Vibrant MTT Cell Proliferation Assay, Cat #V13154, Thermo Fisher Scientific, USA) was used to analyze proliferation. The initial viability of cells without treatment was accepted as 100% for MTT assays performed at a wavelength of 570 nm. Proliferation and the inhibition of proliferation were calculated using the formulas 'Test OD / Control ODX100' and '1- Test OD / Control OD,' respectively, and the data were recorded for statistical analysis (16).

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine DNA gene copies extracted from primary cell cultures, and the level of mRNA (Applied Biosystems 7300/7500 real-time PCR system, Thermo Fisher Scientific, USA; Thermocycling conditions: 2 min at 50˚C, 10 min at 95˚C, 15 sec at 95˚C and 1 min at 60˚C for 40 cycles for each duration). Total RNA was obtained from cultured AF/NP cells using a PureLink™ RNA Mini Kit. A total of 50 ng of RNA was reverse-transcribed using a high capacity cDNA RT kit to obtain cDNA. To determine gene expression profiles, all genes were amplified using TaqMan Gene Expression Assays for the chondroadherin gene (CHAD), β-actin (ACTβ), cartilage oligo matrix protein (COMP), matrix metalloproteinase (MMP) -7, MMP-19, and interleukin-1 beta (IL-1β). As a result of the qPCR experiment, the relative quantity (RQ) values of each sample were obtained using the 7500 Fast SDS program V.2.3 (Thermo Fisher Scientific, USA). ACTβ was used as an endogenous control (ACTβ) to normalize the targeted gene expressions. To obtain comparative results, a reference sample (Group 1, 0 h) was used, and relative quantity values were calculated using the $2^{-\Delta\Delta Cq}$ method (17-19).

Statistical analyses were performed using Minitab (version 18.0) software, and data were evaluated at a 95% confidence interval (CI). Descriptive statistics were presented as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to analyze the interactions of independent variables and the effects of these interactions on the dependent variable. When differences across groups were observed, Tukey's honestly significant difference (HSD) posthoc test was used for multiple pairwise comparisons. The alpha significance value was < 0.05.

RESULTS

Tigecycline administration suppressed proliferation in study group samples at 0h, on day 7 and 21. Proliferation was eliminated in the tigecycline-treated samples by day 21, and cell viability decreased dramatically (Figure 1). MTT and ELISA results were statistically significant ($P < 0.05$).

The RT-qPCR results revealed that CHAD, COMP, and MMP-19 expression increased in the study group samples when compared to the control group samples. IL-1B and MMP-7 expression decreased ($P < 0.05$). All gene expressions decreased in the study group samples when compared to the control group samples at 0h on day 21. The gene expression levels of CHAD, COMP, IL-

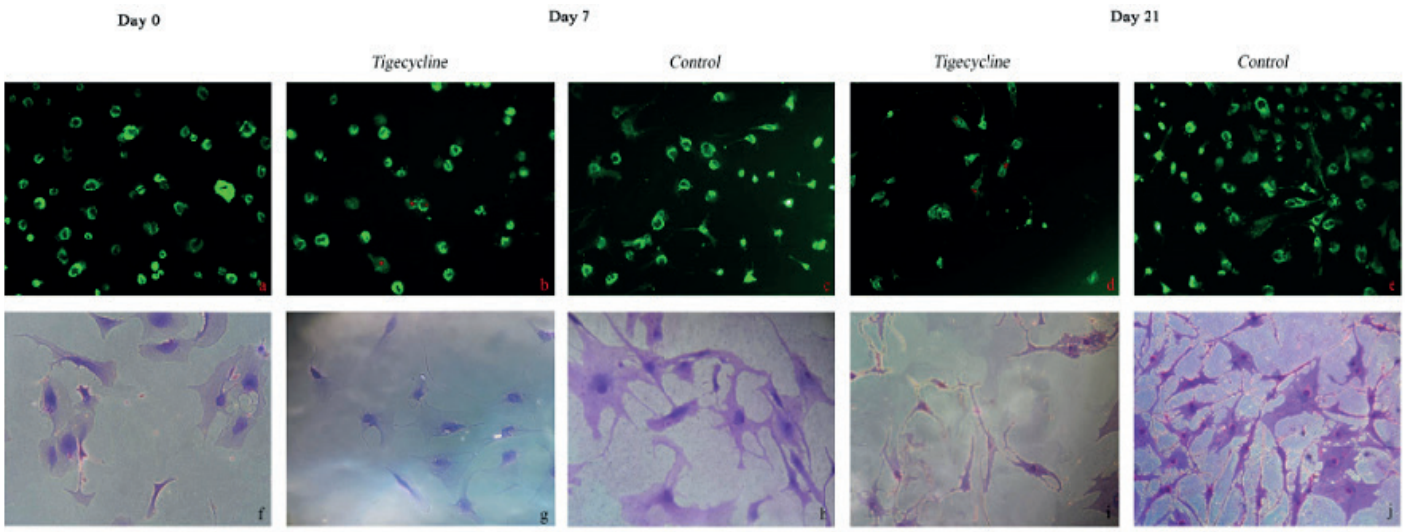

Figure 1. AO/PI staining of AF/NP cell cultures. Control samples and Tigecycline-treated group at 0h and on days 7 and 21

1B, MMP-7, and MMP-19 were 12.11, 9.78, 0.37, 0.76, and 0.14, respectively, on day 21 ($P < 0.05$); however, the expression levels of the relevant genes were eliminated in the tigecycline-treated samples on day 21. These results were statistically significant ($P < 0.05$).

DISCUSSION

Tigecycline was the first glycylcycline antibiotic. Although structurally similar to minocycline, it is a broad-spectrum antibiotic designed to avoid important bacterial resistance mechanisms. The significant increase in resistant microorganisms has led to the common use of tigecycline, which achieves its antimicrobial effects by binding to the bacterial 30S ribosome. Tigecycline is not affected by the Tet (M) protein since its binding point differs from that of tetracycline, and it binds five times more effectively than tetracycline.

The main feature that makes tigecycline more potent than other antibiotics is that it is not affected by common resistance mechanisms, such as ribosomal protection and efflux pumps. Tigecycline is active against gram-positive microorganisms, including *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus faecium,* and *Listeria monocytogenes*, as well as against gram-negative microorganisms, including *Escherichia coli, Klebsiella pneumoniae*, Enterobacter, and *Serratia marcescens*. Tigecycline is also effective against resistant gram-positive microorganisms, including MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), and VRE, as well as against resistant gram-negative microorganisms, including *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. The clinical use of tigecycline has been approved for the treatment of adult patients with complicated intra-abdominal infections and complicated skin and soft-tissue infections. In recent years, tigecycline has been used for postoperative ventriculitis caused by multidrug-resistant *Acinetobacter baumannii* (20). The use of tigecycline for ventriculoperitoneal shunt meningitis caused by multidrug-resistant *Acinetobacter baumannii* has also been reported in the literature (21).

Commercial cell lines (22) or cell cultures established with animal tissues (23) are commonly used to examine the cytotoxicity of antibiotics. Commercial cell lines are known to comprise only a single type cell, therefore, genotype and/or phenotype cannot fully reflect the properties of the originated tissue; thus, the results of studies using cell lines may be misleading (11-15). The sensitivity of animal tissue is known to differ from that of human tissue. Therefore, the results obtained from assays using animal tissues may diverge from those using human tissues, which may cause misleading outcomes (11-19). The present study used human intervertebral disc tissues for the preparation of primary cell cultures; thus, the results obtained are believed to be more reliable.

No studies were retrieved following a comprehensive and systematic search of electronic databases using the keywords 'AF/NP and tigecycline,' and 'intervertebral disc tissue and tigecycline.' A study of the toxicity of tigecycline on human neuron cells was found after scanning electronic databases using the keywords 'cytotoxicity of tigecycline.' This study reported that tigecycline may cause oxidative damage in neuronal cells (24). No studies have examined the effects of tigecycline on AF/NF cells and intervertebral disc tissues; hence, the results of the present study provide new insight into the potential effects of tigecycline.

CHAD is an NP-specific marker (11-13), and the studies conducted to date have provided significant data on catabolic enzymes that cause cartilage degradation. These enzymes are members of the matrix metalloprotease and ADAMTS families, but the ADAMTS-5 enzyme is more specific to cartilage. COMP is an ADAMTS-5 gene (25). The regulation of this gene in cartilage tissue has become

a new research field in the study of OA pathogenesis. Previous studies have reported that inflammatory cytokines increase cartilage damage by stimulating the ADAMTS5 gene (26). The loss of equilibrium in MMP-13, MMP-19, and COMP is responsible for ECM destruction, and proteoglycan loss in the ECM may occur during degeneration. Proteolytic enzymes play a marked role in the pathophysiology of disc degeneration (27,28). The enzymes of the MMP family that destruct ECM by decomposing its components are very important. The increase in fibronectin and its fragments during disc degeneration stimulates MMP production, which has been reported to suppress proteoglycan production and accelerate the degenerative process in intervertebral disc tissue (29).

As a local inflammatory cytokine, IL-1β stimulates acute inflammation by acting on endothelial cells and leukocytes (18) and plays a pivotal role in the healing process after IVD injury.

A significant difference was observed when study group samples were compared with those of the control group after MTT and RT-qPCR assays ($P < 0.05$). Administering tigecycline suppressed and eliminated proliferation in study group samples by day 21 ($P < 0.05$). CHAD, COMP, IL1B, MMP-7, and MMP-19 expression was also eliminated in the study group samples by day 21 ($P < 0.05$).

The present study does have a limitation. The cell cultures were established using the tissues of a small number of patients who were of the same race. However, tissues in the cell cultures were obtained from human intervertebral discs, and all experiments were repeated three times.

CONCLUSION

Medications may have side effects other than those indicated in the package insert.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: The study was carried out with the approval of the local ethics committee (Namik Kemal University -17/26.02.09).

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