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Research Article

In Vitro Assessment of Gentamicin Cytotoxicity on the Selected Mammalian Cell Line (Vero cells)

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Abstract
The aim of this study was to evaluate the *in vitro* cytotoxicity of different concentrations (500-7500 µg/mL) of gentamicin - GENT (aminoglycoside antibiotic) on the selected mammalian cell line (Vero - cell line from African green monkey kidney). Analysis of the cell morphological changes was microscopically evaluated (magnification x 400). Quantification of Ca, Mg and total proteins was performed using spectrophotometry on device Rx Monza (Randox). Quantification of Na, K and Cl was performed on the automatic analyzer EasyLyte. The cell viability was assessed using the metabolic mitochondrial MTT test. Vero cells were able to survive at concentrations of 500 (89.21 %), 1000 (79.54 %) and 2000 µg/mL (34.59 %). We observed statistically significant decrease of vital cell content at concentrations of 2000, 4500, 7500 µg/mL against control group. Vero cell line slightly reacted to the presence of GENT but total proteins and mineral parameters were not significantly affected. Vero cells were highly sensitive to GENT with a significant decrease of viability at concentrations of 2000 and 4500 µg/mL ($P < 0.001$). Our data reveal that GENT has a significant cytotoxic and adverse effect on the cell viability.

Keywords: cytotoxicity, gentamicin, mitochondrial activity, Vero cell line.

Introduction

Aminoglycoside antibiotics were discovered in the middle of last century. Their antimicrobial effects stimulated their large usage in medicine. Aminoglycoside antibiotics (the most commonly used antibiotics worldwide) are effective against gram-negative bacterial infections [1, 2].

Gentamicin (GENT) belongs to the aminoglycoside class of bactericidal antibiotic. Gentamicin is a mixture of C_1, C_{1a}, and the enantiomers C_2 and C_{2a} elaborated by *Micromonospora* spp. with glycosidic linkages at positions 4 and 6 [3] and is active against a broad range of bacterial infections, especially Gram-negative bacteria including *Pseudomonas*, *Proteus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Serratia*, and *Staphylococcus* [1, 4, 5].

The frequent use of gentamicin, not only in medicine, but also in agriculture and veterinary medicine had proved ototoxic [6] and nephrotoxic side effect [7]. Gentamicin-induced nephrotoxicity mainly includes renal inflammatory cascades, high renal oxidative stress, associated pathological signaling mechanisms and renal dysfunction [2]. Also, are known the possible cytotoxic [8, 9, 10] and hepatotoxic [11] effects of gentamicin.

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These effects might be due to differences of gentamicin concentrations. However, there is still a lack of data about the aminoglycosides effect on cell morphology in *in vitro* studies [12].
For this reason, the goal of our study was to evaluate the in vitro cytotoxicity of different concentrations of gentamicin on the selected mammalian cell culture (Vero - cell line from African green monkey kidney).

Material and Methods

In our experiments, we used Vero cell line (cell line from African green monkey kidney). Cell line was obtained from cellular collections of the Department of Bio Preparations, Institute for State Control of Veterinary Biopreparations and Medicines in Nitra (Slovak Republic). Cells were revived according to appropriate protocols (ŠPP ÚŠKVBL Nitra 007) optimized for our laboratories. Cells were transferred into the sterile Roux flasks (DMEM/F12 supplemented with 20% FCS, non-essential amino acids, glutamine, LIF, fibroblast growth factor-2, beta-mercaptoethanol and antibiotics for Vero cells) following revival and cultivated at 37°C. After 24 hours was determined cell density in the monoculture. Cell suspension was prepared cell dilution using Fetal Bovine Serum enriched culture medium. Obtained cell suspensions were transferred into 48 well plates with 500 µl per well. After incubation in Fetal Bovine Serum enriched culture media, the cells were microscopically observed. When a single-layer was coherent, the medium was removed and prepared gentamicin was applied on cells [13, 14].
For testing of Vero cells, we chose gentamicin-GENT (Intervet, MSD Animal Health, South Africa). The concentrations, used in our study, were obtained on the basis of knowledge for the minimum inhibitory concentrations of GENT effect on bacteria and LD50 for laboratory animals. These contents of gentamicin are nontoxic for eukaryotic cells, for that we raised them 1000-times. Then they were modified to concentration, which is toxic (LD100). These concentrations were used as zero dilution, titration continued with a decimal dilution. Chosen concentrations of GENT used in our experiment are showed in Table 1. The final cell culture was cultured for 24h [13, 14]. Concentrations of GENT for all analyses were selected for monitoring its increasing effect.
After application of different concentrations of GENT, we microscopically controlled the condition of cells in the plates (magnification x 400). We observed the structural changes of cells and we assess the quantity of vital, subvital and dead cells [13, 15].

Table 1
Concentrations of gentamicin used for the analyses

Cell vitality	Minerals and TP	Viability
Concentrations of GENT (µg/mL)		
0*	0*	0*
1000	650	1000
2000	1500	2000
4500	4500	4500
7500		

*Control

After 24 hours exposure of selected cells to GENT, cultivating medium was collected for future analyses in micro tubes to -20 °C. Frozen medium was used for total proteins and mineral profile analyses for the purpose of determination of possible antibiotic effect on cell metabolism. Quantification of Calcium (Ca), Magnesium (Mg) and total proteins (TP) was performed using spectrophotometry. Analyses were realized in the biochemical and hematological laboratory from the Department of Animal Physiology of Slovak University of Agriculture in Nitra using commercial sets DiaSys (Diagnostic Systems GmbH, Germany) on semi-automatic analyzer Rx Monza (Randox Laboratories Ltd., United Kingdom). Quantification of Sodium (Na), Potassium (K) and Chloride (Cl) was performed by the automatic analyzer EasyLyte (Medica, Bedford, USA) [16].
Viability of the cells exposed to GENT in vitro was tested using the metabolic activity (MTT) assay [17]. This colorimetric assay measures the conversion of 3- (4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The resulting formazan was measured spectrophotometrically at wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, Thermo Fisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to the antibiotic) [14]. MTT viability test was repeated three times for each antibiotic concentration.
To test for differences between the control and experimental groups was used one-way analysis of variance (ANOVA), with the Scheffe's test. The level of significance was set at ***($P<0.001$); **($P<0.01$); *($P<0.05$).

Results and Discussions

A dose-dependent toxic effect of GENT on cell viability was observed after administration to Vero

cell cultures. The selected cell line exhibited the statistical significant (p < 0.001) degree of susceptibility to the antibiotic at 2000, 4500 and 7500 µg/mL concentrations. On the other hand, Vero cells were resistant to the GENT at 1000 µg/mL concentration (Figure 1). Microscopic observation of the cell viability of Vero cell cultures is shown in Figure 2.

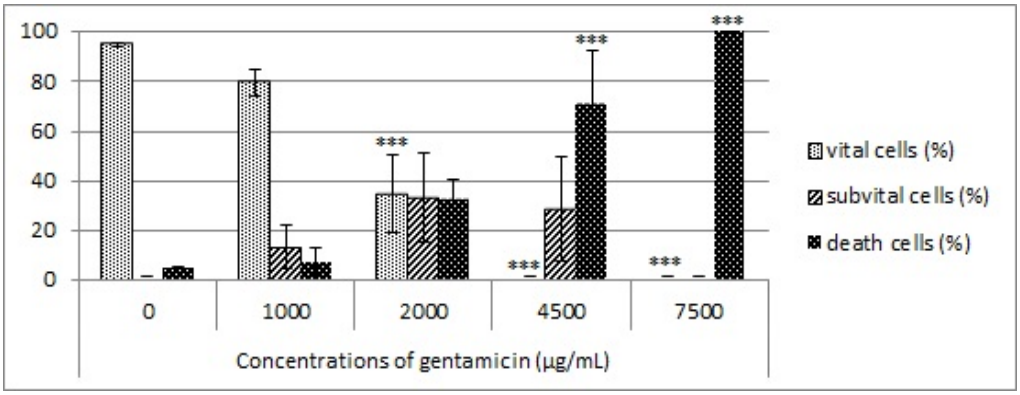

Figure 1. Cell viability (%) evaluation of Vero cell after GENT administration versus control, *(P<0.001); **(P<0.01); *(P<0.05).**

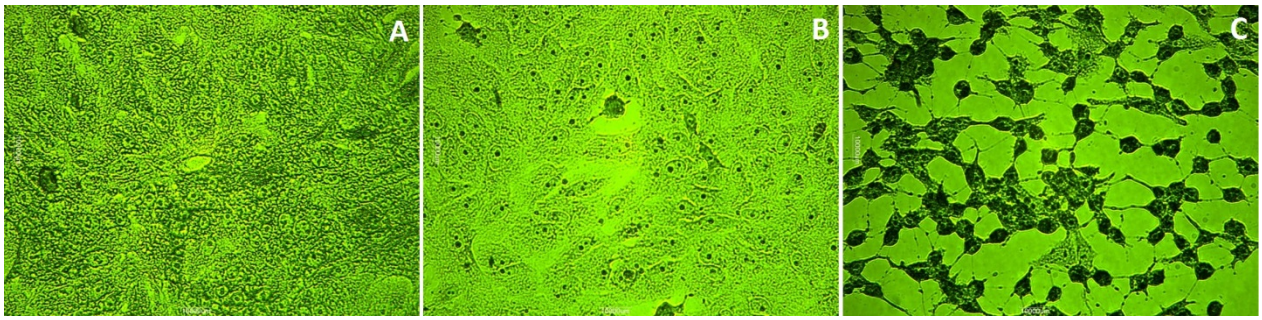

Figure 2. Phase contrast images of Vero cells; A) 0 µg/mL of GENT (control); B) Vero cells after exposure to GENT (1000 µg/mL); and C) Vero cells after exposure to GENT (4500 µg/mL) for 24 hours (magnification x 400)

Although Vero cell lines reacted to the presence of GENT, total proteins content and mineral parameters in the medium were not significantly affected (Figure 3). Yu et al. [18] tested gentamicin on vestibular hair cells (VHCs II) and their findings indicated that increasing of Ca^{2+} could antagonize GENT blocking effect; also, GENT may block the dependent K^{+} channels by impairing calcium influx.

Dose-responses of the gentamicin measured by the MTT test are shown in Figure 4. Gentamicin supplementation revealed to have toxic effects on the Vero cell line. Vero cells exhibited high sensitivity to the antibiotic, as GENT concentrations were higher than 2000 µg/mL and led to a significant decrease (P<0.001) of the mitochondrial activity.

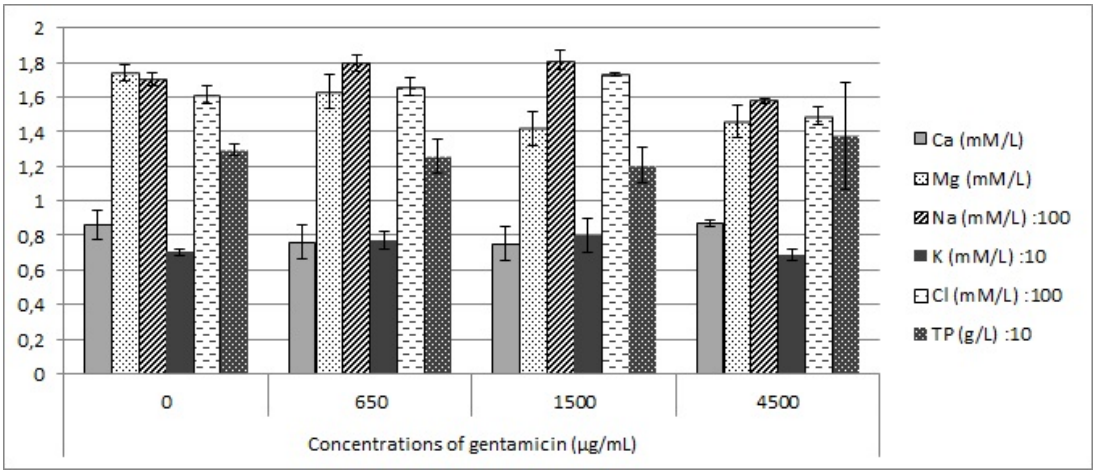

Figure 3. Levels of total proteins and mineral parameters in the medium after GENT application

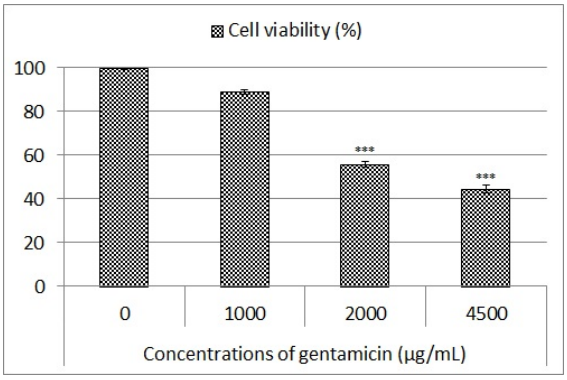

Figure 4. The effect of gentamicin (GENT) on the viability of selected mammalian cells; *(P<0.001); **(P<0.01); *(P<0.05)**

The members of the aminoglycoside antibiotics have the possible impact for nephrotoxicity, ototoxicity, and, infrequent, neuromuscular blockade. The risk of toxicity may be decreased as mechanisms are understood, new dosage strategies introduced, concomitant risk factors avoided, and shorter drug courses used [3].

Aminoglycosides, principally gentamicin induces mesangial cell contraction and proliferation. Simultaneously with the proliferative effect, gentamicin induces mesangial cell apoptosis in renal glomeruli and cultured mesangial cells [1]. Mechanisms of gentamicin nephrotoxicity include several ways: Gentamicin enhances the production of hydroxyradicals [19]; increases in the renal cortical phospholipidosis [20]; gentamicin inhibits Na^+-K^+-ATPase in renal tubule cells [21]; thromboxane A2 (TXA_2) and prostaglandins (PGE) increasing [22]; effect on renal protein synthesis [23]; lysosomal [24] and mitochondrial injury [25]. Combination of these effects could induce acute renal failure [26]. Gentamicin toxicity also induces intracellular oxidative stress [27].

In our previous studies [13, 14, 16] we tested the effect of macrolide antibiotics (tilmicosin, tylosin and spiramycin) on the selected mammalian cell lines (BHK 21, FE and Vero) in vitro. All tested antibiotics showed negative changes in the cell viability and cytomorphology. However, similar effect has been reached at lower concentration of antibiotics in the comparison with gentamicin.

Rathbone et al. [28] tested aminoglycosides (amikacin, tobramycin, and gentamicin), tetracyclines (minocycline, doxycycline) carbapenem, glycopeptide, penicillin and other antibiotics effect on osteogenic cell viability and *in vitro* activity. Human osteoblasts reacted very differently on the antibiotic dose and type. The antibiotics with the highest inhibition included rifampin, tetracyclines and ciprofloxacin, where >75% decrements in cell number were measured at 100 µg/mL. On the other hand, human osteoblast cells showed decline of cell number (>75%) after higher doses of gentamicin (2000 µg/mL), which corresponds with our results.

In vivo study [29] presented kidney injury in rats (cell shrinkage and cytoplasm eosinophilia) after

10 days with 10 mg of gentamicin per kg, where, however was not confirmed correlation between aminoglycoside and induced tubular apoptosis and cortical proliferative with phospholipidosis.

Conclusions

Many factors affect the effect of antimicrobial substances. Observed aminoglycoside (gentamicin) affects cells in different ways. Compared cellular lines reacted differently to each concentration. Another factor affecting chemical substance effect on eukaryotic cells is presence of carriers and excipients. Biologically active substances don't enter the body in clean form, but as a part of medical formula which is extended with adjuvants, which can extend or reduce the ultimate effect of active substance.

Acquired knowledge might be possibly applied in toxicity evaluation of pharmacological effective substances in vitro. Eukaryotic cells respond to chemical substances sensitively under in vitro conditions, therefore these techniques could replace in vivo examinations, which require great numbers of experimental animals.

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