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 vivo 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 detectable (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 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 monolulture assessed and cell density was determined. Cell suspension was prepared by dilution of the cells using FBS enriched culture media. 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 (Fűlőpová et al., 2012; Tvrďá 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 (Fülopová et al., 2012; Tvrđá et al., 2016).

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Cytomorphology</th>
<th>Concentrations (µg/mL) of gentamicin (GENT)</th>
<th>Biochemistry</th>
<th>Viability Test</th>
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<tbody>
<tr>
<td>BHK-21</td>
<td></td>
<td>0; 187.5; 500; 1500; 4500; 7500</td>
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<td>0; 1500; 4500; 6500</td>
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Table 1 Concentrations of gentamicin used for the BHK21 cell line experiments

Cell morphology

After application of various concentrations of GENT, we controlled the condition of cells in the wells macroscopically (magnification x 400). Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells (Fülopová 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 (Tvrđá et al., 2015). This colorimetric assay measures the conversion of 3-(4,5-dimethylthiazol-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) (Tvrđá 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 Ca2+ (P<0.001) 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).
Aminoglycoside antibiotics are substances with relatively narrow spectrum of activity. Antibacterial activity of aminoglycoside antibiotics depends on their specific effect 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 Ca²⁺ 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 (Isefu et al., 2003), whereas other studies have not (Duewelhenke et al., 2007).

In previous studies (Fášlová et al., 2012; Kováčik et al., 2012; Tvrđí et al., 2016), the effect of macroide 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-PK1 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).

**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).

**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).

CONCLUSION

Aminoglycoside antibiotics were discovered in the middle of the hygone century. Their antimicrobial activity found wide use in humane and veterinary medicine. Their use was markedly limited after determination of toxicity on vestibular and glomernular 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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