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Original scientific paper

EVALUATION OF DNA DAMAGE IN RAT LYMPHOCYTES EXPOSED TO TULATHROMYCIN *IN VITRO*

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Maletić J., N. Djelić, M. Radaković, M. Maletić, N. Lakić, V. Kukolj, N. Aleksić, M. Andjelković and Z. Stanimirović (2015*): Evaluation of DNA damage in rat lymphocytes exposed to tulathromycin in vitro.*- Genetika, Vol 47, No. 1, 339-348.

Tulathromycin is a relatively new semi-synthetic macrolide antibiotic, a member of the triamilide group, approved primarly for the treatment of respiratory diseases in cattle and swine. Various genotoxicological studies indicated that tulathromycin is not genotoxic, but no available published data originate from the single-cell gel electrophoresis (Comet) assay. Therefore, the objective of this study was to examine whether it can induce primary DNA damage using *in vitro* Comet assay in isolated rat lymphocytes. Lymphocytes were treated with a broad spectrum of tulathromycin concentrations (from 1 to 100 μM) and co-treatment with an antioxidant, catalase (100 IU/mL and 500 IU/mL) was performed. The highest concentrations of tulathromycin (50 and 100 μM) caused significant increase of DNA damage in rat lymphocytes and catalase did not significantly reduce the DNA-damaging effect of tulathromycin. The results of this study indicate that tulathromycin induces genotoxic effects at high concentrations, that catalase does not exert protective effect in this case.

Key words: comet assay, DNA damage, rat lymphocytes, tulathromycin

INTRODUCTION

Tulathromycin is a triamilide macrolide antibiotic specifically developed for the treatment of bacterial respiratory diseases in cattle and swine (BENCHAOUI *et al.*, 2004; EMEA, 2004; EVANS, 2005). It was also confirmed as highly promising in the treatment of pulmonary abscesses in foals (VENNER *et al.*, 2007) and respiratory infections in goats (WASHBURN *et al.*, 2007; CLOTHIER *et al.*, 2011) and rabbits (ABO-EL-SOOUD *et al.*, 2012).

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Tulatromycin *in vivo* exists in a 9:1 ratio of 15-membered and 13-membered ring structures containing two attached sugar moieties (GALER *et al.*, 2004; NOWAKOWSKI *et al.*, 2004; EVANS, 2005). The unique structural properties of tulathromycin enables its rapid dissemination from the plasma and extended residence time in tissues, primarily the lung, for up to 9 days (ABU-GHARBIEH *et al.*, 2004; GALER *et al.*, 2004; NOWAKOWSKI *et al.*, 2004; EVANS, 2005; YAZAR, 2009). Not unlike other macrolides, it acts by binding to the 50 S subunit of bacterial ribosomes and blocking peptidyl transferase, which results in the dissociation of transfer RNA (tRNA), cessation of peptide translocation, and blocked protein synthesis (BENCHAOUI *et al.*, 2004; EVANS, 2005).This drug is classified as bacteriostatic, but can also exhibit bactericidal activity at higher concentrations (BENCHAOUI *et al.*, 2004; EMEA, 2004; EVANS, 2005).

Tulathromycin may also cause oxidative stress and coagulation disorders (ER *et al.*, 2011). In contrast to other macrolides, which are believed to have antioxidant effects in humans, tulathromycin may cause oxidative damage, possibly due to a different molecular structure and/or doses applied (ER *et al.*, 2011).

The genotoxic potential of tulathromycin was estimated *in vivo* (e.g. the rat micronucleus assay) and *in vitro* (the microbial gene mutation assay, mammalian cell (CHO/HGPRT) gene mutation assays, the human lymphocyte chromosome aberration test). The results indicated that tulathromycin is not genotoxic (EMEA, 2004). However, using flow cytometry WASHBURN *et al.* (2007) showed its genotoxic potential.

Since there is only limited information on the genotoxicity of tulathromycin and that it was not evaluated using single cell gel electrophoresis (Comet) assay, this very sensitive method was applied to assess the DNA damaging effects in rat lymphocytes exposed to tulathromycin in order to provide additional genotoxicological data for this compound.

MATERIALS AND METHODS

Blood samples and treatment

The study was approved by the local Medical Ethics Committee at the Faculty of Veterinary Medicine, University of Belgrade. Heparinised blood samples (4 mL) were obtained from Wistar rats by venepuncture. The average body weight of rats was approximately 200 g. Four animals were used for each experimental point. Heparinised blood samples (sodium heparin, Galenika, Belgrade, Serbia) were immediately processed for isolation of lymphocytes on ficol gradient, as previously described (RADAKOVIC *et al.*, 2014). The 50 μL of lymphocyte suspension was incubated in PBS solution containing tulathromycin, (CAS No 217500-96-4, Draxxin®, Pfizer Animal Healh, USA) at various concentrations, from 1 to 100 μM. The negative control was PBS and the positive 100 μM aqueous solution of H_2O_2. The treated lymphocytes were incubated at 37°C for 1 h. The study was approved by the local Medical Ethics Committee and performed in accordance with The European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes.

The Comet assay

Before the start of the experiment the cells were checked for viability using Trypan blue dye according to the method of PHILLIPS (1973). The Comet assay was performed in three independent experiments on isolated rat peripheral blood lymphocytes. Microscope slides were precoated with 1% double-distilled-water solution of normal agarose (Sigma, St. Louis, MO) and left at room temperature to dry. Lymphocytes were isolated using Amersham ficol gradient as described by

SOLTANI *et al.* (2008). The cell suspension in PBS was treated with tulathromycin at concentrations 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM for 1 h at 37°C. After the treatment the cytotoxicity was determined by Trypan blue exclusion test and the cell suspensions were centrifuged at 2000 rpm for 5 min, the cell pellet mixed with an equal amount of 1% low-melting-point agarose (Sigma) and rapidly placed on the precoated microscope slides, covered with cover-slips and allowed to solidify for 5 min at 4°C. After removing the coverslips 1% agarose layer was added, the coverslips replaced, and left for 5 min at 4°C. Following the final removal of coverslips the slides were placed overnight in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X100 and 10% DMSO, pH 10 adjusted with NaOH). On completion of the lysis, the slides were placed in a buffer (10 M NaOH, 200 mM EDTA, pH 13) for 30 min at 4°C in the dark to allow DNA unwinding. Electrophoresis was carried out for 30 min. at 25 V and 300 mA. Afterwards, the slides were gently rinsed with a neutralising solution (0.4 M Tris base, pH 7.5) three times. The DNA was stained with 50 μL of ethidium bromide (20 μg/mL) per slide. The coded slides were examined and scored blind under a microscope equipped with a device for fluorescence recording at 400× magnification (Zeiss AXIO Imager M1 - Carl Zeiss Hamburg, Germany). DNA damage scoring was performed as described by COLLINS (2002). The cells (50 cells/slide from duplicate slides per sample) were graded visually into five classes according to the tail size: no migration (A), low migration (B), medium migration (C), high migration (D) and extensive migration (E). The total comet scores (TCS), the migration of DNA, were calculated as $TCS = 0 \times A + 1 \times B + 2 \times C + 3 \times D + 4 \times E$.

Statistical analysis

The hypothesis of equality of the effects of different treatments was tested by the comparison of the average TCS. Depending on the values of coefficients of variation, an appropriate method was chosen to test the difference between the average TCSs: for homogenous datasets (c_v<30%) the group test was a parametric test, ANOVA, and for heterogeneous datasets (c_v >30%) a nonparametric, Kruskal Wallis test. Testing the differences between pairs of average TCSs the case of homogenous data in samples (c_v <30%) was done with Tukey test, and in heterogeneous (c_v >30%) with Mann-Whitney U test. Due to large differences in the values for the positive control and other treatments logarithmic scale was used in Figures 1 and 2.

Statistical analysis of the results obtained in the experiment was carried out using statistical software STATISTICA v.6.

RESULTS

Treatment with tulathromycin

In this study the effects of tulathromycin in a wide range of concentrations, from 1 μM to 100 μM, were evaluated. The viability of cells treated with adrenaline was greater than 90% in trypan blue exclusion test at the time of the assay. The value of TCS ranged from 1 in the negative control and cells treated with 1 μM and 10μM of tulathromycin to 332 in the positive control (Table 1). The treatment of lymphocytes with 100 μM aqueous solution of H_2O_2 (positive control) resulted in homogenous TCS values (c_v=11.13%), unlike all other treatments, which produced heterogeneous datasets (coefficient of variation ranged from 33.64 % at 100 μM of tulathromycin to 92.88 % at 20 μM of tulathromycin). Considering this, medians were used to quantify average values: the average value of TCSs varied from 5.5 in the presence of 2 μM of tulathromycin to 279.5 in the positive control.

Table 1. Basic statistical parameters of TCSs in the experiment with various concentrations of tulathromycin (n=4)

Treatment		Median	Min.	Max.	Lower quartil	Upper quartil	Coef. of var.
Negative control (K-)		6.00	1.00	6.00	1.00	6.00	**66.62**
Thulathromicine	2 µM	5.50	2.00	9.00	2.50	8.50	**63.85**
	5 µM	8.50	2.00	11.00	4.00	11.00	**58.12**
	10 µM	7.50	1.00	12.00	2.50	11.50	**76.49**
	20 µM	7.00	2.00	18.00	2.00	15.00	**92.88**
	50 µM	16.00	7.00	19.00	11.00	18.00	**36.28**
	100µM	18.50	11.00	24.00	13.00	23.00	**33.64**
Positive control (K+)		279.50	257.00	332.00	266.50	307.50	11.13

Negative control: 0 µM of tulathormycin (K-); positive control: 100 µM H_2O_2 (K+)

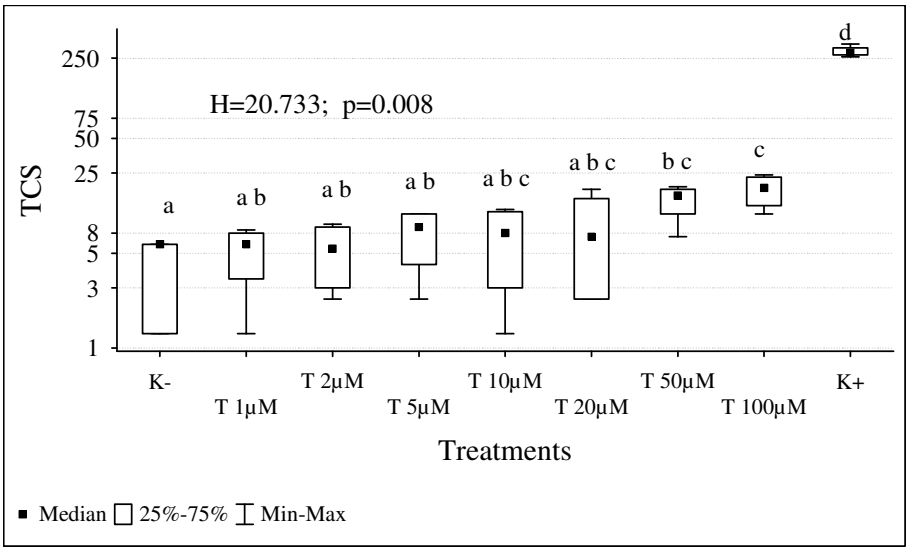

Figure 1. Effects of various tulathromycin concentrations on purified rat lymphocytes as the results of Kruskal-Wallis ANOVA and Mann-Whitney U test

Negative control: 0 µM of tulathormycin (K-); positive control: 100 µM H_2O_2 (K+). The same letters indicate the lack of significant difference ($p>0.05$), while an absence of the same letters denotes the presence of statistical significance between experimental points - based on the results of Mann-Whitney U test.

As the data obtained in this experiment were heterogenous, nonparametric tests were applied. The Kruskal-Wallis test ($H=20.73$, $p=0.008$) showed that treatments exerted very significant different effects on TCSs. The Mann-Whitney U-test clearly showed that tulathromycin

in concentrations from 1 μM to 20 μM did not affect significantly the number of strand breaks in rat DNA in comparison with the negative control (Fig. 1). Only the two highest concentrations of tulathromycin (50 and 100 μM) caused significant increase of DNA damage in comparison to the negative control.

Significantly higher TCS in the positive control in comparison to all other cultures confirmed that the test functioned properly.

Co-treatment with catalase

In order to understand the mechanism of genotoxic effect of tulathromycin, the antioxidant catalase (CAS No 900-1-05-2; Sigma, St. Louis, USA) was used. The concentration of tulathromycin (100 μM) that produced the most severe DNA damage was chosen for co-treatment with the catalase (100 and 500 UI).

In this experiment, co-treatment with catalase, the average value of TCS ranged from 4.0 at 500 IU of catalase to 307.0 in the positive control (Table 2). All data series were homogenous (c_v<30%), and the arithmetic mean, which was used as a measure of central tendency, ranged from 5.0 at 500 IU of catalase to 297 in the positive control.

Table 2. Basic statistical parameters of TCSs in the experiment with co-treatment with tulathromycin and catalase (n=4)

Treatment	Mean	Min.	Max.	Lower quartil	Upper quartil	Coef. of var.
Negative control (K)	5.50	5.00	6.00	5.00	6.00	9.09
T	17.00	16.00	18.00	16.00	18.00	5.88
T+CAT 100IU	8.50	8.00	9.00	8.00	9.00	5.88
T+CAT 500IU	7.50	7.00	8.00	7.00	8.00	6.67
CAT 500IU	5.00	4.00	6.00	4.00	6.00	20.00
CAT 500IU+K^+	58.00	49.00	67.00	49.00	67.00	15.52
Positive control (K^+)	297.00	287.00	307.00	287.00	307.00	3.37

Negative control: 0 μM of tulathormycin (K-); positive control: 100 μM H_2O_2 (K+); 100 μM of tulathormycin (T); 100 and 500 IU of catalase (CAT).

Considering the values of the coefficients of variation parametric tests were used for further analysis. The results of ANOVA ($F=1321.137$, $p<0.001$) showed very significant difference in mean TCS values between the treatments. According to the result of Tukey test, this difference arose from two sources (Fig. 2). Firstly, a significant difference between the two positive controls, that is, the cultures treated with H_2O_2 alone and in addition of catalase, and secondly, the difference between each of these two controls incomparison to all other treatments (tulathromycin alone, tulathromycin in co-treatment with 100 IU and 500 IU of catalase, 500 IU of catalase and negative control). The insignificant difference between the effects of the treatment with tulathromycin only and in co-treatment with catalase proved that this enzyme did not reduce the damage of DNA caused by tulathromycin. The lack of difference between the effect of 500 IU of catalase and the negative control proved that this enzyme did not induce any DNA damage (Fig. 2).

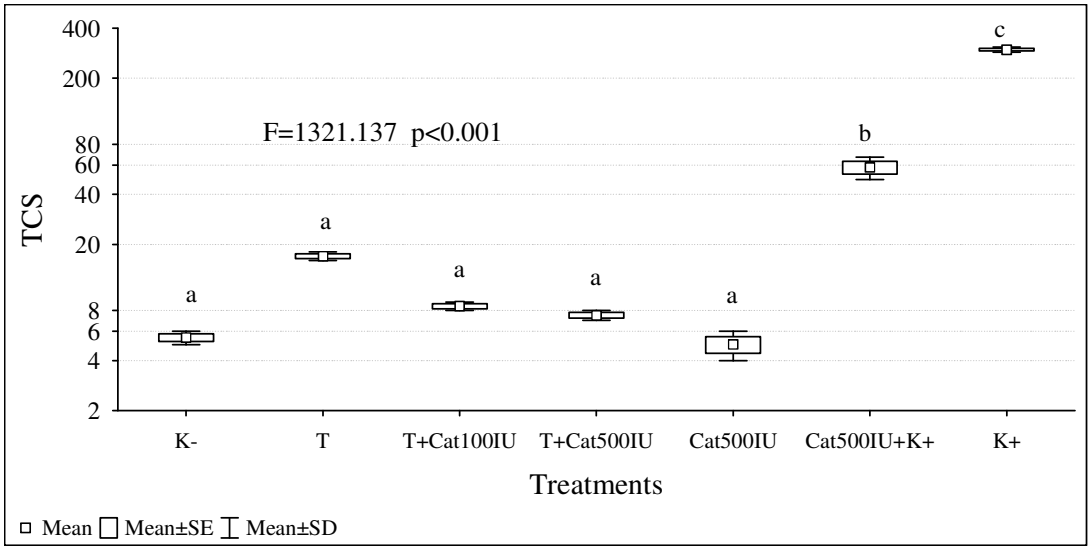

Figure 2. Effects of tulathromycin, catalase, negative and positive control on reduction of DNA damage inpurified rat lymphocytes as results of ANOVA and Tukey test
Negative control: 0 μM of tulathormycin (K-); positive control: 100 μM H_2O_2 (K+); 100 μM of tulathormycin (T); 100 and 500 IU of catalase (CAT). The same letters indicate the lack of significant difference ($p>0.05$), while an absence of the same letters denotes the presence of statistical significance between experimental points - based on the results of Mann-Whitney U test.

DISCUSSION

Tulathromycin is the first member of triamilides, a relatively new subclass of macrolide antibiotics, specifically developed for the treatment of bacterial respiratory diseases in cattle and swine (BENCHAOUI *et al.*, 2004; EMEA, 2004; EVANS, 2005). It accumulates in polymorphonuclear leukocytes and alveolar macrophages, it is also involved in the immune response to infection in the lung and is slowly released into the extracellular space where it acts directly on the pathogens of respiratory infections (ZHANEL *et al.*, 2001; EVANS, 2005, KILGORE *et al*., 2005).

Although there are limited data about the cytotoxicity of macrolide antibiotics, the observed biological effects of tulathromycin and other macrolides point to some cytotoxic effects (FÜLÖPOVÁ *et al.*, 2012). Interestingly, there are differences in the level of cytotoxicity depending on the type of the macrolides and/or the cell line used in the investigations. On the other hand, there are no available data concerninig evaluation of genotoxic effect of tulathromyin using an in vitro comet assay.

In the present research, only the highest concentrations (50 μM and 100 μM) of tulathromycin induced genotoxic effects significantly increasing the level of DNA damage in comparison to the negative control. However, the concentration of 50 μM was much higher than plasma peak concentration of tulathromycin in Holstein male calves (277 ng/g) (COX *et al.*, 2010) treated with therapeutic dose of 2.5 mg/kg b.w. Anyway, the DNA damage caused by

tulathromycin at two highest concentrations is in agreement with the findings of WASHBURN *et al.* (2007), who showed that tulathromycin has a genotoxic potential. They used flow cytometry measured DNA content as a measure of chromosomal abnormalities associated with tulathromycin exposure and showed the increase in half-peak DNA coefficient of variation increased in the treatment group with each subsequent sampling, and that the mean was significantly higher in comparison to the control. They also suggested the need for additional genotoxicological studies of this product (WASHBURN *et al.*, 2007).

Some literature data suggested that tulathromycin causes oxidative stress (ER *et al*., 2011). Oxidative stress is a cell condition which occurs when the production of reactive oxygen species (ROS) exceeds cellular defence mechanisms (DJELIĆ and ANDERSON, 2003; RADAKOVIĆ *et al*., 2011; RADAKOVIĆ *et al*., 2013). ROS can be detrimental to cells due to oxidative damage to DNA, essential enzymes and structural proteins and the induction of an uncontrolled chain reactions (such as lipid peroxidation or auto-oxidation) (COVARRUBIAS *et al*., 2008). Antioxidant enzymes play a role in the removal of various ROS (O'BRIEN, 2000; MAIER and CHAN, 2002; KIRKMAN and GAETANI, 2007). It has been reported that tulathromycin increased the levels of serum malondialdehyde, nitric oxide and superoxide dismutase and decreased the level of antithrombin III in rabbits (ER *et al*., 2011).

In the current research the co-treatment with catalase was performed in order to establish whether $OH^{\bullet}$ induced the damages of DNA. It is known that catalases are enzymes that protect the cell from free radical-induced damage by degradation of hydrogen peroxide, thus preventing its accumulation and toxic action (EJCHART, 2001). We showed that catalase did not reduce the tulathromycin-mediated damage of DNA, which indicates that catalase did not exert significant protective effect. However, it is possible that hydrogen peroxide is not formed in reaction of tulathromycin and molecular oxygen and that some other reactive oxygen species (for example superoxide anion) is generated by tulathromycin.

In conclusion, our data demonstrate that tulathromycin was capable to induce DNA damage only at very high concentrations that exceed therapeutic doses in veterinary medicine. Further analysis, especially under *in vivo* conditions, may provide a new insight in possible genotoxic effects of tulathromycin.

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PROCENA OŠTEĆENJA DNK U LIMFOCITIMA PACOVA POD DEJSTVOM TULATROMICINA *IN VITRO*

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Izvod

Tulatromicin je relativno nov polusintetički makrolidni antibiotik, član grupe triamilida, koji ima primenu u lečenju respiratornih bolesti kod goveda i svinja. Različite genotoksikološe studije pokazuju da tulatromicin nije genotoksičan, ali nema raspoloživih publikovanih podataka u kojima se primenjuje komet test (test elektroforeze pojedinačnih ćelija . Stoga, cilj ovog rada je bio da se ispita da li tulatromicin može izazvati oštećenje DNK primenom *in vitro* Komet testa u izolovanim limfocitima pacova. Limfociti su tretirani širokim spektrom koncentracija tulatromicina (od 1 do 100 uM) i obavljen je ko-tretman sa antioksidansom katalazom (100 IU/mL i 500 IU/mL). Najveća koncentracija tulatromicina (50 i 100 μM) indukovala je značajno povećanje oštećenja DNK u limfocitima pacova, dok katalaza nije značajno redukovala stepen oštećenja DNK pod dejstvom tulatromicina. Rezultati ove studije pokazuju da je tulatromicin indukovao genotoksične efekte pri visokim koncentracijama, a katalaza nije ispoljila protektivan efekat u ovoj studiji.

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