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Research article

Exploring the anti-proliferative and cytotoxic impact of doxycycline on C6 glioma cells

Firuze Unlu Bektas*[1], Mine Kucak[1], Ekin Bektas[1], Tolga Onturk[1], Muhammed Hamza Muslumanoglu[1]

[1] *Yildiz Technical University, Faculty of Arts & Science, Department of Molecular Biology and Genetics, 34220, Istanbul, Türkiye*

Abstract

Doxycycline is a member of the tetracycline group and is a bacteriostatic antibiotic. Therefore, it stops/slows down the reproduction and spread of pathogenic microorganisms and gives the immune system the necessary time to destroy them. In this study, cytotoxic, anti-proliferative, and apoptotic effects of doxycycline on the rat glioma cell line derived from *Rattus norvegicus* were observed. To show the cytotoxicity of doxycycline, MTT test was performed to obtain the IC_{50} value and the dosages of treatment were determined accordingly. With the colony formation test, it was observed that the determined doxycycline dosages reduced the colony formation ability of the single cells. Similarly, wound healing test also showed that doxycycline treatment reduced the ability of cells to migrate. A dose-dependent decrease in the cell number was detected by DAPI staining after doxycycline treatment and the expression levels of cancer related genes were shown by the RT-qPCR method. In conclusion, doxycycline was found to have anti-proliferative and cytotoxic effects in rat glioma cell line, and more comprehensive studies are needed before doxycycline can be used as a complementary agent in cancer treatment.

Keywords: *Cancer; cytotoxicity; doxycycline; proliferation; rat glioma; RT-qPCR*

1. Introduction

Antibiotics, which are amongst the most frequently used drug groups all over the world, have effects other than their antimicrobial effects (Dugray et al., 2001; Singh et al., 2021; Li et al., 2023; Nassar et al., 2023; Duncan et al., 2024; Han et al., 2024). For instance, doxycycline, a tetracycline group antibiotic, has an anti-tumor effect (Lamb et al., 2015; Zhang et al., 2017; Ghasemi and Ghasemi, 2022; Chan and Kamath, 2023; Choi et al., 2024; Mi et al., 2024). Doxycycline is a semi-synthetic antibiotic (Syapin et al., 2016) and was first approved by FDA in 1967. Doxycycline, which has two chemical forms, monohydrate, and hyclate, is synthetically produced from oxytetracycline (Feitosa et al., 2022). Being a bacteriostatic antibiotic, it shows its antimicrobial effect by inhibiting the protein synthesis of bacteria (Feitosa et al., 2022; Shutter and Akhondi, 2023). It binds allosterically to the 30S prokaryotic ribosomal subunit during protein synthesis, preventing the production of the bacteria's essential proteins (Brodersen et al., 2000; Warner et al., 2022). Doxycycline also prevents cell proliferation, induces apoptosis, inhibits matrix metalloproteinases (MMP) and prevents tumor-induced angiogenesis (Yang et al., 2022; Siregar et al., 2023; Wehrli et al., 2023). Gliomas constitute 74.6% of all malignant brain tumors (Souza et al., 2018). Survival of patients with glioblastoma multiforme (GBM) caused by aggressive glioma does not exceed 15 months (Swartz et al., 2014; Romanishin et al., 2024). The C6 cell line is the cell line that most closely resembles the human brain tumors mechanism, according to the comparison between eight rats and it is similar to human glioblastoma in terms of high mitotic index, parenchymal invasion and neo-angiogenesis (Grobben et al., 2002; Barth and

* Corresponding author.
E-mail address: firuze-26@hotmail.com (F. Unlu Bektas).
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Kaur, 2009; Pournajaf et al., 2024). Doxycycline hyclate is a promising agent for use in cancer treatment, as it is currently an FDA-approved drug and has a low cytotoxic effect. This study was conducted to show its effects on cancer cells, which has been a big problem for a long time. Cytotoxic, anti-proliferative and apoptotic effects of doxycycline on cells were observed using proliferation tests and gene expression tests, at the dosages determined by cell viability test, *in vitro*.

2. Materials and methods

2.1. Cell culture

C6 rat glioma cells (ATCC-CCL-107) were provided by Yildiz Technical University (Istanbul, Türkiye). Dulbecco's Modified Eagle Medium-High glucose (DMEM) (Sigma) supplemented with 1% penicillin/streptomycin (Capricorn) and 10% fetal bovine serum (FBS) (Thermo Fisher) was used to grow the cells. They were cultured at 37°C with 5% CO_2. Cell culture was limited to twenty-five passages (P25).

2.2. Cell viability assay

MTT 3-(4,5-dimethyl-thiazolyl 2,5-diphenyltetrazolium bromide) assay was used as previously described to show the viability of the cells after treatment (Kumar et al., 2018). C6 glioma cells (5×10^3) were plated in 96-well culture plates overnight. The experimental cells were treated with 20, 40, 60, 80, 120, and 160 µM concentrations of doxycycline hyclate (Across) for 48 hours. After 48 hours, 10 µL MTT (Goldbio) was added into the wells, and they were incubated at 37°C for 2 hours. 100 µL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, afterwards. The optical density was measured at 570 nm absorbance. GraphPad Prism 9.3.0 was used to calculate the IC_{50} value of doxycycline treatment.

2.3. Colony formation assay

Colony formation assay was used to determine the colony formation ability of a single cell after treatment (Franken et al., 2006). 250 cells per well were plated into 6-well culture plates overnight. After 24 hours, they were treated with 40 µM and 60 µM doxycycline and cultured for 12 days after 48 hours of treatment and the control group did not have any treatment. The medium of the cells was not changed during this period. The colonies were fixed and stained with Gram's crystal violet solution (Merck) after 12 days. ImageJ was used to count the colony number of cells.

2.4. DAPI staining

The same number of cells were plated in T25 flasks and cultured until 70% confluency. Experimental groups were treated with 40 µM and 60 µM doxycycline and they were cultured together with the control group for 48 hours. The cells were washed with 1X PBS (Wisent) and ice-cold methanol (Merck) was used to fixed the cells for 10 minutes. After fixing, the nucleus was stained using an 850 nm DAPI satin solution (Cayman) and protected from light. DAPI solution was removed afterwards, and the cells were washed with 1X PBS. They were captured under a fluorescent microscope (Zeiss) from 5 different areas of the flask. The cell numbers were counted with the image processing program ImageJ.

2.5. Wound-healing assay

Wound healing assay was applied as previously mentioned to observe the changes in cell migration in wound-created cells depending on the treatment (Jonkman et al., 2014). 24-well culture plates were used to plate (5×10^4) cells and the cells were cultured until confluency. A wound was made using a 100-µL sterile pipette tip. The cells were then cultured with 40 µM and 60 µM doxycycline treatment and the control group did not have any treatment. Images of the cells were captured at 0, 24, and 48 hours under the light microscope (Zeiss). The scratch areas were calculated using Wimasis.

2.6. Reverse transcription quantitative PCR assay

After 48 hours of 60 µM doxycycline treatment, the treated and untreated cells were collected. Total RNA was isolated via RiboExTM solution (Geneall) following the provider's instructions and total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific). smART First Strand cDNA Synthesis kit (EURX) was used to synthesize cDNA. The quantitative PCR reaction was performed on Applied Biosystem 7500 with a total volume of 20 µL. Primer sequences, including housekeeping *β-actin*, are shown in Table 1, and qPCR cycles are shown in Table 2. $2^{-\Delta\Delta Ct}$ method was used to show the relative quantification.

Table 1
Primer sequences.

Genes	Forward sequence (5′3′)	Reverse sequence (5′3′)
β-actin	CTCTGTGTGGATTGGTG GCT	GCAGCTCAGTAACAGTC CGC
Mmp9	GATCCCCAGAGCGTTAC TCG	GTTGTGGAAACTCACAC GCC
Bax	GAGGACTCCAGCCACAA AGA	CGAGCTGATCAGAACCA TCA
Bcl-2	TATATGGCCCCAGCATG CGA	GGGCAGGTTTGTCGACC TCA
Caspase3	GGAGCTTGGAACGCGAA GAA	ACACAAGCCCATTTCAG GGT
Parp1	TCTACTTTGCTGATATGG TGTCC	TGGGTAACTTGCTGATG TGAG

Table 2
qPCR protocol.

Step	Cycles	Temperature	Time
Polymerase activation	1	95 °C	2 min
Denaturation		95 °C	5 s
Annealing	40	60 °C	10 s
Extension		72 °C	30 s

2.7. Statistical analysis

Comparisons between replicate groups were calculated with one-way ANOVA by GraphPad Prism 9.3.0. The significance of the results was set at $p < 0.05$.

3. Results and discussion

3.1. Doxycycline reduces the viability of rat glioma cells

The effects of different doxycycline dosages on cell viability after 48 hours were shown with MTT assay. As shown in Fig. 1A, doxycycline inhibited the proliferation ability of rat

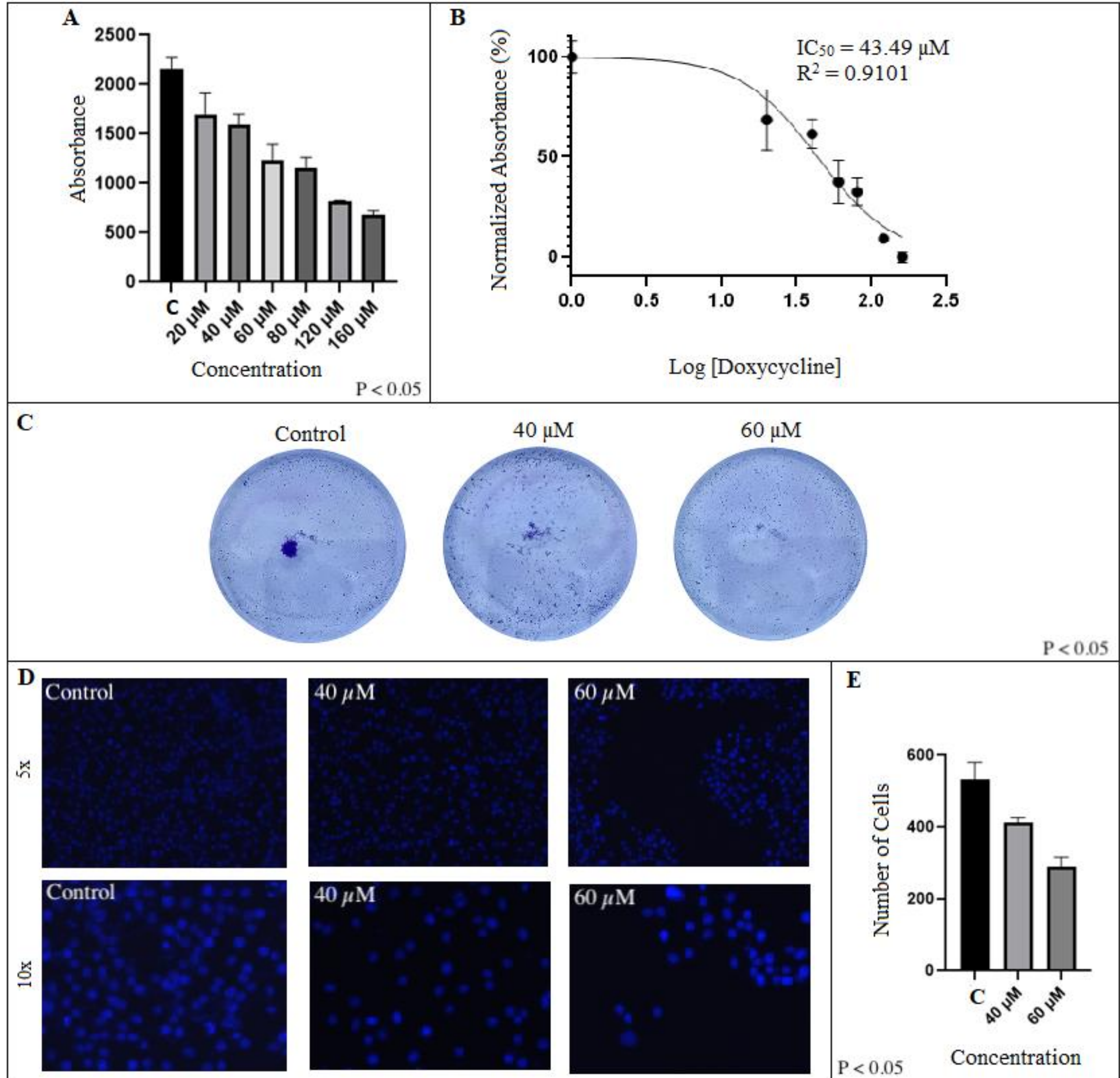

Fig. 1. Doxycycline inhibits cell viability and colony formation ability of rat glioma cells. (A) The viability of the cells after 48h doxycycline treatment decreased in a dosage dependant manner ($P < 0.05$). (B) IC_{50} was calculated as 43.49 µM via GraphPad Prism 9.3.0. (C) Doxycycline notably decreased the colony number in both groups ($P < 0.05$), 40 µM and 60 µM. (D-E) DAPI stained nuclei number was counted at 5x view and the number is significantly reduced in 60 µM doxycycline-treated cells ($P < 0.05$).

glioma cells in a dose-dependent manner. IC_{50} value was found as 43.49 µM (Fig. 1B), considering the viability values of the untreated group cells as 100%. In addition, the R^2 value, the proportion of the variance for a dependent variable, was calculated as 0.9101 (Fig. 1B).

3.2. Doxycycline affects the colony formation capability of rat glioma cells

To confirm the cytotoxicity of doxycycline on the cells, a clonogenic assay was applied. To determine the effects of doxycycline on cells progressively, the test was performed using 40 µM and 60 µM dosages to compare with the control cells. While the number of colonies in control cells is 583, 492 colonies in 40 µM and 419 colonies in 60 µM treated cells were counted, which shows the reduced number of colonies in treated rat glioma cells (15.6% decrease in 40 µM and 28.1% decrease in 60 µM treated cells) (Fig. 1C). Next, DAPI stained nuclei were counted and the number of nuclei reduced in 60 µM doxycycline-treated cells (Fig. 1D & 1E), 22.7% and 45.78% decrease were observed in 40 µM and 60 µM treated cells, respectively. The decrease in the number of cells in 40 µM treated cells was not found statistically significant.

3.3. Doxycycline inhibits the migration of rat glioma cells

Migration ability of rat glioma cells with doxycycline treatment was measured by wound-healing assay. The cells were scratched with a sterile pipette tip in the absence or presence of doxycycline. Gap areas measured after 24 hours, and 48 hours treatment and wound areas were wider of doxycycline-treated cells than in the control group (Fig. 2A). Results showed that 48-hour treatment with doxycycline reduces migration efficiency of the cells by 13,55% and 38,36% in 40 µM and 60 µM doxycycline-treated cells, respectively (Fig. 2B).

3.4. Doxycycline alters the expression of distinct apoptotic genes

Mmp9, a member of the matrix metalloproteinase gene family, is responsible for extracellular matrix degradation in mammals and increased gene expression level is observed in metastatic mammalian cancer cells (Morini et al., 2000). To determine the reduction effect of doxycycline on rat glioma

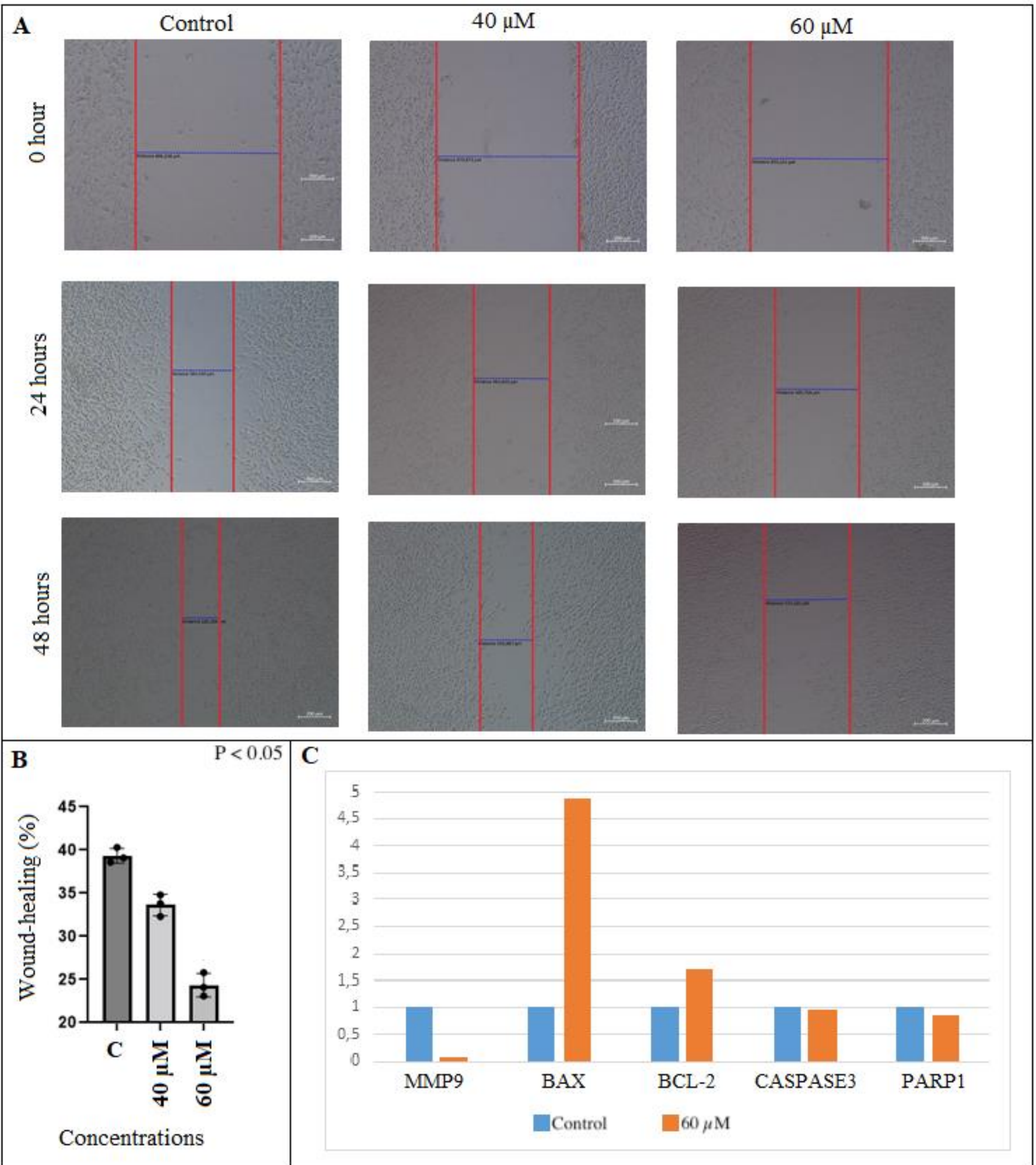

Fig. 2. Doxycycline inhibits migration ability and affects the expression of some apoptotic genes of rat glioma cells. (A) Scratch areas of 40 µM and 60 µM doxycycline-treated cells captured after 24h and 48h. (B) Migration ability was decreased by 13,55% and 38,36%, respectively ($P < 0.05$). (C) Relative quantification of *Mmp9*, *Bax*, *Bcl-2*, *Caspasex3*, and *Parp1* between control and 60 µM treated rat glioma cells.

cells, *Mmp9* expression in 60 µM doxycycline-treated cells for 48 hours was analysed. As demonstrated in Fig. 2C, doxycycline significantly inhibited the expression of *Mmp9* by decreasing it approximately 0.9-fold change. After 48 hours with 60 µM doxycycline treatment, doxycycline strongly induced the expression of *Bax* with approximately 3.5-fold change increase (Fig. 2C), which is a pro-apoptotic gene and has decreased level of gene expression in cancer cells (Miyashita et al., 1994). The expression of *Bcl-2*, which is an anti-apoptotic gene and ensures cell survival (Kelly and Strasser, 2020), was also increased 0.6-fold change in the presence of doxycycline (Fig. 2C). However, doxycycline did not have a significant effect of the expression of *Caspase3* (Fig. 2C), a pro-apoptotic gene (Alnemri et al., 1996), and *Parp1* (Fig. 2C) which is a proliferative gene and its increased gene expression levels detected in cancer cells (Pascal, 2018).

4. Conclusion

Various treatment approaches such as chemotherapy, radiotherapy, immunotherapy, and gene therapy are applied in cancer treatment and the mechanism of action of chemotherapy is primarily to stop the growth of cancer cells and then to kill the cells with a cytotoxic effect (Liu, 2009). However, while chemotherapy drugs can kill cancer cells, they also kill healthy cells where cancer cells form and metastasize, so the side effects of chemotherapeutic drugs can be dangerous for patients (Umfress et al., 2021). Since doxycycline has a bacteriostatic effect, its usage in cancer treatment might prevent the side effects of cancer treatments. Studies showed that doxycycline has high anti-metastatic activity and low cytotoxicity in cancer cells (Sun et al., 2009; Liu et al., 2015). Due to the lack of studies on glioma cells, rat glioma cells were used to investigate the

effects of doxycycline in this study.

In the current study, the cytotoxic effect was measured using MTT assay and IC_{50} value of rat glioma cells were determined as 43.49 µM for 48 hours in a dose-dependent manner. Qin et al. (2015) showed that IC_{50} values of doxycycline on NCI-H446 and A549 cell lines, which are lung cancer cells, were between 1 and 2 µM after 48 hours of application of doxycycline and it was stated that lung cancer is much more sensitive to doxycycline than other cancer cell types. In addition, Liu et al. (2021) demonstrated that for pancreatic cancer cells, Panc-1 and Aspc-1, IC_{50} values were found to be 987.5, 99.64, and 50.02 µM after 48, 72, and 96 hours of doxycycline administration, respectively. Doxycycline also reduces mitochondrial function and protects cells from death caused by hypoxia conditions in glioma cells LNT-229, G55, and U343 and it is effective at high concentrations (Luger et al., 2018). Likewise, anti-proliferative effects occur only at high dosages in U251HF, U87, and LN229 glioma cells and it may be because of GBM tumors being resistant to cytotoxic intervention compared to other solid tumours in clinical settings (Wang-Gillam et al., 2007). Since the dose and time-dependent effects of the determined active substance on different cells are expected in the scientific framework, differences in IC_{50} values in the literature are considered normal.

In this study, the colony formation test was performed on glioma cells with the determined doxycycline dosages and control group. As a result of the test, a significant dose-dependent decrease in colony number was shown. Similarly, Zhang et al. (2017) found that doxycycline significantly reduced colony formation of MCF7 and MDA-MB-468 cell lines, which are breast cancer cells, in a dose-dependent manner. Wound healing testing was performed to consolidate these results. A statistically significant decrease was observed in the wound healing rate depending on the dosage in this study. As Qin et al. (2015) show, doxycycline also decreases wound healing and migration ability of lung cancer cells after 24 and 48 hours. Cells were stained with DAPI after dosage applications for morphological examination. They were captured under microscope to determine the cell numbers and a considerable reduction was shown in the cell number of the 60 µM doxycycline-treated group. As the last step of the experiment, the effects in gene expression levels of the cancer related genes were measured by RT-qPCR compared with the control group. An important reduction in *Mmp* expression levels was determined in studies performed by applying doxycycline to cancer cells. Doxycycline degraded the extracellular matrix and basement membrane and inhibited *Mmp2* and *Mmp9* enzymes, which are involved in the cancer invasion steps, reducing lung cancer metastasis by inhibiting the degradation of the extracellular matrix and basement membrane. Similarly, in this study, a decrease in *Mmp9* expression was observed in the cells treated with 60 µM doxycycline with RT-qPCR test. Wang et al. (2016) showed that, in H446 cells, a small lung cancer line, doxycycline decreased anti-apoptotic gene *Bcl-2* expression and caused an increase in the pro-apoptotic genes *Bax* and *Caspase3* genes. In this study, it was observed that while the *Bax* expression levels increased, the *Bcl-2* level also increased. It is unexpected for genes that often work in reverse. However, the 350% increase in the *Bax* expression level compared to the 60% increase of *Bcl-2* expression level creates a difference in the level between them. Furthermore, the effect of doxycycline is to prevent the cell from proliferating by reducing its metabolism rather than driving it into apoptosis; therefore, an increase in *Bcl-2* level can be expected. Although there was a decrease in *Caspase3* and *Parp1* levels in this study, no significant decrease was observed, which supports the fact that doxycycline reduces cell proliferation instead of causing cell death. The mechanism of action of the active ingredient may vary depending on the cell line examined.

In conclusion, significant results were obtained particularly in cytotoxicity and proliferation tests. In the gene expression test with cancer-related genes, the significant result was observed in the *Mmp9* and *Bax* genes. Doxycycline has been found as a promising agent especially in the prevention of cancer metastasis, since it suppresses the *Mmp9* gene and it does not have a high cytotoxic effect, and reduces the migration ability of cells. In this study, the genes involved in apoptosis were examined, however more cancer-related genes need to be tested for more comprehensive studies.

Additionally, in order to determine whether post-transcriptional modifications have an effect on the mRNA products of the relevant genes, it should be analysed by western blot method, which determines the expression level at the protein level. RNAi and CRISPR/Cas methods can be used to determine the signaling pathways that doxycycline affects to be used in the treatment of glioma cancer, and its effects at the molecular level can be understood in more detail. Doxycycline is considered to have potential use in the treatment of glioma cancer, either alone or as a complementary therapy (Hassan et al., 2022), if promising results are obtained in sufficient quantities from *in vitro* and *in vivo* studies.

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