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REGULAR ARTICLE

EFFECT OF MACROLIDE ANTIBIOTICS ON VARIOUS CELL CULTURES *IN VITRO*: 2. CELL BIOCHEMISTRY

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ABSTRACT

The aim of our study was to evaluate the effect of macrolide antibiotics (tilmicosin, tylosin and spiramycin) on the cellular biochemistry using different cell cultures *in vitro*. Cellular lines from animal tissues (VERO cells - kidney cells of *Macacus Rhesus*, FE cells - feline embryonal cells and BHK21 - cellular line from young hamster kidneys) were used. The effect was assessed after 24 hours of culture. We studied the concentration of calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), chlorides (Cl), total proteins (TP) and cholesterol (Chol). Biochemical analysis of BHK21 cells cultivated with tilmicosin showed a significant decrease in the concentration of Ca, Cl and TP in almost all experimental groups. No significant differences were found in the FE cells. The highest concentrations of tilmicosin led to a significant increase of all analyzed elements and TP in medium in the VERO cells. The effect of tylosin on the BHK21 cell metabolism showed a significant decrease in the concentration of Na and Cl in the all experimental groups and a significant decrease in the concentration of TP in the groups to which more than 700 $\mu g.ml^{-1}$ was added. No significant

differences were found in the FE and VERO cells. Biochemical analysis of BHK21 cells with spyramicin showed a significant decrease in the concentration of Na in the all experimental groups and a significant decrease in the concentration of Cl and TP in the cell cultures with 100 $\mu g.ml^{-1}$, 150 $\mu g.ml^{-1}$, 200 $\mu g.ml^{-1}$, 300 $\mu g.ml^{-1}$ concentrations of spyramycin. The highest concentrations of spyramycin caused a significant increase of Na and a significant decrease of Chol in the FE cells. No significant differences were found in the VERO cells except increased total proteins at the highest concentration of spyramycin.

Keywords: macrolides, cell cultures, biochemistry

INTRODUCTION

In the past, antibiotics definition was limited to chemicals produced by microorganisms, which are able to inhibit the growth or to kill the bacteria and other microorganisms. Nowadays, the concept "antibiotic" is used generally for substances produced by micro-organisms and also for antimicrobial substances produced synthetically. The basic characteristic of antibiotics is the selective toxicity - selective inhibition of growth of infective agents or killing it without breaking the cells of the host organism **(Scholar and Pratt, 2000)**.

The difference between enzymes used for nucleic acids synthesis in prokaryotes and eukaryotes is very important in selective affecting of antibiotics, which inhibit synthesis of nucleic acids, e. g. rifampicin, sulfonamides, trimethoprim, sulfones, pyrimethamine, ciprofloxacin, norofloxacine, nalidixic acid **(Mayer, 2010)**.

Actinomycetes (largest source of natural macrolides) produce more than three hundred 16-memebered macrolides and one hundred 14-membered macrolides. They also produce various sizes of macrolides up to a 60-membered ring, polyene macrolides, macrodiolides, macrotetrolides, and immunosuppressive macrolide lactams **(Ōmura, 2002)**.

Macrolides were the drugs of first choice in the porcine diseases treatment **(Giguère *et al.*, 2006)**. Macrolides have bacteriostatic effect, are absorbed from gastrointestinal tract quickly, are slightly toxic, don´t penetrate to the cerebrospinal fluid and are effective against atypical microbes without cell wall **(Havlík, 2008)**.

Bacteriostatic effect of macrolides depends on dose generally **(Giguère *et al.*, 2006)**. Either clinical importance of possible effect depending on dose, or postantibiotical effect of

some new macrolides against pathogens observed in *in vitro* conditions, were not demonstrated **(Jacobs *et al.*, 2003)**.

Macrolides of first generation are resorbed well. They are distributed into all organs and tissues in the organism and are concentrated in saliva, lymph and parenchyma, tylosin in lungs, derma and mammary gland. Macrolides of second generation, e. g. tilmicosin, are accumulated in phagocytosis cells (lungs macrophages). All macrolides remain relatively long time in cells, 3–4 days. In the liver, they are metabolized primarily by methylation. They are excreted by gall mainly, partly by urine **(Šimůnek and Smola, 2007)**.

The aim of our work was to evaluate the effect of macrolide antibiotics on metabolism parameters of three cell cultures *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. According to cell growth intensity and single layer formation, we either processed cells or let them grow in Roux flask. When single layer of cells was continual, we segregated cells from the base according to ŠPP 007. Cell density was determined. According to the number of cells we diluted suspension to the final rate: BHK – 1.7 x 106 v 1 ml, VERO – 7.6 x 105 v 1 ml a FE 3.6 x 105 v 1 ml. 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 *Maccacus Rhesus*, FE cells - feline embryonic 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. Finally, we chose concentrations, which covered amplitude from minimal, almost zero toxicity, to the concentration, which killed almost 100 % of cells in 24 hours. We repeated this proceeding for all cell lines, because each type of cells is sensitive on different concentration. We obtained final dilutions of antibiotics by diluting of zero concentration of antibiotics in cultivating medium without addition of bovine fetal serum.

Table 1 Concentrations of tilmicosin used for BHK21, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK21	50; 100; 125; 200; 250; 300; 500; 1000; 2000
FE	50; 75; 100; 200
VERO	300; 350; 400; 450;

Table 2 Concentrations of tylosin used for BHK21, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK21	125; 250; 500; 700; 900; 1000; 1500; 2000
FE	650; 700; 750; 800; 900; 1000
VERO	300; 400; 750; 900

Table 3 Concentrations of spiramycin used for BHK21, FE and VERO cell lines

Cell cultures	Concentration µg.ml^{-1}
BHK21	31.25; 62.5; 100; 125; 150; 200; 250; 300; 500
FE	150; 250; 350; 450
VERO	100; 200; 450; 500

Legend: BHK21 - cellular line from young hamster kidneys, FE - feline embryonic cells, VERO - kidney cells of *Macacus Rhesus*

After 24 hour exposure of selected cells to macrolide antibiotics, cultivating medium was drained out by pipette and frozen in eppendorf tubes to -20 °C. Frozen medium was used for biochemical tests to discover the effect of antibiotics on cell metabolism. Concentration of Ca, Mg, Na, K, Cl, total proteins and cholesterol was monitored **(Massányi *et al.*, 2009)**. For Na, K and Cl analysis automatic analyzer EasyLyte (Medica, Bedford, USA) was used, for other parameters semi-automatic analyzer Microlab 300 (Vilat Scientific, Dieren, The Netherlands) was used.

For comparison of results, Scheffe´s and Student´s t-test were used. Evidence supporting differences were evaluated on the surface of the evidential $p < 0.05$; 0.01 a 0.001.

RESULTS AND DISCUSSION

The results were processed by antibiotics, because the aim of our study was to investigate the different effects of specific substances on selected biochemical parameters in animal cells.

Biochemical analysis showed significant decrease in the concentration of calcium in the groups with the highest concentration of tilmicosin (1000 and 2000 $\mu g.ml^{-1}$) in BHK21 cells culture (Figure 1). Chloride concentration showed decreasing tendency in almost all test groups compared to the control. Similarly, the concentrations of total protein were significantly lower ($p < 0.001$) in the experimental groups except the group with the highest concentration of tilmicosin. The concentrations of magnesium, sodium, potassium and cholesterol didn´t achieve significant differences in the experimental groups.

By monitoring of biochemical parameters in the culture medium, no significant differences were detected at FE cells (Figure 2). Magnesium level had a decreasing tendency in media samples with concentrations of 75-200 $\mu g.ml^{-1}$. In contrast, total protein concentration increased in the same samples.

Biochemical changes caused by the presence of tilmicosin are associated with increased release of Ca, Mg, Na, K, Cl and total proteins. In VERO cells culture, we observed significant increase of these parameters concentration in medium with observed cells. Significant increase of Ca concentration ($p < 0.001$), Mg ($p < 0.05$), Na ($p < 0.01$), C ($p < 0.01$), Cl ($P < 0.05$) and total protein ($P < 0.001$) were observed in the group with the highest concentration of tilmicosin (Figure 3).

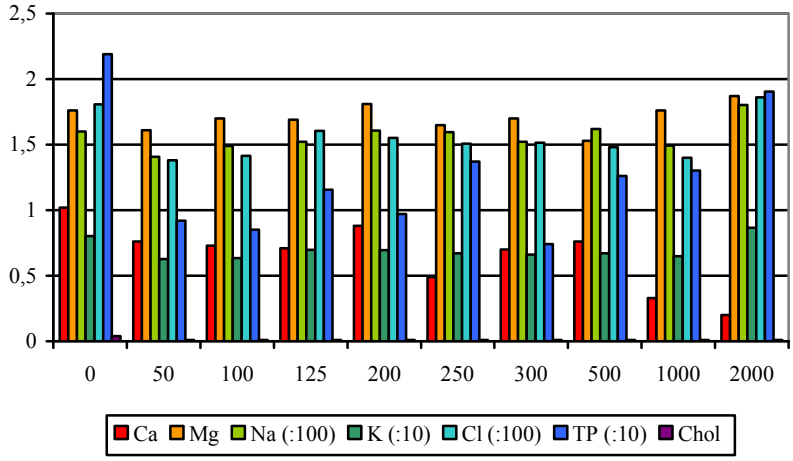

Figure 1 The effect of tilmicosin on the biochemical parameters in the medium – BHK21

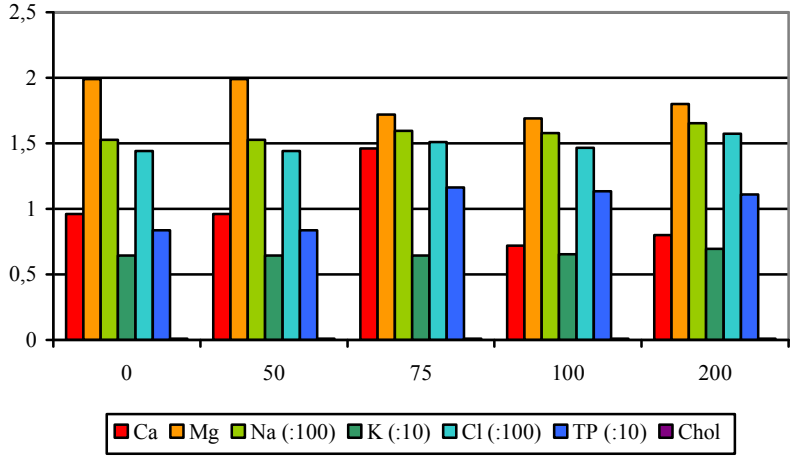

Figure 2 The effect of tilmicosin on the biochemical parameters in the medium – FE

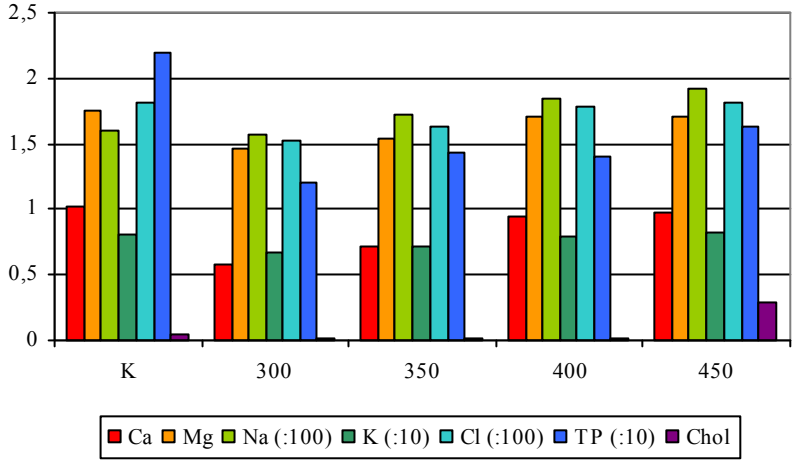

Figure 3 The effect of tilmicosin on the biochemical parameters in the medium – VERO

Although tylosin affects the viability of BHK21 cells in concentration of 700 μg.ml $^{-1}$ **(Fülöpová *et al.*, 2012)**, the metabolism of Na and Cl is affected by tylosin in concentration of 125 μg.ml $^{-1}$. In the culture medium obtained after 24 h cultivation of cells with addition of 700-1000 μg.ml^{-1} total proteins were significantly decreased ($P < 0.01$) compared to control. Significant decrease of Na ($p < 0.001$) and Cl concentration ($p < 0.001$) were observed in the groups with tylosin concentrations from 125 to 1500 μg.ml^{-1} (Figure 4).

By monitoring of biochemical parameters in the culture medium, no significant differences were observed when cultivating FE cells (Figure 5). The level of magnesium and sodium showed an increasing tendency in the media samples with concentrations of 700 - 1000 μg.ml^{-1}. An increase of total protein concentration was observed in the group with tylosin concentration of 750 μg.ml^{-1}.

Biochemical parameters, we studied, showed a rising tendency of some parameters in samples of VERO cell culture medium (Figure 6). Levels of sodium and chloride increased at concentration of 400 μg.ml^{-1}. Concentration of elements in the medium is increased by 10 % and 11 %. At concentration of 750 μg.ml^{-1} it was 4 and 5 %, at concentration of 900 μg.ml^{-1} sodium level increased by 32 % and chloride level by 27 % (Figure 6).

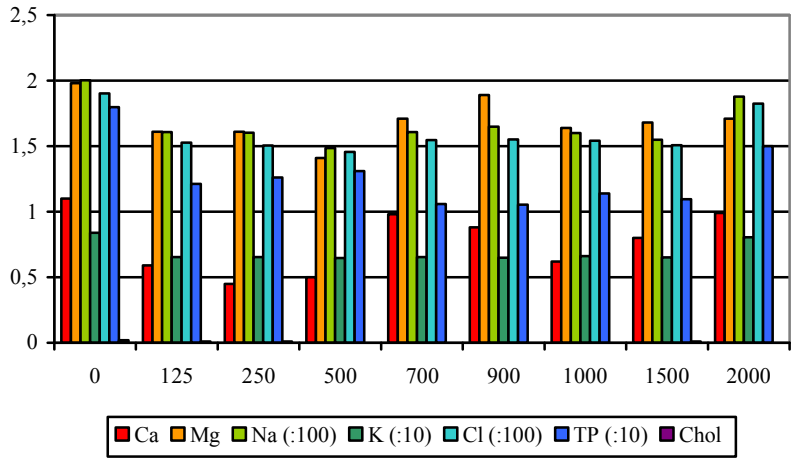

Figure 4 The effect of tylosin on the biochemical parameters in the medium – BHK21

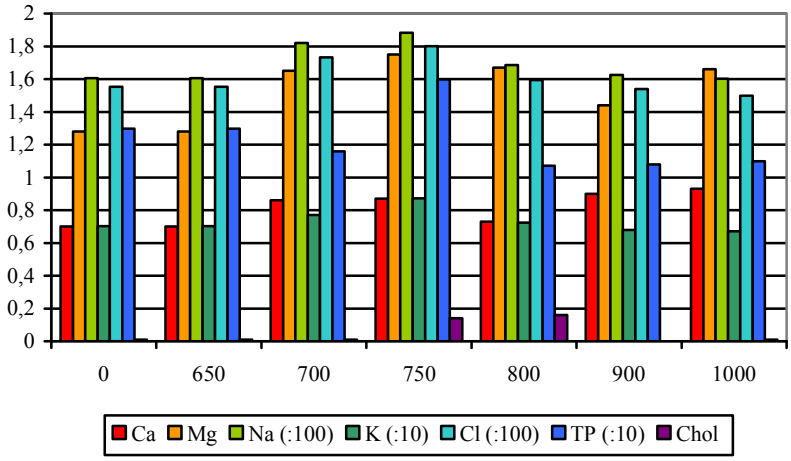

Figure 5 The effect of tylosin on the biochemical parameters in the medium – FE

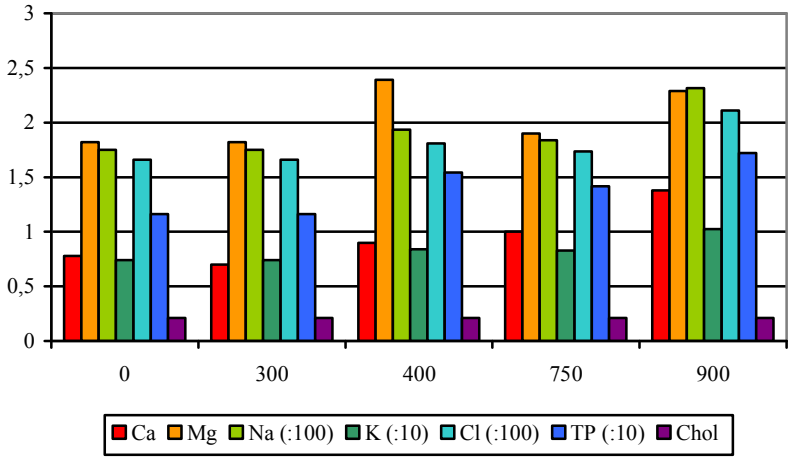

Figure 6 The effect of tylosin on the biochemical parameters in the medium – VERO

Biochemical analysis of BHK 21 culture media taken after 24 hour-culture showed a statistically significant change in sodium concentration ($p < 0.001$) in samples with spiramycin concentration of 31.5 - 300 $\mu g.ml^{-1}$ and decreased levels of chloride concentration in samples with spiramycin in concentration of 100 ($p < 0.01$) , 150 ($p < 0.05$), 200 ($p < 0.05$) and 300 ($p < 0.01$) $\mu g.ml^{-1}$. We also found a decrease of total protein content in all groups compared with the control group, significantly ($p < 0.01$) in groups with spiramycin concentration of 100, 150, 200 and 300 $\mu g.ml^{-1}$ (Figure 7).

Biochemical parameters of FE cells showed only slight deviations compared to the control group. We found significant decrease in the concentration of cholesterol ($p < 0.05$) at

spiramycin concentrations of 250, 350 and 450 µg.ml^{-1}. At higher concentrations, significant increase of the sodium ($p < 0.05$) and calcium concentration ($p < 0.01$) was found (Figure 8).

Biochemical parameters of VERO cells were significantly different from those obtained in the control samples. In all samples, a significant difference in the concentration of total protein ($p < 0.05$) was found in the high-dose group of spiramycin (Figure 9).

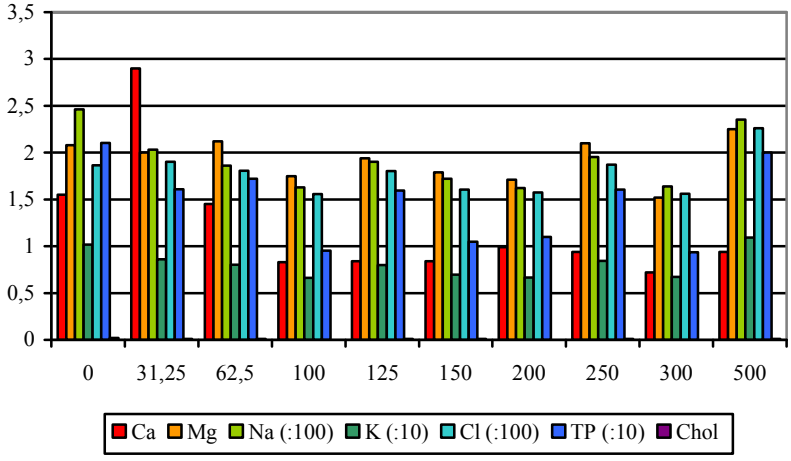

Figure 7 The effect of spiramycin on the biochemical parameters in the medium – BHK

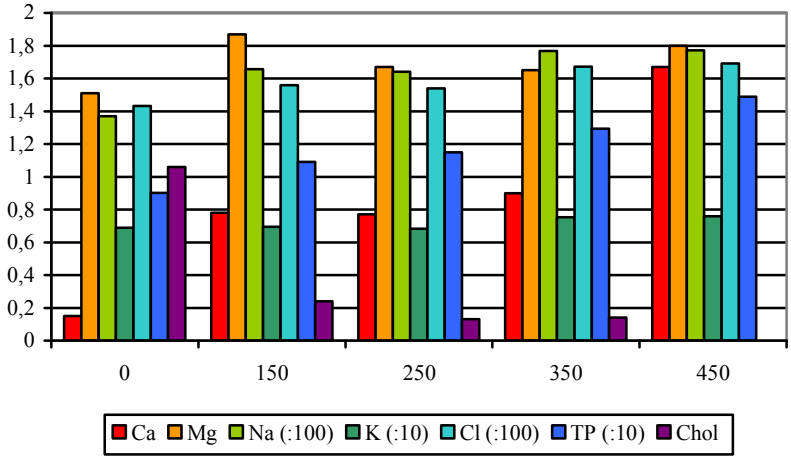

Figure 8 The effect of spiramycin on the biochemical parameters in the medium – FE

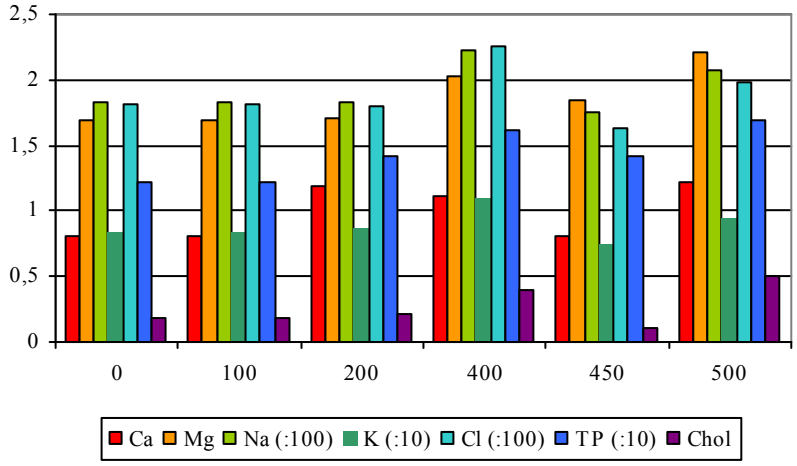

Figure 9 The effect of spiramycin on the biochemical parameters in the medium – VERO

There have been reports of studies of the effects of macrolides on Langerhans cells, epithelial cells, fibroblasts, dendritic cells, T lymphocytes **(Suzuki *et al.*, 1997; Abe *et al.*, 2000; Desaki *et al.*, 2000; Mukaida *et al.*, 1992; Ishida *et al.*, 2007)** and few data about the impact of macrolides on cell morphology, bacteria, soil fauna, cytotoxicity. **(Prescott *et al.*, 1993; Baguer *et al.*, 2000; Shryock *et al.*, 2002; Fülöpová *et al.*, 2012)**.

The macrolides we used were tylosin and spiramycin (1st generation) and tilmicosine (2nd generation). Macrolide antimicrobial agents are used as immunomodulators in the treatment of chronic respiratory tract inflammatory diseases as chronic sinusitis, sinobronchial syndromeanddiffuse panbronchiolitis, etc. **(Yamamoto *et al.*, 1990; Fujii *et al.*, 1995)**

Ishida *et al.* (2007) studied the immune responses of dendritic cells and CD4+ T cells originating from healthy adult volunteers to the P6 protein when the cells were exposed to macrolides. The immune responses of CD4+ T cells to stimulation by P6 protein are inhibited by macrolides. Investigation of the effects of the macrolides on cytokines revealed a tendency for Th1 responses to be inhibited. The macrolides showed a tendency to inhibit the immune responses of Dendritic cells.

Dendritic cells (DCs) are distributed in many tissues of the body, and it is known that they play an important role in inducing immune responses by carrying out antigen presentation to T cells **(Ishida *et al.*, 2007)**.

With regard to the underlying mechanism of macrolides, they have been reported to inhibit the production of mRNA for IL-8 and various other inflammatory cytokines **(Suzuki *et al.*, 1997)**, and there have also been reports that macrolides show inhibitory effects at the levels of the NF-kB and AP-1 transcription factors **(Abe *et al.*, 2000)**.

The recent data in the literature clearly describes macrolides as agents that, far beyond their traditional antimicrobial properties, possess a considerable anti-inflammatory effect. Their exact role in everyday clinical practice for the therapy of chronic respiratory inflammatory conditions depends on results of large randomised clinical trials. With the sole exception of four trials disclosing considerable benefit from the longterm administration of azithromycin in patients with cystic fybrosis **(Giamarellos-Bourboulis, 2008)**.

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CONCLUSION

The group of macrolide antibiotics is known for a long time and well explored, but the effects of these substances have been studied *in vivo* mainly. Because of severe toxicity in animals exploring and usage of these antibiotics has been limited, respectively stopped.

Comparison of the results obtained in our work with the published results is difficult because the studies are usually performed in vivo and examine the impact of substances on tissues and organs.

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