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IMPACT OF TILMICOSIN ON THE RABBIT SPERMATOZOA MOTILITY AND VIABILITY

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ABSTRACT

The aim of this study was to examine the effects of tilmicosin on rabbit spermatozoa viability and motility parameters during short-term *in vitro* incubation at 37°C. Semen samples were collected from adult New Zealand White rabbits and diluted with eight concentrations of tilmicosin: 0.300, 0.350, 0.400, 0.466, 0.500, 0.583, 0.600 and 0.700 mg.ml^{-1} (TIL 1 – 8) diluted in the physiological solution. The motility parameters were evaluated using the Computer Assisted Semen Analyzer system (Sperm Vision®) at five time periods: 0, 1, 2, 3 and 4 hours. Cell viability was measured using the metabolic activity MTT assay. Immediately at the beginning of incubation significantly higher values of motility (MOT) and progressive motility (PRO) were detected in samples TIL2, TIL5 and TIL7 compared to the control. Significantly lower values of tested motility parameters were observed after 1 and 3 hours of incubation. All concentrations of tilmicosin have no significantly negative effect on the parameters of the MOT, PRO and velocity curved line (VCL) after 4 hours of incubation *in vitro* at 37°C. Concentration of tilmicosin 0.350 mg.ml^{-1} has a positive impact on MOT (72.39 ± 15.62%, $p<0.001$) and PRO (54.28 ± 21.23%, $p<0.01$) of rabbit spermatozoa after 4 hours of incubation in vitro at 37°C. Supplementation of tilmicosin led to preservation of the cell over all time periods of the *in vitro* incubation. The results indicate that tilmicosin could be used to semen extenders without negative effects on rabbit spermatozoa motility and viability.

Keywords: Antibiotics, spermatozoa, motility, CASA

INTRODUCTION

Antimicrobial agents are of extraordinary importance for the control of bacterial growth in liquid-preserved ejaculates of farm animals. To counteract increasing bacterial resistance to conventional antibiotics, novel agents with different active mechanisms have to be developed **(Schulze, *et al.*, 2016)**. The bacterial presence in semen samples could be especially problematic in situations where the ejaculates are used for artificial insemination 24 h or more after collection **(Suarez and Pacey, 2006)**. Therefore, it is of great importance that the extended shipped ejaculates maintain the highest quality upon arrival. Bacterial contamination is routinely observed in raw, extended and stored semen produced for artificial insemination when semen is collected by the gloved-hand technique. A study reported that 62.5% of raw ejaculates and 79% of extended boar semen doses showed bacterial contamination **(Maroto Martín *et al.*, 2010)**. The incidence of bacterial presence in the germ line can impact semen quality, also affecting the DNA molecule **(Gonzalez-Marin, *et al.*, 2012)**. The inclusion of antibiotics in semen extenders is recommended for the control of several microorganisms that can be present in spermatozoa **(Bielansky, 2007; Thibier and Guerin, 2000)** and The World Organization for Animal Health recommends the inclusion of antibiotics in extenders used in cryopreservation of spermatozoa, in order to control bacterial contamination **(Madeira, *et al.*, 2014)**.
Tilmicosin, 20-deoxo-20-(3.5-dimethylpiperidin-1-yl) desmycosin, is a semisynthetic derivative of tylosin **(Giguere, *et al.*, 2013)**. Tilmicosin is a macrolide antibiotic developed for veterinary use. It is recommended for treatment and prevention of respiratory diseases in cattle, sheep, pigs, rabbits, chickens and turkeys and for the treatment of other diseases caused by tilmicosin-sensitive microorganisms **(WHO Technical Report Series, 2009)**. Tilmicosin is a narrow spectrum antibiotic and effective against Gram-positive pathogens **(Hogeveen, 2005)**, although some gram-negative bacteria are affected and the drug reportedly has some activity against mycoplasma. Preliminary studies have shown that 95% of studied isolates of *Pasturella haemolytica* are sensitive **(Plamb, 2008)**.
Antibiotics are necessary and mandatory additives in semen extenders for the liquid preservation of several animal species **(Bryła and Trzcińska, 2015)**. Although opportunistic contaminants of semen generally do not incur an important health risk for the inseminated females, they can affect spermatozoa quality (**Yániz *et al.*, 2010**). Fertilizing capacity of spermatozoa can be directly affected by bacteria **(Morrell 2006)** that can impair the spermatoza motility, have the ability to adhere with spermatozoa, and can induce the acrosome reaction **(Qadeer, *et al.*, 2013)**.
The objective of our study was to examine the effects of tilmicosin, macrolide antibiotic, on rabbit spermatozoa viability and motility parameters *in vitro*.

MATERIAL AND METHODS

Biological material

In this study the ejaculates were collected from adult New Zealand White rabbits using artificial vagina **(Massányi *et al.*, 2008)**. The rabbit were kept in individual cages and fed *ad libitum* with a commercial diet.

Sample preparation

Semen was diluted in a ratio of 1 part of semen and 6 parts of physiological solution (Sodium chloride 0.9% Braun, B. Braun Melsungen AG, Melsungen, Germany) – control groups (TILC). At the same ratio semen was diluted with eight different concentrations of tilmicosin (purity 94%, Sigma-Aldrich, St. Louis, USA) solution: TIL1 – 0.300 mg.ml^{-1}; TIL2 – 0.350 mg.ml^{-1}; TIL3 – 0.400 mg.ml^{-1}; TIL4 – 0.466 mg.ml^{-1}; TIL5 – 0.500 mg.ml^{-1}; TIL6 – 0.583 mg.ml^{-1}.; TIL7 – 0.600 mg.ml^{-1}; TIL8 – 0.700 mg.ml^{-1} diluted in the physiological solution. All samples were cultured at 37°C and measured at five time periods: 0, 1, 2, 3 and 4 hours (Time 0 – 4). The experiment was realized in 6 replicates.

Analytical method

Each of thus prepared samples was evaluated using a Computer Assisted Semen Analyzer (CASA) system – Sperm Vision® (Minitub, Tiefenbach, Germany) equipped with a microscope (Olympus BX 51, Japan) to assess the spermatozoa motility. Each sample was placed into Makler Counting Chamber® (depth 10

μm, Sefi–Medical Instruments, Germany). In the present study the following parameters were evaluated – total motile spermatozoa (MOT) [%], progressively motile spermatozoa (PRO) [%] and velocity curved line (VCL) [μm.s^{-1}]. Within each of the measurement by the CASA system were evaluated motility parameters from minimum seven fields of Makler Counting Chamber.

Viability of rabbit spermatozoa exposed to tilmicosin *in vitro* was evaluated by 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. Formazan was measured spectrophotometrically by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed tilmicosin). Viability of the spermatozoa was tested in the samples TIL1, TIL2 and TIL8. Results from the analysis were collected during four repeated experiments for each concentration.

Statistical analysis

Obtained data were statistically analysed by PC program Excel and a statistics package GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, California, USA) using one-way ANOVA with Dennett's posttest. Statistical significance was indicated by *p* values of less than 0.05; 0.01 and 0.001.

RESULTS AND DISCUSSION

The results of CASA analysis of selected motility parameters are presented in Figures 1-3. At the beginning of incubation significantly higher percentage of spermatozoa motility was detected in the samples TIL3 ($p<0.05$), TIL5 ($p<0.01$) and TIL7 ($p<0.001$) compared to control (TIL K). At the same time non-significant differences were observed in the others experimental samples at the Time 0. However after 1 hour of incubation negative effect of tilmicosin on the turkey spermatozoa motility was detected in the samples TIL3, TIL5, TIL6, TIL7 and TIL 8 in comparison to the control. With increasing time of incubation between tested samples and control similar values of motility were observed. Significantly ($p<0.001$) protective effect of tilmicosin on preservation of spermatozoa motility was found in the samples TIL2 (0.350 mg.ml^{-1}). After 4 hours of incubation percentage of motility was 72.69% in the sample TIL2 and 46.10% in the control sample (Figure 1).

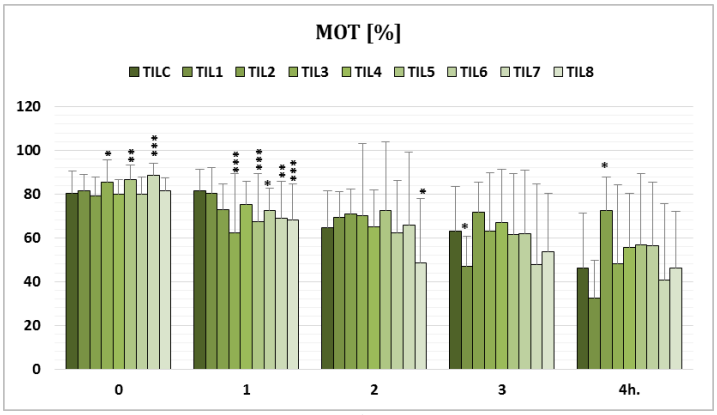

Figure 1 Spermatozoa motility (in %) after tilmicosin addition. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *p<0.05; **p<0.01; ***p<0.001.

Spermatozoa progressive motility (PRO) followed the tendency of spermatozoa motility. Measurement of progressive spermatozoa motility (PRO) showed statistically significant difference immediately at the beginning of incubation (Figure 2). The values of samples TIL3 (76.78%; $p<0.05$), TIL5 (78.43%; $p<0.01$) and TIL7 (81.19%; $p<0.001$) were significantly higher in comparison to the control sample TILK (66.12%). After 2 hours of *in vitro* incubation significant decrease of progressive motility was detected in all tested samples excepr sample TIL1 with the lowest concentration of tilmicosin (0.300 mg.ml^{-1}). With increasing time of incubation values of progressive motility between tested samples and control were balanced. Significantly higher ($p<0.001$) progressive motility was observed only in the sample TIL7 at the Time 4.

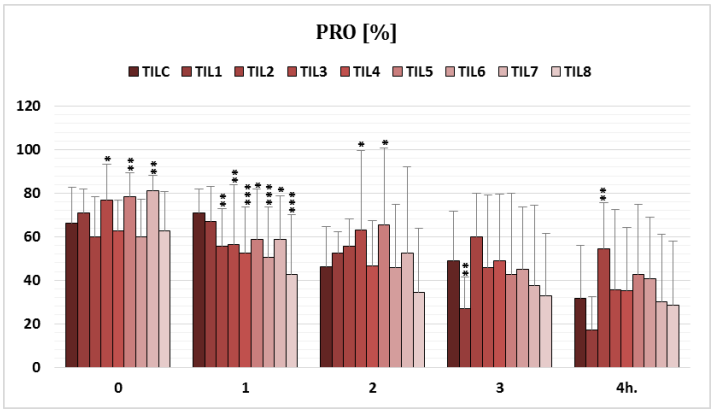

Figure 2 Spermatozoa progressive motility (in %) after tilmicosin addition. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *p<0.05; **p<0.01; ***p<0.001

The initial analysis of velocity curved line (VCL) proved significantly higher values in samples TIL2, TIL5 and TIL7 ($p<0.001$) compared to the control sample TILK. Significantly lower values of VCL were detected in samples TIL2, TIL4 – TIL8 in comparison to the control at the Time 1 and 3 (Figure 3.). At the other time of *in vitro* incubation balanced values of VCL were detected between tested samples and control group.

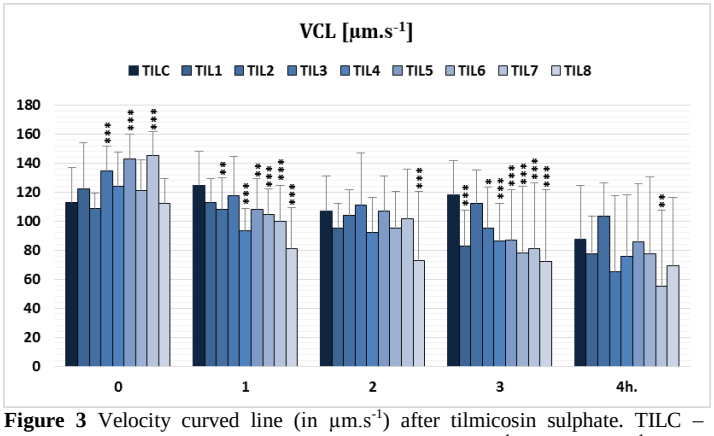

Figure 3 Velocity curved line (in μm.s^{-1}) after tilmicosin sulphate. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *p<0.05; **p<0.01; ***p<0.001

The impact of tilmicosin on viability of rabbit spermatozoa was measured in the samples TIL1 – 0.300 mg.ml^{-1}, TIL2 – 0.350 mg.ml^{-1} and TIL3 – 0 .700 mg.ml^{-1}. During the whole *in vitro* incubation the sample TIL 2 showed the most positive effect on the all monitored spermatozoa motility parameters. According to the MTT assay, significantly decrease of viability was not detected. Nevertheless at the Time 2 increasing of percentage of vital cells was observed (Figure 4). Similar to the CASA analysis, the MTT test revealed stimulation in the cell viability in the samples TIL1 and TIL2. The sample TIL1 (183.3%) showed significantly higher ($p<0.05$) values of viability in comparison to the control sample at the Time 2 and sample TIL2 at Time 4.

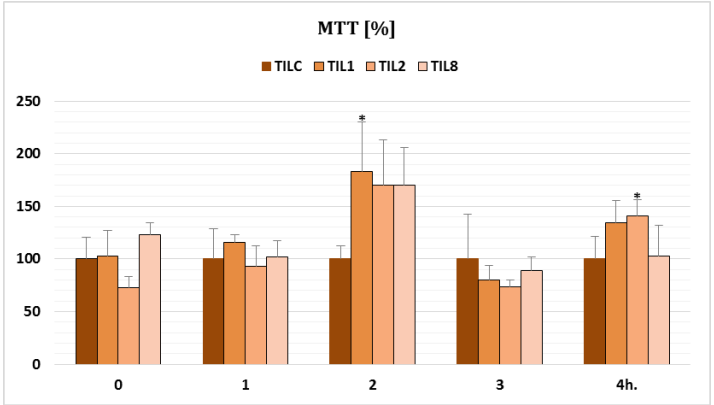

Figure 4 The effect of various doses of tilmicosin on the viability of rabbit spermatozoa. TILC – Control sample, TIL1 – 0.300 mg.ml^{-1}, TIL2 – 0.350 mg.ml^{-1} and TIL3 – 0 .700 mg.ml^{-1}.

The effect of bacteria on spermatozoa quality is still controversial. Semen is an ideal medium for the establishment and growth of several microorganisms including bacteria **(Bryła and Trzcińska, 2015)**. Microbial contamination reduces spermatozoa motility, shortens the storage time of viable semen, and decreases fertility rates, resulting in economic losses for AI facilities **(Bussalleu *et al.*, 2011)**. In contrast, some researchers have found that the presence of bacterial strains in semen samples does not usually impair semen quality **(Cottell *et al.*, 2000)** and that the bacterial contamination of the ejaculate has no significant effect on the farrowing rate or the total number of piglets born (**Reicks and Levis, 2008)**. Therefore it is very important to test not only the effect of bacterial strains on semen quality parameters of *in vitro* conditions but also testing the impact of the commercially used antibiotics.

Several studies have considered the effect of different antibiotics on the quality of stored semen. The data reported in study of **Gloria *et al.* (2014)** reveals that combinations of oflexacin (10-400 $mg.ml^{-1}$) and tylosin (10-250 $mg.ml^{-1}$), seems to have a negligible effect on spermatozoa motility ($p>0.05$). Furthermore, progressive motility was significantly higher for spermatozoa diluted with both antibiotic combinations compared with samples without antibiotics ($p<0.01$). Our results agree with **Gloria *et al.* (2014)** and confirmed that lower concentration of macrolide antibiotics, in our study tilmicosin, have no negative effect on spermatozoa motility. **Madeira *et al.* (2014)** showed that the combination of antibiotics (PES = 100,000 IU/ml penicillin and 0.100 $mg.ml^{-1}$ streptomycin; GTLS: 0.500 $mg.ml^{-1}$ gentamicin; 0.100 $mg.ml^{-1}$ tylosin; 0.300 $mg.ml^{-1}$ lincomycin and 0.600 $mg.ml^{-1}$ spectinomycin; CEF: 0.50 $mg.ml^{-1}$ ceftiofur sodium; ENR: 0.001 $mg.ml^{-1}$ enrofloxacin) in extenders at the tested concentrations did not influence the integrity of either spermatozoa membrane or acrosome, in both cooled and thawed ram spermatozoa. Nevertheless, spermatozoa motility was negatively affected with antibiotic addition. The reduction in spermatozoa motility may be attributed to the reduction of mitochondrial activity in spermatozoa, since quinolone antibiotics inhibit DNA gyrase **(Sárközy, 2001)**. This particular mechanism of action allows the elimination of strains resistant to antibiotics which act on the cell wall, the cytoplasmic membrane or on protein synthesis. **Qadeer *et al.* (2013)** suggested that neomycin, polymyxin, or colistin in combination with penicillin did not deteriorate semen quality and may be suggested to replace streptomycin in traditional antibiotic combination for cryopreservation of Nilli-Ravi buffalo bull spermatozoa. On the other side, combination of gentamycin, tylosin, lincomycin and spectinomycin have slightly significantly ($p<0.05$) negative effect on canine total spermatozoa motility **(Becher *et al.*, 2013)**.

The highest difference of toxicity between the cell lines and the spermatozoa were observed after application of the macrolide antibiotic tilmicosin. According to our measurements, the concentration of tilmicosin 0.350 $mg.ml^{-1}$ has a significantly positive effect on the motility and progressive motility and in the study of VERO cells survival of 25.79% was detected **(Fülöpová *et al.*, 2012)**. For the BHK-21 cells is the lethal dose a concentrations already 0.050 $mg.ml^{-1}$. At the concentration of 0.200 $mg.ml^{-1}$ only one third of cells was vital. The MTT assay showed that even the sample with the highest concentration of tilmicosin (0.700 $mg.ml^{-1}$) had not a negative effect on viability of rabbit spermatozoa. The samples TIL2 (0.350 $mg.ml^{-1}$) even showed significantly increase ($p<0.05$) of percentage of vital spermatozoa at the Time 4, which is in correlation with the results of motility analysis. During whole *in vitro* incubation no significant differences in this concentration of tilmicosin were detected.

CONCLUSION

According to our results it may be concluded that tilmicosin, a macrolide antibiotic, in concentrations from 0.300 to 0.700 $mg.ml^{-1}$ have not significantly negative effect on selected motility parameters after 4 hours of *in vitro* incubation at 37°C. Even concentration of tilmicosin 0.350 $mg.ml^{-1}$ showed significantly positive effect on motility and progressive motility of rabbit spermatozoa. Hereby neither the highest tested concentration of tilmicosin (0.700 $mg.ml^{-1}$) had negative impact on the mitochondrial metabolic activity. Therefore, we can recommend the use of tilmicosin for rabbit semen extenders.

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