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In Vitro Effects of Isoquercitrin on Selected Vitality Markers of Bovine Spermatozoa

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Abstract

The aim of this study was to evaluate the dose- and time-dependent *in vitro* effects of isoquercitrin (ISO), a natural flavonoid with numerous biological properties on bovine spermatozoa during three different time periods (0 h, 2 h, 24 h). Bovine semen samples were diluted and cultivated in physiological saline solution containing 0.5% DMSO together with 200, 100, 50, 10, 5 and 1 µmol/L ISO. Spermatozoa motility was measured using the HTM IVOS CASA (Computer Assisted Semen Analyzer) system. The viability of spermatozoa was assessed by the metabolic (MTT) assay, production of superoxide radicals was quantified using the nitroblue tetrazolium (NBT) test, and chemiluminescence was used to evaluate the generation of reactive oxygen species (ROS). The results of the movement activity showed a significant increase in the motility during long term cultivation in case of concentrations ranging between 5 and 50 µmol/L ISO ($P<0.05$; 24 h). At the same time, ISO supplementation led to a significant preservation of the cell viability ($P<0.05$ in case of 50 µmol/L, $P<0.01$ with respect to 1 and 5 µmol/L, and $P<0.001$ in relation to 10 µmol/L; 24 h). ISO administration at 10 and 50 µmol/L also provided a significantly higher protection against superoxide ($P<0.05$) and ROS ($P<0.001$) overgeneration after a 24 h cultivation. We may suggest that isoquercitrin, particularly at concentrations ranging between 10 and 50 µmol/L, may offer protection to the motility, viability and oxidative status of spermatozoa, particularly notable at 24 h.

1. Introduction

In recent years, oxidative damage that spermatozoa may suffer is receiving an ever-increasing interest. Male gametes seem to be especially sensitive to peroxidative damage due to a relatively high content of unsaturated fatty acids in the phospholipids of the cell membrane and a low antioxidant capacity of the seminal plasma **(Pena *et al.*, 2003)**. Within an aerobic or even a partially aerobic system, the production of reactive oxygen species (ROS) is unavoidable; the damaging ones being the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) **(Bilodeau*et al.*, 2002)**.

In mammalian spermatozoa, excess oxygen during *in vitro* storage results in lipid peroxidation, causing membrane damage and a reduced sperm motility, viability and subsequent subfertility. Extensive effort has been invested to develop liquid storage procedures to hold spermatozoa for 24 h or longer, however fertility is generally lower when insemination is performed with semen stored for more than 6 h **(Donoghue and Donoghue, 1997)**. A successful liquid storage of spermatozoa is dependent on the maintenance of sperm metabolic activity. This is achieved by providing effective environmental conditions for the sperm survival, leading to the evolution of extenders that maintain the membrane integrity, motility, and fertilizing ability of spermatozoa, as well as minimize ROS production **(Coyan *et al.*, 2010)**.

One way to overcome the detrimental effects of ROS on the subsequent sperm performance could be based on adding antioxidant compounds to the extender to block or prevent oxidative stress **(Asadpour *et al.*, 2012)**. Supplementation with natural or synthetic antioxidants could reduce the impact of oxidative damage during sperm storage, and thus improve the resulting quality of semen **(Marti *et al.*, 2003)**.

It is commonly believed that the antioxidant action of flavonoids may play a positive role in various oxidative stress-related diseases such as inflammation, atherosclerosis, and cancer **(Reuter *et al.*, 2010)**. Flavonoids, a class of secondary plant phenols, display antioxidant and chelating properties, and are mostly concentrated in fruits, vegetables, wines and teas **(Awoniyi et al., 2012)**. Isoquercitrin (quercetin-3-O-β-d-glucopyranoside; ISO) is, together with rutin (quercetin-3-O-rutinoside), one of the major glycosidic forms of the natural flavonol quercetin **(Valentová *et al.*, 2014)**. Different studies showed that quercetin and ISO act as powerful antioxidants. Both scavenge H_2O_2, ·OH and O_2^-, and have the ability to reduce the depletion of glutathione, as well as to prevent the loss of of catalase and glutathione peroxidase caused by exposure to ROS **(Jung *et al.*, 2010)**. In the literature, many studies have emphasized on the reduction of oxidative damage and an

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improvement of the overall functional parameters of spermatozoa provided by supplementing flavonoid antioxidants to semen extenders **(McNiven and Richardson, 2006; Taepongsorat *et al.*, 2008; Khaki *et al.*, 2010; Mazzi *et al.*, 2011; Moretti *et al.*, 2012)**.

The purpose of the current study was to investigate the *in vitro* biological and antioxidant activity of isoquercitrin in relation to the motility, viability as well as production of the superoxide radical and reactive oxygen species by bovine spermatozoa.

2. Material and methods

2.1 Semen samples and in vitro culture

Bovine semen samples were obtained from five 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 ISO (Sigma-Aldrich, St. Louis, USA) dissolved in 0.5% DMSO (dimethyl sulfoxide; Sigma-Aldrich) (A – 200; B – 100; C – 50; D – 10; E – 5; F – 1 μmol/L). The control (Ctrl) group was cultured in physiological saline solution containing 0.5% DMSO exclusively. All samples were incubated in the laboratory at room temperature (22–25°C). The control group (medium without ISO) was compared to the experimental groups (exposed to different concentrations of ISO). Specific analyses were carried out at cultivation times of 0 h, 2 h and 24 h.

2.2 Computer-assisted semen analysis

Spermatozoa motility was measured as an indicator of the semen quality. The motion analysis was carried out using the CASA (Computer Assisted Semen Analyzer) system – HTM IVOS (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne, Beverly, USA). 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 **(Tvrdá *et al.*, 2016a)**.

2.3 Viability evaluation

The viability of the cells exposed to ISO *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. The amount of formazan was measured spectrophotometerically. In brief, spermatozoa were stained with the MTT tetrazolium salt (Sigma-Aldrich) dissolved in PBS (Dulbecco's Phosphate Buffer Saline, Sigma-Aldrich) 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, Thermo Fisher Scientific, Finland). The data were expressed in percentage of control (i.e., optical density of formazan from cells not exposed to ISO). The results from the analysis were collected during three repeated experiments at each concentration.

2.4 Quantification of the superoxide production

The nitroblue-tetrazolium (NBT) test was used to quantify the intracellular formation of the superoxide radical, by assessing blue NBT formazan deposits, generated by the reduction of the membrane permeable, yellow-colored, nitroblue tetrazolium chloride (2,20-bis(4-Nitrophenyl)-5,50-diphenyl-3,30-(3,30-dimethoxy-4,40-diphenylene) ditetrazolium chloride) by the superoxide radical. The NBT salt (Sigma-Aldrich) was dissolved in PBS containing 1.5 % DMSO (Sigma-Aldrich) to a final concentration of 1 mg/mL and added to the cells (100 μL per well). After a 1 h incubation spermatozoa were washed twice with PBS and centrifuged at 300 × g for 10 min. Lastly, the cells and formazan crystals were dissolved in 2 mol/L KOH (potassium hydroxide; Centralchem) in DMSO. Optical density was determined at a wavelength of 620 nm against 570 nm as reference by the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.). The data were expressed in percentage of control. The results from the analysis were collected during three repeated experiments at each concentration **(Tvrdá *et al.*, 2016a)**.

2.5 Evaluation of ROS generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino- 2, 3- dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe **(Tvrdá *et al.*, 2016b)**. The tested specimens consisted of luminol (5 μl, 5 mmol/L) and control or experimental samples. Negative controls were prepared by replacing the sperm suspension with 200 μl of the medium. Positive control included 200 μl of the medium and 25 μl H_2O_2 (30%; 8.8 mol/L; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 96-well plate for 15 min using the Glomax Multi+ Combined Spectro-FluoroLuminometer (Promega, Madison, USA). The results were expressed as relative light units (RLU)/sec/10^6 sperm.

2.6 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 advanced statistical evaluations. The level of significance was set at *** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$).

3. Results and discussion

Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by oxidative stress **(Sikka *et al.*, 1995)**. The first indication of the importance of oxidative stress in the etiology of defective sperm function came from studies of correlation between the extent of lipid peroxidation in spermatozoa and a severe motility loss. Thus, exposure of spermatozoa to extracellularly generated ROS induces a loss of membrane integrity that is directly associated with loss of cell viability and subsequent lack of functionality, and fertilizing ability **(Aitken and Baker, 2006)**. Similarly, the loss of motility observed when spermatozoa are subjected to a long-term incubation is related to the ROS level and lipid peroxidation status of the spermatozoa at the end of the incubation period **(Gomez *et al.*, 2002)**. Suppression of lipid peroxidation through the addition of antioxidants, which block the production of ROS

or counteract oxygen toxicity, has been achieved in mammalian sperm with some success **(Donoghue and Donoghue, 1997)**. Currently, many *in vitro* studies have reported that natural antioxidants belonging to the class of flavonoids are able to improve sperm quality of different species **(Purdy et al., 2004; McNiven et al., 2006; Abdallah et al., 2011; Johinke et al., 2014)**. Available facts about the antioxidant properties of this group of substances are associated with enhanced sperm parameters, providing the informative basis for our experiments. This study was performed to investigate the antioxidant effect of different isoquercitrin concentrations on selected bovine spermatozoa parameters.

Our results obtained from the CASA assessment show that ISO administration to the medium increased the spermatozoa motility in comparison with the control group. Significant differences (P<0.05) were observed after 24 h of incubation between the groups containing lower doses of ISO (50, 10 and 5 µmol/L), and the control group. On the other hand, the initial (time 0 h) as well as short-term (time 2 h) motility was slightly lower in experimental groups with high concentrations of ISO (200 and 100 µmol/L) when compared to the control group (Table 1).

Our CASA results are in agreement with different previous *in vitro* studies dealing with antioxidant effects of natural compounds related to ISO on spermatozoa collected from various species. **Moretti *et al.* (2012)** revealed in his study with human sperm, that quercetin, as well as rutin at concentration of 30 µmol/L were able to significantly (P<0.001) improve the sperm motility in samples with induced lipid peroxidation by tert-butylhydroperoxide. Similarly, another experiment with human sperm revealed that supplementation of the cryopreservation medium with quercetin (50 µmol/L) induced a significant improvement in post thaw sperm parameters when compared to the control, particularly with respect to the sperm motility (P = 0.007) and viability (P = 0.008) **(Zribi *et al.*, 2012)**. **Nass-Arden and Breibart (1990)** demonstrated a dual effect of quercetin (0.1 mmol/L) on the ram sperm motility, with quercetin inhibiting motility in the first 2 h of incubation while stimulating the intensity and duration of motility during following 3-4 h.

The interest in sperm ROS formation was created as a possible explanation for poor sperm quality in freshly collected semen, or poor quality of sperm after its processing for reproductive technologies, such as AI, IVF, intracytoplasmic sperm injection, hypothermic nonfrozen storage, and cryopreservation in farm animals, laboratory species, and humans. The rate of intracellular damage would be expected to increase if sperm exposure to ROS increases through its improper storage **(Guthrie and Welch, 2012)**. Disruption of mitochondrial electron transport flow in spermatozoa may result in ROS overgeneration and promotion of oxidative damage associated with a rapid release of hydrogen peroxide into the extracellular space. The induction of ROS production on the matrix side of the inner mitochondrial membrane may lead to peroxidative damage to the midpiece and a loss of sperm movement **(Koppers *et al.*, 2008)**. Reactive oxygen species are conventionally considered as detrimental by-products of cellular metabolism or exposure to xenobiotics, which generate a state of oxidative stress in susceptible cells. Spermatozoa are particularly vulnerable to such stress because they are richly endowed with polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid **(Aitken, 2004)**.

In this study, we recorded that ISO supplementation (time 2 and 24 h) led to a significant enhancing effect on the spermatozoa viability. Specifically, a significantly increased (P<0.05) mitochondrial activity in the experimental groups exposed to 50, 10 and 5 µmol/L ISO were noted at cultivation time 2 h. Furthermore, after 24 h of cultivation, the MTT test revealed significant changes between samples treated with 50 µmol/L (P<0.05), 10 µmol/L (P<0.001) and 5 µmol/L, as well as 1 µmol/L ISO (P<0.01), when compared to the control group without ISO supplementation (Figure 1).

Our NBT data showed non-significant changes in the time 0 h. However, statistically significant results (P<0.05) were observed in the timeframes of 2 h and 24 h with respect to 50 and 10 µmol/L ISO. In both cases we observed a rapid decrease in the production of the superoxide radical (Figure 2). Analyzing our data, we may conclude that concentrations ranging between 50 and 10 µmol/L ISO have positive and protective effects on the sperm metabolism, as they can reduce the production of superoxide radicals, leading to a reduction of the risk of oxidative stress development.

Similar results showed our luminometric assessment after 24 h of *in vitro* cultivation, when ISO administration (50, 10 and 5 µmol/L) provided a significantly (P<0.001) higher protection against ROS overgeneration in comparison with the control group (Table 2).

Our observations related to the superoxide, ROS production and viability of spermatozoa can be supported by several studies. Supplementation of the sperm culture medium with quercetin showed reduced H_2O_2-mediated oxidative damage and plasma membrane disorders in boars **(Desroches *et al.*, 2005)**, stallions **(Gibb *et al.*, 2013)** and also humans **(Zribi *et al.*, 2012)**. **Moretti *et al.* (2016)** observed a higher amount of preserved viability, acrosome and plasma membrane in human spermatozoa with tert-butylhydroperoxide induced lipid peroxidation in the presence of 30 µmol/L and 100 µmol/L of quercetin.

Table 1. Spermatozoa motility (%) in the absence (Ctrl) or presence (A-F) of isoquercitrin during different time periods

Groups/ Time	0	200 µmol/L	100 µmol/L	50 µmol/L	10 µmol/L	5 µmol/L	1 µmol/L
0h	75.67±3.79	65.33±1.98	66.67±1.90	80.33±3.12	79.33±4.79	77.33±2.73	79.33±3.60
2h	61.67±1.54	50.67±6.38	59.67±5.66	64.33±1.54	73.33±2.01	69.33±2.92	62.33±4.51
24h	1.33±0.62	11.33±3.68	14.00±2.55	17.33±3.70*	19.33±1.12*	17.00±2.12*	15.33±1.73

Mean±SEM; * (P<0.05)

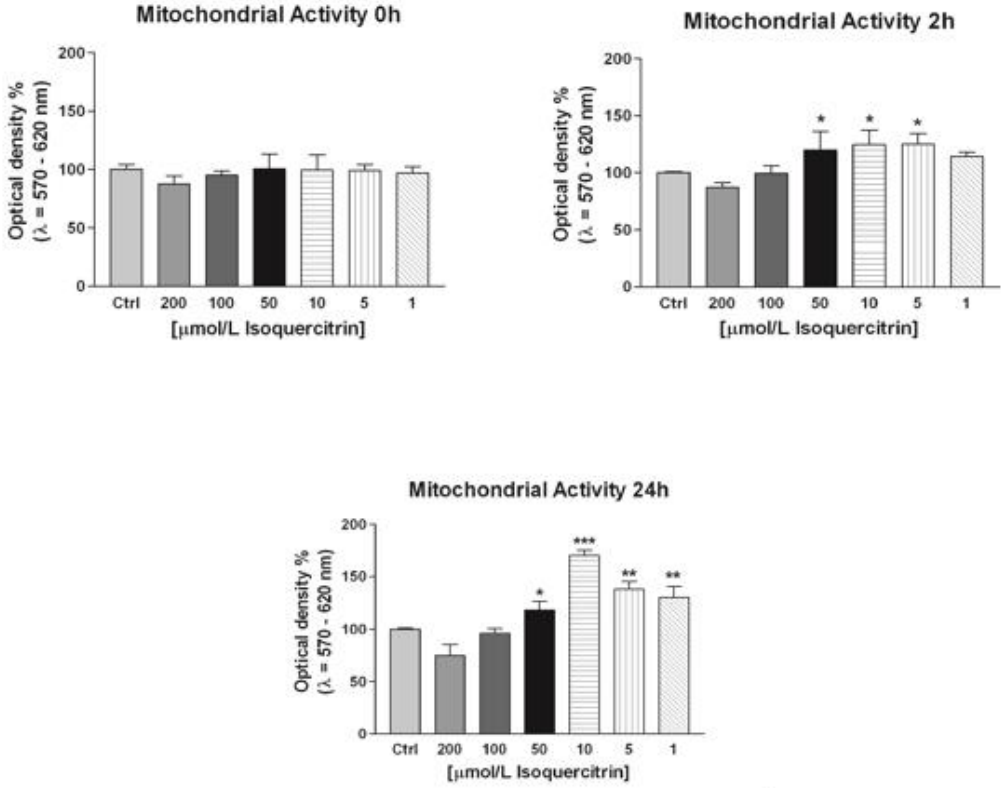

Figure 1. The effect of different ISO doses on the viability of bovine spermatozoa at 0 h, 2 h and 24 h. Each bar represents mean (±SEM) optical density as the percentage of the control, which symbolize 100%. The data were obtained from three independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; *P<0.05.

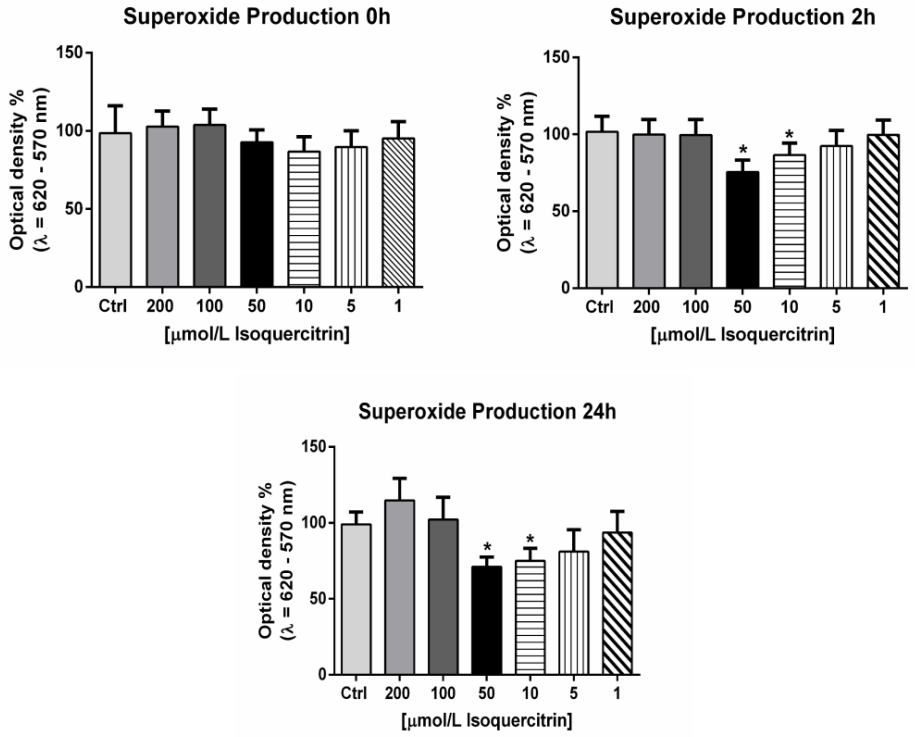

Figure 2. The effect of different doses of isoquercitrin on the superoxide production of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM) optical density as the percentage of control, which symbolize 100%. The data were obtained from three independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; *P<0.05.

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

Groups/ Time	0	200 µmol/L	100 µmol/L	50 µmol/L	10 µmol/L	5 µmol/L	1 µmol/L
0h	2.02±0.23	1.60±0.16	1.47±0.20	1.42±0.10	1.20±0.11	1.29±0.12	1.53±0.25
2h	3.60±0.26	4.36±0.20	3.86±0.15	3.09±0.21	2.66±0.19	3.05±0.24	3.56±0.22
24h	15.46±0.70	16.17±0.87	15.55±0.99	11.49±0.69***	10.92±0.75***	11.44±0.90***	15.27±0.82

Mean±SEM; *** (P<0.001)

4. Conclusion

Developing a defense system against oxidative damage is of practical importance to improve the extended liquid storage of semen for its use in various techniques of artificial insemination or *in vitro* fertilization. The present study demonstrated an improved viability, motility and also a decline of ROS production after short- and long-term storage of bovine spermatozoa following administration of isoquercitrin, particularly in concentrations ranging between 5 and 50 µmol/L, with more significant differences after long-term cultivation (24 h). Our results indicate that isoquercitrin may be able to enhance the vitality of spermatozoa stored for further processing. Ultimately, the fertilizing ability of spermatozoa is most important quality parameter to consider, and this experiment provides a prospective basis for further investigations of isoquercitrin and its effect on spermatozoa.

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Declaration of interest

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

References

1. Abdallah, F.,Zribi, N., Ammar-Keskes, L., 2011. Antioxidative potential of Quercetin against hydrogen peroxide induced oxidative stress in spermatozoa in vitro. Andrologia, 43(4), 261–265. https://doi.org/10.1111/j.1439-0272.2010.01063.x
2. Aitken, R. J., 2004. Human spermatozoa: fruits of creation, seed of doubt. Reprod. Fert. Develop. 16(9), 2. https://doi.org/10.1071/srb04abs002
3. Aitken, R. J., Baker, M. A., 2006. Oxidative stress, sperm survival and fertility control. Mol. Cell. Endocrinol. 250(1-2), 66–69. https://doi.org/10.1016/j.mce.2005.12.026
4. Asadpour, R., Jafari, R., Tayefi-Nasrabadi, H., 2012. The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa. Iran. J. Vet. Res. 13(3), 246-249.
5. Awoniyi, D. O., Aboua, Y. G., Marnewick, J., Brooks, N., 2012. The effects of Rooibos (Aspalathus linearis), green tea (Camellia sinensis) and commercial Rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. Phytother. Res. 26(8), 1231–1239. https://doi.org/10.1002/ptr.3717
6. Bilodeau, J.-F., Blanchette, S., Cormier, N., &Sirard, M.-A., 2002. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology, 57(3), 1105–1122. https://doi.org/10.1016/s0093-691x(01)00702-6
7. Çoyan, K., Başpınar, N., Bucak, M. N., Akalın, P. P., Ataman, M. B., Ömür, A. D., Sarıözkan, S., 2010. Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. Res. Vet. Sci. 89(3), 426–431. https://doi.org/10.1016/j.rvsc.2010.03.025
8. Desroches, N. R., McNiven, M. A., Foote, K. D., Richardson, G. F., 2005. The effect of blueberry extracts and quercetin on capacitation status of stored boar sperm. Cell Preserv. Technol. 3(3), 165–168. https://doi.org/10.1089/cpt.2005.3.165
9. Donoghue, A. M., &Donoghue, D. J., 1997. Effects of water- and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. Poult. Sci. 76(10), 1440–1445. https://doi.org/10.1093/ps/76.10.1440
10. Gibb, Z., Butler, T. J., Morris, L. H. A., Maxwell, W. M. C., Grupen, C. G., 2013. Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and non sorted stallion sperm. Theriogenology, 79(6), 1001–1009. https://doi.org/10.1016/j.theriogenology.2012.06.032
11. Gomez, E., Irvine, D.S., Aitken, R.J., 2002. Evaluation of a spectrophotometric assai for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. Int. J. Androl. 21(2), 81–94. https://doi.org/10.1046/j.1365-2605.1998.00106.x
12. 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
13. Jung, S. H., Kim, B. J., Lee, E. H., Osborne, N. N., 2010. Isoquercitrin is the most effective antioxidant in the plant Thuja orientalis and able to counteract oxidative-induced damage to a transformed cell line (RGC-5 cells). Neurochem. Int. 57(7), 713–721. https://doi.org/10.1016/j.neuint.2010.08.005
14. Khaki, A., Fathiazad, F., Nouri, M., Khaki, A., Maleki, N. A., Khamnei, H. J., & Ahmadi, P., 2010. Beneficial effects of quercetin on sperm parameters in streptozotocin-induced diabetic male rats. Phytother. Res. 24(9), 1285–1291. https://doi.org/10.1002/ptr.3100
15. Koppers, A. J., De Iuliis, G. N., Finnie, J. M., McLaughlin, E. A., &Aitken, R. J., 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J. Clin. Endocrinol. Metab. 93(8), 3199–3207. https://doi.org/10.1210/jc.2007-2616
16. Martí, J., Martí, E., Cebrián-Pérez, J., Muiño-Blanco, T., 2003. Survival rate and antioxidant enzyme aktivity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. Theriogenology, 60(6), 1025–1037. https://doi.org/10.1016/s0093-691x(03)00105-5
17. Mazzi, L., Geminiani, M., Collodel, G., Iacoponi, F., Martini, S., Bonechi, C., &Moretti, E., 2012. Quercetin and rutin: effects of two flavonoids on induced oxidative stress in human ejaculated sperm. Journal of the Siena Academy of Sciences, 3(1), 22-26. https://doi.org/10.4081/jsas.2011.22
18. McNiven, M. A., Richardson, G. F., 2006. Effect of quercetin on capacitation status and lipid peroxidation of stallion spermatozoa. Cell Preserv. Technol. 4(3), 169–177. https://doi.org/10.1089/cpt.2006.4.169
19. Moretti, E., Mazzi, L., Bonechi, C., Salvatici, M. C., Iacoponi, F., Rossi, C., &Collodel, G., 2016. Effect of Quercetin-loaded liposomes on induced oxidative stress in human spermatozoa. Reprod. Toxicol. 60, 140–147. https://doi.org/10.1016/j.reprotox.2016.02.012
20. Moretti, E., Mazzi, L., Terzuoli, G., Bonechi, C., Iacoponi, F., Martini, S., Collodel, G., 2012. Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. Reprod. Toxicol. 34(4), 651–657. https://doi.org/10.1016/j.reprotox.2012.10.002
21. Mosmann, T., 1983. Rapid colorimetric assai for cellular growth and survival: Application to proliferation and cytotoxicity assays. J.

Immunol. Methods, 65(1-2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4
22. Nass-Arden, L., Breitbart, H., 1990. Modulation of mammalian sperm motility by quercetin. Mol. Reprod. Dev. 25(4), 369–373. https://doi.org/10.1002/mrd.1080250410
23. Peña, F. J., Johannisson, A., Wallgren, M., Rodriguez-Martinez, H., 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membráně potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci. 78(1-2), 85–98. https://doi.org/10.1016/s0378-4320(03)00049-6
24. 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 Rumin. Res. 55(1-3), 239–243. https://doi.org/10.1016/j.smallrumres.2004.02.005
25. Reuter, S., Gupta, S. C., Chaturvedi, M. M., Aggarwal, B. B., 2010. Oxidative stress, inflammation, and cancer: How are Theky linked? Free Radic. Biol. Med. 49(11), 1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006
26. Sikka, S. C., Rajasekaran, M., Hellstrom, W. J., 1995. Role of oxidative stress and antioxidants in male infertility. J. Androl. 16(6), 464-468.
27. Taepongsorat, L., Tangpraprutgul, P., Kitana, N., Malaivijitnond, S., 2008. Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats. Asian J. Androl. 10(2), 249–258. https://doi.org/10.1111/j.1745-7262.2008.00306.x
28. Tvrdá, E., Ďuračka, M., Halenár, M., Lukáč, N., Kolesárová, A., 2016 a. In Vitro effects of selected trichothecenes on the rabbit spermatozoa motility behavior – A comparative study. Contemporary Agriculture, 65(3-4). https://doi.org/10.1515/contagri-2016-0013
29. Tvrdá., E., Lukáč, N., Jambor, T., Lukáčová, J., Hashim, F., Massányi, P., 2016 b. In vitro supplementation of lycopene to bovine spermatozoa: effects on motility, viability and superoxide production. Anim. Sci. Pap. Rep. 34(4), 319-328. https://doi.org/10.15414/jmbfs.2015.4.4.336-341
30. Valentová, K., Vrba, J., Bancířová, M., Ulrichová, J., & Křen, V., 2014. Isoquercitrin: Pharmacology, toxicology, and metabolism. Food Chem. Toxicol. 68, 267–282. https://doi.org/10.1016/j.fct.2014.03.018
31. Quideau, S., Deffieux, D., Douat-Casassus, C., &Pouységu, L., 2011. Plant polyphenols: chemici properties, biological activities, and synthesis. Angew. Chem. Internat. Ed. 50(3), 586–621. https://doi.org/10.1002/anie.201000044
32. Zribi, N., Chakroun, N. F., Ben Abdallah, F., Elleuch, H., Sellami, A., Gargouri, J., Keskes, L. A., 2012. Effect of freezing–thawing process and quercetin on human sperm survival and DNA integrity. Cryobiology, 65(3), 326–331. https://doi.org/10.1016/j.cryobiol.2012.09.003