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REGULAR ARTICLE

EFFECT OF MACROLIDE ANTIBIOTICS ON VARIOUS CELL CULTURES *IN VITRO*: 1. CELL MORPHOLOGY

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ABSTRACT

The aim of our study was to evaluate the cytotoxicity of macrolide antibiotics (tilmicosin, tylosin and spiramycin) of various concentrations on different cell cultures *in vitro*. Cellular lines from animal tissues (VERO cells - kidney cells of *Macacus rhesus*, FE cells - feline embryonal cells, BHK 21 cellular line from young hamster kidneys) were used. Tilmicosin effect: BHK cells are most sensitive, significant decrease in vital cells occurs already at the concentration of 50 $\mu g.ml^{-1}$. VERO cells were most resistant, significant decrease of vital cells was observed only at the concentration of 300 $\mu g.ml^{-1}$. Tylosin effect: BHK cells can be considered most sensitive, since at concentrations higher than 500 $\mu g.ml^{-1}$, no vital cells were observed. At the concentration of 1000 $\mu g.ml^{-1}$ were 3.13% of vital and 70.52% of subvital FE cells. In Vero cells, we observed a significant decrease at the concentration of 750 $\mu g.ml^{-1}$. Spiramycin effect: Significant decrease of vital BHK cells was observed at the concentration of 150 $\mu g.ml^{-1}$, at the concentration of 300 $\mu g.ml^{-1}$, no vital cells and only 7.53% of subvital cells were observed. At the concentration of 500 $\mu g.ml^{-1}$ reported 10.34% of vital FE cells. At the concentration of 500 $\mu g.ml^{-1}$ 22.48% of vital and 71.16% of subvital VERO cells were recorded.

Keywords: macrolides, cell cultures, tilmicosin, tylosin, spiramycin

INTRODUCTION

Antibiotics are chemical substances that are toxic to cells in different ways. Toxic effect of antibiotics in healthy eukaryotic cells is often dependent on their concentration, the dose that is injected into the body. Toxicity of antimicrobials may have many forms, varying from mild, short-term, the dramatic, life-threatening cases such as seizures or cardiac arrhythmias. There are five main mechanisms that may explain antimicrobial toxicity: direct effects, hypersensitivity, changes in microbial flora, drug interactions and microbial lysis **(Mandell et al., 2001)**. Using of antibiotics in animal husbandry caused resistant of pathogens to various commercial antibiotics **(Hleba et al., 2011).**

Macrolide antibiotics contain 14-, 15- and 16-membered-ring compounds. These can be subdivided into two major groups —natural products and semisynthetic derivatives **(Bryskier et al., 1999)**. The best known 14-membered-ring macrolide is erythromycin; josamycin and spiramycin are 16-membered-ring macrolides **(Kaiser, 2009)**.

There are different types of effects of subinhibitory concentrations (sub-MIC), which are becoming increasingly recognized as important factors of therapeutic effect **(Krist, 1997)**. They are inhibitors of protein synthesis. Their mechanism of action involves inhibition of bacterial protein synthesis by inhibiting peptidyltransferase to add peptidyl attached to tRNA to another amino acid **(Kaiser, 2009)**. This inhibition is caused by binding to the 50S ribosomal subunit. The binding is reversible, leading to accumulation of di-and tri-peptides in the cell that is able to synthesize the necessary protein and dies. Another potential mechanism of action is premature separation, dissociation of peptidyl-tRNA from the ribosome **(Tenson et al., 2003)**.

Tilmicosin is a semi-synthetic macrolide antibiotic synthesized by a chemical modification of a tylosin related compound and has been approved to control the causal agents of respiratory diseases in farm animals, including Gram-positive bacteria, mycoplasma, and some Gram-negative bacteria **(Shryock et al., 2002; Hunter et al., 2006)**.

Tylosin is an macrolide antibiotic, produced by *Streptomyces fradiae* **(McGuire et al., 1961; Suchodolski et al., 2009, Shimada et al., 2010)** used in veterinary medicine **(Bryskier et al., 1993)**, especially effective against most Gram-positive bacteria (staphylococci, streptococci, *Erysipelotrix rhusiophatiae*) against some Gram-negative bacteria (*Vibrio coli*, *Pasteurella multocida*, *Haemiphillus*) against various strains of pathogenic mycoplasmas and partly against *Treponema hyodysenteriae* **(Potter et al., 1985, Prescott et al. , 1993)**. It

inhibits bacterial protein synthesis in sensitive microorganisms **(Mazzei et al., 1993, Shimada et al., 2010)**.

Spiramycin is a medium-spectrum, macrolide antibiotic widely used in the treatment of respiratory infections **(Rubinstein and Keller, 1996)**. It is mainly produced by certain strains of *Streptomyces ambofaciens*. Spiramycin, a 16-membered macrolide, inhibits translocation by binding to bacterial 50S ribosomal subunits with an apparent 1:1 stoichiometry. This antibiotic is a potent inhibitor of the binding to the ribosome of both donor and acceptor substrates. Spiramycin induces rapid breakdown of polyribosomes, an effect which has formerly been interpreted as occurring by normal ribosomal run-off followed by an antibiotic-induced block at or shortly after initiation of a new peptide **(Brisson et al., 1988)**.

The aim of our study was to evaluate the cytotoxicity of macrolide antibiotics (tilmicosin, tylosin and spiramycin) on different cell cultures (BHK, FE and VERO) *in vitro*.

MATERIAL AND METHODS

In our experiment we used cellular lines BHK 21, FE and VERO stored in the liquid nitrogen in the department of bio preparations ISCVBM Nitra. Cells were revived according to ŠPP ISCVBM Nitra 007. After revival cells were transferred into the sterile Roux flasks and inserted into thermostat by the temperature 37°C. After 24 hours, the intensity of snapping the bottom and the cell multiplication was controlled. Cell density was determined. Suspension was gained by dilution of released cells and bovine fetal serum enriched culture medium.

Obtained suspension was pipetted in 48 well plate in the volume of 500 µl per well. Plates were inserted back to thermostat at temperature of 37°C. Cellular lines from animal tissues: VERO cells - kidney cells of *Macacus Rhesus*, FE cells - feline embryonal cells and BHK 21 cellular line from young hamster kidneys were used.

After incubation of cells in fetal serum enriched culture medium, the cells were checked microscopically. When the single-layer was coherent, medium was decanted and freshly prepared antibiotics were layered on cells.

We chose tilmicosin, tylosin and spiramycin (macrolide antibiotics), which are used in veterinary medicine. Concentrations, used in our experiment, were obtained on the basis of knowledge of the minimum inhibitory concentrations of tylosin effect on bacteria and LD_{50} for laboratory animals. These concentrations are non-toxic for eukaryotic cells, therefore we raised them 1000-fold. Then they were modified to concentration, which is toxic for all cells,

LD_{100}. These concentrations were used as zero dilution, titration continued with a decimal dilution.

Table 1 Concentrations of tilmicosin used for BHK, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK	50; 100; 200; 300; 500
FE	50; 75; 100; 200; 500
VERO	125; 250; 300; 350; 400; 450; 500;

Table 2 Concentrations of tylosin used for BHK, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK	500; 700; 900; 1000; 2000; 3750
FE	500; 700; 800; 900; 1000; 2000
VERO	250; 300; 400; 750; 900; 1000

Table 3 Concentrations of spiramycin used for BHK, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK	31,25; 62,5; 125; 150; 200; 300; 500; 1000
FE	62,5; 125; 150; 250; 350; 450; 500; 1000
VERO	62,5; 100; 200; 450; 500; 1000

Legend: BHK - cellular line from young hamster kidneys, FE - feline embryonal cells, VERO - kidney cells of *Macacus Rhesus*

After application of various concentrations of macrolides, we controlled the condition of cells in the wells microscopically at a magnification of 25x and 40x. Based on the structure of cells, we evaluated the presence of vital, subvital and dead cells. For comparison of results, Scheffe´s and Student´s t-test were used.

RESULTS AND DISCUSSION

We observed the status of cells (Figure 1) via microscopic monitoring of cytotoxicity (morphology), where it is possible to confirm substantial deformation, shape changes and cell destruction by higher concentration of macrolides. Microscopic control of cell viability was done after 48 hours. Such prolonged exposure to any antibiotics killed all cells, therefore data collected after 48 hour incubation were not been processed statistically.

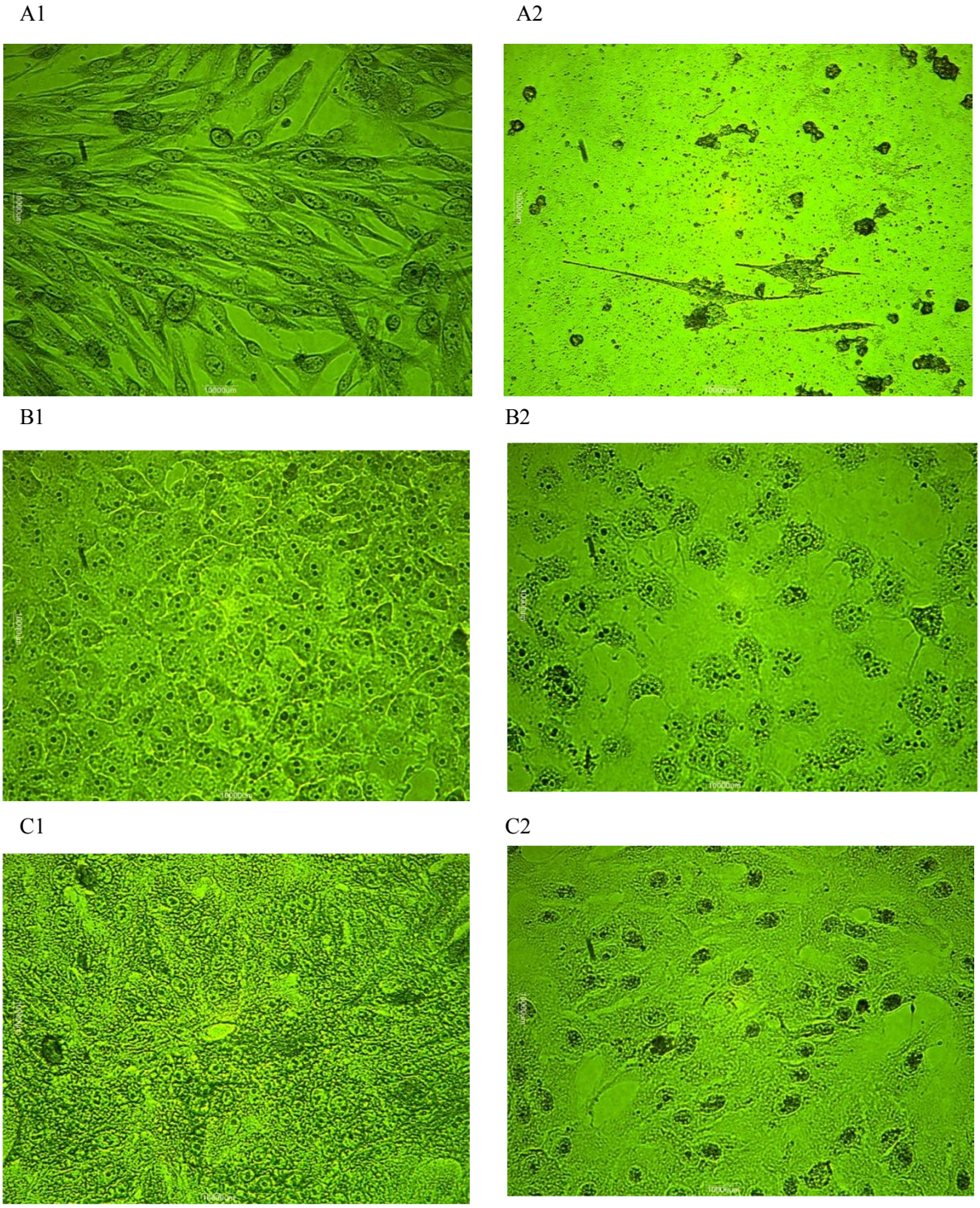

Figure 1 Cell alterations after an experimental administration of macrolides *in vitro*

[A 1,2 – BHK21 cells – Tilmicosin – control vs. 500 µg.ml^{-1}; B 1,2 – FE cells – Tilmicosin – control vs. 200 µg.ml^{-1}; C 1,2 - VERO cells - Tilmicosin – control vs. 450 µg.ml^{-1}]

The results (Figure 2-4) show that tilmicosin negatively affects the living conditions of the cells. The cells change their morphology and begin to die within 24 hours. From the diagrams it is obvious how many cells survived the impact of concentrations of tilmicosin.

BHK cells are most sensitive, significant decrease in vital cells occurs already at the concentration of 50 μg.ml^{-1} (Figure 2). Similar effects were observed in the FE cells. VERO cells were most resistant, significant decrease of vital cells was observed only at the concentration of 300 μg.ml^{-1} (Figure 4).

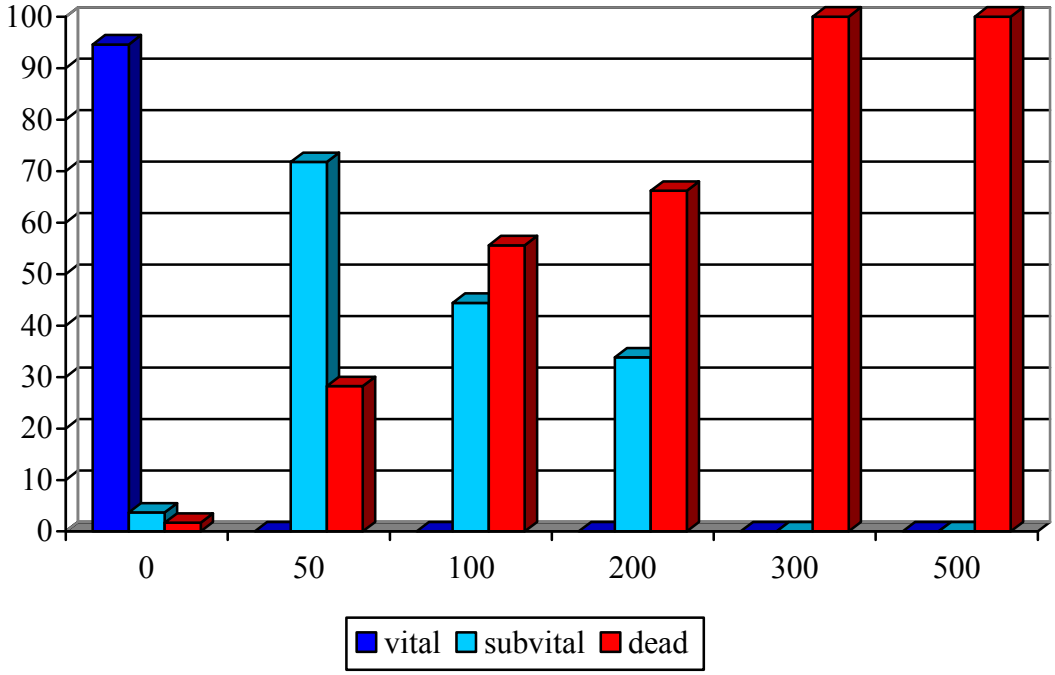

Figure 2 The effect of tilmicosin on the cells quality and cytomorphological changes in medium – BHK

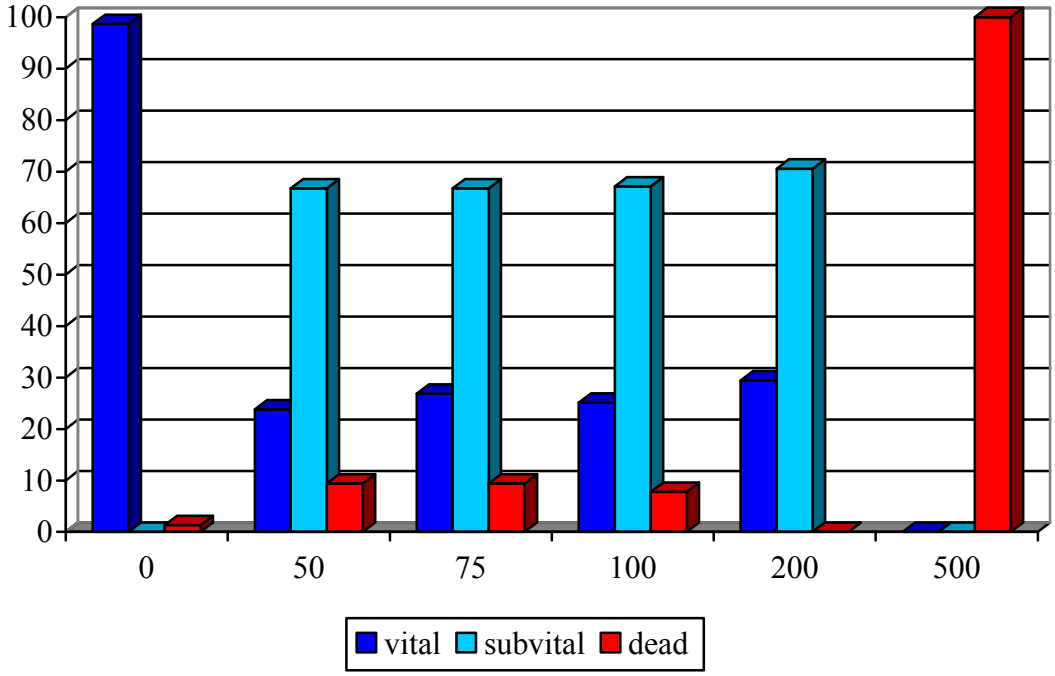

Figure 3 The effect of tilmicosin on the cells quality and cytomorphological changes in medium – FE

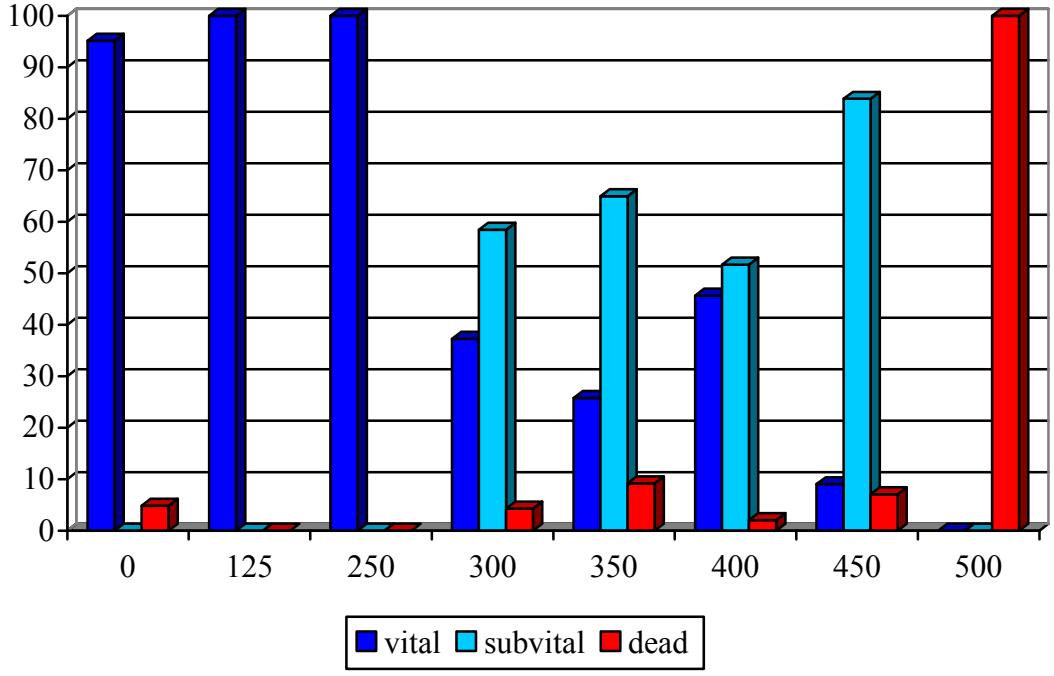

Figure 4 The effect of tilmicosin on the cells quality and cytomorphological changes in medium – VERO

From our findings, it appears that tylosin negatively affects the vitality, the living conditions of the cells (Figure 5, 6, 7). After application of antibiotics to the cells, they started

to change morphology and died within 24 hours. We can clearly see the impact of tylosin concentrations on cell survival in Figures 5 to 7. BHK cells can be considered most sensitive, since at concentrations higher than 500 µg.ml^{-1}, no vital cells were observed (Figure 5). With gradually increasing concentrations a significant decrease of subvital cells was observed. A similar tendency was observed in cells of the FE, where even at a concentration of 1000 µg.ml^{-1} were 3.13% of vital and 70.52% of subvital cells (Figure 6). Significant decrease was recorded at the concentration of 700 µg.ml^{-1}. In Vero cells, we observed a significant decrease at the concentration of 750 µg.ml^{-1}, which can be regarded as the most resistant to tylosin (Figure 7).

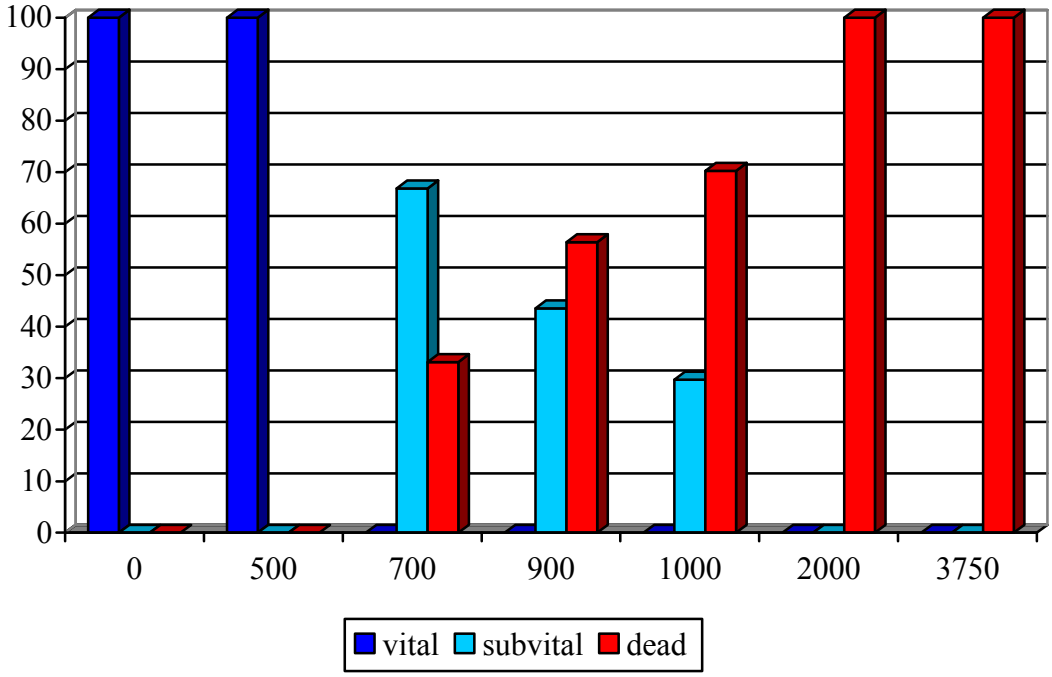

Figure 5 The effect of tylosin on the cells quality and cytomorphological changes in medium – BHK

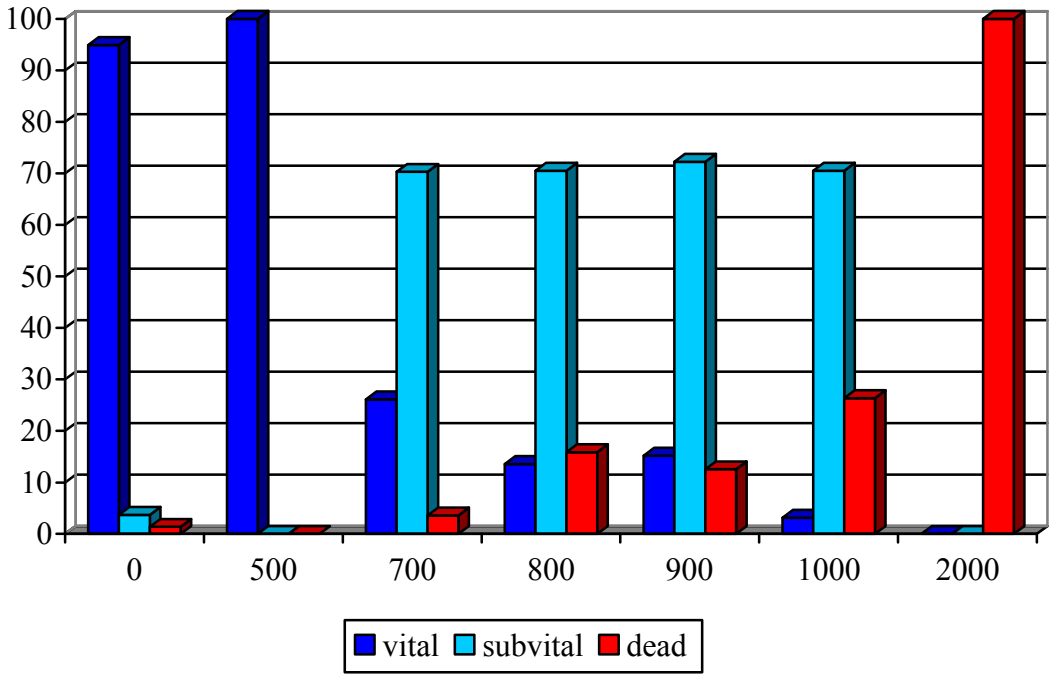

Figure 6 The effect of tylosin on the cells quality and cytomorphological changes in medium – FE

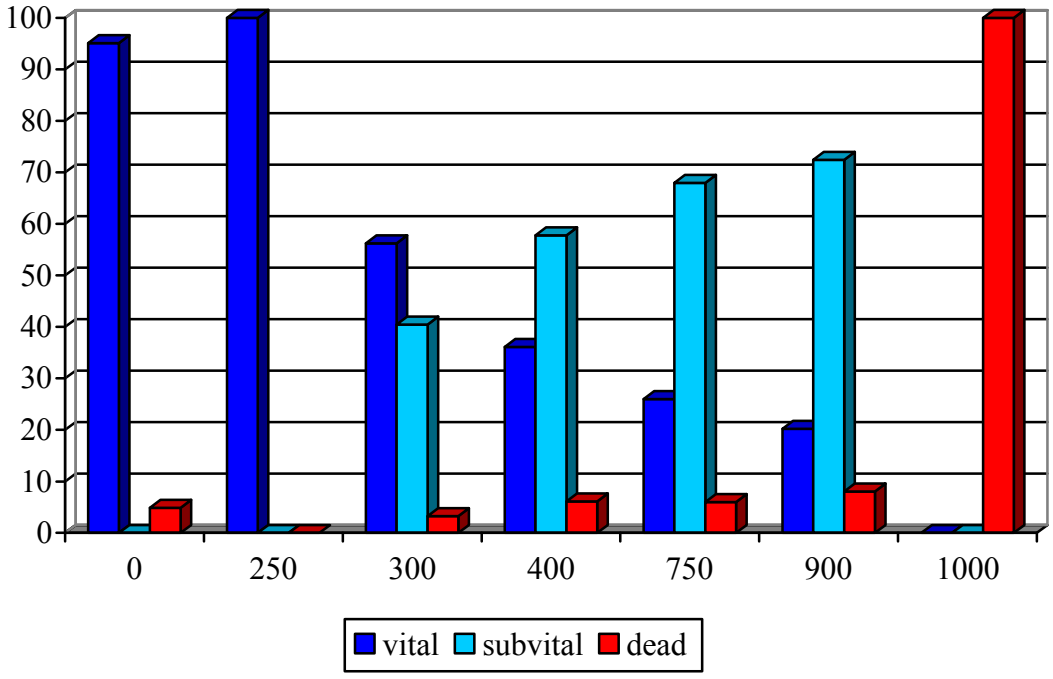

Figure 7 The effect of tylosin on the cells quality and cytomorphological changes in medium – VERO

Even if we spiramycin confirmed the negative impact on the vitality and survival of cells (Figure 8, 9, 10). Significant decrease of vital BHK cells was observed at the concentration of 150 µg.ml^{-1}, and already at the concentration of 300 µg.ml^{-1}, no vital cells and only 7.53% of subvital cells were observed (Figure 8). Effect of spiramycin on FE cells had the same tendency, a significant decrease was recorded at the concentration of 150 µg.ml^{-1}. However, the FE cells can be regarded as more resistant as we still at the concentration of 500 µg.ml^{-1} reported 10.34% of vital cells (Figure 9). VERO cells can be considered the most durable, at the concentration of 500 µg.ml^{-1} 22.48% of vital and 71.16% of subvital cells were recorded.

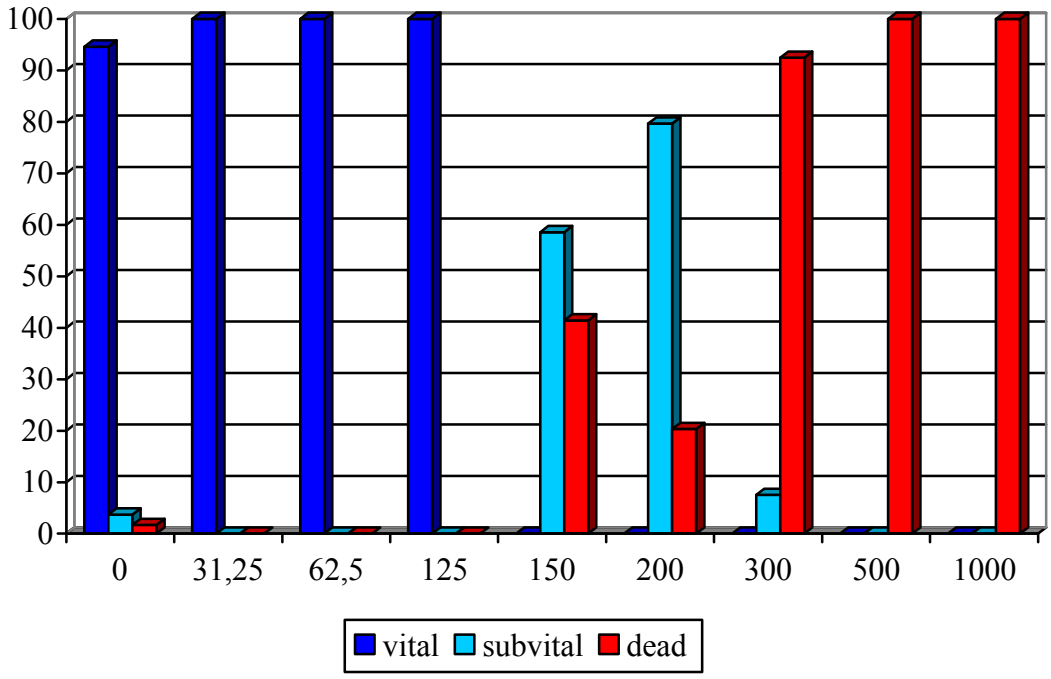

Figure 8 The effect of spiramycin on the cells quality and cytomorphological changes in medium – BHK

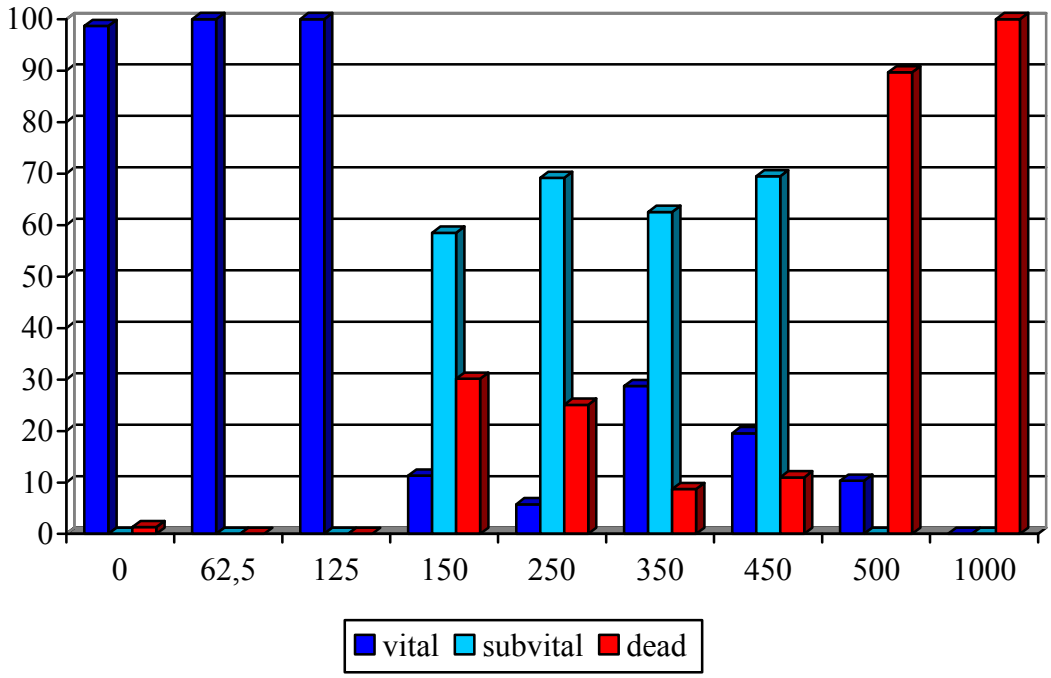

Figure 9 The effect of spiramycin on the cells quality and cytomorphological changes in medium – FE

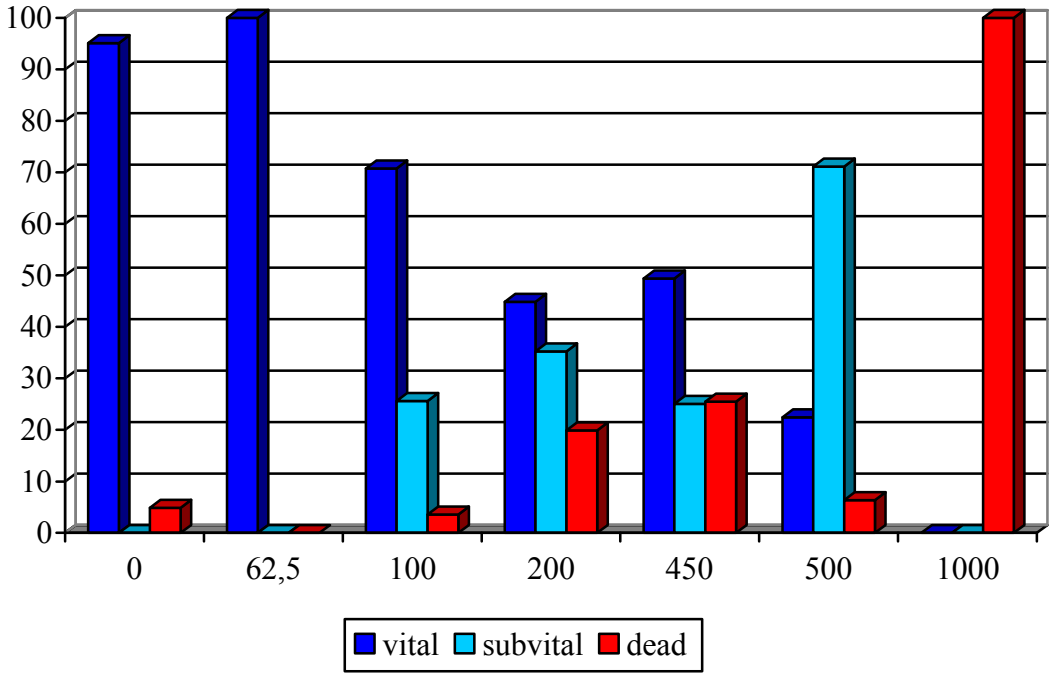

Figure 10 The effect of spiramycin on the cells quality and cytomorphological changes in medium – VERO

There are a few data in the literature about the impact of macrolides on cell morphology, bacteria, leukocytes, soil fauna. **(Prescott et al., 1993; Baguer et al., 2000; Shryock et al., 2002; Ishida et al., 2007)**.

Detection of antibiotics toxicity in animal cells is interesting in terms of bacterial resistance to antimicrobial agents. The increasing resistance can be partly regulated by increasing the dose to the treated organism. It is possible to increase the dose only to a certain concentration of the substance, then toxic effect will prevail over therapeutic effect. If toxicity of treating substance is not significant, the possibility of bacterial resistance to high doses of substance increases. In the case of macrolide antibiotics, it´s due to their method of action, which depends on time, not the dose **(Giguére et al., 2006)**.

Tests of macrolide antibiotics cytotoxicity were performed with human liver cell lines. The study used Chang liver cells to compare the cytotoxicity of three new semisyntetic macrolide antibiotics - roxithromycin, clarithromycin and azithromycin, with three older macrolides - erythromycin carbonate, erythromycin estolate and erythromycin base. The culture plates at a concentration of 5000 or 10 000 cells in 100 microliters were used to perform MTT assay **(Viluksela et al., 1996)**. The results showed that cytotoxicity of macrolides is dose-dependent **(Otoguro et al., 1991)**. In this study, the hepatotoxic potential of macrolides was compared with cytotoxicity of erytromycine derivatives. Cytotoxicity was assessed as the ability of cells to reduce MTT. The level of mitochondrial function also reflects the cell viability **(Supino, 1990)**.

Tilmicosin was developed for a one-off injection in bovine pneumonia treatment caused by *Pasteurella* sp. Effectiveness of tilmicosin was tested on rats, where the antibiotics doses of 50, 250 and 1000 $mg.kg^{-1}$ body weight for three months were used. Signs of toxicity were observed at doses of 250 and 1000 $mg.kg^{-1}$ (reduced viability, reduced food intake, weight loss, organ weight change). Significant mortality was observed at a dose of 1000 $mg.kg^{-1}$ **(Jordan, 1989)**.

Baguer et al. (2000) tested the effects of antibiotics on growth and reproductive ability of individual species of soil fauna (earthworms, springtails), and reported low toxicity of tylosin and oxytetracycline. Significant effect of antibiotics on reproduction was confirmed only at doses over 4000 $mg.kg^{-1}$ or 5000 $mg.kg^{-1}$.

Tylosin toxicity to rodents is low. In the 17-months study, rats were fed by food with the addition of 0, 0.1, 0.3 and 1% tylosin, which is equal to 1, 1000, 3000 and 10000 ppm. Biochemical, haematological, macroscopic or microscopic changes detected, especially enlargement of ovaries and uterus in some females, were not significant compared to control.

These changes were also observed in some control animals, and therefore they can not be definitely attributed to the impact of tylosin **(Lilly, 1990)**. Toxicological studies have shown that tylosin is only slightly toxic for multi-celled organisms, especially when administered orally.

Macrolide antibiotics are clinically useful antibiotics, which are all linked to the large ribosomal subunit near the peptidyl core of tansferase. This core is formed near the RNA and catalyses the formation of peptide bonds during protein elongation **(Tenson et al., 2003)**.

Knowledge of chemical structure of natural antibiotics allowed their modification, usually by binding side chains in order to provide agents with fewer side effects. These modified semi-synthetic antibiotics, including macrolides don´t have many natural disadvantages of erythromycin, which causes nausea, vomiting, and interferes with some other medicinal substances **(Havlík, 2008)**.

CONCLUSION

Active substances cytotoxicity observing in cell cultures allows to compare *in vitro* and *in vivo* effect, because it is known that the substance in a living organism behaves differently than in laboratory conditions. Toxic effects of specific antibiotics varied in concentration as well as in morphology and behavior of cells. Comparison of results obtained in our work with the published results is quite difficult, because toxicological studies are usually performed in vivo and examine the impact of substances on tissues and organs.

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