Original scientific paper
10.7251/AGRENG1801054T
UDC 612.616:579.841

IN VITRO EFFECTS OF *ACINETOBACTER BAUMANNII* AND SELECTED NATURAL BIOMOLECULES ON RABBIT SPERMATOZOA MOTILITY

Eva TVRDÁ[1*], Michal ĎURAČKA[1], Attila KÁNTOR[2], Marek HALENÁR[1], Lukáš HLEBA[3]

[1]Department of Animal Physiology, Slovak University of Agriculture in Nitra, Slovakia
[2]Department of Plant Products Storage and Processing, Slovak University of Agriculture in Nitra, Slovakia
[3]Department of Microbiology, Slovak University of Agriculture in Nitra, Slovakia
*Corresponding author: evina.tvrda@gmail.com

ABSTRACT

The aim of this study was to assess the potential efficiency of selected biologically active substances on the motility behaviour of rabbit spermatozoa subjected to *in vitro* induced *A. baumannii* contamination. The semen samples used for *A. baumannii* detection were collected from 10 New Zealand white male rabbits and the presence of the bacterium was confirmed using MALDI-TOF Mass Spectrometry. For the *in vitro* experiments rabbit spermatozoa were re-suspended in PBS, containing mineral supplements, BSA and glucose in the presence of $3x10^5$ CFU *A. baumannii* and diverse concentrations of selected biomolecules (resveratrol - RES, quercetin - QUE, curcumin - CUR, epicatechin - EPI, isoquercitrin - ISO). The sperm motility was assessed using the computer-aided sperm analysis at 0h, 2h, 4h and 6h. *A. baumannii* significantly decreased the sperm motility ($P<0.001$) at Time 2h and maintained this negative impact throughout the *in vitro* culture. Meanwhile, the motility at Time 2h was significantly higher in the samples subjected to *A. baumannii* together with 10 µmol/L RES ($P<0.01$); 5, 10 and 50 µmol/L QUE ($P<0.001$); 1 µmol/L CUR ($P<0.05$); 10, 50 and 100 µmol/L EPI ($P<0.01$) as well as 50 µmol/L ($P<0.05$) and 100 µmol/L ISO ($P<0.001$) in comparison to the control exposed to the bacterium exclusively. After 4h, the motility remained significantly higher in the groups co-treated with the inoculum and 10 µmol/L RES ($P<0.05$), 50 µmol/L QUE ($P<0.05$) as well as 50 µmol/L EPI ($P<0.05$) when compared to the positive control. Nevertheless, none of the biomolecules was effective against the rapid decline of sperm motility caused by *A. baumannii* during later stages of the experiment (Time 6h). Based on these results, one can conclude that RES, QUE and EPI exhibit antibacterial properties providing a selective advantage to spermatozoa in the presence of *A. baumannii*, particularly during short-term rabbit semen handling.

Keywords: *Acinetobacter baumannii, spermatozoa, biomolecules, contamination, motility.*

INTRODUCTION

A variety of studies have reported that the loss of sperm motility, morphological alterations, acrosome dysfunction, disruption of membrane integrity and oxidative damage may be linked to bacterial contamination of semen under both *in vivo* and *in vitro* conditions (Villegas *et al*., 2005; Fraczek and Kurpisz, 2007; Fraczek *et al.*, 2012). Most data connected to bacteriospermia are related to well-known causative agents of urogenital tract infections, such as *Escherichia coli*, *Staphylococcus aureus*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Chlamydia trachomatis*. Nevertheless, some authors have emphasized that other bacteria, responsible for the colonization of the male urogenital tract, rather than the actual infection, could also contribute to a decreased sperm quality (Fraczek *et al*., 2012).

The genus *Acinetobacter* comprises Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive and oxidase-negative bacteria that are typically short, almost round and rod-shaped. Acinetobacters have been shown to colonize the skin, respiratory and oropharynx secretions of infected individuals (Howard *et al.*, 2012). *A. baumannii* can be an opportunistic pathogen in mammals, affecting individuals with compromised immune systems, and is becoming increasingly important as a nosocomial infection agent. In recent years, the bacterium has been designated as a "red alert" pathogen, generating alarms among the scientific society, arising largely from its extensive antibiotic resistance spectrum (Peleg *et al*., 2008).

Most ejaculates collected from otherwise healthy animals may be contaminated to a certain degree, as semen collection is not a completely sterile process (Bielanski, 2007). As such, antibiotics are commonly added to semen extenders used for artificial insemination to control possible microbial contamination of semen during collection and processing. Since antibiotics themselves may be toxic to spermatozoa, and because of an alarmingly increasing bacterial resistance, there is an urgent need to find alternatives to conventional antibiotics to be used in animal reproduction biotechnologies (Morrell and Wallgren, 2014).

Recent studies have emphasized on the rebirth of naturally occurring compounds with a variety of beneficial properties, rich diversity, complexity and availability, lack of significant toxic effects and intrinsic biologic activity. A broad array of flavonoid and polyphenolic compounds has been shown to possess beneficial properties which could potentially provide a selective advantage to spermatozoa under stress conditions.

This study focused to assess the effects of five natural biologically active compounds (resveratrol, quercetin, curcumin, epicatechin, isoquercitrin) in comparison with three traditional antibiotics (penicillin, gentamycin, kanamycin) on the motion of rabbit spermatozoa subjected to *in vitro* induced *Acinetobacter baumannii* contamination.

MATERIAL AND METHODS

Sample collection

Ten male rabbits (New Zealand white broiler line) were used in the experiment. The animals were 4 months old, with a weight of 4.0±0.2 kg and kept at an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic. The rabbits were housed in a partially air-conditioned rabbit house under a photoperiod of 16L:8D (a minimum light intensity of 80 lux), kept in individual cages and fed with a commercial diet. Water was provided *ad libitum*. The air temperature of 20-24 °C and relative humidity of 65% were maintained in the rabbit house. Institutional and national guidelines on the care and use of animals were followed, and all the experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee. One ejaculate was collected from each rabbit using an artificial vagina. Immediately upon collection, the samples were transferred to the laboratory.

Cultivation and identification of microorganisms

100 µl of each semen sample were transferred into the MacConkey agar (Biomark, Pune) and MRS agar (Biolife, Italy). The cultures were maintained at 37°C during 24h for microorganisms which grew on the MacConkey agar and 37°C during 48-72h for microorganisms which grew on the MRS agar. Purification of all microorganisms was done by four ways streak plate method after the first cultivation. The Chromogenic coliform agar (Oxoid, England) and the URI Select IV (Biolife, Italy) were subsequently used to purify those microorganisms which contaminated the MacConkey agar. Microorganisms which contaminated the MRS agar were repeatedly purified in the MRS agar. All steps of recultivation were done at the same conditions (Hleba *et al.*, 2017).

Matrix-assisted laser desorption/ionization time-of-light (MALDI TOF MS) (Brucker Daltonics, Germany) was used for bacterial identification in the semen samples. Cells from a single colony of fresh overnight culture were used for each isolate to prepare samples according to the manufacturer's recommendations for microorganism profiling using the ethanol-formic acid extraction procedure. Each sample spot was overlaid with 2 µl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and again air-dried for 15 min. To identify the microorganisms, raw spectra obtained for each isolate were imported into the Biotyper software, version 2.0 (Brucker Daltonics), and analyzed without any user intervention (Hleba *et al.*, 2017). *A. baumannii* was identified in seven out of ten semen samples.

The isolated *A. baumannii* was aseptically transferred to the culture medium selected for the *in vitro* experiments and cultured at 36°C for 24 to 48h. Following culture, *A. baumannii* concentration was adjusted to 0.3 McF using a densitometer (DEN^{-1} McFarland Densitometer, Grant-bio, UK). Such inoculum was suitable for the simulation of an *in vivo* environment under *in vitro* conditions taking into consideration an ideal environment for the sperm cells as well as the bacterium.

In vitro experiments

One ejaculate was collected from 10 male rabbits used for previous *in vivo* experiments on a regular collection schedule (twice a week for two consecutive weeks) using an artificial vagina. Only samples with a minimum motility of 60% were used in the experiments.

The resulting semen sample was centrifuged (300 x g) at 25°C for 5 min, seminal plasma was removed and the sperm pellet was washed twice with PBS (Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride; Sigma-Aldrich, St. Louis, MO, USA), resuspended in a culture medium consisting of PBS, mineral supplements for semen cultures (Minitube, Tiefenbach, Germany), 5% glucose (Centralchem, Bratislava, Slovak Republic) and 4% BSA (bovine serum albumin, Sigma-Aldrich) using a dilution ratio of 1:20. Two controls were established – the Negative Control was resuspended in the culture medium exclusively, while the Positive Control contained the culture medium with 0.3 McF *A. baumannii*. Each experimental group was exposed to the bacterium and different concentrations of chosen antibiotics or biomolecules as follows: 300 µg/mL penicillin (PEN; Sigma-Aldrich); 1 mg/mL gentamycin (GEN; Sigma-Aldrich); 80 µg/mL kanamycin (KAN; Sigma-Aldrich); 50, 10 and 5 µmol/L^{-1} resveratrol (RES; Sigma-Aldrich); 50, 10 and 5 µmol/L^{-1} quercetin (QUE; Sigma-Aldrich); 10, 5 and 1 µmol/L^{-1} curcumin (CUR; Sigma-Aldrich); 100, 50 and 10 µmol/L^{-1} epicatechin (EPI; Sigma-Aldrich); 100, 50 and 10 µmol/L^{-1} isoquercitrin (ISO; provided by the Center of Biocatalysis and Biotransformation, Czech Academy of Sciences).

At culture times of 0h, 2h, 4h and 6h the spermatozoa motility (percentage of motile spermatozoa; motility > 5 µm/s; %; MOT) was assessed using the computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). The samples were stained using the IDENT stain, a DNA-specific dye based on Hoechst bisbenzimide (Hamilton-Thorne Biosciences). The IDENT dye provided in Eppendorf tube was diluted with 1 ml of the culture medium and mixed with the sample using a ratio of 1:1. Following 10 min of incubation in the dark, the sample was analyzed under fluorescent illumination. Ten microscopic fields were subjected to each analysis in order to include at least 300 cells.

Statistical analysis

All the data were subjected to statistical analysis using the GraphPad Prism program (3.02 version for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). The results are quoted as the arithmetic mean ± standard error of mean (SEM). The comparative analysis was carried out by a one-way ANOVA with the Dunnett's post test. The level of significance for the analysis was set at * $P<0.05$; ** $P<0.01$; *** $P<0.001$. The comparative analysis was performed as follows: Positive Control (PC) was compared to the Negative Control (NC), while experimental fractions exposed to *A. baumannii* and antibiotics or biomolecules were compared to both Controls.

RESULTS AND DISCUSSION

The initial MOT was lower in the Positive Control when compared to the Negative Control. Moreover, MOT was insignificantly lower in groups exposed to *A. baumannii* together with PEN, GEN, KAN, 50 and 5 µmol/L RES, 10 and 5 µmol/L CUR, 10 µmol/L EPI and 10 µmol/L ISO (Figure 1).

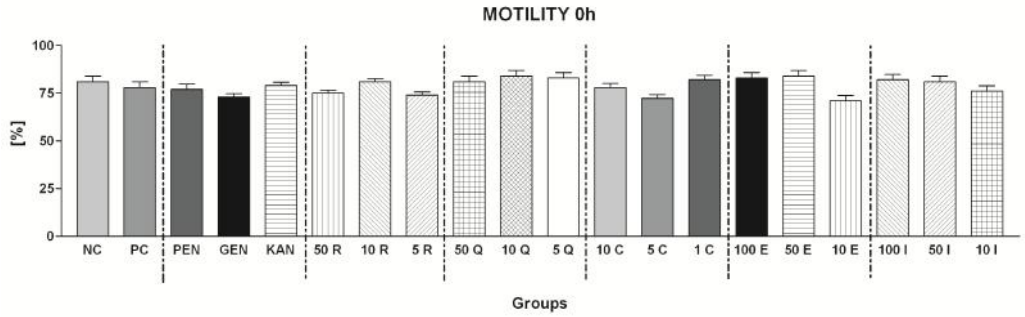

Figure 1. Immediate effects (Time 0h) of *A. baumannii*, selected antibiotics and biomolecules on rabbit spermatozoa motility [%]. Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001. NC – Negative Control; PC – Positive Control; PEN – Penicillin; GEN – Gentamycin; KAN – Kanamycin; R – Resveratrol; Q – Quercetin; C – Curcumin; E – Epicatechin; I – Isoquercitrin.

After 2h the presence of *A. baumannii* significantly decreased MOT (P<0.001) when compared to the untreated Control. Meanwhile, the MOT was significantly higher in the samples subjected to *A. baumannii* with GEN (P<0.01), KAN (P<0.01), 10 µmol/L RES (P<0.01), all QUE doses (P<0.001), 1 µmol/L CUR (P<0.05), all EPI concentrations (P<0.01), 100 µmol/L (P<0.001) and 50 µmol/L (P<0.05) ISO. On the other hand, MOT was significantly decreased in samples exposed to *A. baumannii* with PEN (P<0.01), 50 µmol/L RES (P<0.01), 10 µmol/L and 5 µmol/L CUR (P<0.001) as well as 10 µmol/L ISO (P<0.01).

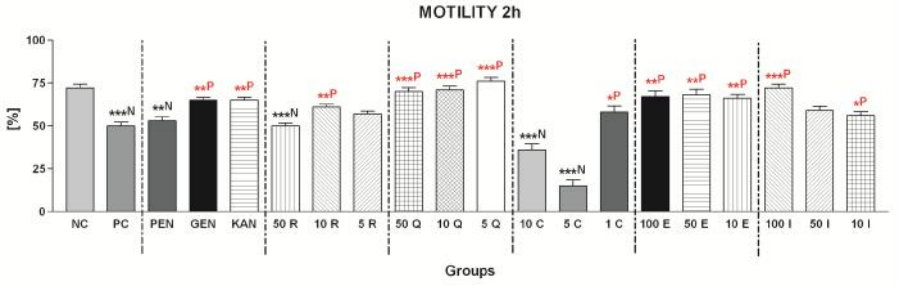

Figure 2. The effects of *A. baumannii*, selected antibiotics and biomolecules on rabbit spermatozoa motility following 2 hours of in vitro culture [%]. Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001. NC – Negative Control; PC – Positive Control; PEN – Penicillin; GEN – Gentamycin; KAN – Kanamycin; R – Resveratrol; Q – Quercetin; C – Curcumin; E – Epicatechin; I – Isoquercitrin. N – vs. Negative (untreated) Control. P – vs. Positive Control (exposed to *A. baumannii* exclusively).

After 4h, the highest MOT was detected in the Negative Control, while the MOT in the Positive Control was significantly lower (P<0.001). While the MOT in all experimental groups was lower when compared to the Negative Control, tit remained significantly higher in the groups co-treated with the inoculum together with PEN (P<0.01), GEN (P<0.001), KAN (P<0.01), 10 µmol/L RES (P<0.05), 50 µmol/L QUE (P<0.05) and 50 µmol/L EPI (P<0.05). In the rest of the experimental groups, the MOT was significantly decreased when compared to the Negative Control (P<0.001).

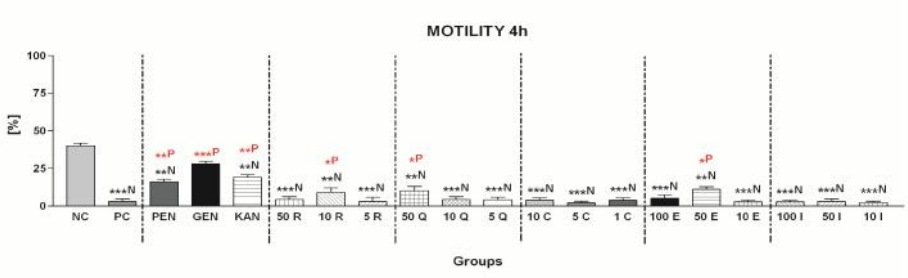

Figure 3. The effects of *A. baumannii*, selected antibiotics and biomolecules on rabbit spermatozoa motility following 4 hours of in vitro culture [%]. Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001. NC – Negative Control; PC – Positive Control; PEN – Penicillin; GEN – Gentamycin; KAN – Kanamycin; R – Resveratrol; Q – Quercetin; C – Curcumin; E – Epicatechin; I – Isoquercitrin. ^{N} – vs. Negative (untreated) Control. ^{P} – vs. Positive Control (exposed to *A. baumannii* exclusively).

The final (Time 6h) assessment revealed that the only group containing actively moving spermatozoa was the Negative Control. Only few moving spermatozoa were detected in the Positive Control (P<0.001). Sperm MOT was preserved to a certain degree by the presence of PEN (P<0.05) and GEN (P<0.01). None of the biomolecules applied was able to maintain the sperm MOT comparable to the Negative Control, and none was effective against the rapid decline of sperm motility caused by the presence of *A. baumannii*.

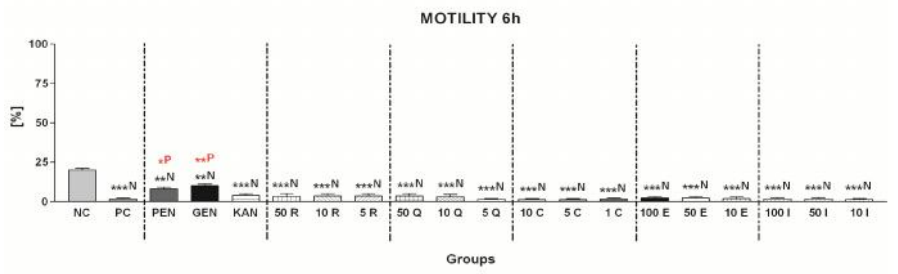

Figure 4. The effects of *A. baumannii*, selected antibiotics and biomolecules on rabbit spermatozoa motility following 4 hours of in vitro culture [%]. Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001. NC – Negative Control; PC – Positive Control; PEN – Penicillin; GEN – Gentamycin; KAN – Kanamycin; R – Resveratrol; Q – Quercetin; C – Curcumin; E – Epicatechin; I – Isoquercitrin. ^{N} – vs. Negative (untreated) Control. ^{P} – vs. Positive Control (exposed to *A. baumannii* exclusively).

A. baumannii has recently emerged as a critical bacterial contaminant of ejaculates. Similarly to our MALDI TOF data, *A. baumannii* was identified in semen by other investigators (Kiessling *et al*., 2008; Sarah *et al.*, 2015) in individuals suffering from oligozoospermia, teratozoospermia and chronic prostatitis. Furthermore, Kiessling *et al.* (2008) performed PCR amplification of bacterial rDNA on 34 semen samples, and identified *Acinetobacter* as one of the largest groups in different specimens. The authors conclude that the abundance of bacteria in semen may influence fertility, and may reflect an inadequate cellular immune response. Moreover Sarah *et al.* (2015) conclude that the presence of *Acinetobacter* in semen samples makes *in vitro* fertilization procedures not viable. Semen extenders with single or combination of antibiotics such as kanamycin, ampicillin, gentamicin, linomycin, penicillin-streptomycin, tylosin and lincospectin have been tried with different success rates in eliminating microbes in animal studies (Hasan *et al.*, 2001). Nevertheless, a large number of antibiotics have been shown to exhibit toxic effects on the sperm motility, viability and DNA integrity. Furthermore, long term use of the same antibiotic may lead to antibiotic resistant varieties. As such, there is an urgent need to search for alternative substances which could provide a selective advantage to male reproductive cells against the stress resulting from microbial contamination (Morrell and Wallgren, 2014). RES has been recently discovered to possess a wide range of cardiovascular, anticancer, anti-inflammatory and protective effects. Our data are contradictory to Collodel *et al.* (2010) who reported that human spermatozoa were more sensitive to the potentially toxic effect of RES with a LD50 between 30 and 50 μmol/L. Moreover, Tvrdá *et al.* (2015b) reported a significant decrease of bull sperm MOT following exposure to 100 or 200 μmol/L RES during a 24h *in vitro* culture. On the contrary, protective effects of lower RES concentrations on spermatozoa are in agreement with Tvrdá *et al.* (2015) who emphasize on the beneficial effects of 5-50 μmol/L RES on the motion behaviour, mitochondrial activity and superoxide production by bovine sperm. Our data are in agreement with Mojica-Villegas *et al.* (2014) who reported that pre-treatment with 15 μmol/L RES prior to incubation with ferrous ascorbate showed an 8.0-fold increase in murine spermatozoa MOT. QUE is a common dietary flavonoid, reported to exhibit a broad variety of favourable biological effects. Protective effects of QUE on rabbit spermatozoa in our study disagrees with earlier reports, according to which QUE compromised human sperm motility and viability (Khanduja *et al.*, 2001). Talking in favour of our results, Tvrdá *et al.* (2016b) emphasized on the protective effects of QUE on bovine sperm motion activity when incubated without the presence of seminal plasma (Tvrdá *et al.*, 2016b), although we must take into consideration that QUE may act dose dependently as either a stimulant at low concentrations or as an inhibitor at high doses. Previous reports on the impact of CUR on male fertility are controversial. Naz (2011) reported that exposure of human and mouse sperm to CUR caused a concentration-dependent decrease of sperm MOT, capacitation and acrosome reaction. Furthermore, our results disagree with Tvrdá *et al.* (2016a) suggesting stimulating and protective effects of CUR on spermatozoa MOT and antioxidant status.

Motility parameters recorded by our IDENT CASA technique partially complement previous findings by Bucak *et al.* (2010) demonstrating a significant motion improvement of ram spermatozoa supplemented with CUR. Interestingly, their later study on CUR administration to bovine semen revealed non-significant differences in the sperm motion (Bucak *et al.*, 2012).

EPI is a flavonoid and antioxidant commonly found in green tea and cocoa. Jamalan *et al.* (2016) evaluated the effects of different flavonoids including EPI on the recovery of sperm MOT and prevention of membrane damage from aluminium, cadmium and lead. Contrary to our study, the report revealed that EPI did not protect spermatozoa from heavy metal-mediated damage, rather, it showed inhibitory effects on the sperm motion associated with co-incubation with selected heavy metals. Inversely Purdy *et al.* (2004) revealed that EPI may aid in maintaining the MOT of cooled goat sperm in a dose dependent manner.

ISO is found in a variety of medicinal plants, likely contributing to their pharmacological qualities (Appleton, 2010). According to our data, although being structurally similar to QUE, the molecule did not exhibit beneficial effects on the sperm MOT. On the other hand, numerous animal studies emphasize on potential ameliorative effects of plant extracts containing ISO on the testicular structure and function, as well as sperm concentration, motility and morphology (Awoniyi *et al.*, 2011). As such, we may suggest that more specific experiments on the roles of ISO are to be designed in order to elucidate its beneficial or harmful roles in male reproduction.

CONCLUSIONS

Based on our preliminary CASA results it can be concluded that resveratrol, quercetin and epicatechin exhibit antibacterial properties providing a selective advantage to the male gametes in the presence of *Acinetobacter baumannii*, particularly during short-term rabbit semen handling. On the other hand, curcumin and isoquercitrin did not prove to possess significant protective or beneficial effects on the *in vitro* survival of rabbit spermatozoa in the presence of uropathogenic bacteria. Last but not least, more experiments will be necessary to unravel specific molecular mechanisms of action of *A. baumannii*, antibiotics and/or natural biomolecules on the structure and function of male reproductive cells.

ACKNOWLEDGMENT

This research was supported by the Research Center AgroBioTech built in accordance with the project Building Research Centre “AgroBioTech” ITMS 26220220180 and by the Slovak Research and Development Agency Grants no. APVV-0304-12 and APVV-15-0544. The authors are grateful to Prof. Dr. Vladimir Kren, FRSC from the Center of Biocatalysis and Biotransformation, Czech Academy of Sciences, for the provision of isoquercitrin.

REFERENCES

Appleton J. (2010). Evaluating the bioavailability of isoquercetin. Journal of Natural Medicines, vol. 2, pp. 1-4.

Awoniyi D.O., Aboua Y.G., Marnewick J.L., du Plesis S.S., Brooks N.L. (2011). Protective effects of rooibos (Aspalathus linearis), green tea (Camellia sinensis) and commercial supplements on testicular tissue of oxidative stress induced rats. African Journal of Biotechnology, vol. 10, pp. 17317-17322.

Bielanski A. (2007). Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. Theriogenology, vol. 68, pp. 1-22.

Bucak M.N., Sariozkan S., Tuncer P.B., Sakin F., Atessahin A., Kulaksız R., Cevik M. (2010). The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Research, vol. 89, pp. 24-30.

Bucak M.N., Basspinar N., Tuncer B.P., Coyan K., Sariozkan S., Akalin P.P., Buyukleblebici S., Kucukgunay S. (2012). Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. Andrologia, vol. 44, pp. 102-109.

Collodel G., Federico M.G., Geminiani M., Martini S., Bonechi C., Rossi C., Figura N., Moretti E. (2010). Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. Reproductive Toxicology, vol. 31, pp. 239-246.

Fraczek M., Kurpisz M. (2007). Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. Journal of Andrology, vol. 28, pp. 325-333.

Fraczek M., Piasecka M., Gaczarzewicz D., Szumala-Kakol A., Kazienko A., Lenart S., Laszczynska M., Kurpisz M. (2012). Membrane stability and mitochondrial activity of human-ejaculated spermatozoa during in vitro experimental infection with Escherichia coli, Staphylococcus haemolyticus and Bacteroides ureolyticus. Andrologia, vol. 44, pp. 315-329.

Hasan S., Andrabi S.M., Muneer R., Anzar M., Ahmad N. (2001). Effects of a new antibiotic combination on post-thaw motion characteristics and membrane integrity of buffalo and sahiwal bull spermatozoa and on the bacteriological quality of their semen. Pakistan Veterinary Journal, vol. 21, pp. 6-12.

Hleba L., Kmeť V., Tóth T., Kačániová M. (2017). Resistance in bacteria and indirect beta-lactamase detection in *E. coli* isolated from Culex pipiens detected by matrix-assisted laser desorption ionization time of flight mass spectrometry. Journal of Environmental Science and Health, Part B, vol. 52, pp. 64-69.

Howard A., O'Donoghue M., Feeney A., Sleator R.D. (2012). Acinetobacter baumannii: An emerging opportunistic pathogen. Virulence, vol. 3, pp. 243-250.

Jamalan M., Ghaffari M.A., Hoseinzadeh P., Hashemitabar M., Zeinali M. (2016). Human sperm quality and metal toxicants: protective effects of some flavonoids

on male reproductive function. International Journal of Fertility and Sterility, vol. 10, pp. 215-223.

Khanduja K.L., Verma A., Bhardwaj A. (2001). Impairment of human sperm motility and viability by quercetin is independent of lipid peroxidation. Andrologia, vol. 33, pp. 277-281.

Kiessling A.A., Desmarais B.M., Yin H.Z., Loverde J., Eyre R.C. (2008). Detection and identification of bacterial DNA in semen. Fertility and Sterility, vol. 90, pp. 1744-1756.

Mojica-Villegas M.A., Izquierdo-Vega J.A., Chamorro Cevallos G., Sanchez-Guiterrez M. (2014). Protective effect of resveratrol on biomarkers of oxidative stress induced by iron/ascorbate in mouse spermatozoa. Nutrients, vol. 6, pp. 489-503.

Morrell J.M., Wallgren, M. (2014). Alternatives to antibiotics in semen extenders: a review. Pathogens, vol. 3, pp. 934-946.

Naz R.J. (2011). Can curcumin provide an ideal contraceptive? Molecular Reproduction and Development, vol. 78, pp. 116-123.

Peleg A.Y., Seifert H., Paterson D.L. (2008). Acinetobacter baumannii: Emergence of a successful pathogen. Clinical Microbiology Reviews, vol. 21, pp. 538-582.

Purdy P.H., Ericsson S.A., Dodson R.E., Sternes K.L., Garner D.L. (2004). Effects of the flavonoids, silibinin and catechin, on the motility of extended cooled caprine sperm. Small Ruminant Research, vol. 55, pp. 239-243.

Sarah A., Saif Q., Ruhi K., Asfia S. (2015). Semen culture in patients with chronic prostatitis syndrome: A valuable diagnostic tool. International Journal of Current Microbiology and Applied Sciences, Special Issue 1, pp. 236-242.

Tvrdá E., Lukáč N., Lukáčová J., Hashim F., Massányi P. (2015). In vitro supplementation of resveratrol to bovine spermatozoa: effects on motility, viability and superoxide production. Journal of Microbiology, Biotechnology and Food Sciences, vol. 4, pp. 336-341.

Tvrdá E., Tušimová E., Kováčik A., Paál D., Greifová H., Abdramanov A., Lukáč, N. (2016a). Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. Animal Reproduction Science, vol. 172, pp. 10-20.

Tvrdá E., Tušimová E., Kováčik A., Paál D., Libová Ľ., Lukáč N. (2016b). Protective effects of quercetin on selected oxidative biomarkers in bovine spermatozoa subjected to ferrous ascorbate. Reproduction in Domestic Animals, vol. 51, pp. 524-537.

Villegas J., Schulz M., Soto L., Sanchez R. (2005). Bacteria induce expression of apoptosis in human spermatozoa. Apoptosis, vol. 10, pp. 105-110.