Archives of Ecotoxicology, Vol. 2, No. 3, pp. 30-34, 2020

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Archives of Ecotoxicology

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The Effect of Penicillin on the Vitality of Bull Spermatozoa

Ján Kováč[a*], Nishonoy Khasanova[b], Tomáš Slanina[a], Peter Massányi[a], Eva Tvrdá[a]

[a] *Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic*

[b] *Department of Biotechnology, Faculty of Biology, National University of Uzbekistan, VUZ Gorodok, 700174, Tashkent, Uzbekistan*

Article info
Received 25 March 2020
Revised 3 June 2020
Accepted 16 September 2020
Published online 30 September 2020

Regular article

Keywords:
Antibiotic
Sperm motility
Acrosomal integrity
Mitochondrial activity
Membrane integrity
in vitro

Abstract

Antibiotic supplementation into semen extenders is an important way to control several microorganisms that can affect semen quality by their presence. The objective of the present work is to estimate the effect of two different concentrations (300 µg/mL and 600 µg/mL) of penicillin on the selected quality parameters of spermatozoa collected from bulls (motility, mitochondrial activity, acrosome integrity and membrane integrity) after 0, 2 and 24 h of *in vitro* culture. Sperm motion was examined using HTM IVOS computer-aided sperm analysis (CASA), cell viability was assessed with the metabolic activity (MTT) assay. The acrosomal integrity was evaluated following the fast green – rose bengal staining protocol and the eosin – nigrosin staining method was used to assess the functional integrity of the sperm membrane. Our results indicate that penicillin at lower amount significantly ($p>0.05$) decreased the sperm motility, mitochondrial activity and membrane integrity after 24 h of *in vitro* culture. Supplementation of higher doses of this substance led to a significant decrease of the sperm motion during 0, 2 ($p>0.05$) as well as after 24 h ($p>0.01$), of the viability after 2 h ($p>0.05$) and 24 h ($p>0.01$), of the acrosomal integrity after 24 h ($p>0.05$) and of the membrane integrity at 24 h ($p>0.001$) too. We can consider, that the effect of penicillin addition to bovine spermatozoa during *in vitro* incubation is time and dose dependent.

1. Introduction

Semen quality may be highly challenged by the presence of bacteriospermia. Microorganisms cause lower reproductive functions of males, by agglutination of motile sperm, by alternations in cell morphology and by reducing the occurrence of acrosome reaction (**Azawi and Ismaeel, 2011**). Natural mating or artificial insemination (AI) are a way for transmission of bacteria that can negatively affect the reproductive function of females and decrease the fertility rates (**Akhter *et al.*, 2007**). Semen samples are usually collected from the farm animals using an open-ended artificial vagina. This process can be easily compromised with bacterial contamination, which eventually causes lower semen quality during storage and contaminates the female reproductive tract (**Yániz *et al.*, 2010**). The presence and the multiplication of the bacteria in semen may deteriorate the semen samples during *in vitro* culture (**Moretti *et al.*, 2009**).
Over the last 60 years bovine semen samples have been extended in the presence antibiotics to control bacterial growth. Bacterial populations that could be present in semen samples are controlled by antibiotics present in semen extenders (**Boonthai *et al.*, 2015; Avilés *et al.*, 2019**). The most common combination of antibiotics added to bovine semen are penicillin and streptomycin, followed by gentamycin, tylosin, lincomycin and spectinomycin (**Visser *et al.*, 1999**), but some microorganisms may have a resistance to them (**Morrell and Wallgren, 2014**).

There is currently limited data regarding the effectiveness of antibiotics on the semen sample quality. The aim of this study was to investigate the effect of two different concentrations of penicillin during *in vitro* culture on the bovine reproductive cells.

2. Material and methods

2.1 Semen sample collection and processing

Semen samples (n=20) were collected from four adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). Ejaculates were collected regularly (once a week for five consecutive weeks) from each bull using an artificial vagina. After the collection, semen concentration and motility were measured by phase-contrast microscopy (200 x). For further experiments, we chose ejaculates with required quality (min. 70 % motility and concentration of 1 x 10^9 sperm/mL). Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.
Samples were diluted in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma Aldrich) containing various concentrations of the penicillin (300; 600 µg/mL) using a dilution ratio of 1:40. The samples were cultured at laboratory temperature (22-25°C). After

*Corresponding author: jan.johnny.kovac@gmail.com
https://doi.org/10.36547/ae.2020.2.3.30-34

culture periods of 0, 2, 24h, sperm motility, membrane integrity, acrosome integrity and mitochondrial activity were assessed in each group

2.2 Spermatozoa motility analysis

Spermatozoa motion parameters were evaluated using the computer-aided sperm analysis CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). The system was set up as follows: frame rate – 60 Hz; minimum contrast – 20; static head size – 0.25 – 5.00; static head intensity – 0.40 – 2.00; static elongation – 20 – 100; default cell size - 4 pixels; default cell intensity – 40. Ten µL of each sample were placed into the Makler counting chamber (depth 10 µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and spermatozoa motility (MOT; percentage of motile spermatozoa; motility > 5 m/s; %) was evaluated immediately. Ten microscope fields were subjected to each analysis to include at least 300 cells **(Tvrdá *et al.*, 2018).**

2.3 Eosin nigrosin staining

The functional integrity of the sperm membrane was assessed using the eosin - nigrosine staining method. This technique is based on the ability of eosin to penetrate into non – viable cells **(Moskovtsev and Librach, 2013)**. Five microliters of each sample were placed on tempered glass slide, mixed with 10 µL 5 % eosin (Sigma – Aldrich), followed by 10 µL 10 % nigrosine (Sigma – Aldrich). The mixture was smeared on glass slide and left to air – dry at 37 °C. The slides were observed using bright field microscopy at x 1000 using oil immersion. At least 200 sperm per slides were identified as either dead (red heads) or live (white heads), and expressed as a percentage rate. All slides were evaluated blindly by one observer.

2.4 Acrosomal integrity

The fast green – rose bengal staining protocol designed by **Pope *et al.* (1991)** was used to evaluate the acrosomal status of spermatogenic cells. This single – step staining method applies a mixture consisting of 1 % fast green (Sigma - Aldrich), 1 % rose Bengal (Sigma – Aldrich) and 40 % ethyl alcohol (Centralchem, Bratislava, Slovak Republic) in 0.1 M citric acid – 0.2 M disodium phosphate buffer (Sigma – Aldrich). Twenty microliters of the thawed sample were mixed with 20 µL of the staining solution and incubated for 70 s at room temperature. Ten microliters of the mixture were smeared on the tempered glass slide and air – dried at 37 °C. Acrosomal integrity was assessed using bright field microscopy at x 1000 using oil immersion. At least 200 cells per slide were evaluated for the presence or absence of acrosome, and expressed as a percentage rate. All slides were followed blindly by one observer.

2.5 Mitochondrial activity (MTT test)

Mitochondrial activity of the bovine reproductive cells was assessed using the colorimetric metabolic activity (MTT) test, which is based on the conversion of a yellow tetrazolium salt (3 – (4,5 – dimetylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide; MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. The tetrazolium salt (Sigma – Aldrich) was dissolved in PBS (Dulbecco's Phopshate Buffer Saline without calcium chloride and magnesium chloride; Sigma – Aldrich) at 5 mg/mL. Ten µL of the tetrazolium solution was mixed with each sperm suspension. After a 2 h incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO_2), the formazan crystals were dissolved in 80 µL of acidified (0.08 mol/L HCl; Centralchem) isopropanol (Centralchem). Optical density was determined at a wavelength of 570 nm against 620 nm as reference using a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are expressed as percentage of the control set to 100 % (**Knazická *et al.*, 2012).**

2.6 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com). Initially, descriptive statistical characteristics were evaluated. One-Way ANOVA with the Dunnett's test were used for specific statistical evaluations, based on the assumption that values in each row represent paired observations. The level of significance was set at $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

3. Results

Our experiment focused on the *in vitro* effects of penicillin on four quality parameters of bovine sperm. All assessments we performed at 0, 2 and 24 h using working solutions with 600, 300 µg/mL of penicillin administration.

The bovine sperm motility was performed using the CASA system. According to our results we can say, that higher concentration of penicillin (600 µg/mL) had a negative effect by decreasing of activity of the sperm cells at 0 and 2 h ($p>0.05$) as well as after 24 h ($p>0.01$) of *in vitro* culture. The lower dose addition had a similar effect, after 24 hours ($p>0.05$) when compared to the control group (Figure 1).

The second measured parameter was the mitochondrial activity, as mitochondria are the source of energy for the movement of the reproductive cells. At the beginning we did not register any significant changes. At 2 h, the higher applied concentration, significantly decreased ($p < 0.05$) mitochondrial viability. After 24 h of incubation, we observed a significantly decreasing mitochondrial activity ($p < 0.05$; $p < 0.01$) at both of the given doses (Figure 2).

The eosin – nigrosin staining was used as a method to assess membrane integrity of the bovine spermatozoa. Penicillin addition showed a decreasing membrane integrity at 2 h, but after 24 h of *in vitro* culture, administration of this substance led to a significantly decreased membrane integrity at the lower dose ($p < 0.05$) and at the higher dose ($p < 0.001$) as well (Figure 3).

The evaluation of the acrosomal status was prepared by the fast green - rose bengal staining method. Our results indicate, that exposure to penicillin led to a significantly decreased acrosome integrity at a higher dose application at 2 h ($p < 0.05$) and after 24 h ($p < 0.05$) (Figure 4).

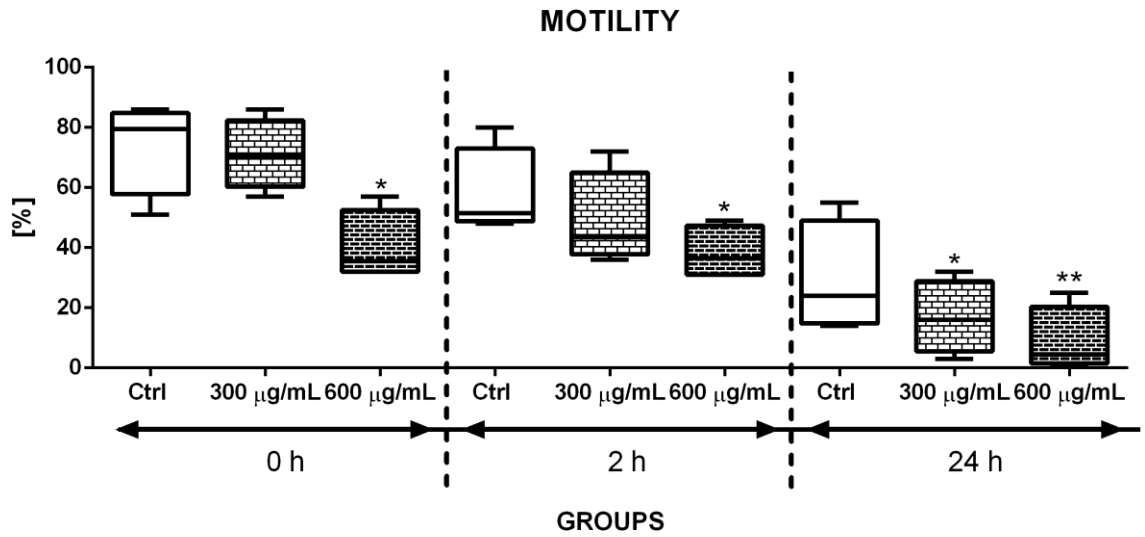

Figure 1. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$; ** $p < 0.01$.

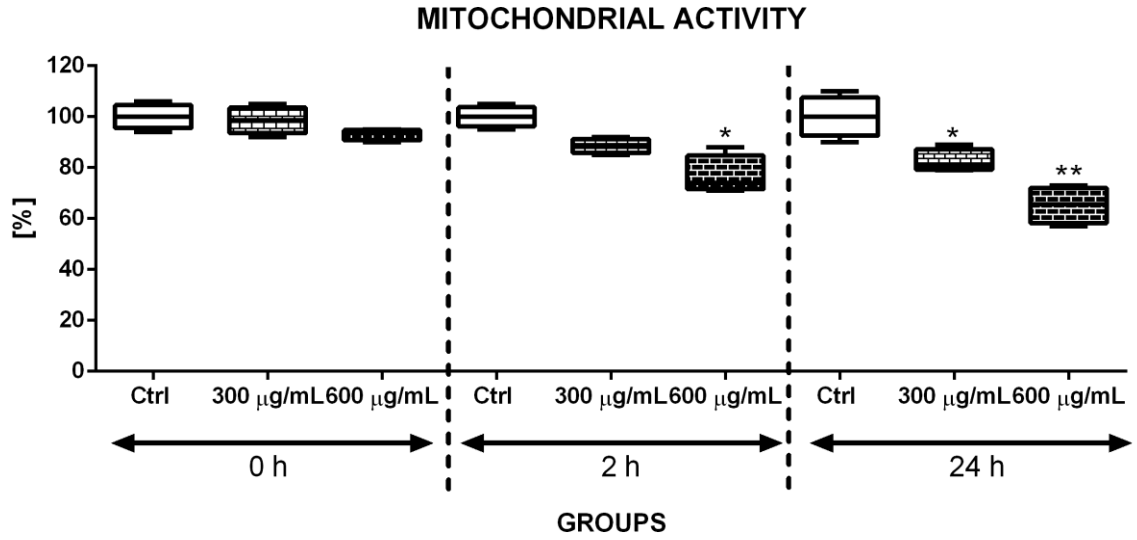

Figure 2. The effect of various concentrations of the penicillin on the mitochondrial activity of bovine spermatozoa at 0h, 2 and 24 h. The level of significance was set at * $p < 0.05$; ** $p < 0.01$.

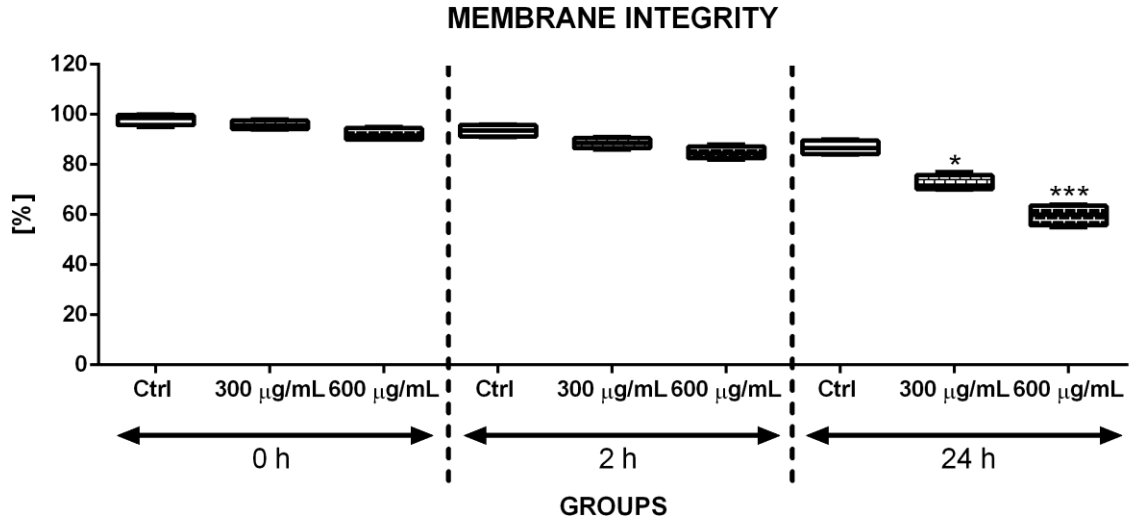

Figure 3. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$; *** $p < 0.001$.

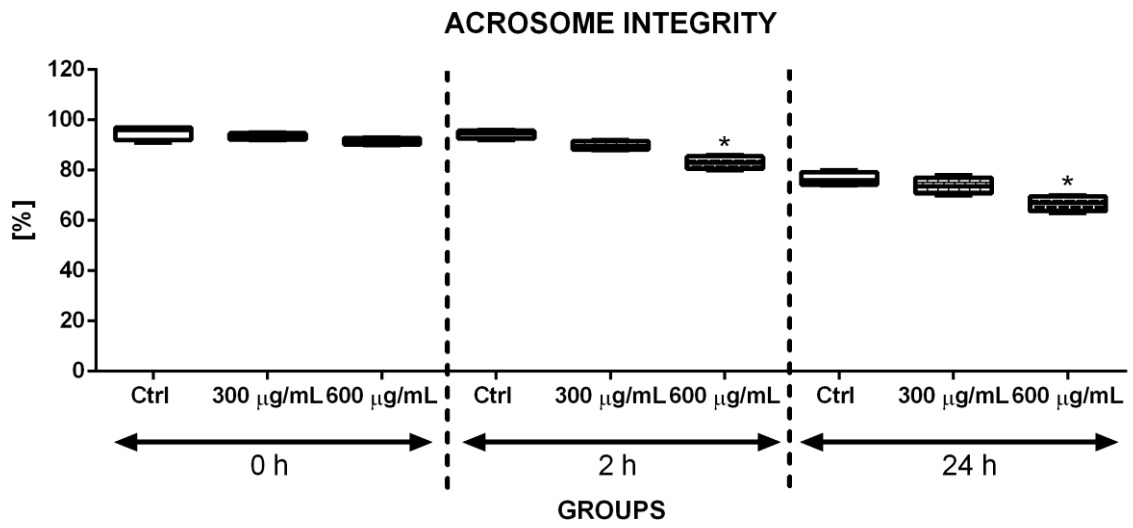

Figure 4. The effect of various concentrations of the penicillin on the motility of bovine spermatozoa at 0 h, 2 h and 24 h. The level of significance was set at * $p < 0.05$.

4. Discussion

In our study we found negative effects of penicillin. The same results were observed by **Stoss *et al.* (1978)**. They applied penicillin and streptomycin to rainbow trout semen, leading to bacterial growth inhibition, but with a detrimental effect on the spermatozoa too. Furthermore, **Schlegel *et* al. (1991)** monitored the effect of therapeutic doses of penicillin G and cephalothin on mammals. The authors recorded a gonadotoxic effect of both agents expressed as spermatogenesis arrest by this 8-day antibiotic treatment in rats.

Inversely, **Miraglia *et al.* (2003)** compared four antibiotics used to inactivate leptospires in Holstein Friesian bull semen. During their experiment, egg yolk citrate (EYC) extender was used with different concentrations of antibiotics. After 24 h there were no significant effects on the progressive motility or individual progressive motility of spermatozoids, but penicillin-streptomycin addition showed the best way to inactivate leptospires.

The first study focused on the effect of this antibiotic on male gametes was published by **Almquist *et al.* (1946).** Their experiment showed that the addition of penicillin into diluted semen of bulls with relatively high breeding efficiency did not cause any significant changes in the quality of bull semen. Subsequently, **Almquist (1949)** tested the impact of penicillin on the fertility of spermatozoa from relatively infertile bulls. Surprisingly the report showed that the administration of this agent in different amounts (500 and 1000 units per ml of extender) highly significantly increased the fertility of the inseminated cows. This conclusion was explained by the fact, that bulls with quite lower fertility might contain types of bacteria which can cause breeding difficulties, and penicillin may raise efficiency of the bovine semen only by eliminating the microorganisms which cause a disbalanced internal milieu for semen viability.

Alavi-Shoushtari *et al.* (2007) investigated the effects of various antibiotics on controlling bacterial contaminations in tris-egg yolk diluted buffalo bull semen. Each semen sample was exposed to the recommended and twice the recommended dose of antibiotic. The next step was to find out the impact of these additives on the buffalo spermatozoa diluted in the same extender (tris-egg yolk +glycerol) after freezing and thawing. They summarized, that penicillin G revealed to be an effective substance at both doses against bacterial growth and proved to be suitable as an additive to the semen extenders. The discrepancies between their report and our data may be explained by the fact that our results were measured at laboratory temperature (22-25 °C) during 24h, revealing no positive effects of the antibiotic on the bull semen quality parameters.

On the other hand, **Oplinger and Wagner (2015)** studied the ability of penicillin and streptomycin to reduce bacterial coldwater disease in Rainbow Trout semen. After 15 min exposure of these antibiotics in extender mixed with spermatozoa, the bacterial amount was reduced and the sperm motility was not changed. But, during production-scale trials, the authors observed a reduction of fertilized egg production by stored sperm in the antibiotic containing extender. This decreasing ability of fertilization can be caused by the penicillin content what finally points to the negative effect of this antibiotic on the basic semen parameters such as sperm motility, acrosome integrity, mitochondrial activity and membrane integrity as was showed in our results. On the other hand, the effects of penicillin and streptomycin sulphate were examined by **Rahimi *et al.* (2016)**. They focused on the application of these two antibiotics to semen samples gained from caspian brown trout during 12 days short-term storage at 4°C. After the experiment, the results showed, that the percentage and duration of semen viability were significantly higher in the samples enriched with antibiotic than in the antibiotic free group. They concluded that application of these antibiotics may improve the viability of caspian brown trout during short-term storage.

The effects of antibiotics on the sperm survival have become a fruitful topic in animal andrology. **Aurich and Spergser (2007)** investigated the impact of gentamicin addition to the semen extender on the motility, velocity and membrane activity. Addition of this antibiotic during cooled storage decreased the motility and velocity and may negatively affect the sperm functions in extended semen samples.

While our study showed a negative effect of penicillin, **Akhter *et al.* (2007)** tried to find out the effect of two antibiotic combination (streptomycin and penicillin; SP) (gentamycin, tylosin, lincomycin and spectinomycin; GTLS) in extender on the bacterial control and sperm quality of liquid bovine semen stored at 5 °C. After three days of storage at 5 °C, there were no differences in the sperm motility, longevity and plasma membrane integrity, but after five days, the measured semen parameters were significantly better in extender containing SP compared to GTLS and control group without antibiotic addition. The same conclusion was determined by **Dissanayake *et al.* (2014)**. The objective of their study was to detect the effect of penicillin and streptomycin in the elimination of bacteria and to

the human sperm motility, survivability and pregnancy rates. They found that this antibiotic combination did not seem to have any harmful effects on human reproductive cells.

Tylosin is an antibiotic belonging to macrolides. **Slanina *et al.* (2016)** examined the effects of tylosin on selected motion parameters of turkey spermatozoa during short-term *in vitro* incubation at 41 °C. Their results indicated, that tylosin did not have any negative effects on the motility parameters of the sperm samples. Finally, **Sone *et al.* (1982)** reported that after seven days of storage at 15 °C, about 80 % of the semen samples were without any bacterial growth and the mean amounts of the motility and normal acrosomes were 75.4 % and 82.7 %, in the presence of dibekacin.

5. Conclusion

In our study we focused on the determination of the *in vitro* effect of the penicillin on male reproductive markers: sperm motility, mitochondrial activity, membrane integrity and acrosomal integrity. The higher added concentration of the antibiotic (600 µg/mL) significantly decreased the motility as well as mitochondrial activity after 2 h. After 24 h both of the applied doses showed a detrimental effect on the viability and sperm metabolism of the bovine spermatozoa. The protective dose (300 µg/mL) significantly reduced the membrane integrity, same as the higher dose did, after 24 h of incubation. The acrosome integrity was significantly lower after 2 h as well as after 24 h in the presence of the higher applied dose. We may conclude that penicillin addition, as an antibiotic control against bacteriospermia, can significantly inhibit the selected parameters, revealing its potential negative effect on the bovine reproductive cells.

Acknowledgments

This study was supported by the VEGA grant no. 1/0239/20, APVV grant no. APVV-15-0544 and and by the European Community Project no. 26220220180: Building Research Centre „AgroBioTech“. Experiments of this study were performed with support of the “CeRA” Team of Excellence.

Declaration of interest

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

References

1. Akhter, S., Ansari, M. S., Andrabi, A. M. H., Ullah, N., Qayyum, M. 2007. Effect of Antibiotics in Extender on Bacterial and Spermatozoal Quality of Coolode Buffalo (Bubalus bubalis) Bull Semen. *Reproduction in Domesstic Animals*, 43(3), 272-278. https://doi.org/10.1111/j.1439-0531.2007.00890.x
2. Alavi-Shoustari, S. M., Ahmadi, M., Shahvarpour, S., Kolahain, S. 2007. Effects of Tiamulin, Tetracycline, Fluorophemicol, Penicillin G, Linco-Spectin, Erythromycin and Oxytetracycline on Controlling Bacterial Contaminations of the River Buffalo (Buballus bubalis) Semen. *Pakistan Journal of Biological Sciences*, 10 (18), 3200-3204. ISSN 1028-8880
3. Almquist, J. O., Thorp, W. T. S., Knodt, C. B. 1946. The Effect of Penicillin upon the Livability, Fertility and Bacterial Content of Bull Semen. *J Animal Sci*, 5, 400-401.
4. Almquist, J. O. 1949. The Effect of Penicillin upon the Fertility of semen from Relatively Infertile Bulls. *Journal Series of the Pennsylvania Agricultural Experiment Station*, 32(11), 950-954. https://doi.org/10.3168/jds.S0022-0302(49)92143-8
5. Aurich, C., Spergser, J. 2007. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology*. 67(5), 912-918. https://doi.org/10.1016/j.theriogenology.2006.11.004
6. Azawi, O. I., Ismaeel, M. A. 2011. Influence of addition of different antibiotics in semen diluent on viable bacterial count and spermatozoal viability of Awasi ram semen. *Vet. World*, 5(2), 75-79. https://doi.org/10.5455/vetworld.2012.75-79
7. Boonthai, T., Khaopong, W., Sangsong, j., Nimrat, S., Vuthiphandchai, V. 2015. Effect of antibiotic supplementation on the quality of cryopreserved fish sperm of silver barb (Barbodes gonionotus): Sperm motility and viability, bacterial quality and fertilization. *Animal Reproduction Science*, 166, 36-46. https://doi.org/10.1016/j.anireprosci.2016.01.002
8. Dissanayake, D. M. A. B., Amaranath, K. A., Perera, R. R. D. P., Wijesinghe, P. S. 2014. Antibiotics supplemented culture media can eliminate non-specific bacteria from semen during sperm preparation for intra uterine insemination. *Journal of Human Reproductive Sciences*, 7(1), 58-62. https://doi.org/10.4103/0974-1208.130859
9. Avilés, C. H., Love, Ch. C., Serafini, R., Teague, S, R. 2019.Suppemental Antibiotics in a Commercial Extenders for Stallion Semmen. *Journal of Equine Veterinary science*, 80, 33-35. https://doi.org/10.1016/j.jevs.2019.06.014
10. Knazicka, Z., Tvrda, E., Bardos, L. & Lukac, N. (2012). Dose- and timedependent effect of copper ions on the viability of bull spermatozoa in different media. *Journal of Environmental Science and Health, Part A*, 47(9), 1294-1300. https://doi.org/10.1080/10934529.2012.672135
11. Miraglia, F., Morais, Z. M., Cortez, A., Melville, P. A., Marvullo, M. F. V., Richtzenhin, L. J., Visintin, J. A., Vasconcellos. 2003. Comparison of four antibiotics for inactivating leptospires in bull semen diluted in egg extender and experimentally inoculated with Leptospira santarosai serovar guaricura. *Brazilian Journal of Microbiology*, 34, 147-151. ISSN 1517-8382.
12. Moretti, E., Capitani, S., Figura, N., Pammolli, A., Federico, M. G., Giannerini, V. 2009. The presence of bacteria species in semen and sperm quality. *Journal of Assisted Reproduction and Genetics*, 26, 47-56. https://doi.org/10.1007/s10815-008-9283-5
13. Morrell, J. M., Wallgren, M. 2014. Alternatives to Antibiotics in Semen Extenders: A Review, *Pathogens*, 3, 934-946. https://doi.org/10.3390/pathogens3040934
14. Moskovtsev, S. I., Librach, C. L. 2013. Methods of sperm vitality assessment. Carrell DT, Aston KI (EDS): Spermatogenesis: Methods and Protocols, Methods in Molecular Biology. Springer Science + Business Media, New York. 13-19.
15. Oplinger, R. W., Wagner, E. J. 2015. Use of penicillin and streptomycin to reduce spread of bacterial coldwater disease I: antibiotics in sperm extenders. *J Aquat Anim Helath*, 27(1), 25-31. https://doi.org/10.1080/08997659.2014.966211
16. Pope, C. E., Zhang, Y. Z., Dresser, B. L. 1991. A simple staining method for quantifying the acrosomal status of cat spermatozoa. *Journal of Zoo and Wildlife Medicine* 2, 87-95.
17. Rahimi, R., Hajirezaee, S., Shaluei, F., Katadj, J. K. 2016. Antibiotics, Penicillin and Streptomycin improve semen quality indices of endagered Caspian brown trout, Salmo trutta caspius (Kessler, 1870) during in vitro short-term storage. *Aquaqulture Research*, 47, 3662-3666. https://doi.org/10.1111/are.12819
18. Schlegel, P. N., Chang, T. S. K., Marshall, F. F. 1991. Antibiotics: potential hazards to male fertility. Fertility and Sterility, 55 (2), 235-242.
19. Slanina, T., Brandysova, M., Massanyi, P. 2016. Effect of Tylosin on turkey spermatozoa motility. Animal Physiology 2016, Mendel University in Brno, 326. ISBN978-80-7509-416-2
20. Sone, M., Ohmura, K., Bamba, K. 1982. Effects of various antibiotics on the control of bacteria in boar semen. *Veterinary Record*, 111, 11-14. http://dx.doi.org/10.1136/vr.111.1.11
21. Stoss, J., Buyukhatipoglu, S., Holtz, W. 1978. Short-term cryopreservation of rainbow trout (Salmo gairdneri Richardson) sperm. *Ann. Biol. anim. Bioch. Biophys*, 18(4), 1077-1082.
22. Tvrdá, E., Konečná, N., Zbyňovská, K. & Lukáč, N. (2018). Antioxidant Effects of Peppermint (Mentha piperita) Extract on the Oxidative Balance of Rabbit Spermatozoa. *Journal of Advanced Agricultural Technologies*, 5(2), 117-122. http://dx.doi.org/10.18178/joaat.5.2.117-122
23. Visser, I. J. R., Laak, E. A., Jansen, H. B. 1999. Failure of antibiotics gentamycin, tylosin, lincomycin and spectinomycin to eliminate Mycopladma bovis in artificially infected frozen bovine semen. *Theriogenology*, 51(4), 689-697. https://doi.org/10.1016/S0093-691X(99)00018-7
24. Yániz, J. L., Marco-Aguado, M. A., Mateos, J. A., Santolaria, P. 2010. Bacterial contamination of ram semen, antibioti sensitives, and effects on sperm quality during storage at 15 °C, *Animal Reproduction Science*, 122, 142-149. https://doi.org/10.1016/j.anireprosci.2010.08.006