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INFLUENCE OF GENTAMICIN ON THE SPECIFIC CELL CULTURE (BHK-21) *IN VITRO*

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ABSTRACT

Gentamicin (GENT) is an aminoglycoside antibiotic commonly used against Gram-negative bacterial infections. GENT is probably the most commonly used antibiotic of all aminoglycosides. The aim of our study was to evaluate the *in vitro* toxicity of different concentrations of GENT on selected mammalian cell culture (BHK-21 – baby hamster kidney cells). After application of various concentrations of GENT, we controlled the condition of cells in the wells microscopically (magnification x 400). Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells. Cell medium was used for biochemical analyses (Calcium - Ca, Magnesium – Mg, total proteins - TP, Sodium - Na, Potassium - K and Chloride – Cl). Viability of the cells exposed to selected antibiotic *in vitro* was evaluated using the metabolic activity (MTT) assay. BHK-21 cells were able to survive at a concentration 187.5; 500; 1500; 4500 µg/mL. We found statistically significant decrease ($P<0.001$) of vital cells in comparison with control in all concentrations of GENT higher than 500 µg/mL. We also found significant increase in the number of subvital and death cells compared to control group in all concentrations of GENT higher than 500 µg/mL. Biochemical parameters observed in the medium were significantly affected in all concentrations of GENT. Content of Na^+ and Cl^- was the most importantly affected in all observed groups against control group ($P<0.001$). A statistically significant decrease of Ca ($P<0.01$) was detected (control vs 937.5 µg/mL resp. 7500 µg/mL of GENT). The mitochondrial activity of the BHK21 cells was significantly ($P<0.001$) decreased after the administration of all concentrations of GENT when compared to the Control. In conclusion, the exposure of Baby Hamster Kidney fibroblasts (BHK-21) to gentamicin at our concentrations resulted in severe cell damage. Acquired knowledge is possible to apply in toxicity evaluation of pharmacological effective substances in vitro.

Keywords: Gentamicin, BHK21, cell morphology, biochemistry, mitochondrial activity

INTRODUCTION

Fifty years of experience with aminoglycoside antibiotics has confirmed their usefulness in many infections with gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp. as well as *Staphylococcus aureus* and some streptococci (**Vakulenko and Mobashery, 2003**). The increased knowledge about molecular structure, pharmacology and pharmacokinetics has resulted in reduced risks for severe toxic damage in kidneys (nephrotoxicity) (**Mingeot-Leclercq and Tulkens, 1999; Khan *et al.*, 2009; Com *et al.*, 2012**) and in the ear (ototoxicity) (**Wersäll, 1995; Garetz and Schacht, 1996; Forge and Schacht, 2000; Selimoglu, 2007; O´neil, 2008**). Despite their toxicity, aminoglycosides play an increasingly important role in the management of serious infections. Their toxicity has led to comparatively restrained usage, but they remained effective against many pathogens. Aminoglycosides are valuable drugs for symptomatic treatment of gram-negative sepsis, for the management of serious infections caused by *Pseudomonas aeruginosa*, and as agents in the treatment of endocarditis (**Turnidge, 2003**).

Gentamicin is an aminoglycoside antibiotic commonly used against Gram-negative bacterial infections (**Priuska and Schacht, 1995; Rao *et al.*, 2006**). Gentamicin (GENT) is probably the most commonly used antibiotic of all aminoglycosides (**Balakumar *et al.* 2010**). GENT is administered for meningitis, pneumonia, Pseudomonas infections, septicemia, E. coli infections, Staphylococcus infections, listeria, tularemia, brucellosis, endocarditis, respiratory tract infections, urinary tract infections, bone infections, cystic fibrosis, diverticulitis, neutropenia, and sepsis and necrotizing entercolitis in newborns, for peritonitis, topical treatment for burns and skin infections, opthamalic drops for eye infections, intratympanic injection for Meniere's disease (**Xie *et al.*, 2011**). Numerous studies documented cytotoxicity of GENT (**Cuppage *et al.*, 1977; Spiegel *et al.*, 1990; Crann *et al.*, 1992; Dehne *et al.*, 2002; Chung *et al.*, 2006**). The aim of our study was to evaluate the *in vitro* toxicity of different concentrations of GENT on selected mammalian cell culture (BHK-21 – baby hamster kidney cells).

MATERIAL AND METHODS

Cell culture

In our experiment we used BHK-21 (Baby Hamster Kidney fibroblasts) cell line stored at the Department of Bio Preparations, Institute for State Control of Veterinary Bio preparations and Medicines in Nitra. Cells were revived according to relevant protocols. 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 FE cells) following revival and cultivated at the 37°C. After 24 hours, the monoculture assessed and cell density was determined. Cell suspension was prepared by dilution of the cells using FBS enriched culture medium. Prepared suspensions were transferred into 48 well plates at 500 µl per well. After further incubation in FBS enriched culture media, the cells were assessed microscopically. When a single-layer was coherent, the medium was discarded and freshly prepared antibiotics were layered on cells (**Fülöpová *et al.*, 2012; Tvrdá *et al.*, 2016**).

Antibiotic

For testing of BHK-21 cells we chose gentamicin-GENT (Intervet, MSD Animal Health, South Africa). Concentrations, used in our experiment, were obtained on the basis of knowledge of the minimum inhibitory concentrations of gentamicin effect on bacteria and LD50 for laboratory animals. These concentrations are non-toxic for eukaryotic cells therefore we raised them 1000-times. Consequently, they were modified to concentration, which is toxic for all cells (LD100). These concentrations were used as zero dilution, titration continued with a decimal dilution. Selected concentrations used in our experiment are displayed in Table 1 (**Fülöpová *et al.*, 2012; Tvrdá *et al.*, 2016**).

Table 1 Concentrations of gentamicin used for the BHK21 cell line experiments

	Cytomorphology	Biochemistry	Viability Test
Cell culture	**Concentrations (µg/mL) of gentamicin (GENT)**		
BHK-21	0; 187.5; 500; 1500; 4500; 7500	0; 937.5; 1875; 3750; 7500	0; 1500; 4500; 6500

Cell morphology

After application of various concentrations of GENT, we controlled the condition of cells in the wells microscopically (magnification x 400). Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells (**Fülöpová *et al.*, 2012**).

Biochemical test

After 24 hours exposure of selected cells to GENT, cultivating medium was drained out by pipette and frozen in micro tubes to -20 °C. Frozen medium was used for biochemical 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 photometry. Analyses were realized in the biochemical and hematological laboratory at the Department of Animal Physiology of SUA 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) (**Kováčik *et al.*, 2012**).

Cell viability (MTT)

Viability of the cells exposed to selected antibiotic *in vitro* was evaluated using the metabolic activity (MTT) assay (**Tvrdá *et al.*, 2015**). 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 can be measured spectrophotometrically at a measuring 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) (**Tvrdá *et al.*, 2016**). Results from the analysis were collected during three repeated experiments at each concentration.

Statistical methods

The significance of differences between the control and experimental groups was evaluated by one-way analysis of variance (ANOVA), with the Scheffe's test. The level of significance for the comparative as well as correlation analysis was set at ***(P<0.001); **(P<0.01); *(P<0.05).

RESULTS AND DISCUSSION

Morphology and survival of the BHK-21 cell line were affected by the concentration higher than 500 µg/mL of GENT. Number of subvital and death cells were directly proportional to elevation of the gentamicin content in the culture medium. We recorded a lethal dose for all cells in the medium with the highest content of GENT (7500 µg/mL). Analysis of morphological changes of BHK-21 cells is shown in Figure 1.

Figure 1 Changes of BHK-21 cell morphology after exposure to gentamicin. Concentrations of gentamicin: A) 0 µg/mL (control); B) 1500 µg/mL; C) 4500 µg/mL (magnification x 400)

BHK-21 cells were able to survive at a concentration 187.5; 500; 1500; 4500 µg/mL. We found statistically significant decrease (P<0.001) of vital cells in comparison with control in all concentrations of GENT higher than 500 µg/mL. We also found significant increase in the number of subvital and death cells compared to control group in all concentrations of GENT higher than 500 µg/mL (Figure 2).

Biochemical parameters observed in the medium were significantly affected in all concentrations of GENT. Content of Na^+ and Cl^- was the most importantly affected in all observed groups against control group (P<0.001). A statistically significant decrease of Ca (P<0.01) was detected (control vs 937.5 µg/mL resp. 7500 µg/mL of GENT) (Figure 3).

The mitochondrial activity of the BHK21 cells was significantly (P<0.001) decreased after the administration of all concentrations of GENT when compared to the Control (Figure 4).

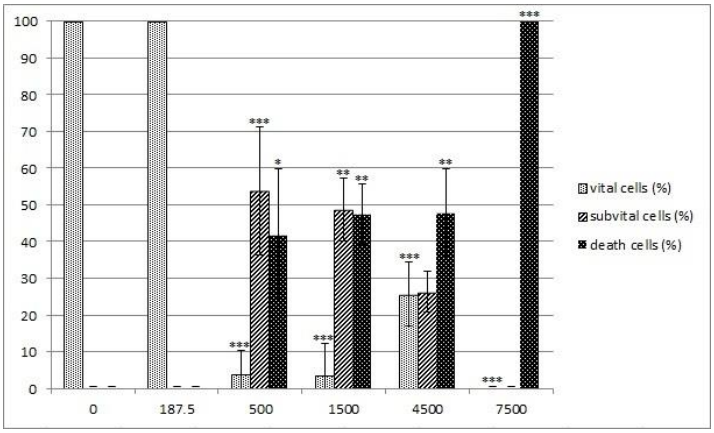

Figure 2 Values (%) of BHK-21 cell morphological changes after GENT application (GENT concentrations: 187.5; 500; 1500; 4500; 7500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

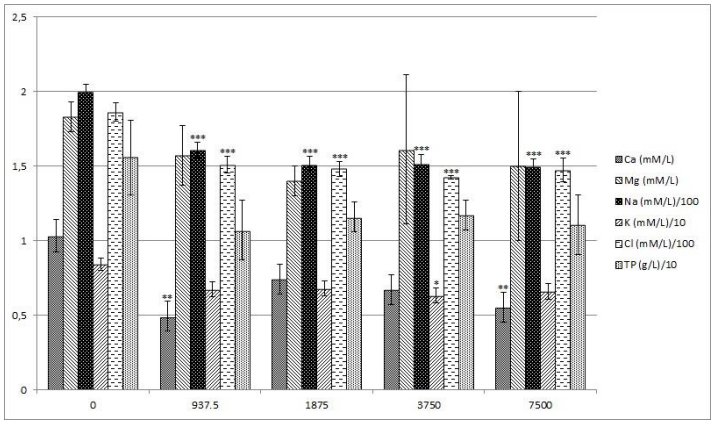

Figure 3 Biochemistry parameters levels in medium after GENT application (GENT concentrations: 937.5; 1875; 3750; 7500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

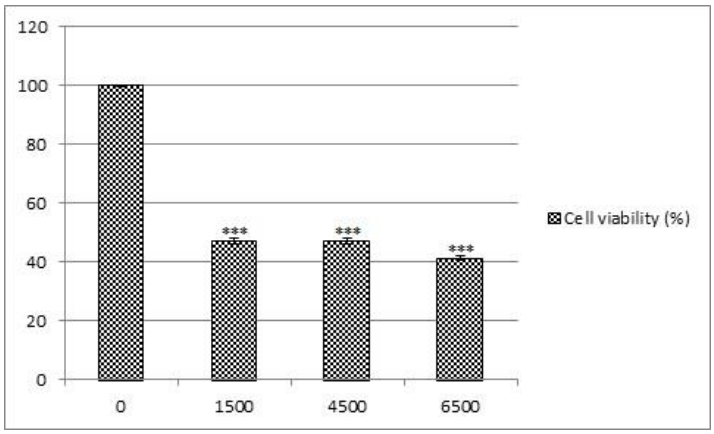

Figure 4 Effect of gentamicin on the viability of BHK-21 cells (MTT test) (GENT concentrations: 1500; 4500; 6500 µg/mL) against control (GENT concentration: 0 µg/mL)
***(P<0.001); **(P<0.01); *(P<0.05).

Aminoglycoside antibiotics are substances with relatively narrow spectrum of activity. Antibacterial activity of aminoglycoside antibiotics depends on their effective concentration in extracellular space. Nephrotoxicity induced by aminoglycosides manifests clinically as renal failure (**Mingeot-Leclercq and Tulkens, 1999**). GENT has been tested as a typical model for the study of nephrotoxicity (**Cuppage *et al.*, 1977; Mondorf *et al.*, 1978**). There are a few data in the literature about the effect of the gentamicin and other aminoglycosides on the cell lines metabolic activity (**Ford *et al.*, 1994; Yagi *et al.*, 1999; El Mouedden *et al.*, 2000; Duewelhenke *et al.*, 2007)**. We demonstrated that GENT in high concentrations may be cytotoxic for Baby Hamster Kidney cells (BHK-21). The MTT assay provided information about the overall metabolic activity (**Berridge *et al.*, 2004**).
Yu *et al.* (2014) tested GENT on vestibular hair cells (VHCs II) and their findings indicated that increasing of Ca^{2+} could antagonize gentamicin blocking effect; also gentamicin may block the dependent K^+ channels by impairing calcium influx. The effect of GENT to organisms and cell lines have been claimed – some studies have reported negative significant effects (**Isefuku *et al.*, 2003**), whereas other studies have not (**Duewelhenke *et al.*, 2007**).
In previous studies (**Fülöpová *et al.*, 2012; Kováčik *et al.*, 2012; Tvrdá *et al.*, 2016**), the effect of macrolide antibiotics (tilmicosin, tylosin and spiramycin) was tested on the specific mammalian cell lines (BHK 21, FE, VERO) *in vitro*. Effects of these antibiotics have a similar tendency for all measured parameters as GENT, but at lower concentrations (150 µg/mL; 500 µg/mL).
El Mouedden *et al.* (2000) tested exposure of GENT to three cell types (Embryonic Rat Fibroblasts, MDCK and LLC-PK_1 cells) and confirmed intrinsic capability of inducing apoptosis in cells after systematic administration. The murine C2C12 cells cultured with different concentrations of gentamicin (12.5 - 800 µg/ml) for 48 days showed negative changes in cell viability and alkaline phosphatase activity, although the cell number showed no significant changes (**Ince *et al.*, 2006**).

CONCLUSION

Aminoglycoside antibiotics were discovered in the middle of the bygone century. Their antimicrobial activity found wide use in humane and veterinary medicine. Their use was markedly limited after determination of toxicity on vestibular and glomerular apparatus.
In conclusion, the exposure of Baby Hamster Kidney fibroblasts (BHK-21) to gentamicin at our concentrations resulted in severe cell damage. The cytotoxicity of antimicrobial agents evaluated in mammalian cell cultures enables us to provide better understanding to their specific *in vitro* and *in vivo* properties. These results raise questions as to the feasibility of using gentamicin. Acquired knowledge is possible to apply in toxicity evaluation of pharmacologically effective substances *in vitro*. In this regard we must be aware that any biologically active substance, antibiotics, toxicants, heavy metals, natural extracts behave differently in *in vivo* experiments in comparison to *in vitro* conditions.

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