The Effect of *Enterococcus faecium*-Induced *in vitro* Infection in Bovine Semen

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Abstract
Bacterial contamination of semen samples represents potential threat to the sperm quality. *Enterococcus faecium* was identified in our previous study concerning bacteriocenosis of fresh bovine ejaculates. The objectives of this study are focused on the effect of *Enterococcus faecium*-induced bacteriospermia on sperm motility, mitochondrial membrane potential and reactive oxygen species generation. Bovine semen samples came from breedeng bulls kept in a breeding centre (Slovak Biological Services, a.s., Lužianky, Slovakia). Immediately, samples were subjected to density gradient separation using Percoll PLUS to separate seminal plasma along with seminal bacteriocenosis. An 18-hours culture of E. faecium isolated from bovine semen was diluted to 0.5 McFarland and co-incubated with spermatozoa, while held at 37°C during 4 hours. One-way ANOVA followed by Dunnett's comparison test were used to express arithmetic mean ± standard error of mean of obtained data. Data obtained using the Computer-assisted sperm analysis showed that spermatozoa motility slightly decreased when treated with *E. faecium*. However, a significant decrease ($P<0.01$) in mitochondrial membrane potential was recorded after 4 hours treated with E. faecium. After 2 and 4 hours were observed significant increases of ROS levels in the groups with added *E. faecium*, when compared to control. We may conclude that increased concentrations of *Enterococcus faecium* may trigger oxidative burst in spermatozoa resulting in decreased mitochondrial membrane potential and thus impaired fertilizing ability of bovine spermatozoa.

Keywords: bacteriospermia, *Enterococcus faecium*, spermatozoa, bulls, ROS

1. Introduction

An enormous economic pressure is exerted on the success of artificial insemination. The demand for insemination doses is related to a success rate of artificial insemination in livestock. Therefore, bacterial contamination of the insemination dose is an undesirable factor in terms of the birth rate reduction and with regards to the recipient's health [1]. There are several sources of bacterial contamination, including intrinsic bacteria of the male urogenital tract [2], during semen collection or laboratory processing [3]. Mehta et al. [4] recorded enterococci in above 50% of semen samples obtained from male partners from infertile couples. Although, the enterococci belong to major bacterial species colonizing the intestinal flora of human and animals, they may cause several serious infectious diseases including urinary tract infections.

Some pathogenic bacteria, such as *Mycoplasma*, *Ureaplasma* and *Chlamydia* are believed to be responsible for increased reactive oxygen species (ROS) generation in semen [5]. Although, low production of ROS is essential for sperm hyperactivation and acrosome reaction, increased

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concentrations cause oxidative stress (OS). OS results in mitochondrial damage, membrane disruption, subsequent motility and fertilizing ability decrease [6]. Therefore, the aim of this study was to reveal the effect of *Enterococcus faecium*-induced bacteriospermia on the sperm motility, mitochondrial membrane potential and ROS generation in bovine semen samples.

2. Materials and methods

Sample collection and processing

Semen samples (n=7) were collected from Holstein Friesian breeding bulls at Slovak Biological Services (Nitra, Slovakia). The cattle were kept under uniform conditions. Semen samples were obtained using a sterile artificial vagina. The samples were transported to the laboratory within 20 minutes and subjected to the Percoll PLUS (Sigma-Aldrich, St. Louis, MO, USA) gradient separation. Single layer centrifugation was used in order to separate seminal plasma together with intrinsic bacteria and damaged sperm cells. The Percoll PLUS solution (PPS) was prepared according to Parrish et al. [7] and 1×10^9 spermatozoa was gently layered on the top of a 9 mL 70% PPS column. After centrifugation, supernatant was removed and sperm cells were diluted in Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium in case of control group. In addition to sperm cells and PBS, the experimental group contained 1.5×10^8 *Enterococcus faecium*, which was previously isolated from the bovine ejaculate. The samples were cultured at 37°C. At times of 0, 2 and 4 hours, the computer-assisted sperm analysis (CASA), mitochondrial membrane potential assessment and ROS production were measured.

The CASA assessment

Spermatozoa motility was analysed using the CASA system (Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten µL of sample solution were placed into the pre-warmed Makler counting chamber (37°C; depth 10 µm; Sefi Medical Instruments, Haifa, Izrael). Sperm motility was analysed in 10 displayed fields and at least 30 cells were analysed per field. 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.

Mitochondrial membrane potential assay

The Mitochondrial Membrane Potential Assay Kit (Cayman Chemicals, Ann Arbour, MI, USA) was used for the mitochondrial membrane potential (MMP) assessment. The lipophilic cation dye JC-1 forms polymers in healthy cells with a high MMP, emitting a red fluorescence. On the other hand, the JC-1 dye remains as a monomer form in cells with a low MMP, emitting a green fluorescent light. The measurements were performed using the Glomax Multi+ combined spectro-fluoro-luminometer (Promega, USA) on dark 96-wells plate. The results were expressed as the rate of JC-1 polymers to JC-1 monomers [8].

Reactive oxygen species measurement

Reactive oxygen species production was measured using the chemiluminescence method as described by Agarwal et al. [9]. The blank comprised of 100 µL of PBS (Sigma Aldrich, USA). Negative control contained 100 µL PBS and positive control contained extra 12,5 µL H_2O_2 (30%; 8.8 M; Sigma Aldrich, USA). One hundered of tested samples, negative and positive control was mixed with luminol (5mM; Sigma-Aldrich, St. Louis, MO, USA). The chemiluminescence was measured on 96-wells plate and the results are expressed as relative light units/s/1×10^6 spermatozoa.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism program (version 8.0 for Mac, GraphPad Software Inc., San Diego, CA, USA). Paired t-test was applied for specific statistical analysis. The level of significance was set at ** $P<0.01$.

3. Results and discussion

The aim of present study was to investigate samples of bovine ejaculates exposed to 1.5×10^8 *Enterococcus faecium* at 37°C and at times of 0, 2 and 4h the motility assessment, mitochondrial membrane potential and reactive oxygen species production were evaluated. The CASA assessment results are summarized in Figure 1. The initial assessment (Time 0h) showed no significant difference between the control group and the

group with added *E. faecium*. After 2h, we observed a slight decrease in the *E. faecium* group when compared to the control group. Although the observed decrease was not statistically significant even after 4 h, this discrepancy may be explained by the ability of bacteria to agglutinate sperm cells. According to Prabha et al. [10] sperm ligand on bacteria adhere to sperm heads and tails, agglutinate sperm cells and thereby deteriorate sperm motility, morphology and viability. Authors of this study observed during the CASA assessment that the sperm agglutinates were not evaluated during the motility analysis because the software was unable to capture, recognize and evaluate the movement of agglutinated spermatozoa. Therefore, the software evaluated only the movement of individual sperm cells, which, unlike the agglutinated spermatozoa, were motile. The agglutinated spermatozoa are displayed in Figure 2, observed using bright field microscopy (Leica DMI6000B; Wetzlar, Germany). Verstegen et al. [11] described similar experiences when evaluated post-thawed spermatozoa. The sperm cells tend to agglutinate when they undergo a cryopreservation process.

The mitochondrial membrane potential assessment may provide a more accurate reflection on the effect of *Enterococcus faecium* on the spermatozoa quality, as mitochondrial activity is directly linked to sperm motility. At initial time, there were no significant difference between the observed groups. After 2 h, a slight decrease of MMP was observed in the group treated with *E. faecium* when compared to the control group. After 4 h, spermatozoa observed in the group with added *E. faecium* exhibited significantly decreased ($P<0.01$) MMP in comparison to the control group. Schulz et al. [12] observed the effect of *Escherichia coli* on mitochondrial membrane potential, phosphatidylserine translocation, motility and viability on human spermatozoa. In agreement to our study, their results showed significantly lower MMP ($P<0.001$) when co-incubated with 0.35×10^7 CFU/mL at 37°C during 1 h. Moreover, motility and viability were significantly decreased ($P<0.05$) when compared to the control group. They reported that the bacterial adhesion phenomenon is only one of several adverse effects of *E. coli* in semen. Soluble factors released by the bacteria may play a significant role. The loss of sperm motility is related to α-hemolysin. This soluble toxin may form pores on the surface of mitochondrial membrane and rapidly releases potassium, and at the same time occurs influx of calcium, mannitol and sucrose, resulting in osmotic lysis of the cell. In addition to the presence of hemolysin, 8 other virulence factors have been identified to contribute bacterial aggregation, biofilm formation and immune modulation [13]. As sperm motility depends on a proper mitochondrial functionality, recent study reported that mitochondrial membrane potential may predict the sperm motility 4 hours in advance [14]. Other study reported significant ($P<0.001$) positive correlations between MMP and total sperm motility and progressive motility [15].

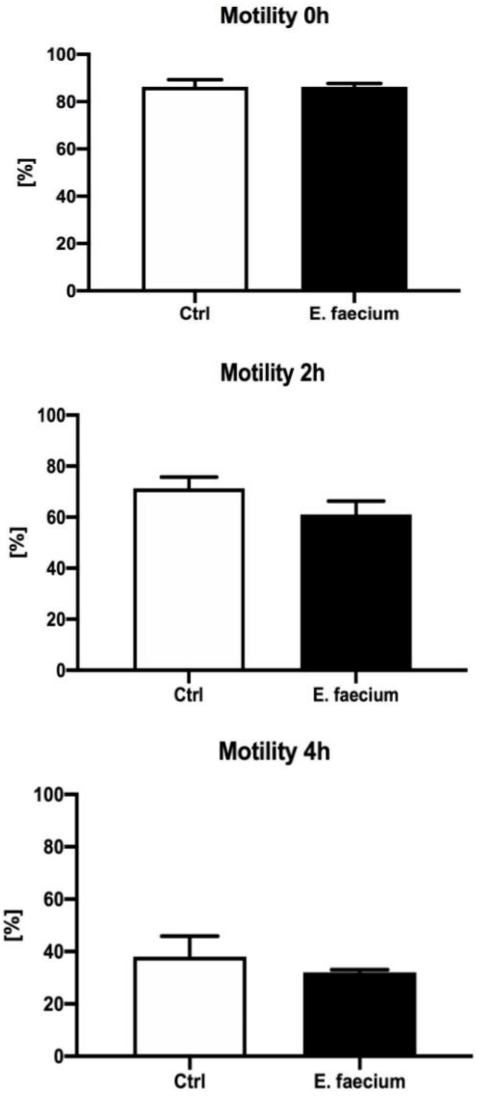

Figure 1. The development of the sperm motility of *E.faecium*-induced bacteriospermia compared to the control group during 4 hours incubation at 37°C

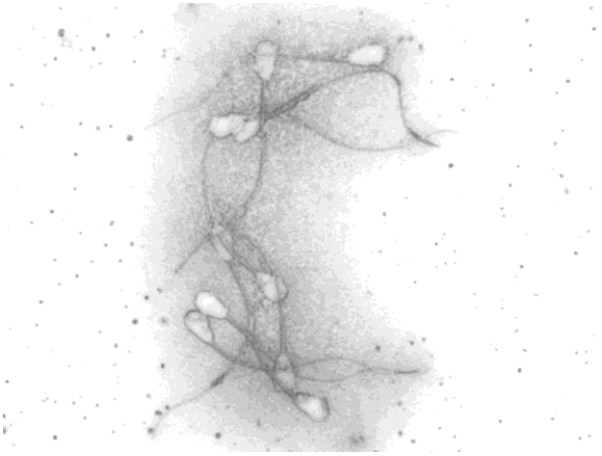

Figure 2. Sperm agglutination observed after 4 h co-incubation of bovine spermatozoa with 1.5×10^8 *Enterococcus faecium*

Although, low concentrations of reactive oxygen species are responsible for the trigger point of fertilization in normozoospermic individuals, hyperactivation. Increased levels of ROS are well-known for their harmful effect at the level of the sperm membrane [6]. Our study showed no significant difference when evaluating at the initial time. However, a significant increase ($P<0.01$) of ROS levels was observed after 2 h in the group with *E. faecium*. Similarly, the group administered with *E. faecium* exhibited significantly elevated ($P<0.01$) levels of ROS after 4 h co-incubation. Similar results were recorded in recent study, when rabbit semen samples were co-incubated with *E. faecalis* at concentration of 0.9×10^8 [16]. It appears that extracellular ATP (eATP) may play an important role during bacteriospermia. At physiological conditions, ATP is occurring in nanomolar concentrations in the extracellular space. Once the sperm membrane is disrupted, the concentration of eATP is elevated from nanomolar to millimolar amounts [17]. This activates P2X7 receptors in the sperm membrane and increase calcium infux, resulting in increased activity of NADPH-oxidase, and thereby increased ROS production [18]. The result of increased concentrations of ROS is oxidative stress, which leads to lipid peroxidation, sperm DNA damage and cytochrome C-mediated apoptosis. Moreover, Fraczek et al. [19] observed that both, apoptosis and necrosis, may be the result of bacterial contamination of human semen.

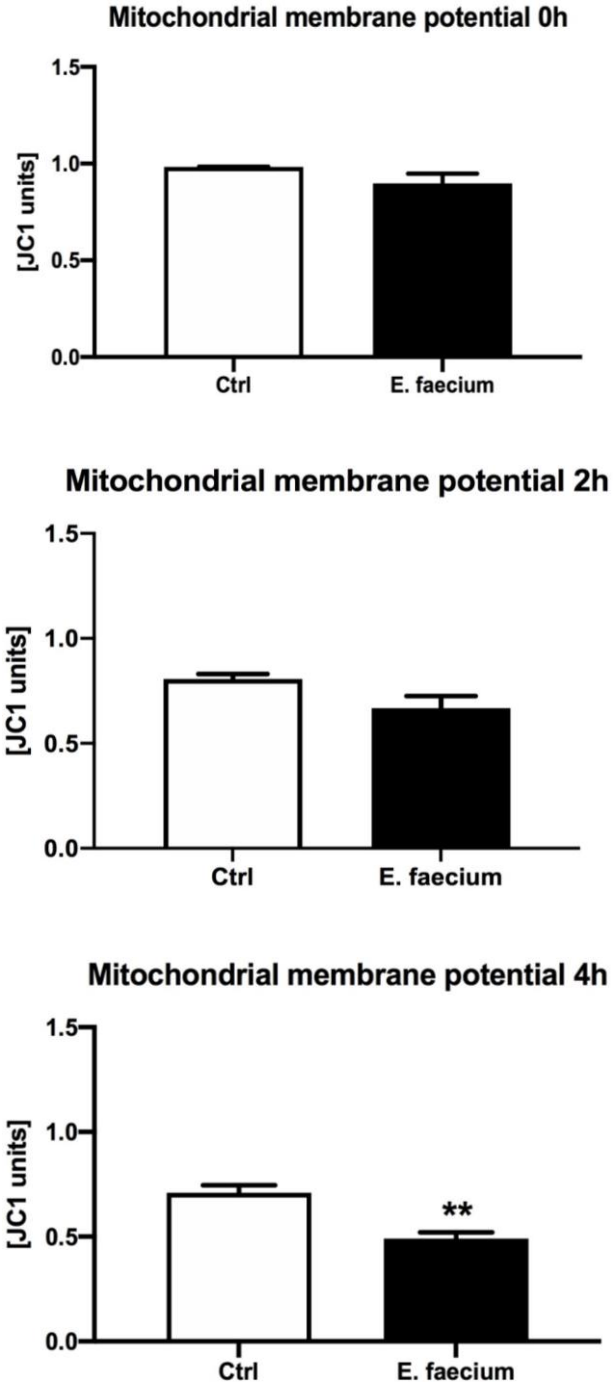

Figure 3. The development of mitochondrial membrane potential in *E.* faecium-induced bacteriospermia compared to the control group during 4 hours incubation at 37°C. ** $P<0.01$.

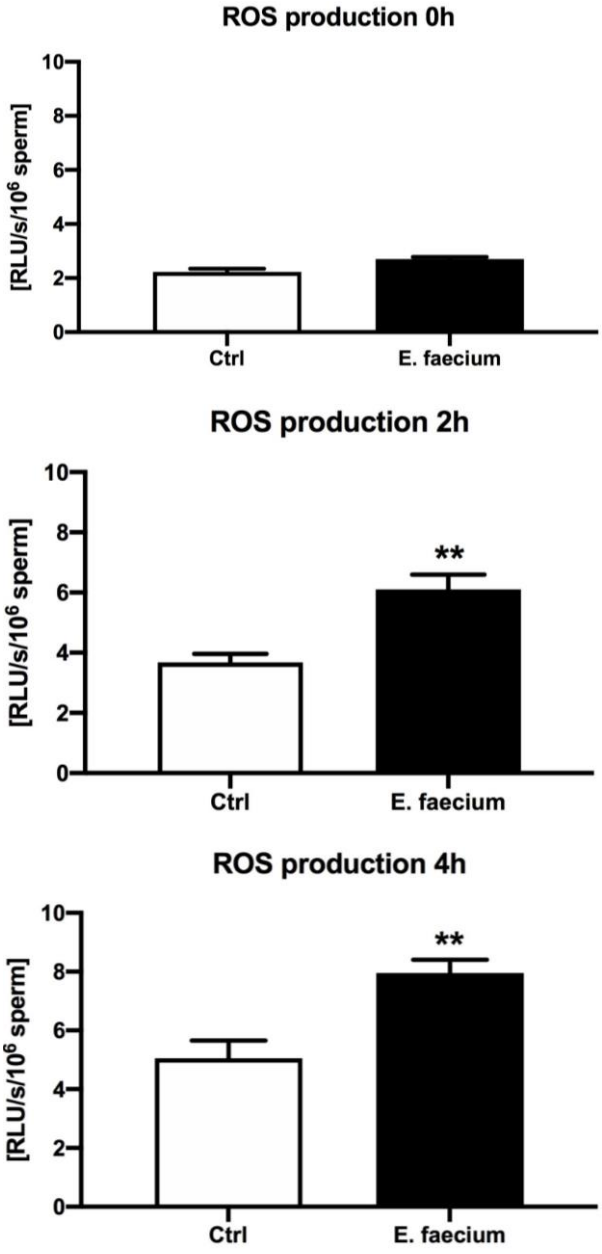

Figure 4. The development of mitochondrial membrane potential in *E.* faecium-induced bacteriospermia compared to the control group during 4 hours incubation at 37°C. ** P<0.01

4. Conclusions

In summary, our *in vitro* study revealed that the presence of *Enterococcus faecium* in bovine semen may increase reactive oxygen species levels and decrease sperm mitochondrial membrane potential. Although, the motility assessment did not provide any significant differences, the authors explained this discrepancy with errors in the software evaluation of the CASA system, which occurred due to sperm agglutination. Further studies are needed to prove mechanisms of harmful effects of *E. faecium* on spermatozoa.

Acknowledgements

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References

1. Thibier, M., Guerin, B., Hygienic aspects of storage and use of semen for artificial insemination, Animal Reproduction Science, 2000, 62:233-251.
2. Marcus, S., Bernstein, M., Ziv, G., Glickman, A., and Gipps, M., Norfloxacin nicotinate in the treatment of Pseudomonas aeruginosa infection in the genital tract of a bull, Veterinary Research Communication, 1994, 15:331-336.
3. Rana, N., Vaid, R. K., Phulia, S. K., and Singh, P., Assessment of bacterial diversity in fresh bubaline semen, Indan Journal of Animal Sciences, 2012, 82:596-598.
4. Mehta, R. H., Sridhar, H., Kumar, B. R. V., and Kumar, T. C. A., High incidence of oligozoospermia and teratozoospermia in human semen infected with the aerobic bacterium Streptococcus faecalis, Reproductive BioMedicine Online, 2002, 5:17-21.
5. Rybar, R., Prinosilova, P., Kopecka, V., Hlavicova, J., Veznik, Z., Zajicova, A., and Rubes, J., The effect of bacterial contamination of semen on sperm chromatin integrity and standard semen parameters in men from infertile couples, Andrologia, 2011, 44:410-418.
6. Agarwal, A., Virk, G., Ong, C., and du Plessis, S. S., Effect of Oxidative Stress on Male Reproduction, The World Journal of Men's Health, 2014, 32:1-17.
7. Parrish, J. J., Krogenaes, A., and Susko-Parrish, J. L., Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development, Theriogenology, 1995, 44:859-869.
8. Agnihotri, S. K., Agrawal, A. K., Hakim, B. A., Vishwakarma, A. L., Narender, T., Sachan, R., and Sachdev, M., Mitochondrial membrane potential (MMP) regulates sperm motility, In Vitro Cellular & Developmental Biology - Animal, 2016, 52:953-960.
9. Agarwal, A., Gupta, S., and Sharma, R., Reactive Oxygen Species (ROS) Measurement. In: Andrological Evaluation of Male Infertility. Agarwal, A., Gupta, S., Sharma, R., Springer, 2016, pp. 155-163.
10. Prabha, V., Gupta, T., Kaur, S., Kaur, N., Kala, S., and Singh, A., Isolation of spermatozoal immobilization factor from Staphylococcus aureus

filtrates, Canadian Journal of Microbiology, 2009, 874-879.
11. Verstegen, J., Iguer-Ouada, M., and Onclin, K., Computer assisted semen analyzers in andrology research and veterinary practice, Theriogenology, 2002, 57:149-79.
12. Schulz, M., Sánchez, R., Soto, L., Risopatrón, J., and Villegas, J., Effect of Escherichia coli and its soluble factors on mitochondrial membrane potential phosphatidylserine translocation, viability, and motility of human spermatozoa, Fertility and Sterility, 2010, 94:619-623.
13. Madsen, K. T., Skov, M. N., Gill, S., and Kemp, M., Virulence Factors Associated with Enterococcus Faecalis Infective Endocarditis: A Mini Review, The Open Microbiology Journal, 2017, 11:1-11.
14. Alamo, A., De Luca, C., Mongioì, L. M., Barbagallo, F., Cannarella, R., La Vignera, S., Calogero, A. E., and Condorelli, R. A., Mitochondrial Membrane Potential Predicts 4-Hour Sperm Motility, Biomedicines, 2020, 8:1-9.
15. Yang, Y., Fan, Y., Kuang, Y. P., and Lyu, Q. F., Correlations of Sperm Mitochondrial Membrane Potential with Semen Parameters and Male Obesity, Reproductive and Developmental Medicine, 2018, 2:116-119.
16. Duracka, M., Lukac, N., Kacaniova, M., Kantor, A., Hleba, L., Ondruska L., and Tvrda, E., Antibiotics Versus Natural Biomolecules: The Case of In Vitro Induced Bacteriospermia by Enterococcus Faecalis in Rabbit Semen, 2019, 24:1-20.
17. Franke, H., Illes, P. Involvement of P2 receptors in the growth and survival of neurons in the CNS, Pharmacology & Therapeutics, 2006, 109:297-324.
18. Barlett, R., Yerburry, J. J., and Sluyter, R., P2X7 Receptor Activation Induces Reactive Oxygen Species Formation and Cell Death in Murine EOC13 Microglia, Mediators of Inflammation, 2013, 2013:1-18.
19. Fraczek, M., Hryhorowicz, M., Gaczarzewicz, D., Szumala-Kakol, A., Kolanowski, T. J., Beutin, L., and Kurpisz, M., Can apoptosis and necrosis coexist in ejaculated human spermatozoa during in vitro semen bacterial infection?, Journal of Assisted Reproduction and Genetics, 2015, 32:771-779.