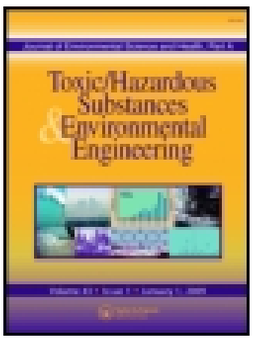

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Cytotoxic effect of aminoglycoside antibiotics on the mammalian cell lines

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ABSTRACT

Aminoglycoside antibiotics have been used for treating serious but also routine infections in veterinary and human medicine for many years. The basic aim of this work is to evaluate the cytotoxicity of dihydrostreptomycin and neomycin *in vitro* on three cell cultures - BHK-21 (Syrian golden hamster kidney fibroblast), VERO (African green monkey kidney fibroblast) and FEA (feline embryonic fibroblast) cells. The morphological changes were examined by Giemsa staining. Cells were dried and visualized under fluorescence microscope. After the exposure to different experimental doses of dihydrostreptomycin (812.5-20000 μg/mL) and neomycin (1000-20000 μg/mL) during 24 h, the viability of BHK-21, FEA and VERO cell lines were evaluated by MTT assay. Viability of BHK-21 cells significantly ($P < 0.001$) decreased after treatment with 3500; 5500 and 7500 μg/mL of dihydrostreptomycin and 9000; 10000 and 20000 μg/mL of neomycin. The FEA cell viability decreased significantly ($P < 0.001$; $P < 0.01$) at 2500 and 3000 μg/mL dihydrostreptomycin and at 3000 μg/mL of neomycin treatment. Only the highest concentration of dihydrostreptomycin (20000 μg/mL) reduced VERO cell viability significantly ($P < 0.01$). Based on or results we can assume the effect of different antibiotics in different concentrations on cell lines is various. Detection of antibiotic toxicity to animal cells is very important because of the increasing resistance of bacteria. One of the solutions is drug dose increasing, but only to a certain concentration, since the toxic effect over the therapeutic one will prevail, which we have also shown in this work.

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Dihydrostreptomycin; neomycin; BHK21; VERO; FEA; cytotoxicity; MTT assay

Introduction

Aminoglycoside antibiotics have been used for treating serious but also routine infections in veterinary and human medicine for many years. Their importance is noticeable mainly in treating infections caused by gram-negative bacilli, including bacteria that may be resistant to other agents. They became a very useful class of drugs because of their chemical and pharmacokinetic properties, fast bactericidal activity, relatively low rate of resistance and large spectrum of effects in a large variety of animal species. On the other hand, their use poses a risk of potential toxicity and production of chemical residues in food-producing animals.[1–3]

The way aminoglycoside antibiotics act has been studied since the development of the first, streptomycin. The result has been a remarkable number of ways in which these substances act on bacteria: ribosome blockade, mistranslation, membrane damage, and irreversible antibiotic absorption.[3,4] The mechanism of the antibacterial effect is given by a disorder of proteosynthesis (error in genetic information reading), which, however, at lower concentrations only affects growing bacteria. In particular, it is a misreading of the mRNA on the 30S subunit of the ribosome. This leads to inaccurate amino acid incorporation into the polypeptide, thereby damaging the cell membrane or inhibiting synthesis.[2,3,5] The effect of higher concentrations is a bactericidal effect induced by the synthesis of fake proteins, which can be incorporated into the cytoplasmic membrane and cause functional disorders. Membrane permeability increases and bacteria lose essential components. The antibacterial spectrum is very broad.[5]

The significant clinical toxicities of aminoglycosides are ototoxicity, nephrotoxicity and less often neuromuscular toxicity.[6–8] Aminoglycosides may affect/damage the proximal renal tubule cells by several mechanisms. Lysosomal dysfunction, decreasing of mitochondrial respiration, interaction with the proximal tubule cell plasma membrane's phospholipids and enzymes and at the level of the glomerulus.[3]

The basic aim of this work is to evaluate the cytotoxicity of aminoglycoside antibiotics (dihydrostreptomycin - DHS, neomycin - NEO) *in vitro* on three cell lines - BHK-21

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Table 1. Concentrations of dihydrostreptomycin and neomycin used for morphological evaluation.

Cell culture	Concentrations of dihydrostreptomycin (μg/mL)	Concentrations of neomycin (μg/mL)
BHK-21 (baby hamster kidney fibroblast)	812.5; 1625; 2500; 3500; 5500; 7500; 15000	3125; 6250; 8000; 9000; 10000; 20000; 25000
FEA (feline embryo fibroblast)	1500; 2000; 2500; 3000; 3250; 7000	1000; 1500; 2000; 3000; 3125; 6250
VERO (African green monkey kidney fibroblast)	10000; 15000; 16000; 17000; 18000; 20000	1562.5; 3125; 4000; 5000; 8000; 15000; 25000

Table 2. Concentrations of dihydrostreptomycin and neomycin used for cytotoxic evaluation.

Cell culture	Concentrations of dihydrostreptomycin (μg/mL)	Concentrations of neomycin (μg/mL)
BHK-21 (baby hamster kidney fibroblasts)	3500; 5500; 7500	9000; 10000; 20000
FEA (cat domestic embryo fibroblast)	2000; 2500; 3000	1500; 2000; 3000
VERO (african green monkey kidney fibroblast)	17000; 18000; 20000	5000; 8000; 15000

(Syrian golden hamster kidney fibroblast), VERO (African green monkey kidney fibroblast) and FEA (feline embryonic fibroblast) cells.

Material and methods

Chemicals

Dihydrostreptomycin sesquisulfate (≥98%) and neomycin solution were purchased from Sigma-Aldrich (St. Louis, USA) and prepared using cell culture medium without fetal bovine serum (FBS) as solvent. Both DHS and NEO were used in this study not only to suppress the growth of bacteria in cell culture media but also to define the potential impact of experimental concentrations on exposed cells and intracellular parameters. The integral part of the complex study was to clearly specify the effect of another aminoglycosides (gentamicin, neomycin) and macrolides (tylosin, spiramycin) related to the cellular mechanism.

Cell lines

BHK-21 (Syrian golden hamster kidney fibroblast), VERO (African green monkey kidney fibroblast) and FEA (feline embryonic fibroblast) cell lines were obtained from the Institute for State Control of Veterinary Biologicals and Medicine. Selected cell lines are commonly used in cytotoxic evaluation *in vitro*. As a suitable model are able to reflect the different mechanism of action in specific aminoglycosides and macrolides. In addition, experimental procedures were designed on the basis of intensive collaboration with the Institute for State Control of Veterinary Biologicals and Medicaments Nitra (Slovak Republic).

Cell cultures and treatments

The BHK-21, FEA and VERO cell lines were purchased from the central collection of cell cultures, and they were chosen as the experimental model in our *in vitro* study. Cell lines were grown in DMEM/Hamś F12 (Dulbeccós modified Eaglés medium, Sigma-Aldrich, USA) supplemented with 10% FBS (fetal bovine serum, BiochromAG, Germany), 2% L-glutamine (Sigma-Aldrich, USA), 1% MEM non-essential acid solution (Sigma-Aldrich, USA) together with FBF-2 (fibroblast growth factor-2, Sigma-Aldrich, USA) and 1% penicillin-streptomycin solution (100 IU/mL penicillin and 100 μg/mL streptomycin, Sigma-Aldrich, USA). The cells were planted in sterile 48-well plates (Nunclon, Denmark) adjusted to a final volume of 500 μL/well. The final cell concentration - as determined by the growth profile of cell lines - was 1.7×10^6 cells/mL (BHK-21), 7.6×10^5 cells/mL (VERO) and 3.6 x.10^5 cells/mL (FEA). After pre-cultivation, cells were cultured in the presence of different experimental doses of dihydrostreptomycin (812.5-20000 μg/mL) and neomycin (1000-25000 μg/mL) during 24 h. All incubations were carried out at 37 °C under humidified atmosphere of 95% air and 5% CO_2.

Assessment of morphological changes with Giemsa staining

The BHK-21, FEA and VERO cell lines were grown to 70% confluency in 75 cm^2 flasks for 24 h. Then, cells were seeded in 24-well plates and incubated with certain doses of dihydrostreptomycin (812.5-20000 μg/mL) and neomycin (1000-25000 μg/mL) during 24 h (Table 1). Subsequently, the morphological changes were examined by Giemsa staining. After the exposition, cells were washed with PBS and fixed with cold methanol for 10 min. Methanol was removed and cells were air-dried. Then, fixed cells were stained with 10% Giemsa (Sigma-Aldrich, USA) solution for 20 min and washed with tap water. Cells were dried and visualized under fluorescence microscope using 25 x and 50 x objective. The morphological changes were observed, and photographs were taken.

Cytotoxicity screening – MTT assay

Cytotoxicity induced by dihydrostreptomycin and neomycin was assessed by MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay which measures the reduction of a yellow tetrazolium salt to insoluble blue formazan in viable cells according to Mosmann[9] with slight modifications. Briefly, each of cell line was cultured with experimental doses of dihydrostreptomycin and neomycin during 24 h (Table 2). After treatment, the medium was removed, cells were washed once with sterile PBS (phosphate buffered saline, Sigma-Aldrich, USA) and incubated at 37 °C in 5% CO_2 under humidified atmosphere of 95% air for 3 h with 1 mg/mL of MTT tetrazolium salt (Sigma-Aldrich, USA) dissolved in culture DMEM/Hamś F12

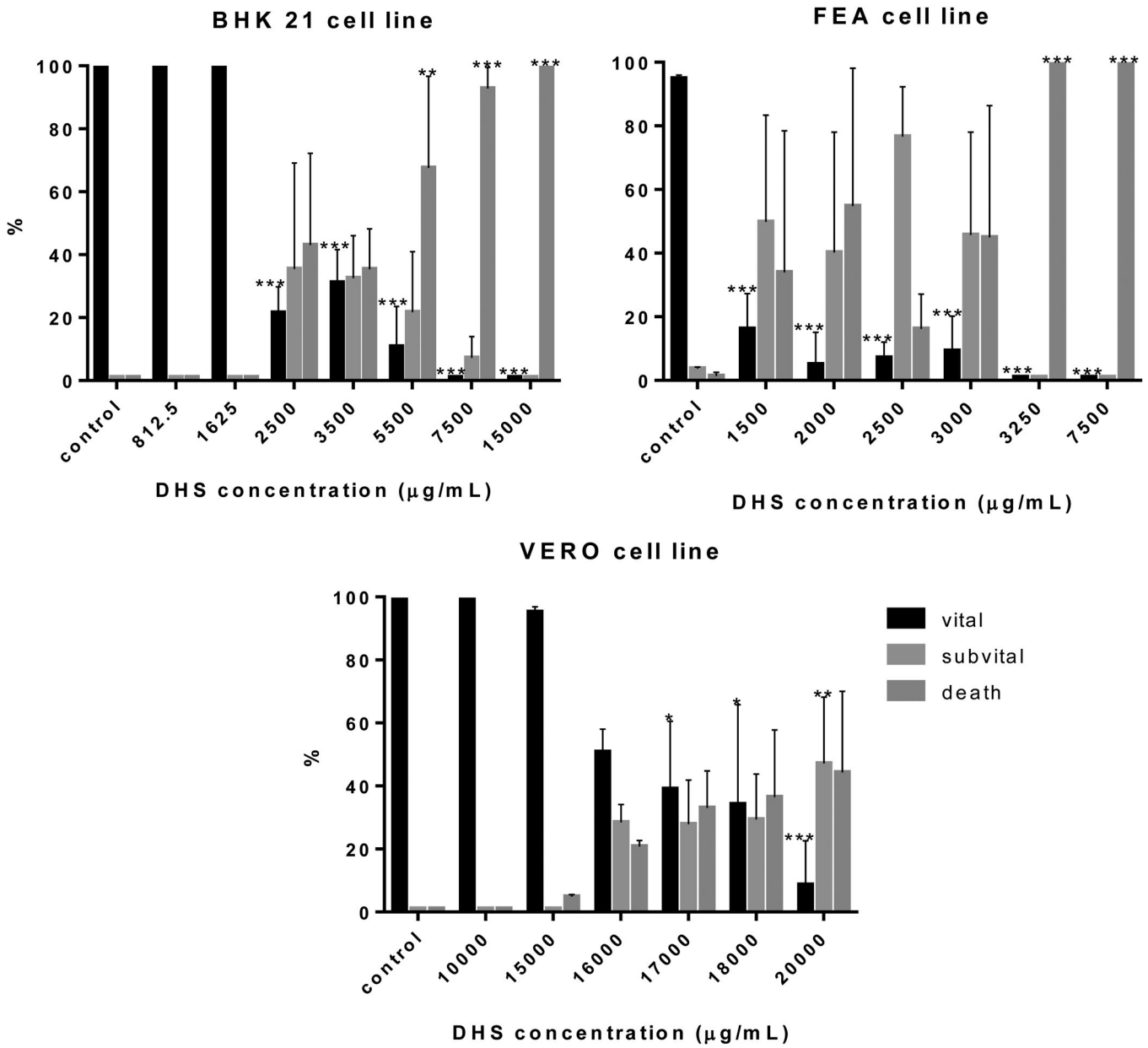

Figure 1. Cytomorphological changes analysis values – Dihydrostreptomycin (DHS). Each bar represents the mean vitality (± SD) % of control (untreated) and treated groups. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$) between control and experimental groups.

medium. Subsequently, the supernatants were removed and formed formazan deposits were dissolved by adding 300 µL/well of isopropanol (p.a. CentralChem, Slovak Republic). Aliquots of the resulting solutions were transferred to 96-well plates and evaluated spectrophotometrically (Multiscan FC, ThermoFisher Scientific, Finland) at 570 nm against 620 nm wavelengths. All data were expressed in percentage of control (untreated) group.[10]

Statistical analysis

All experiments were performed three times in triplicates. Statistical analyses were performed using the GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego, California, USA). All obtained data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk test. Analysis of variance (ANOVA) with the Dunnett's multiple comparison test was used to evaluate the significance of differences between the control and experimental groups. Results were expressed as the mean ± standard deviation of the mean (SD). The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).

Results

Figures 1 and 3 represent the selected cell lines reaction on the dihydrostreptomycin concentrations after 24 hours incubation. The significant decrease ($P < 0.001$) of BHK 21 vital cells was observed after the administration of 2500 µg/mL DHS when compared to the Control. The statistically

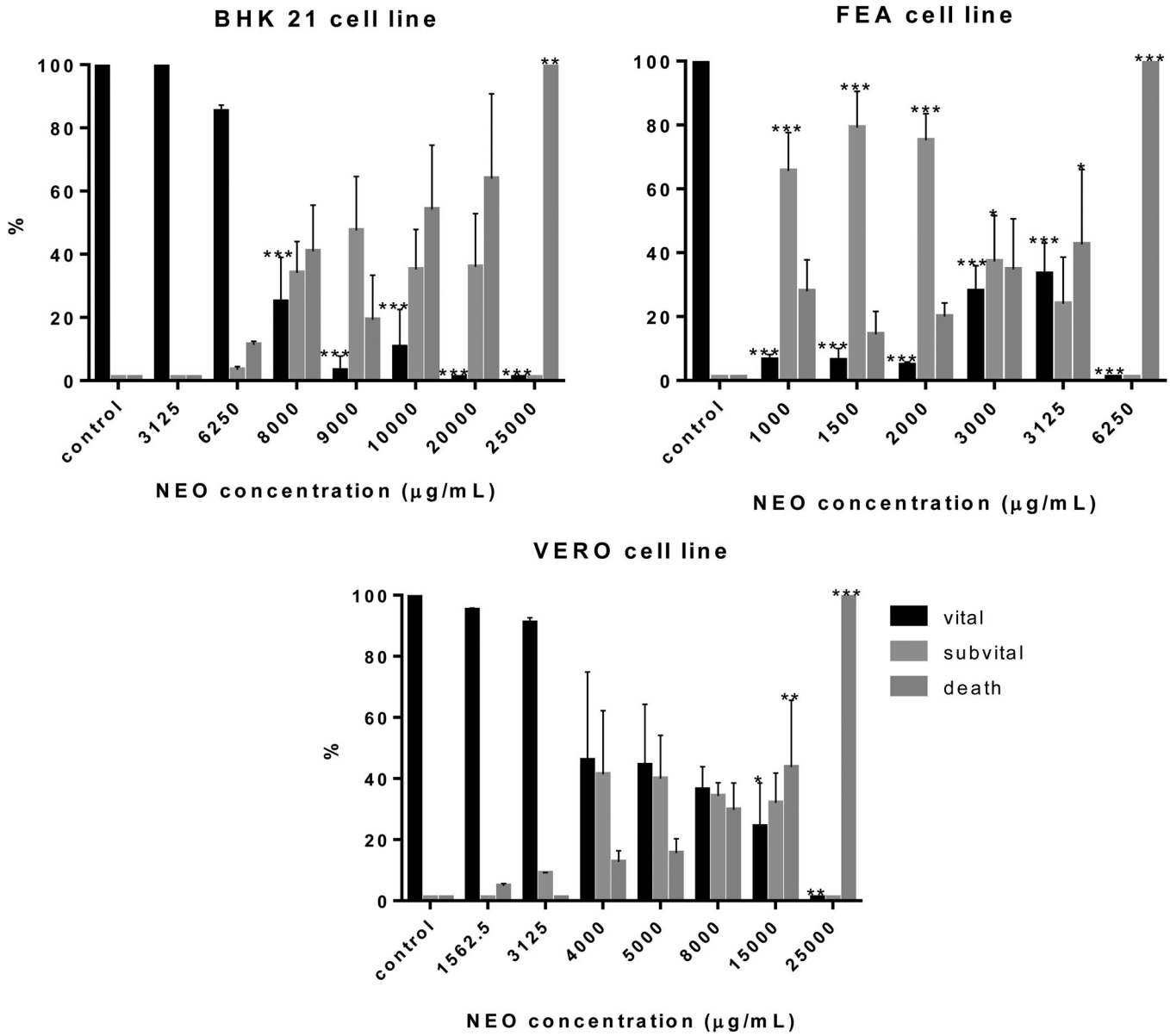

Figure 2. Cytomorphological changes analysis values – Neomycin (NEO). Each bar represents the mean vitality (± SD) % of control (untreated) and treated groups. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$) between control and experimental groups.

significant increase ($P < 0.01$ rep. $P < 0.001$) of dead cells occurred at higher concentrations (5500, resp. 7500 and 15000 µg/mL) of DHS. FEA cells were more sensitive to the added antibiotic than BHK 21 cells. Concentrations 3250 and 7500 µg/mL caused the death of all cells ($P < 0.001$). The cut-off concentration between the sub-lethal and lethal dose was 3000 µg/mL. High cell resistance was recorded in a VERO cell line. In this case, DHS concentrations of 16000 µg/mL killed or devitalized about 50% of the cells. At higher concentrations (17000 and 18000 µg/mL) a significant decrease ($P < 0.05$) of vital cells was recorded. Nearly 90% of the VERO cells were killed at 20000 µg/mL ($P < 0.001$).

Neomycin (NEO) concentrations (from 8000 to 20000 µg/mL) increased BHK 21 cells mortality significantly ($P < 0.001$), however lethal effect was observed at 25000 µg/mL (Figure 2). In a FEA cell line NEO caused toxicity in a dosage of less than eight times the BHK 21. Cell vitality was significantly ($P < 0.001$) reduced at concentration 1000 µg/mL. From 100% of vital cells in control, the percentage decreased to 6.43% at this concentration. Concentration 6250 µg/mL caused the death of all cells ($P < 0.001$). VERO cells were slightly affected (non-significant) by NEO at concentration 1562.5 µg/mL. The concentrations from 3125 to 8000 µg/mL reduced cell viability gradually (Figures 2 and 4). Significant decrease ($P < 0.05$) of vital cells was recorded at 15000 µg/mL. Total devitalization was caused by a dose of 25000 µg/mL ($P < 0.01$). Structural changes of selected cell lines after DHS and NEO administration are shown in Figures 3 and 4.

After the exposure to different experimental doses of dihydrostreptomycin (812.5-20000 µg/mL) and neomycin (1000-20000 µg/mL) during 24 h, the viability of BHK-21, FEA and VERO cell lines were evaluated by MTT assay (Figure 5). The viability of exposed cells was calculated based on intracellular succinate dehydrogenase activity, when 3500; 5500 and 7500 µg/mL of dihydrostreptomycin

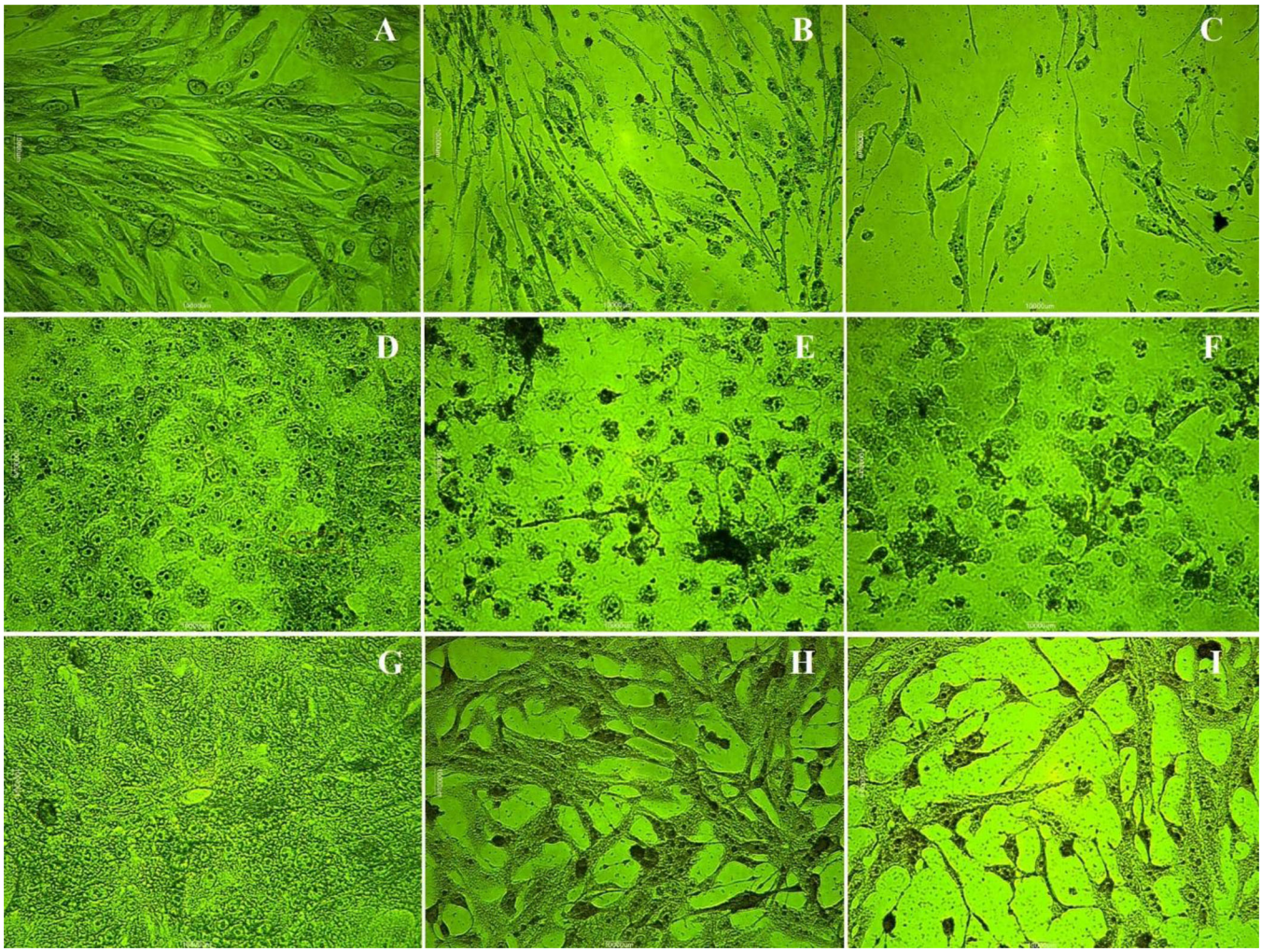

Figure 3. Structural changes of selected cell cultures after dihydrostreptomycin administration (magnification: 400 x). BHK21 (A – control; B – 2500 μg/mL; C – 5500 μg/mL), FEA (D – control; E – 2500 μg/mL; F – 3250 μg/mL), VERO (G – control; H – 16000 μg/mL; I – 20000 μg/mL).

significantly ($P < 0.001$) decreased this parameter. Significant ($P < 0.001$) reduction was also observed after neomycin treatment at 9000; 10000 and 20000 µg/mL in BHK-21 cells. The FEA cell viability did not change significantly when the concentrations of dihydrostreptomycin and neomycin were 2000 µg/mL and 1500 or 2000 µg/mL, respectively. Conversely, the cell viabilities decreased significantly ($P < 0.001$; $P < 0.01$) at 2500 and 3000 µg/mL dihydrostreptomycin and at 3000 µg/mL of neomycin treatment. The viability of VERO cells remained significantly unaltered at 17000 and 18000 µg/mL of dihydrostreptomycin and at 5000, 8000 and 15000 µg/mL neomycin. However, only the highest concentration of dihydrostreptomycin (20000 µg/mL) reduced cell viability significantly ($P < 0.01$).

Discussion

In our study, selected aminoglycoside antibiotics were tested, which are widely used in human and veterinary medicine.[3] Each antibiotic was used in sub toxic concentration (LD_{50} for bacteria) and multiple concentrations. Antibiotic solution was poured onto the monolayer and the cells were observed in exact time intervals (24 hours). If there were no changes after this time, we considered the concentration to be non-toxic. The changes we have observed were degenerative changes in culture cells, a cytopathic effect. These changes can range from local to complete tissue destruction. The acute toxicity observed in the LD50 and the symptoms of substance action are often used to characterize chemicals and classify them in toxicological categories.[11] Cytotoxic effect of the other group of antibiotics - macrolides and aminoglycoside gentamicin on mammalian cell lines was confirmed in our previous studies.[12–14] Mingeot-Leclercq and Tulkens[15] and El Mouedden et al.[16] reported that aminoglycosides are capable of inducing apoptosis that is associated with lysosomal phospholipidosis. Apoptosis was induced by gentamicin added to the medium to LLC-PK1 cells at 2 µM for 2 days. Neomycin has a similar mechanism of action like gentamicin. To date, the majority of studies of neomycin have focused on aquatic or terrestrial animals or on its germicidal activity *in vivo* and *in vitro*. The most of them cannot confirmed mutagenicity, carcinogenicity and teratogenicity, confirming its unlikely genotoxic potential.[17] However, the ototoxicity of neomycin is a significant obstacle to their wider clinical application. The generation of

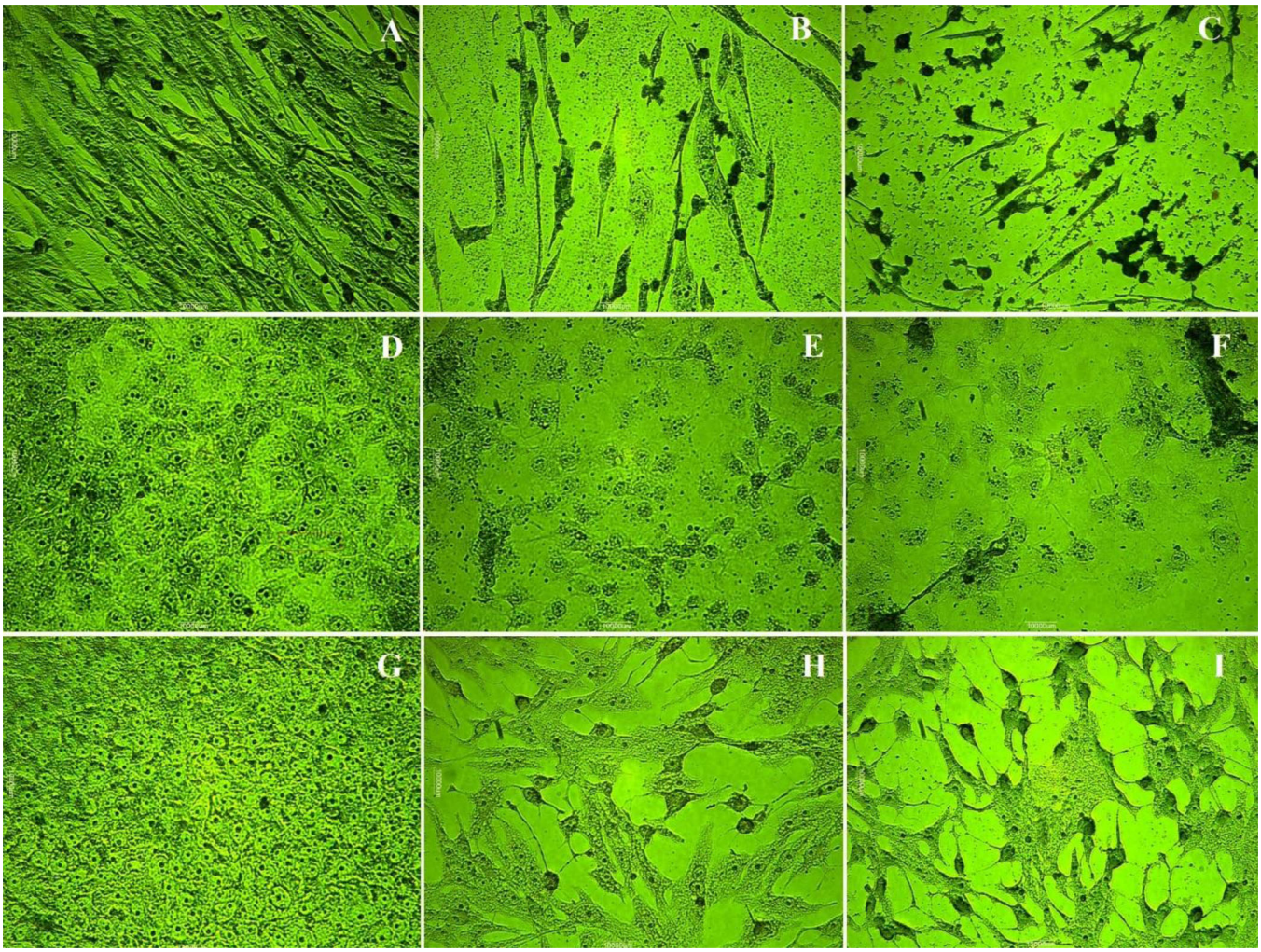

Figure 4. Structural changes of selected cell cultures after neomycin administration (magnification: 400 x). BHK21 (A – control; B – 9000 μg/mL; C – 20000 μg/mL), FEA (D – control; E – 1500 μg/mL; F – 3000 μg/mL), VERO (G – control; H – 4000 μg/mL; I – 8000 μg/mL).

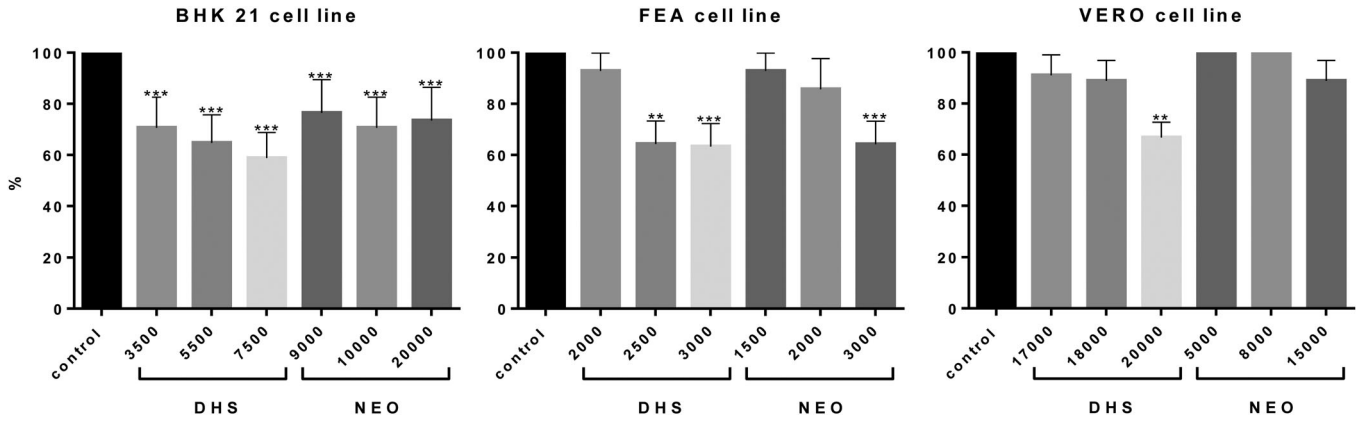

Figure 5. The potential impact of DHS and NEO on the cell viability *in vitro*. Each bar represents the mean (± SD) viability (%) of control (untreated) and treated groups. The level of significance was set at *** ($P < 0.001$) and ** ($P < 0.01$) between control and experimental groups.

reactive oxygen species (ROS) is presumed to be a principal mechanism responsible for ototoxicity. The increasing level of ROS overwhelms the redox status and skews cell metabolism toward the activation of intrinsic apoptosis, which is regulated by the combined actions of pro- and anti-apoptotic members of the Bcl-2 family.[18] Neomycin ototoxicity[1], was also evaluated as higher than gentamicin, dihydrostreptomycin, amikacin, neamine and spectinomycin effect. Ototoxicity has been reported after doses of 30-300 mg/kg daily subcutaneously in mice, 10-60 mg/kg daily subcutaneously in guinea pigs and 24-96 mg/kg daily intramuscularly in dogs.[19] Nowadays, the production and application of streptomycin are decreasing worldwide, while dihydrostreptomycin production gradually increased. It is

attributed to higher stability than streptomycin, almost similar biological activity, and lower toxicity.[20] Allergic reactions to streptomycin have been observed after administration of antibiotic contaminated feed or by intramammary infusion in cattle.[21,22] Van Brujinsvoort et al.[23] confirmed, that DHS tend to penetrate the milk of treated cows and, at increased concentration, negatively affects starter cultures in the dairy industry. In addition, a large proportion of the honeys of foreign origin tested in this study contained dihydrostreptomycin. The European commission have established maximum residue limits for DHS in cattle, sheep, pigs and poultry for muscle (500 µg/kg), liver (500 µg/kg), kidney (1000 µg/kg), fat (500 µg/kg) and milk (200 µg/l). DHS have relatively low toxicity after oral administration to rodents (LD_{50} 9000-25000 mg/kg bw/day).[24] The zebrafish is emerging as a suitable model for genetic analysis of hair cell development and function both in vitro and in vivo. Olt et al.[25] evaluated the effect of 0.1- and 1-mM DHS on larval zebrafish hair cells. The membrane potential of exposed cells was significantly more hyperpolarized compared to the control, suggesting that small factions of transducer channels open at rest directly contributes to cell resting membrane potential in larval zebrafish. Marcotti et al.[26] studied the blocking mechanism of the mechano-electrical transducer channel by the dihydrostreptomycin in acutely dissected organs of Corti from CD-1 mice. Their study confirmed that the DHS rapidly and reversibly blocks the current flowing though mechano-electrical transducer channels in mouse cochlear outer hair cells. Aminoglycoside antibiotics such as DHS or NEO enter the inner ear by crossing the blood-labyrinth barrier through a yet not entirely clear mechanism. However, the inhibition of Kv7.4 channels has been proposed to contribute on the aminoglycoside antibiotics – induced outer hair cell degeneration. Although cochlear hair cells are the primary site of damage induced by mentioned antibiotics on the inner ear, the stria vascularis is also susceptible to this group of drugs.[27] In this work, the effect of antimicrobials was influenced by several factors. The monitored ATBs, despite the same origin, act on cells differently, especially in different concentrations. The compared cell lines responded differently to individual concentrations. Another factor influencing the effect of chemicals on eukaryotic cells is the presence of carriers and excipients. Because the active ingredients are not administered in pure form, but the final forms include excipients that can prolong or alleviate their effect.

Aminoglycosides still play a significant role in coping with serious infections. Their toxicity led to a relatively restrained use and, despite widespread resistance, remained active against many pathogens.[2] Aminoglycosides are valuable drugs for the symptomatic treatment of sepsis caused by gram-negative bacteria, for the management of severe infections caused by *Pseudomonas aeruginosa* and as a supportive agent for endocarditis.[7,28]

Conclusion

Detection of antibiotic toxicity to animal cells is of great importance in terms of bacterial resistance observations to antimicrobial agents. With increasing resistance, it is also possible to fight partly by increasing the dose of the drug administered to the treated organism. This increase is possible only to a certain concentration of the substance, since the toxic effect over the therapeutic one will prevail, which we have also shown in this work. When evaluating drugs, it is necessary to know the highest level of a substance that does not cause adverse reactions in order to compare it with MIC (minimum inhibitory concentration) and thus to determine whether the substance is active against pathogenic microorganisms and at the same time harmless to the treated animal or human. Despite their toxicity, aminoglycosides are useful and remain the first-choice drug in the treatment of serious infections.

Declaration of interest

The authors report no conflicts of interest.

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