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The *In Vitro* Effect of Kanamycin on the Behaviour of Bovine Spermatozoa

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Abstract

The use of antibiotics is a common part of animal biotechnologies. Especially, the use of antibiotics in semen extenders is necessary. However, the effect of antibiotics on the spermatozoa structure and function is still not completely examined. Therefore, the aim of our study was to investigate the effect of kanamycin on bovine spermatozoa at concentrations of 80 and 160 µg/mL during the 24 h *in vitro* cultivation. Semen samples were collected from clinically healthy Holstein-Friesian bulls. At times of 0, 2 and 24 h the motility assessment, mitochondrial activity, acrosomal and membrane integrity evaluation were performed. The sperm motility was measured using the Computer-assisted sperm analysis (CASA). Mitochondrial activity was evaluated through the Mitochondrial Toxicity Test (MTT). The acrosomal status was determined using the fast green/rose bengal staining on slides. Similarly, the membrane integrity was analysed using the eosin-nigrosin staining. Our results revealed the dose- and time-dependent effect of kanamycin under the *in vitro* conditions. In conclusion, the selected concentrations of kanamycin may have adverse effects on the motility, mitochondrial activity, acrosomal and membrane integrity during semen processing. Considering the relatively low concentrations used, we do not recommend to use kanamycin as a supplement in bovine semen extenders.

1. Introduction

The collection of bovine semen samples is not a sterile process. Even thorough sanitary precautions cannot prevent the potential microbial load in semen. Not only during semen collection, but also during processing and storage of semen the sample may be contaminated. Even freezing is not an obstacle to the bacterial survival, colonization of semen sample and an outbreak of infection disease inside the recipient's uterus **(Paray *et al.*, 2018)**. Therefore, antibiotic treatment in semen storage media is necessary. However, the use of an optimal concentration of antibiotics is as important as the choice of antibiotic supplementation. The same concentration of the same antibiotic in the semen extender may, depending on the species, improve or deteriorate spermatozoa quality **(Morell and Walgren,2014)**.

Aminoglycosides are popularly used as supplements of semen extenders **(Morrell and Wallgren, 2014; Khaki, 2015)** due to their selective mechanism of action. Kanamycin (KAN) irreversibly binds to the bacterial 16s rRNA, causing misalignment of the amino acids during proteosyntesis leading to the breakdown of the polysomes into non-functional monosomes **(Wieninger *et al.*, 2011)**. Nowadays, various types of antibiotics or thier combinations are used to manage bacteriocenosis in ejaculates and potential risk of harmful effects of bacteria. Previous study recommended the dose of 80 µg/mL of KAN in semen extenders **(Di Iorio *et al.*, 2014)**. However, there are no studies about the effect of KAN on spermatozoa during their *in vitro* cultivation **(Stoianov, 1987)**. Therefore, the aim of our study was to evaluate the effect of the recommended concentration (80 µg/mL) and double concentration (160 µg/mL) of KAN on the motility, mitochondrial activity, membrane and acrosomal integrity of bovine spermatozoa during 0, 2 and 24 h.

2. Material and methods

2.1 Sample collection and processing

Semen samples (n=10) were collected from clinically healthy Holstein-Fiesian breeding bulls (Slovak Biological Services, a.s., Lužianky, Nitra, Slovakia). Each sample was subjected to the quality criteria depending on the breed. After collection, the semen samples were transported to the laboratory within 15 min. in constant temperature. Samples were diluted in physiological saline solution (PS; sodium chloride 0.9% w/v, Bieffe Medital, Grosotto, Italia) in a dilution ratio of 1:40. Kanamycin (KAN; Kanamycin sulfate, Sigma-Aldrich, St. Louis, USA) dissolved in PS was added to the semen samples at final concentrations of 80 and 160 µg/mL. The control group (Ctrl) consisted of PS and spermatozoa. Semen samples were incubated during 24 hours at room temperature (20-22°C). At times of 0, 2 and 24 h, motility assessment, mitochondrial activity, membrane and acrosomal integrity were analysed.

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2.2 Motility assessment

Basic analysis of the sperm quality, motility assessment, was carried out using the Computer-assisted semen analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten µL of each sample were placed into the Makler counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and subjected to sperm motility (MOT) analysis. Only spermatozoa moving at least 5 µm/s is considered to be motile. The percentage of motile spermatozoa was evaluated at a minimum of 300 cells in each sample (**Tvrdá et al., 2016**).

2.3 Mitochondrial activity assessment

Mitochondrial metabolic activity was analysed using Mitochondrial Toxicity Test (MTT). This colorimetric method is based on enzymatic conversion of a yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan particles by succinate dehydrogenase of intact mitochondria within living cells. Twenty µL of tetrazolium salt (Sigma-Aldrich, St. Louis, USA) dissolved in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich, St. Louis, USA) to concentration of 5 mg/mL were added to each sample and incubated during 1 h at 37°C. Subsequently, formazan crystals were dissolved using 80 µL of isopropanol (propan-2-ol; Centralchem, Bratislava, Slovakia). Optical density was measured using the Glomax Multi+ (Promega Corporation, Madison, WI, USA) at a wavelength of 570 nm against 620 nm as reference. The results are expressed as percentage of the Ctrl group set to 100% (**Tvrdá et al., 2016**).

2.4 The sperm membrane integrity

The standard eosin-nigrosin staining protocol was performed to evaluate the functional maintenance of the sperm membrane. The assay is based on the ability of eosin to penetrate into the cells and to distinguish non-viable cells from viable. Five µL of each sample were placed on a glass slide and immediately mixed with 10 µL 5% eosin (Sigma-Aldrich, USA), followed by 10 µL 10% nigrosin (Sigma-Aldrich, USA). The mixture was smeared on a glass slide and left to air-dry. Using the bright-field microscopy (1000 ×; minimum of 200 cells in each sample were evaluated based on a ratio of red heads (dead)/white heads (live). The results are expressed as a percentage of live spermatozoa (**Agarwal et al., 2016**).

2.5 The acrosomal status

Acrosomal integrity of sperm cells was evaluated according to staining protocol by **Pope *et al.* (1991)**. The staining solution consisted of 1% fast green, 1% rose bengal (both Sigma-Aldrich, USA) and 40% ethanol (Centralchem, Slovakia) dissolved in 0.1M citric acid–0.2M disodium phosphate buffer (Sigma-Aldrich, USA). Twenty µL of each sample was mixed with 20 µL of staining solution and incubated during 70 s at room temperature (20-22°C). Ten µL of the mixture was smeared on a glass slide and left to air-dry. The presence or absence of acrosome was performed by one observer using the bright-field microscopy (1000 ×). Minimum of 200 cells in each sample were counted. The results are expressed as a percentage of sperm with intact acrosome.

2.6 Statistical analysis

The GraphPad Prism program (Version 6.0 for MS Windows; GraphPad Software, www.graphpad.com) was used for statistical evaluation. All data are expressed as the arithmetic mean (AM) ± standard error of mean (SEM). One-way ANOVA with following Dunnett's test was used for specific statistical evaluations. The significance level was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

3. Results

Two concentrations of kanamycin were used to reveal the effect of kanamycin on the motility, mitochondrial activity, membrane and acrosomal integrity of spermatozoa. The CASA assessment (Figure 1) revealed already at initial time a significant decrease (P<0.01) in the group treated with 160 µg/mL of KAN when compared to the control group. Only a slight decrease was observed in the group treated with 80 µg/mL. After 2 h, spermatozoa motility significantly decreased in both experimental groups (P<0.01 in case of 80 µg/mL; P<0.001 in case of 160 µg/mL). Similar results were obtained after 24 h. When compared to Ctrl, spermatozoa motility decreased by treating 80 µg/mL (P<0.05) as well as 160 µg/mL (P<0.001) of KAN.

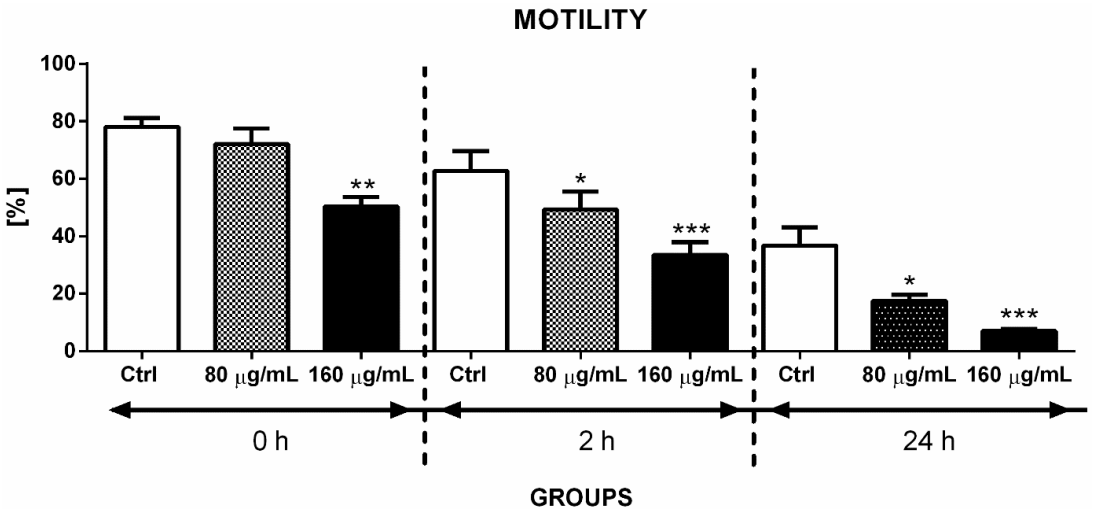

Figure 1. The development of spermatozoa motility in the groups treated with 0 (Ctrl), 80 and 160 µg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

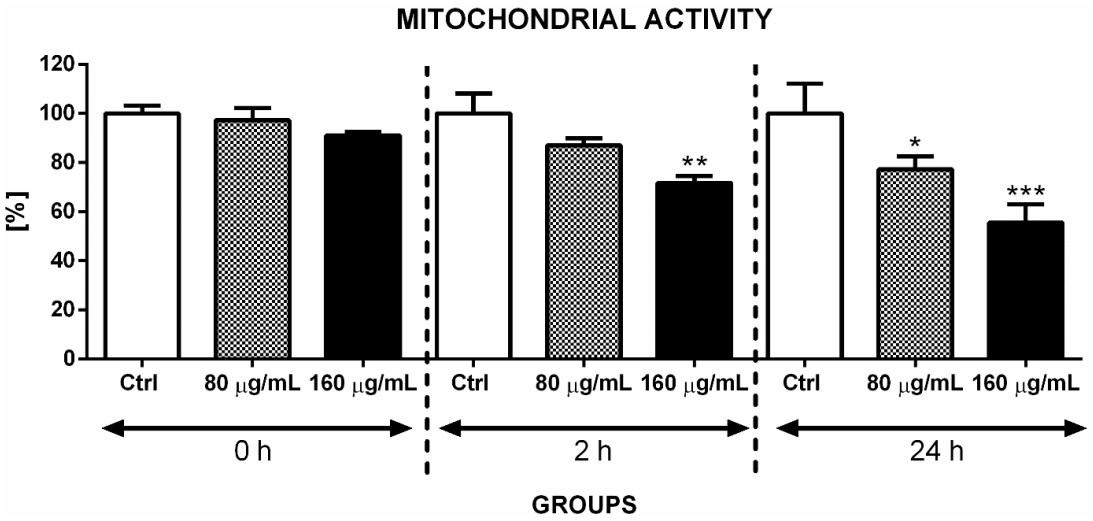

Figure 2. The development of mitochondrial activity of spermatozoa in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

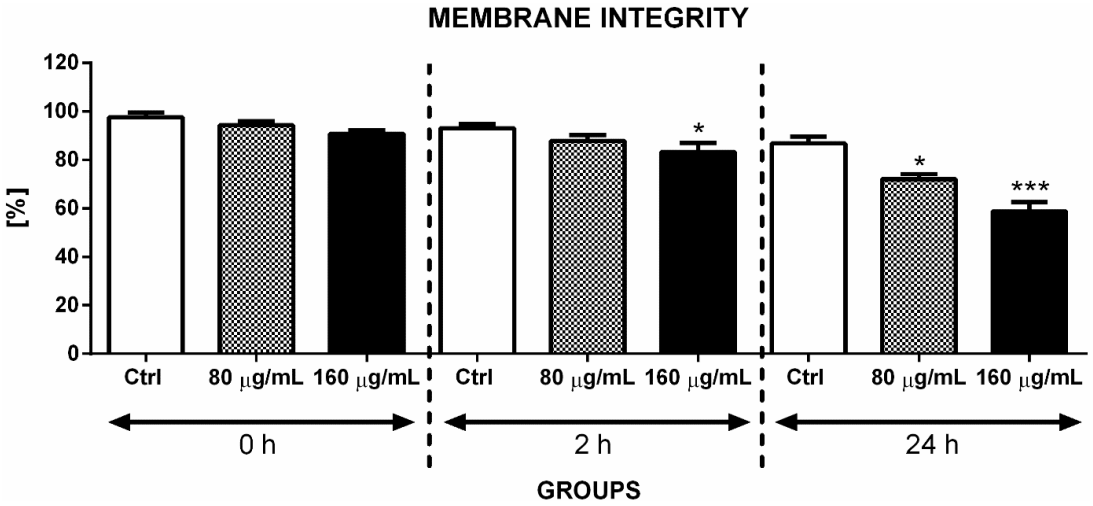

Figure 3. The development of the percentage of spermatozoa with intact membrane in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

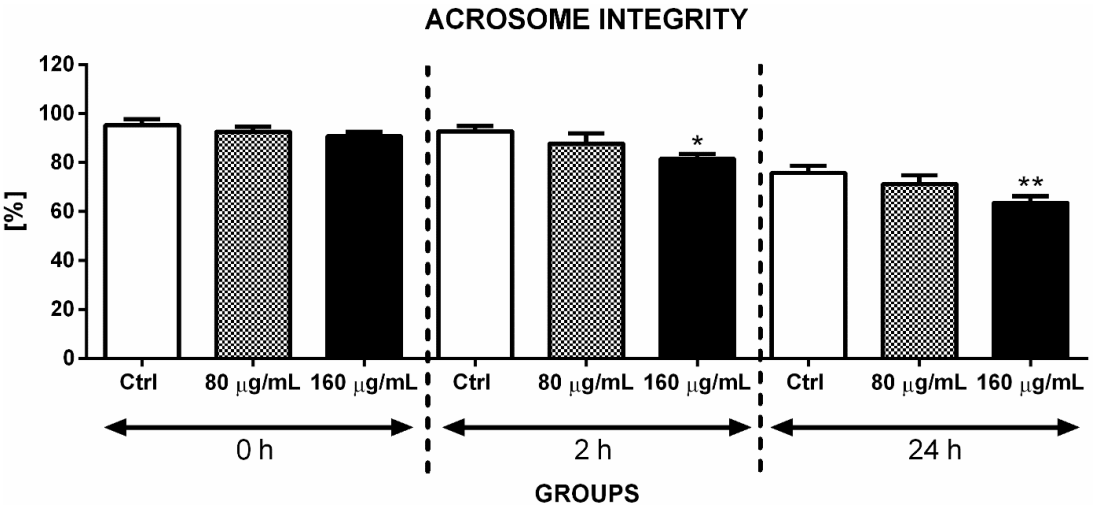

Figure 4. The development of the percentage of spermatozoa with intact acrosome in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

The results of mitochondrial activity assessment (Figure 2) showed the dose-dependent decreasing trend at the initial time with no significant differences when compared to Ctrl. The set decreasing trend continued also after 2 h with observable significant decrease in the group treated with 160 µg/mL of KAN. Finally, both concentrations of KAN significantly inhibited mitochondrial activity ($P<0.05$ in case of 80 µg/mL and $P<0.001$ in case of 160 µg/mL).

The eosin-nigrosin staining did not reveal any significant differences during the initial assessment (Figure 3). After 2 h, the percentage of sperm cells with damaged membrane integrity significantly increased in the group treated with 160 µg/mL. The results of final measurement showed significantly decreased percentage of sperm cells with intact membrane integrity in the group treated with 80 µg/mL ($P<0.05$) as well as the group treated with 160 µg/mL ($P<0.001$).

Similarly, the evaluation of acrosomal status (Figure 4) did not bring with any significant differences during the initial measurement. After 2 h, the percentage of sperm cells with damaged acrosomal integrity significantly increased in the group treated with 160 µg/mL. The dose-dependent trend was observed also after 24 hours. However, only in the group treated with 160 µg/mL significantly decreased ($P<0.01$) the amount of spermatozoa with intact acrosome.

4. Discussion

Lack of studies about the direct *in vitro* effect of KAN on spermatozoa gave rise to investigate basic structural and functional quality of bovine spermatozoa under these conditions. According to previous *in vivo* study on rats, aminoglycosides decreased the seminal vesicle weight. Moreover, production of abnormal spermatids was increased, depending on a dose, leading to increased sperm abnormalities. These structural abnormalities were reflected in a decreased sperm count and sperm motility. Decreased activities of superoxide dismutase, catalase and glutathione peroxidase in semen suggested that KAN may damage spermatogenesis and spermatozoa through inducing oxidative stress in the male reproductive tract **(Narayana, 2008)**. As unsaturated fatty acids are richly represented in mammalian sperm membranes, they are particularly sensitive to increased concentrations of reactive oxygen species (ROS), leading to the alteration of membrane fluidity **(Mahanta *et al.*, 2012)**, thus damage of membrane integrity.

Schlegel *et al.* (1991) reported that probably the premature termination of meiosis at the stage of primary spermatocytes may be responsible for harmful effects of aminoglycosides during spermatogenesis. On the other hand, the effect on mature spermatozoa was according to this study negligible. Furthermore, they reported that aminoglycosides had no direct effect on the viability and/or motility of spermatozoa under the *in vitro* conditions. Kanamycin, streptomycin, amikacin, gentamycin, tobramycin and neomycin had no detrimental effects at concentrations up to 1000 µg/mL. In contrast to this statement, our results proved detrimental effect of kanamycin at concentration of 160 µg/mL during the initial motility measurement. Other parameters, such as mitochondrial activity, membrane and acrosomal integrity, slightly decreased, but there was no significant difference.

Aminoglycosides can induce also programmed cell death. The formation of ROS begins with the entry of the aminoglycoside into the cell and binding with the iron cation to the aminoglycoside-Fe complex, which catalyzes the production of ROS in the presence of unsaturated fatty acids **(Lesniak *et al.*, 2005)**. One of the ROS-activated pathways is the c-Jun N-terminal kinase pathway, which translocates to the nucleus and activates genes responsible for apoptosis. Subsequently, a signal is sent to the mitochondria which induces the release of cytochrome c. Cytochrome c reports information to caspases on the initiation of cell death **(Davis, 2000; Rybak and Ramkumar, 2007)**.

Acrosomal integrity is largely influenced by the level of ROS. During physiological conditions, ROS play an important role in the fusion of male gamete with the oocyte **(El-Taieb *et al.*, 2015)**. However, if concentration of kanamycin is too high, then ROS are pathologically increased which leads to premature acrosomal reaction and therefore to the decrease of fertilizing potential of the semen sample.

Kalghatgi *et al.* (2013) studied detrimental side effects of bactericidal antibiotics in various cell lines. Clinically relevant doses of kanamycin had induced intracellular ROS production dependent from dose and time. Mitochondrial superoxide, a precursor of major forms of ROS, and concentration of released H_2O_2 were significantly increased in human MCF10A cell line. Therefore, we may hypothesize, that if there was the excess of kanamycin-induced ROS in sperm mitochondria, activity of succinate dehydrogenase was inhibited **(Andreyev *et al.*, 2015)**, thus the respiratory chain inhibited production of ATP resulting in decrease of spermatozoa motility.

4. Conclusion

Based on our findings, kanamycin had dose- and time-dependent effect on bovine spermatozoa under *in vitro* conditions. Concentration of 160 µg/mL of kanamycin had immediately detrimental effect on motility, which reflected after 2 h in decreased mitochondrial activity, damaged membrane and acrosomal integrity. There was only slight effect of kanamycin at concentration of 80 µg/mL immediately after mixing with spermatozoa. However, the use of kanamycin as a supplement during cryoconservation process could show in decreased total sperm quality during post-thaw process, and thereby could decrease fertilizing potential of bovine spermatozoa in the process of artificial insemination. Therefore, we do not suggest the use of kanamycin at observed concentrations as an antimicrobial agent in bovine semen preservation medium. The question remains whether lower concentrations of kanamycin would have the desired antimicrobial effect and at the same time would not damage the structural and functional properties of bovine spermatozoa.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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