JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

NARINGENIN, CURCUMIN AND ISOQUERCITRIN MITIGATE DAMAGES IN SPERMATOZOA CAUSED BY *STAPHYLOCOCCUS AUREUS* DURING BACTERIOSPERMIA *IN VITRO*

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https://doi.org/10.55251/jmbfs.9489

ARTICLE INFO

Received 31. 7. 2022
Revised 4. 11. 2022
Accepted 15. 11. 2022
Published 21. 12. 2022

Regular article

ABSTRACT

The presence of bacterial contamination in bovine insemination doses may lead to unexpected economic loss. Currently used antimicrobial supplements are often ineffective due to antibiotic resistance of the common bacterial contaminants. *Staphylococcus aureus* belongs to the frequent inhabitant of bovine semen with a deleterious effect on spermatozoa quality. Bioactive substances including naringenin, curcumin and isoquercitrin were previously studied with various beneficial effects on spermatozoa. Therefore, the study aimed to simulate bacteriospermia in vitro using *S. aureus*, which was previously isolated from the bovine ejaculate, and to test if the bioactive substances mentioned above would bring some protective properties against the bacterial damage. Density gradient centrifugation was used to avoid contamination by naturally present bacteria. *S. aureus* was inoculated in BGM-3 medium, and co-incubated with pre-washed spermatozoa and bioactive substances. At times of 0, 2, and 4 h, progressive motility, the membrane potential of mitochondria, reactive oxygen species (ROS) formation, and sperm DNA damage were evaluated. The results showed that *S. aureus* significantly increased ROS production, sperm cells with damaged mitochondrial membrane potential, and sperm DNA integrity which led to a decrease in the progressive movement of spermatozoa. On the other hand, all three bioactive substances significantly scavenged elevated ROS. Especially, the groups treated with naringenin and isoquercitrin showed preserved mitochondrial membrane, DNA integrity inside the sperm head, and improved sperm progressive movement under bacteriospermia *in vitro*.

Keywords: *Staphylococcus aureus*, spermatozoa, ROS, progressive motility, sperm DNA, mitochondrial membrane potential

INTRODUCTION

Artificial insemination represents a modern strategy of livestock production with an effective way of spreading valuable genotypes without spreading sexually transmitted diseases. The success of artificial insemination is limited by the quality of the neat semen sample. Despite that animals are kept in a strict hygiene environment, semen collection is not a sterile process, and various bacterial species are often part of ejaculates. Preputial fluid, penile skin, urine, contaminated tools, and laboratory equipment may be a source of ubiquitous bacteria (**Rana *et al.*, 2012**).
Recent studies showed that the presence of bacteria complexly affects spermatozoa structure leading to affecting its functional activity (**Ďuračka and Tvrdá, 2018; Ďuračka *et al.*, 2020; Ďuračka *et al.*, 2021a**). The qualitative as well as quantitative bacterial composition play role in semen quality. From a global perspective, higher bacterial load and more variable bacterial composition led to the increased immune response and production of reactive oxygen species (ROS). *Staphylococcus* genus significantly contributes to the bacterial composition of bovine semen. Especially, *S. aureus* was frequently identified in poor-quality semen samples in larger doses. Bacteriospermia, increased levels of bacteria in semen, is associated with reduced spermatozoa motility (**Ďuračka *et al.*, 2021b**). A previous study on human ejaculates revealed that *S. aureus* produces a protein with a sperm-agglutinating activity (**Ohri *et al.*, 2005**). The adhesive properties of *S. aureus* were also observed using scanning electron microscopy (**Agarwal and Prabha, 2006**). Especially, sperm tails are prone to form sperm-bacteria adhesion. In addition, mitochondria give rise to ROS production, its membrane potential predicts sperm motility 4 hours in advance (**Alamo *et al.*, 2020**). **Wang *et al.* (2003)** showed that increased ROS production is associated with decreased mitochondrial membrane potential (MMP). Spermatozoa have been known for their limited ability to cope with elevated ROS production. Oxidative stress leads to pathological alterations in membranous structures as well as DNA molecules (**Tvrdá *et al.*, 2011**). Recent study investigated the effect of different *Staphylococcus* species on the sperm DNA integrity, while *S. aureus* caused the most damage to the DNA integrity amongst other species (**Ďuračka *et al.*, 2021b**). The use of antioxidants of plant origin in semen preservation media brings beneficial effects regarding to the maintenance of high semen quality, even after thawing. Naringenin, a bioactive flavonoid richly represented in citrus fruits, was previously observed as a favourable additive in extended boar semen during 72h cultivation (**Tvrdá *et al.*, 2020**). Curcumin is well-known for its strong antibacterial and antioxidant properties. Especially, at low concentrations curcumin proved protective properties to bull spermatozoa when subjected to oxidative stress (**Tvrdá *et al.*, 2016**). Isoquercitrin is a derivate of quercetin with higher bioavailability than quercetin (**Valentová *et al.*, 2014**) and so far, only a little information is available considering sperm quality. Mitochondrial toxicity test proved its protective properties towards mitochondria (**Benko *et al.*, 2019**).
This study aimed to reveal if selected bioactive substances can protect sperm cells from bacterial damage during *in vitro* simulated bacteriospermia.

MATERIAL AND METHODS

Collection and processing of biological material

Semen samples from Holstein Friesian breeding bulls were gathered at the Slovak Biological Services (Nitra, Slovakia). Each ejaculate (n=5) was gathered using the sterilized artificial vagina and transported within 20 min. to the laboratory in the pre-warmed thermos (37°C). Each semen sample met a minimum of 70% spermatozoa motility to be used for these experiments. Semen was processed using Percoll density gradient media (Cytiva, Marlborough, MA, USA). A discontinuous gradient of 45 and 90% (v/v) Percoll diluted with BGM-3 was pre-warmed to 37°C and covered by approximately 1×10^9 sperm cells. Falcon tubes (15 mL) were centrifuged at 400 × g for 15 min. Non-motile spermatozoa prevailed within the top layer and 45% Percoll fraction. Similarly, the upper part of 90% Percoll fraction contained non-motile spermatozoa. The pellet located at the bottom of the falcon tube contained predominantly motile spermatozoa and was used for our

experiments. Each fraction was separately discarded with Pasteur's pipettes to avoid contamination of the pellet. The pellet was resuspended with 3 mL BGM-3 medium and subjected to centrifugation (400 × g; 5 min.) to remove residual colloid. Afterward, spermatozoa concentration was adjusted to 50 × 10^6 cells/mL with *S. aureus*-contaminated or uncontaminated BGM-3. *S. aureus* was inoculated to the BGM-3 medium at a concentration of 0.5 McFarland, as this concentration was suitable for short-term *in vitro* bacterial toxicology experiments (**Ďuračka *et al*., 2021b**). The negative control (NC) group contained only spermatozoa without added bacteria. The positive control (SA) was contaminated with *S. aureus*. The experimental groups were contaminated with *S. aureus* and treated separately with naringenin (NAR), curcumin (CUR), and isoquercitrin (ISOQ) at a concentration of 1 µmol/L. At times of 0, 2, and 4 hours, progressive motility, mitochondrial membrane potential, reactive oxygen species and sperm DNA fragmentation were evaluated.

Evaluation of sperm progressive motility

Sperm progressive motility (PROG) was analysed using the computer-aided semen analysis (CASA) HTM TOX IVOS II (Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten microliters of sample were put into Makler counting chamber (depth 10 µm; Sefi Medical Instruments, Haifa, Israel) pre-warmed to 37°C. Objective measurement of spermatozoa motion behaviour was performed in 10 microscope view fields, when at least 300 cells were analysed. The progressive movement of the sperm cells was expressed in the percentage of the sperm cells moving ≥25 µm/s.

Membrane potential in mitochondria

The sperm mitochondrial membrane was exposed to cationic lipophilic JC-1 dye at a concentration of 5 mg/mL. The JC-1 dye (Cayman Chemical, Ann Arbour, MI, USA) was dissolved in Dulbecco's phosphate buffered saline (DPBS) solution (Sigma-Aldrich, St. Louis, MO, USA) and at least 1 × 10^6 sperm cells were stained in each group. The principle of the assessment is based on the ability of JC-aggregates formation in cells with healthy mitochondrial membranes. The JC-1 dye stays in monomeric form in cells with low mitochondrial membrane potential. Fluorometric analysis was performed using Glomax Multi+ (Promega, USA) on 96-well dark plate. The JC-1 aggregates emit red fluorescent light, while the monomeric form emits green fluorescence (**Agnihotri *et al*., 2016**). The results are stated as the rate of JC-1 aggregates to JC-1 monomers.

Production of oxygen radicals

Chemiluminescent analysis was performed to determine reactive oxygen species (ROS). Luminol (5 mM; 5-amino-2,3-dihydro-1,4-phthalazinedione) was used as a probe in tested samples, with negative and positive controls. Blank, negative and positive controls contained 100 µL of DPBS. Hydrogen peroxide (30%; 9.8 M) was added to the positive controls (**Agarwal *et al*., 2016**). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Glomax Multi+ system (Promega, USA) measured chemiluminescence. The results are stated as the relative light units/s/1 × 10^6 spermatozoa.

Sperm chromatin dispersion test

The Halomax® kit (Halotech DNA, Madrid, Spain) was used to detect sperm DNA fragmentation. Before analysis, sperm concentration was adjusted to 15-20 × 10^6 sperm/mL. Diluted sperm samples were attached to a glass slide in an agarose matrix and left to solidify in the refrigerator (5 min.). A series of solutions were applied to lyse and fix samples: lysis solution (5 min.), distilled water (5 min.), ethanol (70%, 90%, 100% - each for 2 min.). The sperm cells were stained with the Brightfield Staining Kit (HT-BFS; Halo-tech DNA, Madrid, Spain). The presence of a big, dispersed halo around the sperm heads defines sperm with fragmented DNA inside, while a small compact halo represents sperm cells carrying undamaged DNA (**González-Marín *et al*., 2011**). Minimum of 300 sperm cells were evaluated in each sample as per the manufacturer's recommendation. The results are specified as the percentage of the sperm cells with fragmented DNA.

Statistical evaluation

The GraphPad Prism (version 8.0 for Mac, GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis of obtained results. Data were subjected to one-way ANOVA and followed by the Tukey multiple-comparisons test. The significance level was set at *P<0.05, **P<0.01, and ***P<0.001.

RESULTS AND DISCUSSION

Staphylococcus species were previously present in bovine ejaculates with compromised semen quality (**Ďuračka *et al*., 2021a**). Besides alterations in the structures of spermatozoa including sperm head, neck, and tail, fluctuations in the biochemical composition of seminal plasma were also detected. Particularly, magnesium and calcium loss were observed during *Staphylococcus*-induced bacteriospermia (**Ďuračka *et al*., 2021b**). Our study showed that the presence of *S. aureus in vitro* had a deleterious effect on sperm progressive motility in time. A partial explanation may be found in mitochondrial damage detected as increased representation of JC-monomers. Mitochondrial membrane potential is considered one of the best sperm mitochondrial indicators evaluating its function and reflecting energy status (**Paoli *et al*., 2011**). **Alamo *et al*. (2020)** found out that mitochondrial membrane potential may predict sperm motility 4 hours in advance. Similar results to our study were previously published by **Fraczek *et al*. (2012)** during experimental in vitro infection induced by *Staphylococcus haemolyticus* as well as our previous study demonstrated loss of mitochondrial membrane potential in the presence of *S. aureus*. **Gupta and Prabha (2012)** approached to a molecular mechanism of sperm immobilization factor isolated from *S. aureus*. This protein inhibited mitochondrial Mg^{2+}-ATPase activity in a dose-dependent manner, while sperm flagellar motion is just dependent on ATP.

Any significant differences were not observed during the initial CASA measurement between the control, SA group and the treated groups. However, the group contaminated with *S. aureus* showed after 2 hours significant decrease when compared to the uncontaminated group (NC; P<0.05). The group treated with NAR and ISOQ showed significantly higher sperm motility when compared to the SA group (P<0.05). After 4 h of the cultivation, the SA group showed a significant decrease when compared to the NC group (P<0.01). Contrarily, significantly higher sperm motility (P<0.05) was recorded in the NAR-treated group when compared to SA.

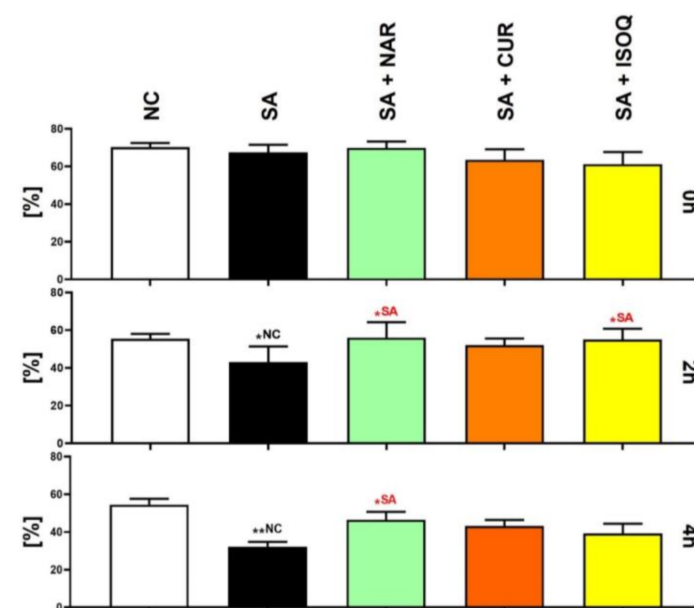

Figure 1 Evaluation of progressive movement (≥25 µm/s) of spermatozoa cultivated under different conditions for 4 hours at 37°C. NC – negative control: no treatment; SA – spermatozoa were treated with *S. aureus*; SA + NAR – spermatozoa were treated with *S. aureus* and naringenin; SA + CUR – spermatozoa were treated with *S. aureus* and curcumin; SA + ISOQ – spermatozoa were treated with *S. aureus* and isoquercitrin. *NC – significant difference (P<0.05) when compared to NC; **NC – significant difference (P<0.01) when compared to NC; *SA – significant difference (P<0.05) when compared to SA.

Similar to the sperm motility analysis, no changes in mitochondrial membrane potential were recorded amongst the observed groups during the initial measurement (Figure 2). A substantial reduction of the mitochondrial membrane potential was observed after 2 h in the SA group compared to NC (P<0.01). A growing trend of adverse effects of *S. aureus* on the mitochondrial membrane (P<0.001) was noticed after 4 h incubation, while the treatment with bioactive molecules mitigates these adverse effects. In particular, CUR- and ISOQ-treated groups showed a significantly higher rate of JC-aggregates (P<0.01). Also, NAR significantly mitigated (P<0.05) the damage to the mitochondrial membrane caused by the presence of *S. aureus*, but a significant decrease has been shown when compared to the NC group (P<0.05). NAR was previously reported to depolarize membrane potential in mitochondria of endometriosis cells (**Park *et al*., 2017**). The study of **Tvrdá *et al*. (2020)** showed that exposure of boar spermatozoa during long-term preservation to NAR maintained higher mitochondrial succinate dehydrogenase activity when compared to the untreated group. **Ďuračka *et al*. (2019)** showed that NAR scavenges mitochondrial-produced superoxide in boar spermatozoa. A recent report revealed that NAR stimulates the resting phase of mitochondrial respiration, not the active phase (**Ferramosca *et al*., 2021**). Elevated ROS production caused by bacterial contamination is strongly correlated with the formation of JC-1 monomers which are typical for depolarized membranes (**Zhao and Drlica, 2014**). As previously shown in our study (**Ďuračka *et al*., 2019b**),

CUR particularly emphasized the protection of mitochondrial membrane potential under bacteriospermia induced by *Enterococcus faecalis*.

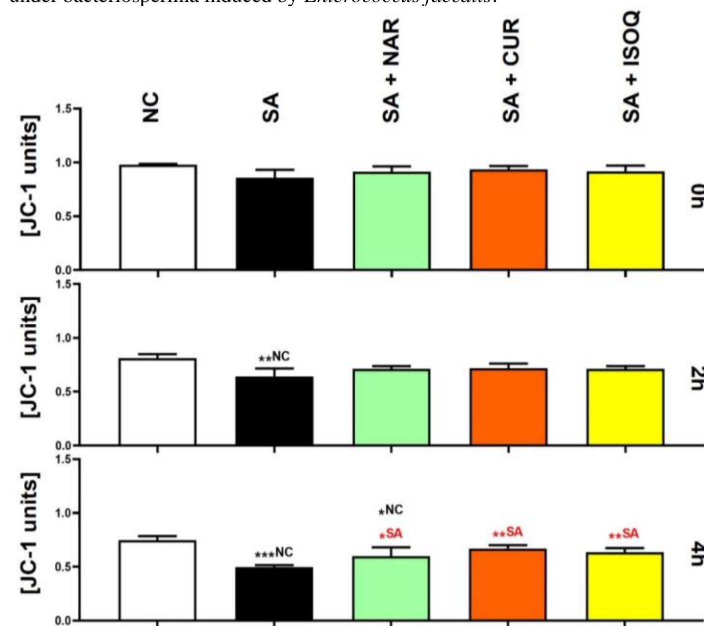

Figure 2 Evaluation of mitochondrial membrane potential of spermatozoa cultivated under different conditions for 4 hours at 37°C. NC – negative control: no treatment; SA – spermatozoa were treated with *S. aureus*; SA + NAR – spermatozoa were treated with *S. aureus* and naringenin; SA + CUR – spermatozoa were treated with *S. aureus* and curcumin; SA + ISOQ – spermatozoa were treated with *S. aureus* and isoquercitrin. *NC – significant difference (P<0.05) when compared to NC; **NC – significant difference (P<0.01) when compared to NC; ***NC – significant difference (P<0.001) when compared to NC; *SA – significant difference (P<0.05) when compared to SA; **SA – significant difference (P<0.01) when compared to SA.

The initial analysis (Figure 3) showed an increased level of free oxygen radicals in the SA group when compared to the uncontaminated group (P<0.01). At the same time, the ISOQ-treated group showed significantly reduced ROS concentration when compared to the SA group (P<0.05). After 2 h, the ROS production has significantly grown in SA (P<0.001 compared to NC), while the groups containing bioactive compounds showed significant mitigation of ROS concentration (P<0.01). The final assessment demonstrated similar results: a significant rise in ROS concentration (P<0.001) was recorded in the SA group, while in the presence of bioactive substances was ROS levels reduced (P<0.01 in case of ISOQ; P<0.05 in case of NAR and CUR). However, when compared to the uncontaminated group, ROS levels were significantly increased (P<0.05). NAR played a particular role in our experiments in relation to oxidative balance. The presence of *S. aureus* promoted oxidative stress during incubation, while the NAR-treated group showed a significantly reduced concentration of global ROS. Curcumin (CUR) is a well-studied antioxidant with the potential to increase total antioxidant capacity **(Jakubczyk *et al*., 2020)**. Our study showed that bacterial contamination and resulting high ROS levels were suppressed by the antioxidant activity of CUR. **Tvrdá *et al*. (2016)** induced oxidative stress in bovine spermatozoa using ferrous ascorbate. In the end, they concluded that CUR exhibits an exceptional antioxidant activity, concerning mitochondrial superoxide generation as well as activity of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic glutathione. So far, there are in vitro studies testing the effect of ISOQ on spermatozoa in a physiologically normal environment **(Ďuračka *et al*., 2017; Benko *et al*., 2019)**. The authors observed changes against the control group in mitochondrial metabolic activity, motility analysis as well as ROS production, particularly when analysed after long-term incubation (24 hours). Our study shows that ISOQ can also actively scavenge free radicals in the environment with induced oxidative stress by bacteria.

The sperm chromatin dispersion test (Figure 4) showed similar fragmentation of DNA in the sperm head in each group at the beginning. The SA group showed significant damage to sperm DNA after 2 h (Figure 5) of incubation when compared to NC (P<0.001). The contaminated groups treated with CUR and ISOQ did not differ significantly from both, NC and SA. However, the group treated with NAR showed a significantly lower percentage of spermatozoa with damaged DNA when compared to the SA group (P<0.05). The sperm DNA damage was significantly lower also after 4 h of incubation in the case of the NAR and ISOQ treated group (P<0.05). Sperm DNA integrity represents a crucial male factor in successful fertilization. **Agarwal *et al*. (2018)** reported that excessive ROS elevation during bacteriospermia leads to sperm DNA fragmentation. A recent study **(Ďuračka *et al*., 2021a)** observed bull semen quality concerning the bacterial presence and showed that sperm DNA fragmented with increasing colony-forming units, particularly in the presence of *S. aureus*.

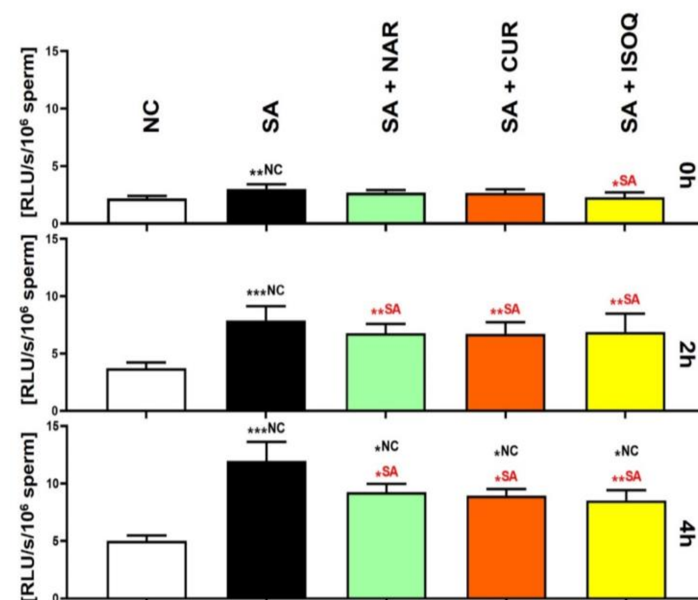

Figure 3 Formation of reactive oxygen species in the observed groups under different conditions during 4 hours at 37°C. NC – negative control: no treatment; SA – spermatozoa were treated with *S. aureus*; SA + NAR – spermatozoa were treated with *S. aureus* and naringenin; SA + CUR – spermatozoa were treated with *S. aureus* and curcumin; SA + ISOQ – spermatozoa were treated with *S. aureus* and isoquercitrin. *NC – significant difference (P<0.05) when compared to NC; **NC – significant difference (P<0.01) when compared to NC; ***NC – significant difference (P<0.001) when compared to NC; *SA – significant difference (P<0.05) when compared to SA; **SA – significant difference (P<0.01) when compared to SA.

The nucleopores are well-permeable for ROS. Therefore, the DNA molecule with its specific bonding system is a perfect target for free radicals **(Villani *et al*., 2010)**. Antioxidant supplementation may improve the sperm DNA fragmentation status and thereby fertilization potential. Particularly, NAR and ISOQ showed potent antioxidant properties leading to a lower percentage of spermatozoa with damaged DNA. In accordance with our results, DNA-protective properties of NAR were previously proved in boar spermatozoa during 72-h preservation **(Ďuračka *et al*., 2019a; Tvrdá *et al*., 2020)**. **Adana *et al*. (2018)** hypothesized that NAR may affect the reparation enzymes of sperm DNA strand breaks or stimulate the antioxidant mechanism to neutralize overproduced ROS. Although CUR showed in our analysis non-significant differences between the NC and SA groups, results published by **Tvrdá *et al*. (2018)** provided evidence about the DNA-protective properties of CUR against oxidative stress induced by cryopreservation of bovine semen. The beneficial effects of quercetin on the maintenance of sperm DNA integrity are well-known **(Tvrdá *et al*., 2020)**. So far, the effect of ISOQ on sperm DNA was not considered. **Boligon *et al*. (2012)** evaluated the protective effects of flavonoids isolated from *Scutia buxifolia* against H_2O_2-induced chromosomal damage in human lymphocytes. They concluded that ISOQ recovered the mitotic index and chromosomal instability better than another glycoside derivate, quercitrin.

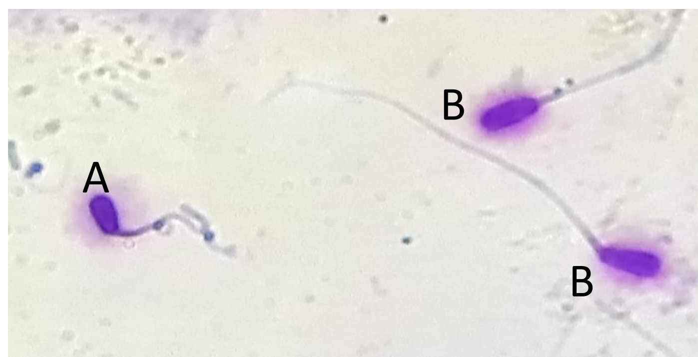

Figure 4 The sperm chromatin dispersion test – staining pattern of sperm DNA fragmentation. **A** – sperm with fragmented DNA, sperm showing nucleoids with a large and spotty halo of chromatin dispersion. **B** – sperm without fragmented DNA, sperm showing nucleoids with a small and compact halo of chromatin dispersion.

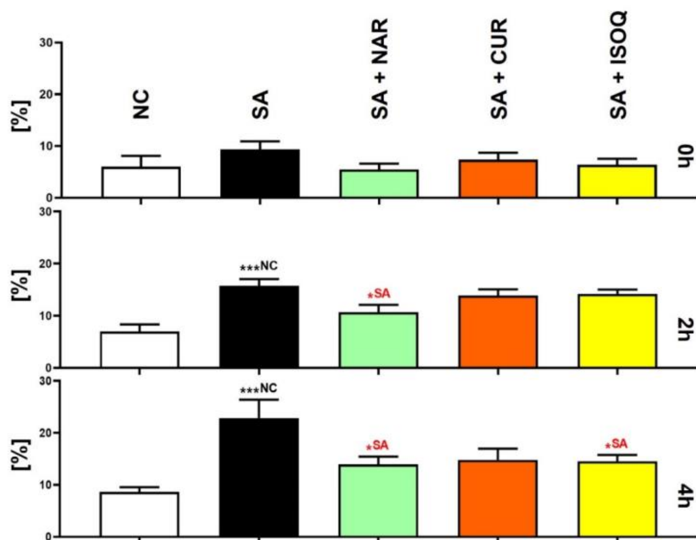

Figure 5 The sperm DNA damage under the different conditions for 4 hours at 37°C. NC – negative control: no treatment; SA – spermatozoa were treated with *S. aureus*; SA + NAR – spermatozoa were treated with *S. aureus* and naringenin; SA + CUR – spermatozoa were treated with *S. aureus* and curcumin; SA + ISOQ – spermatozoa were treated with *S. aureus* and isoquercitrin. *NC – significant difference (P<0.05) when compared to NC; ***NC – significant difference (P<0.001) when compared to NC; *SA – significant difference (P<0.05) when compared to SA.

CONCLUSION

The overuse of antibiotics and bacterial resistance to uniformly added antimicrobials in insemination doses brings debates about the effects of these bacterial contaminants on spermatozoa quality. *Staphylococcus* genus, particularly *S. aureus*, represents a major part of bacterial contaminants in bovine semen. The presented study showed that bacteriospermia induced by *S. aureus* increased ROS production with a direct impact on the mitochondrial structure and thereby sperm progressive motility. Besides the mitochondrial damage, elevated ROS significantly increased the proportion of spermatozoa with damaged DNA inside the sperm head. Plant-based bioactive substances with antioxidant potential may prevent the damage caused by bacteriospermia. All tested biological compounds provided a substantial decrease in ROS concentration by scavenging them. These beneficial effects of naringenin, curcumin, and isoquercitrin improved the function of sperm mitochondria under bacteriospermia, which led to the maintenance of sperm progressive movement. Moreover, these selected bioactive substances provided DNA-protective properties, which probably originate from their antioxidant activity. Especially, naringenin and isoquercitrin showed significant protection against oxidative damage of sperm DNA.

Acknowledgments: The study was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic VEGA project no. 1/0239/20 and by the Cultural and Educational Grant Agency project no. KEGA 008SPU-4/2021. This publication was supported by the Operational Program Integrated Infrastructure within the project: Demand-driven Research for the Sustainable and Innovative Food, Drive4SIFood 313011V336, co-financed by the European Regional Development Fund.

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