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# The apoptotic and proliferative effects of tulathromycin and gamithromycin on bovine tracheal epithelial cell culture

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**Abstract:** Gamithromycin and tulathromycin are commonly used in the treatment of bovine respiratory bacterial diseases. The current work was undertaken to establish the apoptotic, necrotic, and cytotoxic effects of these antibiotics in the target animal. Cells with apoptosis and necrosis were determined by dual staining method, cytotoxic effects were determined by MTT assay, cell proliferative effects were examined by XCelligence real-time cell analysis system (RTCA-SP). The comparison between gamithromycin and tulathromycin concentrations on tracheal cells in terms of % cell viability was found to be significantly different. While the cell viability percentage of gamithromycin was higher at 150 µg/mL, 180 µg/mL, and 240 µg/mL than tulathromycin, and at 2 µg/mL, 4 µg/mL, 10 µg/mL, 20 µg/mL, and 50 µg/mL concentrations tulathromycin cell viability was higher than gamithromycin ($p < 0.05$). When the staining method data were evaluated, the difference between the results of % apoptotic index at 20 µg/mL concentration was significant and it was found that gamithromycin had more apoptotic effect than tulathromycin ($p < 0.05$). It was seen that tulathromycin and gamithromycin applied on tracheal epithelial cells at concentrations of 2 and 10 µg/mL increased the viability depending on time. The increase in epithelial cell proliferation of gamithromycin and tulathromycin due to time shows that these antibiotics can maintain long-term prophylactic treatment against diseases.

**Key words:** Bovine trachea, epithelial cell, gamithromycin, proliferation, tulathromycin

## 1. Introduction

Macrolides have been widely used in skin, respiratory and gastrointestinal systems and soft tissue infections since the second half of the 20th century [1,2]. Since macrolides are lipophilic weak basic drugs [3], they easily penetrate tissues by passive diffusion [4].

The concentration of macrolides in peripheral tissues is higher than serum concentrations, they have long half-lives, wide distribution to tissues, and their concentration in the lung are long-lasting, making them one of the first antibiotics used in the treatment and prevention of respiratory diseases [5–7]. New generation macrolides such as gamithromycin and tulathromycin, which were developed in recent years, are used in the treatment of respiratory bacterial diseases of cow and pig [2, 8–11].

Macrolides have the ability to penetrate and accumulate in many eukaryotic cell types. Though not as much as neutrophils and macrophages, they can accumulate in epithelial-like cell lines [12]. Airway epithelium is a physicochemical barrier that plays a vital role in host defence against inhaled pathogens (bacteria, virus, etc.) and irritants [13,14]. When respiratory epithelial cells are subject to inflammatory mediators, macrolide antibiotics have a protective impact against epithelial injury [15]. Because of these properties, they show a therapeutic effect against intracellular organisms [12].

Macrolide group antibiotics are known to regulate the functions of immune system cells as well as their antimicrobial effect. Studies conducted in recent years show that some macrolide group antibiotics cause apoptosis [16,17]. To our knowledge, there is no study conducted on bovine tracheal epithelial cells cytotoxic, apoptotic, necrotic, and proliferative effects of gamithromycin and tulathromycin. The aim of this study was to investigate cytotoxic, necrotic, apoptotic, and cell proliferative effects of tulathromycin and gamithromycin on in vitro bovine tracheal epithelial cells.

## 2. Materials and methods

### 2.1. Chemicals

Tulathromycin (Sigma-Aldrich SLM2107, Germany) and gamithromycin (Sigma-Aldrich 32161, Germany) used

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in the experiments were dissolved in dimethyl sulfoxide (DMSO, Isolab, Germany).

**2.2. Bovine tracheal epithelial cell isolation and cell culture**

Tracheal epithelial tissue samples, from 12–36 months old *Simmental* breed male cattle, slaughtered in a local slaughterhouse, were transported to the cell culture laboratory with a centrifuge tube (50 mL, Corning, USA) containing a transport solution [99% DMEM, (Dulbecco's modified eagles medium), 1% penicillin-streptomycin, Biological Industries, Israel]. To isolate epithelial cells, samples were mechanically scraped with a sterile scalpel from tracheal epithelial tissue and cut into very small pieces. Then these samples were placed in 25 $cm^2$ culture flasks (Corning, USA) with 3 mL of cell medium [79% DMEM, 20% foetal bovine serum (Biological Industries, Israel), 1% penicillin-streptomycin] was added and incubated in the incubator (5% $CO_2$, 37°C). The cell medium (89% DMEM, 10% foetal bovine serum, 1% penicillin-streptomycin) was changed every 48 h due to the proliferation of tracheal epithelial cells [18, 19].

**2.3. Cytotoxicity assays**

After counting bovine tracheal epithelial cells; 10 $\times 10^3$ cells (100 µL) were seeded to each well in a 96-well plate (Corning, USA). 2, 4, 10, 20, 50, 100, 120, 150, 180, 210, 240, 300 µg/mL of gamithromycin or tulathromycin were applied on the cells with triplicates for each test group. Doses were selected according to the treatment and target concentration in the tissue. As the control group; 0.5% DMSO medium mixture was added to the cells. Cells were incubated again for 24 h. At the end of the incubation, the cell media in 96-well plated was discarded and 50 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Ambresco-Life Science, USA) solution was added to the wells. Cells were incubated in the incubator for 2 h. At the end of the incubation, the cell media in 96-well plated was discarded, 100 µL of isopropanol (Sigma, Germany) was added and it was analysed in plate reader (Biotek, USA) at 570 nm wavelength. The cytotoxicity test method was carried out in accordance with ISO 10993-5 protocol [20]. The MTT assay was repeated three times.

**2.4. Determination of apoptosis and necrosis by dual staining method**

Tracheal epithelial cells were seeded at 10 $\times 10^3$ cells in a 48-well plate. 2, 4, 10, 20, 50, 100 µg/mL of gamithromycin or tulathromycin were applied on the cells in triplicates for each test group. As the control group; 0.5% DMSO medium mixture was added to the cells. Cells were incubated again for 24 h. At the end of incubation, cell media in 48-well plated wells were discarded and 70 µL of double staining solution (2 mg/mL hoechst 33342, 100 µg/mL ribonuclease A, 2 µg/mL propidium iodide, Serva, Israel) were added. At the end of this 15-min incubation; apoptotic cells were evaluated using the DAPI filter (340–488 nm wavelength) and necrotic cells were evaluated using the FITC filter (480–520 nm wavelength) by inverted microscope (Leica DM6000B, Germany) [21]. The dual staining method was repeated three times.

**2.5. Determination of cell proliferation by real-time cell analysis system**

Gamithromycin or tulathromycin between 2 µg/mL and 10 µg/mL concentrations were applied on the cells seeded at 5 $\times 10^3$ cells per 96-well e-plate in triplicate. DMSO medium mixture (0.5%) was added to the cells as control group; 20% DMSO medium mixture was added to the cells in the positive control group. E-plate was placed in the RTCA-SP (Roche, Germany) and cultured for 96 h and real-time impedance measurement was taken from the system every 10 min. After 96-h of incubation, graphs showing time-dependent cell proliferation were obtained [21].

**2.6. Statistical analysis**

The data from the cytotoxicity, apoptosis and necrosis assays were calculated using Microsoft Excel (Microsoft Office 2010) program. GraphPad Prism (Software ver. 6) was used to calculate $IC_{50}$ (the half-maximal inhibitory concentration) from the MTT assay. The data obtained in the study were given as arithmetic mean ± standard error. Statistical analyses were done by SPSS 15.0 package program. Student t-test was performed to evaluate the difference between gamithromycin and tulathromycin groups. $p < 0.05$ value was accepted as significant.

**3. Result**

**3.1. Cytotoxic effects of tulathromycin and gamithromycin**

Cytotoxicity results of tulathromycin and gamithromycin applied on bovine tracheal epithelial cells are given in Figure 1 and Figure 2. While the viability of gamithromycin at the highest concentration (300 µg/mL) was 25.701 ± 3.37%, the viability of tulathromycin was 20.093 ± 2.47%. At a low concentration (2 µg / mL), the viability of tulathromycin (172.197 ± 3.23%) was higher than that of gamithromycin (128.251 ± 11.45%). $IC_{50}$ values of gamithromycin and tulathromycin were calculated according to the results of the MTT method, in bovine tracheal epithelial cells. $IC_{50}$ value of gamithromycin was 156 ± 9 µg/mL and the $IC_{50}$ value of tulathromycin was 134.7 ± 7.1 µg/mL. When the same concentrations of gamithromycin and tulathromycin are compared; statistical significance was found between 2 µg/mL, 4 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL, 150 µg/mL, 180 µg/mL, and 240 µg/mL concentrations in terms of cell viability. While the cell viability percentage of gamithromycin was higher at 150 µg/mL, 180 µg/mL, and

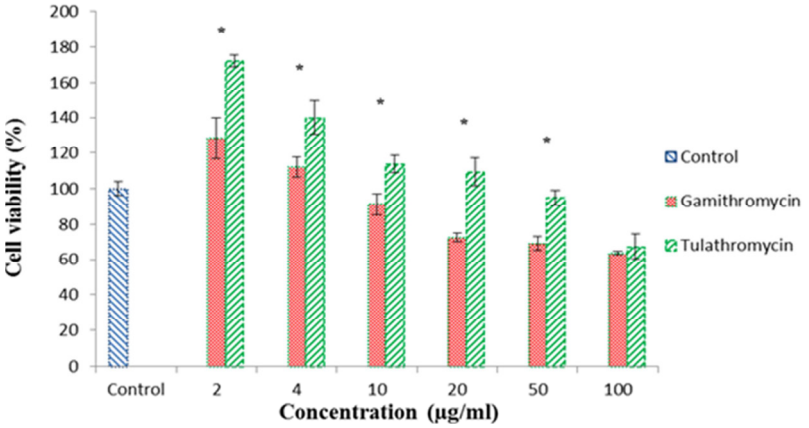


**Figure 1.** The effect of tulathromycin and gamithromycin on the cell viability of tracheal epithelial cells at concentrations between 2 µg/mL–100 µg/mL. *,The difference between the groups is significant ($p < 0.05$).

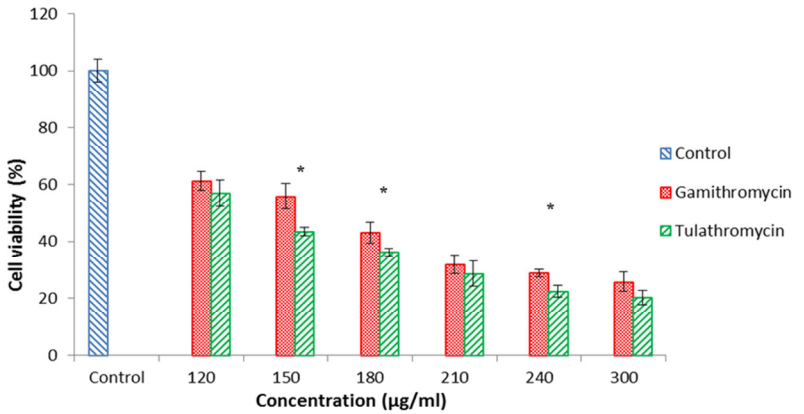


**Figure 2.** The effect of tulathromycin and gamithromycin on the cell viability of tracheal epithelial cells at concentrations between 120 µg/mL–300 µg/mL. *, The difference between the groups is significant ($p < 0.05$).

240 µg/mL than those of tulathromycin; at 2 µg/mL, 4 µg/mL, 10 µg/mL, 20 µg/mL, and 50 µg/mL concentrations tulathromycin cell viability was higher than gamithromycin ($p < 0.05$) (Figure 1, Figure 2).

### 3.2. Cell apoptosis and necrosis of gamithromycin and tulathromycin

The percentage (%) apoptotic and necrotic index results of tracheal epithelial cells of tulathromycin and gamithromycin are given in Figure 3 and Figure 4, respectively. As the concentration of both antibiotics increased, apoptotic and necrotic cell percentage (%) index results were increased. However, at 20 µg/mL concentration, the difference in apoptotic percentage (%) index results between gamithromycin and tulathromycin was significant and the percentage of apoptotic cells was higher in gamithromycin than that of tulathromycin ($p < 0.05$). According to the necrotic cell percentage (%) index results, the difference between 20 and 100 µg/mL concentrations was statistically significant and it was found that the percentage of necrotic cells was higher in gamithromycin than that of tulathromycin ($p < 0.05$). Apoptotic and necrotic cell images of tracheal epithelial cells in the control group and gamithromycin, tulathromycin groups are shown in Figure 5.

### 3.3. Cell proliferation of gamithromycin and tulathromycin

After plating the tracheal epithelial cells to E-plate the cell proliferation graphics were obtained for approximately 96 h. Concentrations of gamithromycin and tulathromycin (2 and 10 µg/mL) were administered at the 24$^{th}$ h. A comparative proliferation graph of gamithromycin and tulathromycin applied on tracheal epithelial cells at 2 µg/mL is given in Figure 6. In the first 10 h following antibiotic administration to tracheal epithelial cells, cell proliferation was found to be higher in tulathromycin when compared to gamithromycin. Gamithromycin was found to have

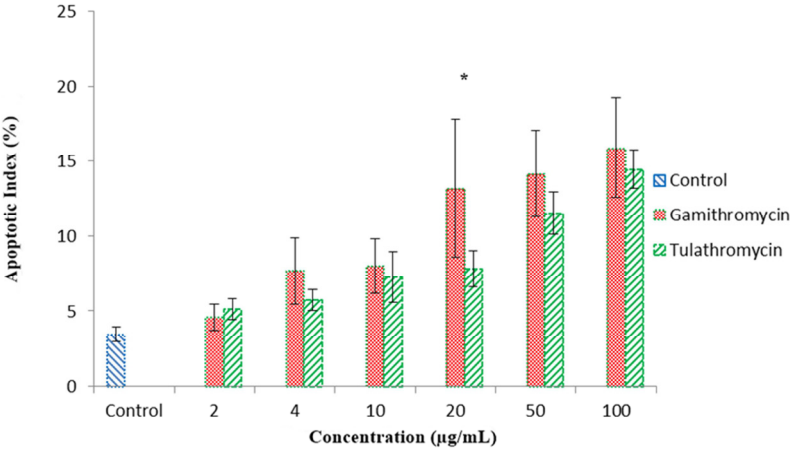


**Figure 3.** Apoptotic cell percentage (%) index of tracheal epithelial cells at concentrations between 2 µg/mL–100 µg/mL of tulathromycin and gamithromycin. *, The difference between the groups is significant ($p < 0.05$).

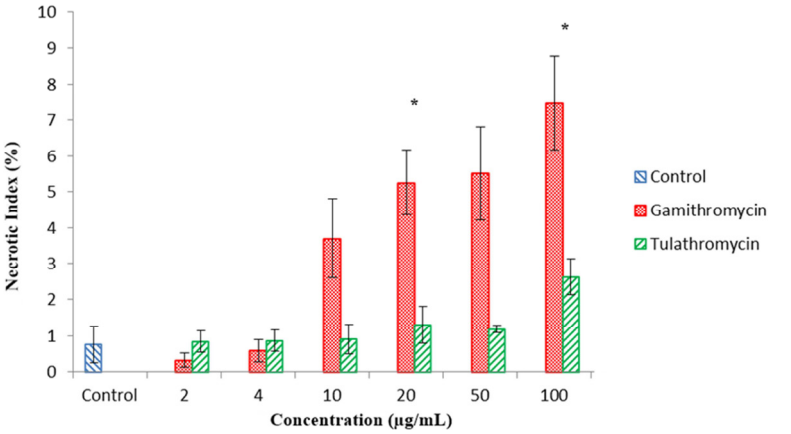


**Figure 4.** Necrotic cell percentage (%) index of tracheal epithelial cells at concentrations between 2 µg/mL–100 µg/mL of tulathromycin and gamithromycin. *, The difference between the groups is significant ($p < 0.05$).

a higher cell proliferation rate than tulathromycin for approximately 50 h after 10 h. In addition, after application to tracheal epithelial cells, a continuous increase in proliferation was observed in gamithromycin and tulathromycin (2 µg/mL) while proliferation decreased after a certain time in the control groups.

A comparative proliferation graph of gamithromycin and tulathromycin applied on tracheal epithelial cells at 10 µg/mL is given in Figure 7. The first 30 h after gamithromycin and tulathromycin were applied to tracheal epithelial cells; it was found that cell proliferation was higher in tulathromycin than that of gamithromycin. On the other hand, after 30 h, the cell proliferation rate was higher in gamithromycin than tulathromycin. In addition, while proliferation increased in tracheal epithelial cells for approximately 54 h after gamithromycin and tulathromycin application, a decrease in cell proliferation was observed after 78 h. Tracheal epithelial cell proliferation in the negative control group was increased in the first 52 h, while a decrease was observed after the 52nd hour. Cell proliferation in the positive control group; increased in the first hours, then stopped and decreased at last.

## 4. Discussion and conclusions

Cell viability and/or proliferation rates are the best parameters that give information about the health status of the cells. Generally, the health and metabolism of cells are affected by factors such as concentration of physical or chemical substances, temperature, and application time. If the chemical agents have different mechanisms such as destroying the cell membrane, and irreversible binding to receptors, toxicity occurs in the cells. Therefore,

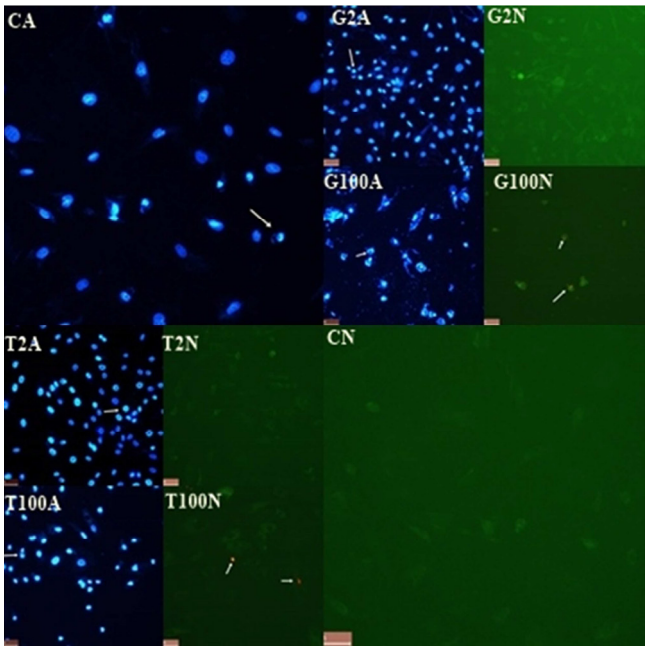


**Figure 5.** Apoptotic and necrotic cell image in the tracheal epithelial cells of gamithromycin, tulathromycin, and control group. CA: Control group apoptotic cell image, CN: Control group necrotic cell image, G2A: Gamithromycin 2µg/mL, apoptotic cell image, G2N: Gamithromycin 2µg/mL, necrotic cell image, G100A: Gamithromycin 100µg/mL, apoptotic cell image, G100N: Gamithromycin 100µg/mL, necrotic cell image, T2A: Tulathromycin 2µg/mL, apoptotic cell image, T2N: Tulathromycin 2µg/mL, necrotic cell image, T100N: Tulathromycin 100µg/mL, apoptotic cell image, T100N: Tulathromycin 100µg/mL, necrotic cell image, (photos were taken at 20X magnification under Leica DM6000 Inverted Microscope).

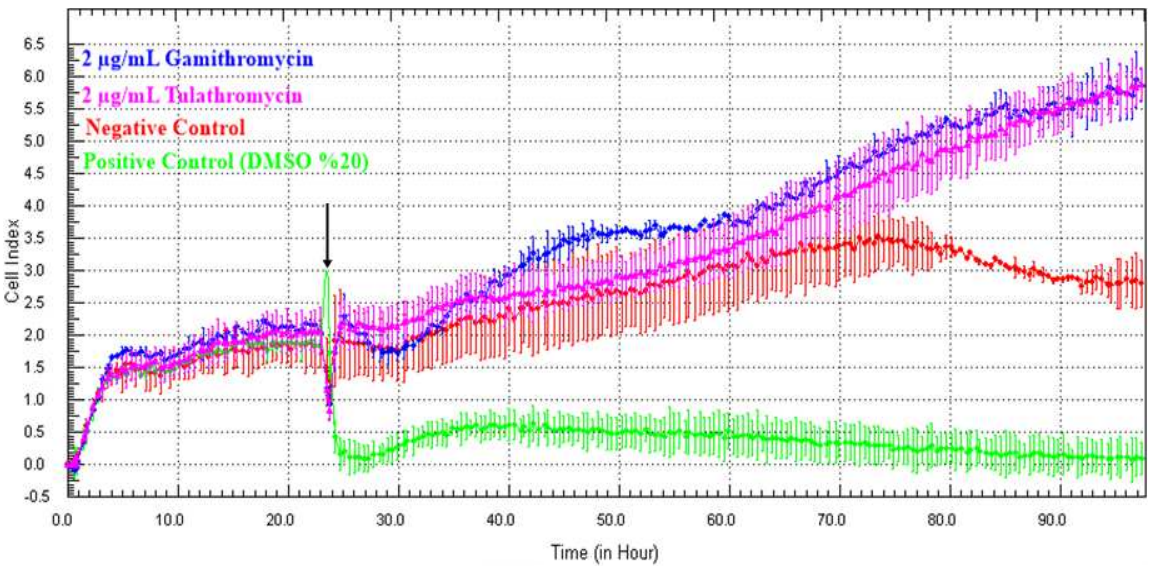


**Figure 6.** Comparative proliferation graph of tracheal epithelial cells at a concentration of 2 µg/mL of gamithromycin and tulathromycin.

cell viability or cytotoxicity assays provide an important advantage in determining the cytotoxic effects of drugs and chemicals [22].

Duewelhenke et al. [23] reported an increase in the cytotoxic effect of the macrolide group antibiotic azithromycin and roxithromycin on the primary human

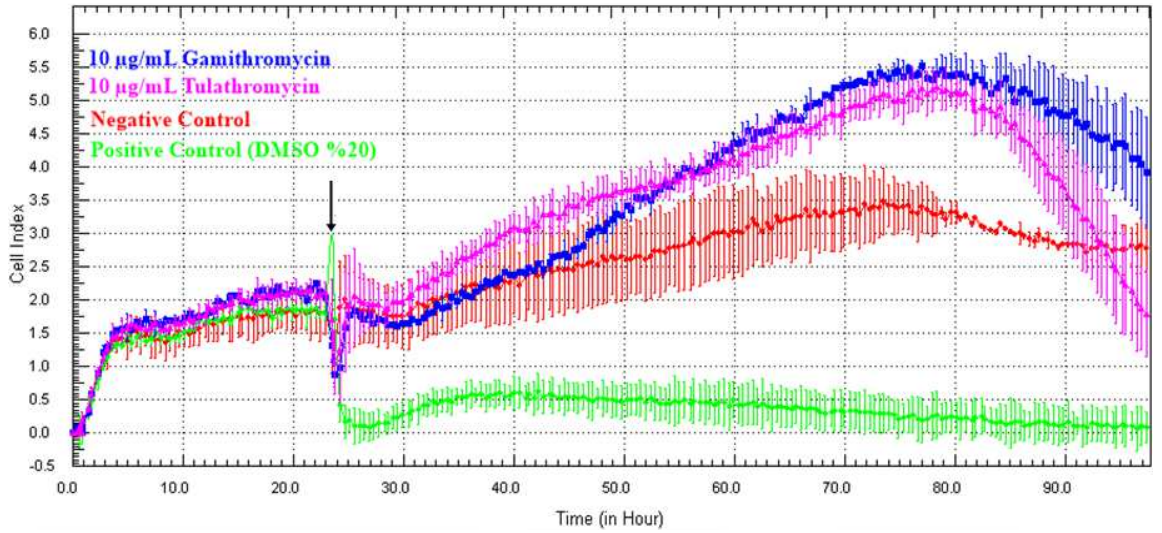


**Figure 7.** Comparative proliferation graph of tracheal epithelial cells at a concentration of 10 µg/mL of gamithromycin and tulathromycin.

osteoblast cells depending on the concentration (100 µg/mL 20%–30%, 200 µg/mL 30%–45%, 400 µg/ mL 40%–60%).

Viluksela et al. [24] researched the cytotoxic effects of macrolide antibiotics on liver cells. They declared erythromycin estolate has the most cytotoxic effect on liver cells after 4–48 h incubation, and this macrolide is followed by erythromycin 11,12-cyclic carbonate, clarithromycin, roxythromycin, erythromycin base, azithromycin, respectively. The findings of the present study are consistent with the findings of Duewelhenke et al. and Viluksela et al. [23,24]. Thus this study also showed that cytotoxicity of tulathromycin and gamithromycin varies depending on the concentration of the substances and time. When $IC_{50}$ values are evaluated according to MTT results, the $IC_{50}$ value of gamithromycin (156 ± 9 µg/mL) is higher than the $IC_{50}$ value (134.7 ± 7.1 µg/mL) of tulathromycin. According to these values, it can be concluded that gamithromycin has higher confidence intervals in terms of cytotoxicity on tracheal epithelial cells than tulathromycin.

Recent studies have reported that cytotoxicity caused by some drugs is due to the cells' mitochondrial toxicity. With the increase of mitochondrial toxicity in cells formed by drugs; organ toxicity may occur in the liver, skeletal muscle, kidney, and heart. The drugs that cause mitochondrial toxicity are cholesterol-lowering drugs, antidiabetics, pain relievers (NSAIDs), some antibiotics (fluoroquinolones, macrolides), and anticancer drugs [25]. Jiang et al. [26] declared that due to the increased concentration of azithromycin; the proliferation of human fibroblast and MCF-12A cells is reduced and caused mitochondrial toxicity. Although the cell lines are different, in accordance with the presented study, high concentrations of both active substances cause a decrease in cell viability. Therefore; toxicity on tracheal epithelial cells induced by gamithromycin and tulathromycin is thought to occur as a result of mitochondrial toxicity, as induced by other macrolide group antibiotics.

Apoptosis plays a significant role in the development and maintenance of all mammalian tissues. The damage or aging is eliminated with programmed cell death without harming the mammal by apoptosis [27]. Data obtained from human and animal lung biopsies show that apoptosis has a significant effect on the development of cell proliferation and various respiratory diseases [28].

Macrolides have been shown to cause apoptosis on macrophages and epithelial cells [17]. Duquette et al. [29] reported that neutrophil and monocyte-derived macrophages isolated from porcine blood samples induce apoptosis depending on the concentration and duration of exposure to tulathromycin. In another study, Moges et al. [30] stated that the apoptosis induced by tylosin (0.1, 1, and 10 µg/mL) on neutrophil and monocyte-derived macrophages from pig blood varies with time and concentration. In the present study, apoptosis and necrosis on the tracheal epithelial cells caused by gamithromycin and tulathromycin were changed depending on the concentration. Although the drugs and cell lines used in the study are different, the results are compatible with the results of Duquette et al. and Moges et al. [29, 30] studies showing an increase in the percentage of apoptosis on tracheal epithelial cells due to an increase in the concentration of the drugs.

Apoptosis is an important key for the regeneration event. The proliferative aspect of the regeneration event,

including blastema formation, is stimulated by signals from apoptotic cells [31]. According to the results of the real-time cell analysis system, apoptosis was observed in tracheal epithelial cells and it is thought that proliferation seen in the concentrations of both substances at 2 and 10 µg/mL may be caused by signals from apoptotic cells.

Real-time cell analysis system provides real-time monitoring of changes in cell proliferation and toxicity of tulathromycin ($t_{1/2:}$ about 73 h) and gamithromycin ($t_{1/2:}$ about 60 h) [7,32]. According to the results of the cell analysis system performed in the present study, epithelial cell proliferation increased depending on the time in low concentrations of gamithromycin and tulathromycin. It is suggested that gamithromycin and tulathromycin may have a therapeutic effect against epithelial damage that may occur in tracheal epithelial cells like other macrolide group antibiotics due to their cell proliferation enhancing properties.

As a result; the effects of macrolide antibiotics may differ depending on their chemical structure and concentrations and the cell and animal species to which they are applied. The cytotoxicity of gamithromycin (2 and 4 µg/mL) and tulathromycin (2, 4, and 10 µg/mL), which has the ability to penetrate and accumulate into the cell, is very low in tracheal epithelial cells and thus can be used safely. Between these antibiotics, the confidence interval is higher in gamithromycin. The apoptosis caused by both antibiotics can increase the proliferation effects of the cells and protect the cells against damage that may occur in epithelial cells, where the disease agent was first encountered. The results of the real-time cell analysis system revealed that gamithromycin and tulathromycin increased epithelial cell proliferation depending on the time; which is beneficial besides its antimicrobial activity.

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