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

Published by Faculty of Biotechnology and Food Sciences

DOSE- AND TIME-DEPENDENT EFFECTS OF EPICATECHIN ON BOVINE SPERMATOZA IN VITRO

Hana Greifová, Eva Tvrdá, Tomáš Jambor, Norbert Lukáč*

Address(es): Ing. Hana Greifová
Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, +42137 641 4288.

*Corresponding author: hgreifova@gmail.com

doi: 10.15414/jmbfs.2017/18.7.3.235-239

ARTICLE INFO

Received 2. 2. 2017
Revised 24. 8. 2017
Accepted 15. 11. 2017
Published 1. 12. 2017

Regular article

OPEN ACCESS

ABSTRACT

The purpose of this study was to determine the effect of epicatechin (EPI) on the motility, viability, and reactive oxygen species (ROS) production of bovine spermatozoa in the presence of various EPI doses during different time periods (Time 0 h, 2 h, 6 h, 24 h). Semen samples were cultivated in physiological saline solution containing 200, 100, 50, 10, 5 and 1 μM/L of EPI dissolved in 0.5% DMSO. Spermatozoa motility was determined using the HTM IVOS and CASA (Computer Assisted Semen Analyzer) system. The cell viability was measured by the MTT (metabolic activity) assay and chemiluminescence was evaluated to quantify the ROS generation. Initial (0 h) viability was significantly improved ($P<0.05$) in experimental samples supplemented with the lowest concentration of EPI (1 μM/L). Motility after 2 h of cultivation showed significant increase ($P<0.05$) values only at concentration 100 μM/L. In the case of ROS production and even spermatozoa viability after 2 h of cultivation was not observed significant differences. Prolonged cultivation (6 h) showed significant improvements in all evaluated parameters in the case of concentrations ranging between 1 and 50 μM/L EPI ($P<0.001$ in concentration 5 μM/L for viability; $P<0.01$ in concentrations 10 and 50 μM/L for motility, viability; and ROS production even in concentration 5 μM/L; $P<0.05$ in concentrations 1 and 5 μM/L for motility and ROS production). 24 h of cultivation confirmed the protective effect of EPI in experimental groups after comparison with the control group with significant differences ($P<0.001$) for motility, as well as for viability and ROS production in concentrations ranging between 1 and 50 μM/L. Higher concentrations of EPI (100 and 200 μM/L) also showed significant changes ($P<0.01$) in values of viability and ROS production.

Keywords: Epicatechin, bovine spermatozoa, CASA system, MTT assay, ROS production

INTRODUCTION

Though a lot of studies have shown upturn of sperm quality and fertilizing ability after antioxidant administration, current attention has been aimed on natural substances and extracts with different beneficial properties including antioxidant effect. Indeed, there is a large number of medicinal plants known to control various health problems, used by indigenous people as part of a traditional medicine, especially in Asian countries (**Fatma *et al.*, 2009**).
Catechins are known as flavonoids present in various natural sources. Catechin, epicatechin and epicatechin gallate are the essential polyphenols occurring in fruit wine. High concentrations of epicatechin are in green tea, apples, blackberries, broad beans, cherries, black grapes, pears, raspberries, and cocoa beans (**Gadkari and Balaraman, 2015**). Nowadays these natural compounds are becoming increasingly popular in association with their potential as biological active substances, such as antimutagenic and antitumorigenic activity. Catechins and other flavonoids are considered effective antioxidants capable to scavenge oxygen radicals. Large number of researches have proved that catechins mitigate the impact of oxidative stress on biological tissues and subcellular structures. (**Terao *et al.*, 1994**). Epicatechin is able to scavenge hydroxyl radicals, peroxyl radicals, superoxide radicals, and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, there was also observed that catechin and epicatechin have a peroxyl radical scavenging activity 10 times higher than those of L-ascorbate or beta-carotene (**Yilmaz and Toledo, 2004**). It is known that the antioxidant activity of polyphenols such as epicatechin is mediated by hydrogen-donating capacity of their phenolic groups. Another antioxidant property is their metal-chelating potential which may also play a key role in the protection against iron- and copper-induced free radical reactions. (**Rice-evans *et al.*, 1995**).
It is generally accepted that the generation of low ROS levels provide optimal conditions for several main sperm functions, including capacitation, acrosome reaction, *zona pellucida* binding and oocyte fusion. However, overproduction of ROS and imbalance between ROS concentration and the antioxidant capacity of cells leads to the development of pathological condition known as oxidative stress (**Moretti *et al.*, 2012**). Membranes of sperm cells are rich in polyunsaturated fatty acid, what may lead to peroxidation of membrane lipids because of high susceptibility of spermatozoa to oxygen-induced damage. (**Sikka, 2001**). Oxidative stress is a major aspect in the etiology of bad sperm quality, functions as motility and causes morphological changes and oxidative alterations to DNA, membranes, and proteins (**Moustafa *et al.*, 2004**). Spermatozoa and seminal plasma contain a wide range of protective enzymatic antioxidants, such as the glutathione peroxidase/reductase system, superoxide dismutase, catalase and low-molecular weight antioxidants, vitamin E, vitamin C, urate, and albumin, which are able to scavenge ROS and provide prevention of potential cellular defects mediated by ROS overproduction. Though, it is known that long-term exposure to seminal plasma is harmful for sperm functions, including motility (**Tremellen, 2008**).
One of the strategies for mitigate the impact of ROS overproduction during processing of sperm is to enhance the antioxidant capacity of medium; for example, administration of natural substances with antioxidant activity to utilized sperm buffer, such as curcumin (**Tvrdá *et al.*, 2015**), lycopene (**Tvrdá *et al.*, 2016**) and many others. Also supplementation of freezing extenders with natural antioxidants was used in many studies dealing with improving of overall sperm parameters by inhibiting of oxidative stress (**Bucak *et al.*, 2010; Bucak *et al.*, 2015; Omur and Coyan, 2016**).
This study was carried out to test the antioxidant effects of different epicatechin concentrations during *in vitro* cultivation at several time periods on the motility, sperm viability and production of reactive oxygen substances of bovine spermatozoa.

MATERIAL AND METHODS

Semen samples and *in vitro* culture

Bovine semen samples were obtained from 20 adult breeding bulls (Slovak Biological Services, Nitra, Slovakia). The samples had to accomplish the basic criteria given for the corresponding breed. After collection, the samples were stored in the laboratory at room temperature (22–25°C) for later analysis. Each

sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medital, Grosotto, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration. Spermatozoa were incubated with various concentrations of epicatechin ((−)-Epicatechin; Sigma-Aldrich, St. Louis, USA) dissolved in 0.5% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) (A – 200; B – 100; C – 50; D – 10; E – 5; F – 1 μM/L). The control (Ctrl) group was cultured with physiological saline solution and all samples were cultivated in the laboratory at room temperature (22–25°C). The control group (medium without EPI) was compared to the experimental groups (exposed to different concentrations of EPI).

Computer-assisted semen analysis

Spermatozoa motility was measured as an indicator of semen quality. The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – HTM IVOS (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne) at cultivation times 0 h, 2 h, 6 h and 24 h. Each sample was placed into the Makler Counting Chamber (depth 10 mm, Sefi-Medical Instruments, Haifa, Israel) and the percentage of motile spermatozoa (motility > 5 μm/s; MOT) was evaluated. This study was performed in three replicates at each concentration and time. At least 1000 spermatozoa were analyzed in each sample (**Lukáč *et al.*, 2011**).

Viability evaluation

The viability of the cells exposed to epicatechin *in vitro* was evaluated by the metabolic activity (MTT) assay (**Mosmann, 1983**). This colorimetric assay measures the conversion of a yellow water-soluble tetrazolium salt [3-(4,5-dimetylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide] (MTT) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The amount of formazan was measured spectrophotometerically. In brief, the cultured cells in 96-well plates were stained with MTT tetrazolium salt (Sigma, St. Louis, MO, USA). MTT was dissolved in PBS (Dulbeccos Phosphate Buffer Saline, Sigma, St. Louis, USA) and added to the cells (20 μL per well). After 1 h of incubation (37°C), the cells and the formazan crystals were dissolved in 80 μL of isopropanol (2-propanol, p.a. CentralChem, Bratislava, Slovakia). Optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiscan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e., optical density of formazan from cells not exposed to EPI). The results from the analysis were collected during three repeated experiments at each concentration.

Evaluation of ROS generation

ROS levels in samples were assessed by chemiluminescence assay using luminol (5-amino- 2, 3- dihydro-1, 4-phthalazinedione; Sigma, St Louis, MO) as the probe (**Tvrdá *et al.*, 2016**). The tested samples consisted of luminol (5 μL, 5 mM) and control or experimental samples (dilution ratio 1:40). Negative controls were prepared by replacing the sperm suspension with 200 μL of the extender. Positive control included 200 μL of the extender and 25 μL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich, St. Louis, USA) in triplicates. Chemiluminescence was measured on a 96-well plate for 15 min using the Glomax Multi+ Combined Spectro-FluoroLuminometer (Promega). The results were expressed as relative light units (RLU)/sec/10^6 sperm.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA with Dunnett's posttest was used for statistical evaluations. The level of significance was set at ∗∗∗ (P<0.001); ∗∗ (P<0.01); ∗(P<0.05).

RESULTS AND DISCUSSION

The initial spermatozoa motility (0 h) showed increased value only in experimental groups C (50 μM/L EPI) and E (5 μM/L EPI) in comparison to the control group without EPI, though without any statistical significance (P>0.05) (Table 1). Significant difference (P < 0.05) was observed after 2 h of incubation between the group containing higher dose of EPI – experimental group B (100 μM/L EPI) and the control group without EPI. No significant differences were found between others experimental groups and control group at the time 2 h. After 6 h of *in vitro* cultivation were observed several significant differences in spermatozoa motility. At this time, significant increased value of motility was noted at the dose 50 μM/L EPI- experimental group C (P<0.01), 10 μM/L EPI- experimental group D (P<0.01) and 5 μM/L EPI- experimental group E (P<0.05) in comparison with the control. At the end of the experiment (24 h), significantly elevated (P<0.001) motility showed all experimental groups (1-200 μM/L EPI; experimental group A-F), after a comparison with the control group (Table 1).

Table 1 Spermatozoa motility (%) in the absence (Ctrl) or presence (A-F) of epicatechin during different time periods (Mean±SEM)

Groups/ Time	Ctrl	A	B	C	D	E	F
0h	86.33±1.76	83.00±4.08	84.33±4.96	87.00±3.78	86.33±4.17	88.67±3.37	73.33±3.42
2h	69.67±2.24	71.33±3.62	79.67±1.57*	76.67±2.02	73.67±2.26	76.33±3.48	58.33±2.17
6h	50.00±1.51	52.67±2.38	56.33±3.84	66.00±2.24**	66.67±1.83**	60.67±2.84*	48.00±1.94
24h	8.33±0.81	25.67±1.75***	31.00±1.93***	35.00±1.66***	37.33±1.21***	36.33±1.25***	34.00±1.09***

∗∗∗ (P<0.001); ∗∗ (P<0.01); ∗ (P<0.05)

The viability of bovine spermatozoa as detected by the MTT cytotoxicity assay was initially (time 0 h) significantly increased only at the lowest dose of EPI (1 μM/L)- experimental group F (P<0.05) but paradoxically, all experimental groups after 2 h of *in vitro* cultivation were without any statistical significance in comparison with the control group (Figure 1). EPI administration at 6 h led to a significant improvement of cell viability at dose 50 and 10 μM/L EPI- experimental groups C, D (P<0.01) and at dose 5 μM/L EPI- experimental group E (P<0.001; Figure 1). After 24 h of *in vitro* cultivation the spermatozoa mitochondrial activity increased in all experimental groups with EPI treatment with significant differences (P<0.01) in experimental groups A and B (200 and 100 μM/L EPI) and also at lower doses (50, 10, 5 and 1 μM/L EPI) in experimental groups C, D, E and F were found significant improved values of viability when compared to the control group (Figure 1). Slight inhibition in the cell viability of bovine spermatozoa was determined only in experimental group A (200 μM/L EPI) after 2 h of incubation, although without any statistical significance (Figure 1).

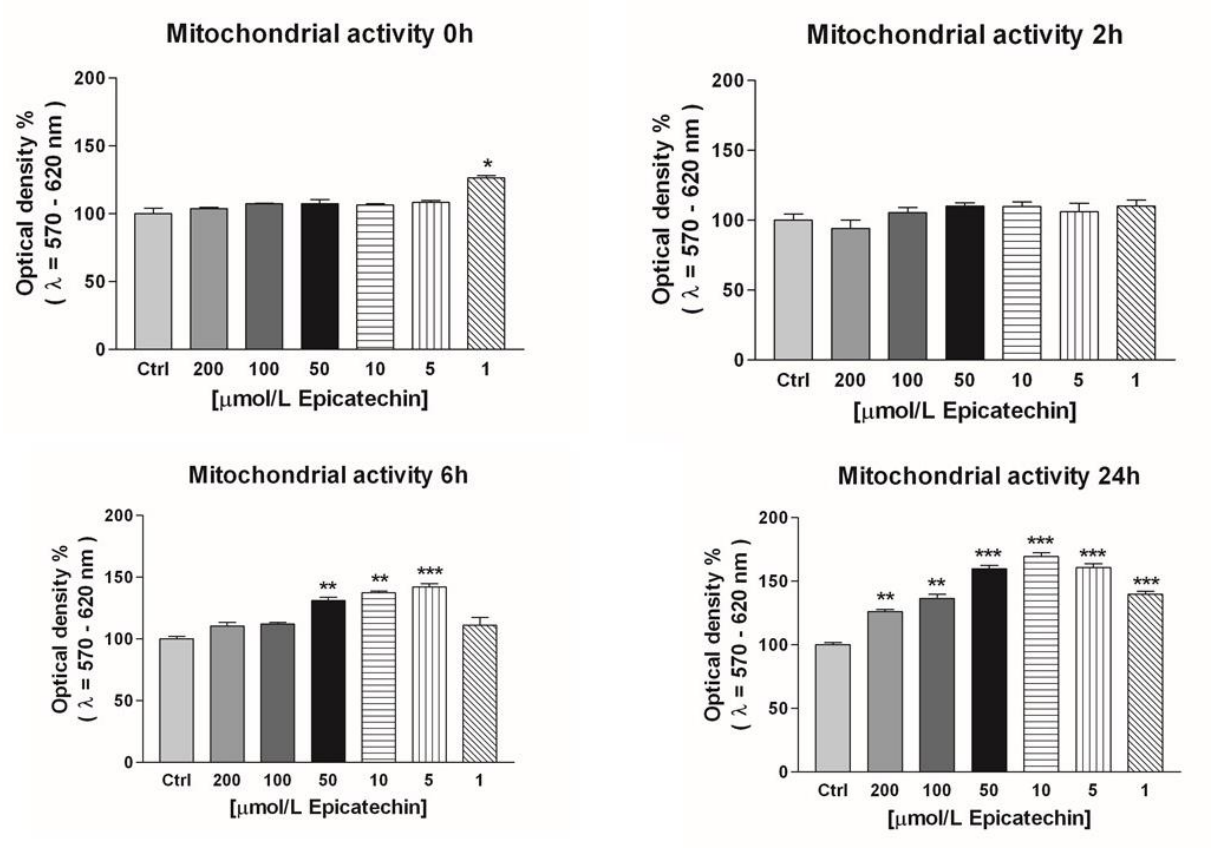

Figure 1 The effect of different doses of epicatechin on the viability of bovine spermatozoa (n=20) at 0h, 2h, 6h, and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from three independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; *P<0.05. Ctrl – 0; A – 200; B – 100; C –50; D – 10; E – 5; F – 1 μM/L EPI.

Luminometric evaluation showed that epicatechin administration provided protection against ROS overgeneration with significantly decreased values at cultivation time 6 h in experimental group F (P<0.05), as well as in groups C, D and E (P<0.01) when compared to the samples from control group without EPI treatment (Table 2). Significant changes in ROS production were also observed after 24 h of *in vitro* cultivation at the all doses of epicatechin; for experimental groups A and B (P<0.01) and experimental groups C, D, E, F (P<0.001) too (Table 2).

Table 2 ROS production (RLU/sec/10^6 sperm) in the absence (Ctrl) or presence (A-F) of epicatechin during different time periods (Mean±SEM)

Groups/ Time	Ctrl	A	B	C	D	E	F
0h	2.88±0.22	2.25±0.15	2.09±0.10	2.02±0.09	2.08±0.13	2.09±0.13	2.62±0.14
2h	5.13±0.29	4.32±0.21	4.28±0.33	3.82±0.21	3.99±0.23	4.00±0.24	4.39±0.24
6h	8.83±0.89	7.29±0.33	7.17±0.60	6.13±0.44**	6.02±0.43**	6.08±0.30**	6.93±0.35*
24h	18.49±0.99	15.07±0.65**	14.94±0.87**	12.84±0.59***	11.84±0.67***	11.56±0.78***	13.22±0.87***

*** (P<0.001); ** (P<0.01); * (P<0.05)

Recently, antioxidant substances have aroused great interest due to their ability to minimize the deleterious effects of reactive oxygen species (ROS) on several biological and pathological processes. ROS are necessary for the normal physiological function of sperm, although their concentration must be kept under strict control to avoid deleterious effects, such as damage to cell structures: lipids and membranes, proteins, and DNA (**Dias *et al.*, 2014**). There is a growing interest in enlightening the role of ROS formation in sperm as they are responsible for lower sperm quality in freshly collected semen and poor quality of sperm after processing for usage in reproductive technologies, such as artificial insemination, *in vitro* fertilization, or cryopreservation (**Guthrie and Welch, 2012**). Short- and long-term storage of viable spermatozoa in a liquid state in medium would have implications for the transport of spermatozoa to distant laboratories, and be advantageous for repeated insemination procedures using fresh spermatozoa. Therefore, establishment of optimal composition for sperm storage is of extreme relevance, as these cells are highly dependent on the supply of exogenous substrates and, due to their high metabolic rates, produce elevated amounts of ROS (**Sato and Ishikawa, 2004**). Free radicals and reactive oxygen species, which are generated by spermatozoa and leukocytes during storage, induce alterations in sperm hyperactivation, capacitation, and acrosome reaction (**Wittayarat *et al.*, 2013**). It has been demonstrated that polyphenols such as epicatechin prevents spontaneous mutations, LDL oxidation, and chromosomal damage induced by ROS in somatic cells, and in the context of male reproduction, catechins showed protective action against several deleterious effects by minimizing oxidative stress (**Roy *et al.*, 2003**). It was reported that catechins therapy protects against testicular ischemia-reperfusion injury through its antioxidant activity (**Sugiyama *et al.*, 2012**), and in human spermatozoa maintained at 37 °C, motility and viability can be improved by the addition of catechins to the extracellular media at low concentrations (**De Amicis *et al.*, 2012**).

The aim of this study was to evaluate the antioxidant effects of epicatechin on the bovine spermatozoa motility, viability, and ROS production *in vitro*. Our results agree with findings of **Purdy *et al.* (2004)** with cooled goat sperm, when detected motility between control and various concentrations (25-100 μM/L) of catechin was significantly improved in experimental groups after 96 h cultivation. Another study with boar semen also evaluated that the motility of spermatozoa supplemented with catechin (25 a 50 μM/L) was higher than that of the control group after 24 and 48 h. In addition, after incubating the sperm for 24 and 72 h, sperm viability assessed by eosin exclusion test showed that experimental groups supplemented with catechin were significantly higher than that of the control group. Also, malondialdehyde concentration was significantly lower in samples with catechin after 24, 48 and 72 h (**Boonsorn *et al.*, 2010**). **Moretti *et al.* (2012)** examined, that epicatechin have concentration-dependent effect on sperm viability and motility. Epicatechin at 400 μM/L concentration caused a decrease in sperm progressive motility concomitant with a significant increase in non-

progressive motility. A possible explanation for the reduced progressive motility is via estrogen receptors. Spermatozoa motility closely relates with mitochondrial activity, because spermatozoa contain many mitochondria helically arranged around the mid-piece axoneme, thus mitochondria play a key role in the energy production (the generation of ATP) and maintenance of spermatozoa motility (**Jansen and Burton, 2004**). Our experiment indicates that the concentrations lower than 100 µM/L EPI after 6 h of incubation, and all concentrations after 24 h, significantly increased spermatozoa mitochondrial activity. On the other hand, all doses of EPI were not effective in the case of mitochondrial activity after 2 h incubation. **Wittayarat *et al*. (2012)** investigated the effect of vitamin C (0.5 or 1 mM) along with 0.75 mg/mL green tea polyphenol to semen extender provided significantly higher percentages of canine sperm motility and viability (assessed by a live/dead stain combination) during cold storage at 5°C for 4 weeks compared to unsupplemented semen.

The specific mechanism through which polyphenol inhibits oxidative stress during semen preservation is not clear. Polyphenols may bind to components of the sperm membrane and prevent the lipid membrane oxidation induced by free radicals (**Hyon, 2004**). The epididymis and spermatozoa are highly rich in polyunsaturated fatty acids and thus susceptible to injury induced by ROS what is associated with lipid peroxidation. Subsequently, products of membrane lipids peroxidation such as malondialdehyde, are highly toxic to spermatozoa and causes an irreversible damage of spermatozoa motility and integrity (**Aitken, 1995**). **Chen *et al*. (2003)** demonstrated that tea catechins significantly increased the cell viability, decreased the intracellular Ca^{2+} level and ROS formation, and improved the mitochondrial membrane potential of PC12 cells (a neuron cell model). Also data obtained from study with catechins-rich grape extract showed, that the incubation of thawed bovine spermatozoa with 2 µg/mL and 5 µg/mL of the extract for 2 hours resulted in a significantly better maintenance of viable spermatozoa with intact acrosome and same concentrations kept the levels of malondialdehyde production in significantly lower level, compared to the other groups, after 2 hrs and 4 hrs of incubation (**Sapanidou *et al*., 2014**). These findings related to membrane lipid peroxidation could also support our results obtained from assessment of ROS production, when levels of ROS were significantly decreased after 6 and 24 h of incubation, especially in experimental groups where the concentrations of EPI varied between 1 and 50 µM/L.

CONCLUSION

Based on results obtained from this study it may be concluded that epicatechin, especially at lower doses (1-50 µM/L), is able to prevent the development of oxidative stress by overproduction of ROS in semen samples cocultivated with this natural substance. Antioxidant activity of epicatechin is in this experiment also supported by results obtained from evaluating of spermatozoa motility, which is closely related to the function maintenance and fertilizing ability of sperm cells. Epicatechin seems to protect bovine spermatozoa against the impairment caused by reactive oxygen species and thus increase the percentage of viable cells during short-, as well as during long-term incubation. Administration of natural antioxidants, in this case epicatechin, to semen extenders or medium could therefore be one of the most effective method of improving the sperm parameters and prolong the time of spermatozoa storage for later processing or using in various techniques of artificial insemination or *in vitro* fertilization.

Acknowledgments: This work was co-funded by the European Community under the Project no. 26220220180: Building Research Centre „AgroBioTech", the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and of the Slovak Academy of Sciences VEGA Project no. 1/0857/14 and by the Slovak Research and Development Agency Grant no. APVV-15-0544 and APVV-0304-12-D3.

REFERENCES

Aitken, R. J. (1995). Free radicals, lipid peroxidation and sperm function. *Reproduction, Fertility and Development, 7*(4), 659. https://doi.org/10.1071/rd9950659

Boonsorn, T., Kongbuntad, W., Narkkong, N., Aengwanich, W. (2010). Effects of catechin addition to extender on sperm quality and lipid peroxidation in Boar semen. *American-Eurasian Journal of Sustainable Agriculture 7*(3), 283-288.

Bucak, M. N., Ataman, M. B., Başpınar, N., Uysal, O., Taşpınar, M., Bilgili, A., & Akal, E. (2014). Lycopene and resveratrol improve post-thaw bull sperm parameters: sperm motility, mitochondrial activity and DNA integrity. *Andrologia, 47*(5), 545–552. https://doi.org/10.1111/and.12301

Bucak, M. N., Sariozkan, S., Tuncer, P. B., Sakin, F., Atessahin, A., Kulaksiz, 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 89*(1), 24-30. http://dx.doi.org/10.1016/j.smallrumres.2009.11.015

De Amicis, F., Santoro, M., Guido, C., Russo, A., & Aquila, S. (2012). Epigallocatechin gallate affects survival and metabolism of human sperm. *Molecular Nutrition & Food Research, 56*(11), 1655–1664. https://doi.org/10.1002/mnfr.201200190

Dias, T. R., Alves, M. G., Tomás, G. D., Socorro, S., Silva, B. M., & Oliveira, P. F. (2014). White Tea as a Promising Antioxidant Medium Additive for sperm storage at room temperature: A comparative study with green tea. *Journal of Agricultural and Food Chemistry, 62*(3), 608–617. https://doi.org/10.1021/jf4049462

Fatma, B. A., Nozha, Ch. F., Ines, D., Hamadi, A., Basma, H., & Leila, A. K. (2009). Sperm quality improvement after date seed oil in vitro supplementation in spontaneous and induced oxidative stress. *Asian Journal of Andrology, 11*, 393-398. https://doi.org/10.1038/aja.2008.6

Gadkari, P. V., & Balaraman, M. (2015). Catechins: sources, extraction and encapsulation: A review. *Food and Bioproducts Processing, 93*, 122–138. https://doi.org/10.1016/j.fbp.2013.12.004

Guthrie, H. D., & Welch, G. R. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology, 78*(8), 1700-1708. https://doi.org/10.1016/j.theriogenology.2012.05.002

Hyon, S. H. (2004). A non-frozen living tissue bank for allotransplantation using green tea polyphenols. *Yonsei Medical Journal, 45*(6), 1025. https://doi.org/10.3349/ymj.2004.45.6.1025

Chen, L., Yang, X., Jiao, H., & Zhao, B. (2003). Tea catechins protect against lead-induced ROS formation, mitochondrial dysfunction, and calcium dysregulation in PC12 cells. *Chemical Research in Toxicology, 16*(9), 1155–1161. https://doi.org/10.1021/tx0340605

Jansen, R. P. S., & Burton, G. J. (2004). Mitochondrial dysfunction in reproduction. *Mitochondrion, 4*(5-6), 577–600. https://doi.org/10.1016/j.mito.2004.07.038

Lukac, N., Bardos, L., Stawarz, R., Roychoudhury, S., Makarevich, A. V., Chrenek, P., & Massanyi, P. (2010). *In vitro* effect of nickel on bovine spermatozoa motility and annexin V-labeled membrane changes. *Journal of Applied Toxicology, 31*(2), 144-149. https://doi.org/10.1002/jat.1574

Moretti, E., Mazzi, L., Terzuoli, G., Bonechi, C., Iacoponi, F., Martini, S., Rossi, C., & Collodel, G. (2012). Effect of quercetin, rutin, naringerin and epicatechin on lipid peroxidation induced in human sperm. *Reproductive Toxicology, 34*(4), 651-657. http://dx.doi.org/10.1016/j.reprotox.2012.10.002

Moustafa, M. H., Sharma, R. K., Thornton, J., Mascha, E., Abdel-Hafez, M. A., Thomas, A. J., & Agarwal, A. (2004). Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Human Reproduction, 19*(1), 129-138. https://doi.org/10.1093/humrep/deh024

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1-2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Omur, A. D., & Coyan, K. (2016). Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. *Veterinární Medicína, 61*(1), 10-16. https://doi.org/10.17221/8677-vetmed

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, 55*(1-3), 239–243. https://doi.org/10.1016/j.smallrumres.2004.02.005

Rice-evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., & Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research, 22*(4), 375–383. https://doi.org/10.3109/10715769509145649

Roy, M., Chakrabarty, S., Sinha, D., Bhattacharya, R. K., & Siddiqi, M. (2003). Anticlastogenic, antigenotoxic and apoptotic activity of epigallocatechin gallate: a green tea polyphenol. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 523-524*, 33-41. https://doi.org/10.1016/s0027-5107(02)00319-6

Sapanidou, V. G., Margaritis, I., Siahos, N., Arsenopoulos, K., Dragatidou, E., Taitzoglou, I. A., & Tsantarliotou, M. P. (2014). Antioxidant effect of a polyphenol-rich grape pomace extract on motility, viability and lipid peroxidation of thawed bovine spermatozoa. *Journal of Biological Research-Thessaloniki, 21*(1), 19. https://doi.org/10.1186/2241-5793-21-19

Sato, M., & Ishikawa, A. (2004). Room temperature storage of mouse epididymal spermatozoa: exploration of factors affecting sperm survival. *Theriogenology*, *61*(7-8), 1455–1469. http://dx.doi.org/10.1016/j.theriogenology.2003.07.013

Sikka, S. C. (2001). Relative impact of oxidative stress on male reproductive function. *Current Medicinal Chemistry*, *8*(7), 851-862. https://doi.org/10.2174/0929867013373039

Sugiyama, A., Chiba, M., Nakagami, T., Kawano, S., Sanada, Y., Tajiri, T., & Toki, A. (2012). Beneficial effects of (−)-epigallocatechin gallate on ischemia-reperfusion testicular injury in rats. *Journal of Pediatric Surgery*, *47*(7), 1427-1432. https://doi.org/10.1016/j.jpedsurg.2012.01.069

Terao, J., Piskula, M., & Yao, Q. (1994). Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Archives of Biochemistry and Biophysics*, *308*(1), 278-284. https://doi.org/10.1006/abbi.1994.1039

Tremellen, K. (2008). Oxidative stress and male infertility- a clinical perspective. *Human Reproduction Update*, *14*(3), 243-258. https://doi.org/10.1007/978-1-61779-776-7_16

Tvrdá., E., Lukáč, N., Jambor, T., Lukáčová, J., Hashim, F., & Massányi, P. (2016). *In vitro* supplementation of lycopene to bovine spermatozoa: effects on motility, viability and superoxide production. *Animal Science Papers and Reports*, *34*(4), 319-328.

Tvrdá., E., Lukáč, N., Jambor, T., Lukáčová, J., & Massányi, P. (2015). Curcumin in male fertility: effects on spermatozoa vitality and oxidative balance. *The Journal of Microbiology, Biotechnology and Food Sciences*, *04*(2), 120-124. https://doi.org/10.15414/jmbfs.2015.4.special2.120-124

Tvrdá, E., Mackovich, A., Greifová, H., & Norbert Lukáč. (2016). Lycopene offers protection against oxidative damage in frozen-thawn bovine semen. *Scientific Papers: Animal Science and Biotechnologies*, *49*(1), 115-118.

Wittayarat, M., Ito, A., Kimura, T., Namula, Z., Luu, V. V., Do, L. T. K., & Otoi, T. (2013). Effects of green tea polyphenol on the quality of canine semen after long-term storage at 5°C. *Reproductive Biology*, *13*(3), 251–254. https://doi.org/10.1016/j.repbio.2013.07.006

Wittayarat, M., Kimura, T., Kodama, R., Namula, Z., Chatdarong, K., Techakumphu, M., Sato, Y., Taniguchi, M., & Otoi, T. (2012) Long-term preservation of chilled canine semen using vitamin C in combination with green tea polyphenol. *Cryo Letters*, *33*(4), 318-326.

Yilmaz, Y., & Toledo, R. T. (2004). Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. *Journal of Agricultural and Food Chemistry*, *52*(2), 255–260. https://doi.org/10.1021/jf030117h