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IN VITRO EFECTS OF SELECTED BIOLOGICALLY ACTIVE COMPOUNDS ON RABBIT SPERMATOZOA MOTILITY BEHAVIOUR

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ABSTRACT

The aim of this study was to evaluate the *in vitro* effects of selected pure naturally occuring biologically active compounds (resveratrol-RES, quercetin-QUE, curcumin-CUR, epicatechin-EPI, isoquercitrin-ISO) on rabbit sperm motility using the IDENT staining method and the computer-aided sperm (CASA) analysis. Semen samples were collected regularly from 10 male rabbits, pooled, the seminal plasma was removed and the sperm fraction was exposed to different concentrations (1, 5, 10, 50 and 100 µmol/L) of chosen biomolecules. At culture times of 0h, 2h, 4h, 6h and 8h, the spermatozoa motility was assessed using the TOX IVOS II. CASA system and the IDENT fluorescent staining method. The motility assessment revealed different behavior patterns, specific and unique to each of the studied biomolecules. After 8h of *in vitro* culture, the highest sperm motility was detected in experimental groups subjected to 10 µmol/L RES ($P<0.05$); 1-10 µmol/L QUE ($P<0.01$ with respect to 1 µmol/L QUE; $P<0.001$ in case of 5 and 10 µmol/L QUE); 1 µmol/L CUR ($P<0.01$); 1-100 µmol/L EPI ($P<0.01$ in relation to 50, 10 and 5 µmol/L EPI; $P<0.001$ with respect to 100 and 1 µmol/L EPI) and 10 µmol/L ISO ($P<0.05$) when compared to the untreated Control. Our data provide evidence on specific toxic and beneficial concentrations as well as exposure periods applicable for each biomolecule, which may be useful for future *in vitro* farmagological or toxicological studies on male reproduction in rabbits.

Keywords: Spermatozoa, rabbit, resveratrol, quercetin, curcumin, epicatechin, isoquercitrin

INTRODUCTION

Current evidence suggests that oxidative stress (OS) represents an important factor in male reproductive dysfunction. An increased concentration of abnormal spermatozoa generating free radicals (FRs) and a reduced antioxidant capacity of semen, which has been reported in animals and humans, are two dominant factors underlying seminal OS (**Aitken *et al.*, 1991; Tvrdá *et al.*, 2011**). At the same time, a specific cellular structure leaves spermatozoa to be uniquely vulnerable to oxidative insults. Sperm plasmatic membranes contain large quantities of polyunsaturated fatty acids, while their cytoplasms lack significant amounts of FR scavengers (**Buettner, 1993**), leading to an icreased risk of oxidative damage, and subsequently a decreased semen quality (**de Lamirande and Gagnon, 1993**). Furthermore, FR overproduction may result in increased morphological defects, alterations to sperm capacitation and acrosome reaction, all of which are related to a compromised fertility (**Agarwal *et al.*, 2014**).

Recently, a number of reports have emphasized on the positive effects of oral antioxidant administration on male fertility in animals and humans (**Donnely *et al.*, 1999**; **Agarwal and Sekhon, 2010**). On the other hand, studies focused on the *in vitro* effects of antioxidants on spermatozoa are still very sparse, controversial or contradictory. The in vitro data are nevertheless important, as it has been shown on numerous occasions that external antioxidants may protect spermatozoa against oxidative injury and a subsequent dysfunction. Such knowledge is essential for spermatozoa processing protocols performed in medical or veterinary andrology for spermatozoa cryopreservation or assisted reproductive technologies (**Saleh and Agarwal, 2002**).

Administration of synthetic antioxidants to cell cultures is an efficient strategy to prevent OS. However, the safety of synthetic additives has been under a constant debate, raising the potential of naturally occurring compounds with antioxidant properties because of their chemical diversity, structural complexity, availability, lack of significant toxic effects and intrinsic biologic activity (**Alarcón de la Rastra, 2008**).

In this study, we followed a systematic approach to assess the *in vitro* effects of a wide range of pure flavonoid (quercetin, epicatechin, isoquercitrin) and polyphenolic (resveratrol, curcumin) compounds on rabbit sperm motility, one of the physiological manifestations of their fertilizing ability. Furthermore, we have validated the fluorescent-based rabbit sperm motility assessment using the IDENT staining method and the computer-aided sperm (CASA) analysis.

MATERIAL AND METHODS

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 on a regular collection schedule (twice a week for two consecutive weeks) using an artificial vagina. Immediately upon collection, the sperm concentration and motility were assessed in each ejaculate. Only samples with a minimum motility of 60% were used in the experiments. Individual ejaculates were mixed together in order to acquire a pooled sample of rabbit semen.

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, 5% glucose (Centralchem, Bratislava, Slovak Republic) and 4% BSA (bovine serum albumin, Sigma-Aldrich). Each experimental group was exposed to a different concentration (1, 5, 10, 50 and 100 µmol/L) of a chosen biomolecule (resveratrol, quercetin, curcumin, epicatechin, isoquercitrin; Sigma-Aldrich) using a dilution ratio of 1:20. The Control group carried no supplementation. The samples were cultured at 37 °C.

At culture times of 0h, 2h, 4h, 6h and 8h, the spermatozoa motility (percentage of motile spermatozoa; motility > 5 μm/s; %) was assessed using the computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). In order to standardize a fluorescent assessment of sperm activity, 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 a 10 min incubation in the dark, the sample was analyzed under fluorescent illumination. The system was set up as follows: frame rate - 30 at 60 Hz, dark field; minimum contrast - 50; static head size - 0.28-4.30; static head intensity - 0.12-2.92; static elongation - 8-97; minimum cell size - 7 pixels; default cell intensity - 70, magnification - 1.75, illumination intensity - 2198. Ten μL of each sample were placed into the Makler counting chamber (depth 10 μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. 10 microscopic fields were subjected to each analysis in order to include at least 300 cells.

All the data were subjected to statistical analysis using the GraphPad Prism program (a 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.

RESULTS AND DISCUSSION

Resveratrol (RES)

The CASA assessment revealed a continuous decrease of spermatozoa motility and in all groups over the course of a 8h *in vitro* culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups supplemented with 5 and 50 μmol/L RES when compared to the Control group, although without any statistical significance (P>0.05). Despite being statistically insignificant, a motion-promoting effect of RES remained notable after 2h, specifically following the addition of 1, 5 and 50 μmol/L RES. A significantly higher (P<0.05) MOT was detected in case of 1 and 5 μmol/L RES following a 4h culture. After 6h, the decline of spermatozoa MOT was significantly slowed down following the supplementation of a concentration range of 1-10 μmol/L RES (P<0.001). At the end of the experiment (8h), elevated motility was observed in the experimental group supplemented with 5 μmol/L RES, being significantly higher in comparison with the Control (P<0.05) (Table 1).

Table 1 Time- and dose-dependent effects of resveratrol (RES) on rabbit spermatozoa motility [%]

	Ctrl	100 μmol/L RES	50 μmol/L RES	10 μmol/L RES	5 μmol/L RES	1 μmol/L RES
0h	80.55±2.33	68.97±6.59	81.90±8.21	73.98±5.55	82.98±8.32	73.95±6.87
2h	69.03±4.02	61.09±7.00	72.99±6.98	69.09±4.76	78.99±7.89	73.56±8.00
4h	59.12±3.33	53.98±4.44	58.90±6.09	68.89±3.44	73.98±6.02*	69.98±5.99*
6h	37.55±2.99	43.98±3.99	46.97±3.98	56.09±5.55**	63.79±5.96***	60.98±5.99***
8h	20.04±1.88	11.98±2.09	15.04±0.67	14.90±1.23	29.99±2.09*	23.19±2.00

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

RES has recently emerged as a complex biomolecule due to its wide range of cardiovascular, anticancer, antiinflamatory and protective effects **(Calabrese *et al.*, 2010)**. Although no distinct toxicity was reported *in vivo* **(Cottart *et al.*, 2010)**, *in vitro* experiments show that low RES doses improve cell survival, while high doses increase cell death **(Brown *et al.*, 2009)**.

Our CASA results are contradictory to **Collodel *et al.* (2010)** who evaluated the effects of RES on human spermatozoa. Unlike our results, 100 μmol/L RES exerted cytotoxic activities against spermatozoa in a dose dependent manner. The LD50 was defined as 50 μmol/L RES, spermatocytes were more sensitive to the harmful effect of RES with a LD50 between 30 and 50 μmol/L. While at 100 μmol/L RES the rabbit sperm motility was decreased in a non-significant manner, human sperm motility in the above mentioned study was absent. Moreover, **Tvrdá *et al.* (2015b)** reported a significant decrease of bull sperm motility following exposure to 100 or 200 μmol/L RES during a 24 *in vitro* culture. The differences in our results may be explained by the sample type which was collected from rabbits as opposed to humans or bulls respectively, as well as a shorter exposure to the biocompound. On the other hand, the stimulating effects of lower (1-5 μmol/L) RES concentrations detected by our IDENT CASA technique are in agreement with **Tvrdá *et al.* (2015a;b)** who emphasize on the beneficial and protective effects of a concentration range of 5-50 μmol/L RES on the motion behavior, mitochondrial activity and intracellular superoxide production by bovine sperm cells. Furthermore, in an *in vitro* human study, progressive motility reached high values between 6 and 15 μmol/L RES **(Collodel *et al.*, 2010)**.

Our CASA records are in agreement with **Mojica-Villegas *et al.* (2014)** who reported that a pretreatment with 15 μmol/L RES 15 min prior to incubation with ferrous ascorbate (FeAA) showed an 8.0-fold increase in murine spermatozoa motility. On the other hand, spermatozoa motility diminished in spermatozoa cryopreserved with RES comparably to the control in the studies by **Pasqualotto *et al.* (2006)** and **Garcez *et al.* (2010)** Furthermore **Silva *et al.* (2012)** reported that the addition of RES before cryopreservation did not significantly affect progressive motility, vigor, acrosome integrity, or plasma membrane integrity. In the meantime, **Sarlos *et al.* (2002)** recorded a higher motility and acrosome integrity of ram spermatozoa supplemented with RES after their storage at 5 C for 6 days. Similar results were reported in studies focused on swim-up selected human spermatozoa where low RES doses led to a higher progressive motility **(Collodel *et al.*, 2010).**

Quercetin (QUE)

The CASA examination revealed an immediate (Time 0h) but insignificant (P>0.05) motion-promoting effect of QUE, specifically in experimental groups exposed to 1 and 10 μmol/L of this flavonoid (Table 2). Beneficial effects of QUE remained visible although non-significant (P>0.05) at Time 2h, and covering all the concentrations applied to the experimental groups. After 4h, spermatozoa motion was significantly higher in the experimental groups supplemented with 1, 5 (P<0.05) and 100 (P<0.01) μmol/L QUE. Nevertheless, motility evalution at 6h revealed a rapid decline of rabbit sperm motion exposed to the highest QUE concentration, resulting in a significantly lower MOT in comparison with the Control (P<0.01). At the same time, sperm motility promoting properties of low QUE concentrations were confirmed, leading to a higher MOT (P<0.001 in relation to 1 μmol/L QUE; P<0.05 with respect to 5 μmol/L QUE; Table 2) when compared to the Control. At the end of the experiment (8h), a significantly higher spermatozoa MOT was observed in experimental groups supplemented with a range of 1-10 μmol/L QUE (P<0.01 with respect to 1 μmol/L QUE; P<0.001 in case of 5 and 10 μmol/L QUE). Meanwhile, the MOT assessment revealed a decreased bias in the experimental group supplemented with the highest concentration of QUE (100 μmol/L), after a comparison with the Ctrl group (P<0.001).

Table 2 Time- and dose-dependent effects of quercetin (QUE) on rabbit spermatozoa motility [%]

	Ctrl	100 μmol/L QUE	50 μmol/L QUE	10 μmol/L QUE	5 μmol/L QUE	1 μmol/L QUE
0h	80.55±2.33	76.16±6.09	79.98±5.55	80.80±9.01	77.55±3.23	81.45±2.90
2h	69.03±4.02	74.55±4.55	77.34±3.99	77.77±6.09	74.23±2.90	77.64±3.39
4h	59.12±3.33	73.22±3.99**	68.09±5.22	67.67±5.02	73.89±3.22*	72.76±4.44*
6h	37.55±2.99	16.90±1.20**	20.90±3.33	29.12±2.31	47.66±1.99*	60.77±4.44***
8h	20.04±1.88	9.09±0.35***	18.07±1.77	41.21±2.33***	42.24±2.12***	39.03±2.22**

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Quercetin is a common dietary flavonoid, reported to exhibit a broad variety of favorable biological effects (**Aherne and O'Brien, 2000**), hence it is not a surprise to find reports focused on assessing its impact on male reproductive performance.

Improved motility recorded after QUE administration to the rabbit sperm medium in our study disagrees with earlier reports, according to which QUE compromised human sperm motility (at 5–200 μmol/L QUE) and viability (50-100 μmol/L QUE), coinciding with Ca^{2+}-ATPase downregulation, and leading to the loss of sperm motion (**Khanduja *et al.*, 2001**). In addition, this biomolecule exhibited a significant inhibitory impact on the hyaluronidase activity and sperm penetration ability of non-capacitated, capacitated and acrosome-reacted *Cynomolgus* monkey sperm in a dose-dependent manner (**Li *et al.*, 1997**). Talking in favor of

our results, **Tvrdá *et al.* (2014; 2016b)** did suggest protective effects of QUE on bovine sperm motion activity when incubated over a period of 24 h (**Tvrdá *et al.*, 2014**) or without the presence of seminal plasma (**Tvrdá *et al.*, 2016**), although we must acknowledge that in case of a broader concentration range, QUE may act dose dependently as either a stimulant at low concentrations or as an inhibitor at high doses (**Taepongsorat *et al.*, 2008**). This controversy was validated *in vivo* when **Aravindakshan *et al.* (1985)** revealed that treatment with higher QUE doses (300 mg/kg body weight) reduced the fertility rate of male rats, while **Taepongsorat *et al.* (2008)** showed that intramuscular administration of 90 and 270 mg QUE/kg body weight/day led to significant improvements in the spermatozoa concentration, motility and viability.

Mazzi *et al.* (2011) and **Moretti *et al.* (2012)** compared the potential of quercetin and resveratrol on human sperm incubated with tert-butylhydroperoxide (TBHP). On the one hand, QUE showed a higher toxicity with respect to the sperm motility and viability than RES, and on the other hand, its antioxidant activity was stronger, as evidenced by fluorescent and electron microscopy. QUE was particularly active in preserving sperm membranes, chromatin texture and acrosomes, which were compromised by TBHP. Similarly, **Tvrdá *et al.* (2016b)** found that QUE was capable of preventing the decline of spermatozoa vitality and functional activity as a consequence of FeAA-associated oxidative damage. Quercetin concentrations ranging between 50 and 100 µmol/l were particularly effective in protecting the spermatozoon against the damage caused by free radical overgeneration through prevention of lipid peroxidation, protection and maintenance of spermatozoa metabolism as reflected by the motility and mitochondrial activity.

Curcumin (CUR)

In case of CUR, the MOT evaluation revealed that the initial (Time 0h) MOT was higher in experimental groups supplemented with 5 and 100 µmol/L CUR when compared to the Control group (0 µmol/L CUR), although without any statistical significance (P>0.05). Although statistically insignificant, a motion-promoting effect of CUR was visible after 2h, specifically in experimental groups exposed to 5 and 10 µmol/L CUR. At the same time, 50 and 100 µmol/L CUR caused a significant decrease of the spermatozoa motility (P<0.001). After 4h, the decline of spermatozoa motion was significantly decreased following the administration of 50 and 100 µmol/L CUR in comparison with the Control. Furthermore, a significantly increased sperm MOT was observed in experimental groups supplemented with 1, 5 (P<0.01) and 10 µmol/L CUR (P<0.05). Examination at 6h of *in vitro* culture showed that the spermatozoa motility was significantly increased following exposure to 1 and 5 µmol/L CUR (P<0.001) when compared to the Control. At the same time, an insignificantly higher (P>0.05) motion was recorded in the group supplemented with 10 µmol/L CUR, while a significant (P>0.001) decline was observed in the case of 50 and 100 µmol/L CUR. At the end of the experiments (8h), the highest motility was observed in the experimental group supplemented with 1 µmol/L CUR, being significantly higher in comparison with the Control (P<0.01), while MOT was significantly decreased (P<0.001) in the groups supplemented by the highest concentrations of CUR, after a comparison with the Ctrl group (P<0.001; Table 3).

Table 3 Time- and dose-dependent effects of curcumin (CUR) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L CUR	50 µmol/L CUR	10 µmol/L CUR	5 µmol/L CUR	1 µmol/L CUR
0h	80.55±2.33	82.31±7.56	77.88±6.78	79.98±6.77	85.98±5.98	75.33±5.32
2h	69.03±4.02	0.00±0.00***	44.45±3.33***	76.77±7.07	78.86±4.44	75.65±3.33
4h	59.12±3.33	0.00±0.00***	26.24±2.11***	70.09±5.55*	74.98±6.08**	75.77±4.67**
6h	37.55±2.99	0.00±0.00***	1.09±0.09***	42.43±3.99	74.57±5.45***	57.87±5.78***
8h	20.04±1.88	0.00±0.00***	0.00±0.00***	17.56±1.90	17.90±2.02	37.09±1.11**

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Previous reports on the impact of CUR on male fertility are debatable. **Naz (2011)** revealed that exposure of human and murine sperm to CUR caused a concentration-dependent decrease of sperm motility, capacitation and acrosome reaction. At high CUR concentrations, a complete inhibiton of spermatozoa motility and function was observed. Studies on the impact of CUR on the spermatozoa activity have indicated its role in the energy metabolism of male reproductive cells. **Reddy and Aggarwal (1994)** suggest that CUR has the ability to inhibit protein kinase C (PKC), which is believed to have a role in modulating spermatozoa flagellar movement **(Rotem *et al.*, 1990a,b)**. As such, the CUR-induced PKC inhibition may be responsible for its possible spermatozoa-immobilizing activities **(Rithaporn *et al.*, 2003)**. On the other hand, our results agree with **Salashoor *et al.* (2012)** and **Tvrdá *et al.* (2016b)** suggesting stimulating and protective effects of CUR on spermatozoa motility and antioxidant status. Moreover **Salashoor *et al.* (2012)** reported that increasing doses of CUR significantly increased the sperm concentration, motility and testosterone content in rats.

The first report on the useful effects of CUR **(Bucak *et al.*, 2008)** on the motility, morphology and antioxidant activities of cooled ram spermatozoa has given rise to its use as an antioxidant additive to the freezing extender. Motility parameters recorded by our IDENT CASA technique complement previous findings by **Bucak *et al.* (2008; 2010)** demonstrating a significant improvement in the motion of cryopreservad ram spermatozoa supplemented with CUR. Interestingly, their later study focused on CUR administration to a cryopreservation extender for bovine semen led to non-significant differences in the sperm motion characteristics **(Bucak *et al.*, 2012)**. Nevertheless, our results correlate with the report by **Soleimanzadeh and Saberivand (2013)** as well as **Tvrda *et al.* (2016c)** on frozen-thawed semen, where CUR addition had a positive impact on both motility and viability.

Epicatechin (EPI)

In the case of EPI, the initial MOT assessment (Time 0h) revealed a non-significantly increased sperm motion in all experimental groups supplemented with EPI (P>0.05) in comparison to the Control group (Table 4). After 2h, a significant motion-promoting effect was noted in case of 1 µmol/L and 50 (P<0.01) µmol/L EPI (P<0.001). This beneficial effect remained visible after 4h and furthermore included a broader concentration range when compared to the Control (P<0.05 with respect to 50 µmol/L EPI; P<0.01 in terms of 100 and 5 µmol/L EPI; P<0.001 in case of 1 µmol/L EPI). After 6h, the decline of MOT associated with the *in vitro* culture was slowed down significantly in all experimental groups exposed to EPI (P<0.05 with respect to 5 µmol/L EPI; P<0.001 in case of 100, 50, 10 and 1 µmol/L EPI), and this MOT-stimulating and protective effect extended throughout the end of the *in vitro* experiment (Time 8h; P<0.01 in relation to 50, 10 and 5 µmol/L EPI; P<0.001 with respect to 100 and 1 µmol/L EPI).

Table 4 Time- and dose-dependent effects of epicatechin (EPI) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L EPI	50 µmol/L EPI	10 µmol/L EPI	5 µmol/L EPI	1 µmol/L EPI
0h	80.55±2.33	83.89±8.01	80.77±7.65	81.98±7.43	80.98±7.89	83.24±67
2h	69.03±4.02	75.67±7.07	80.08±6.78**	76.77±5.67	77.09±5.09	82.76±7.77***
4h	59.12±3.33	74.67±4.78**	73.56±8.00*	68.65±3.21	76.67±6.56**	80.98±8.00***
6h	37.55±2.99	72.22±6.65***	60.97±5.56***	58.46±4.44***	49.79±3.67*	73.98±7.44***
8h	20.04±1.88	42.77±3.33***	36.09±6.07**	38.98±3.87**	38.33±2.99**	40.48±7.09***

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Epicatechin is a flavonoid and antioxidant commonly found in green tea and, most of all, in cocoa **(Katz *et al.*, 2011)**. Since it is known that spermatozoa are sensitive to OS, several studies have examined potential roles of *in vitro* EPI supplementation in protecting sperm from ROS overproduction. **Jamalan *et al.* (2016)** evaluated the effects of different flavonoids including EPI on the recovery of sperm motility and prevention of membrane damage from aluminum chloride, cadmium chloride, and lead chloride. The study revealed that catechin behaved in an unexpected manner as it did not protect spermatozoa from heavy metal-mediated damage, nor it did not exhibit any protective effects, rather, it showed inhibitory effects on the sperm MOT associated with a co-incubation with selected heavy metals. Following the application of increased catechin concentrations from 0 to 1000 µmol/L, a gradual and dose-dependent decrease in sperm motility compared to the untreated control group was observed. Similarly, **Moretti *et al.* (2012)** showed that none of the selected EPI concentrations selected (20, 30, 50, 100, 200, 400 µmol/L) was not effective as an antioxidant to protect swim-up selected human sperm against tert-butylhydroperoxide induced lipid peroxidation. Furthermore, the study showed that sperm progressive motility and viability were significantly reduced after incubation with EPI at 400

µmol/L. Recently, differential protective effects of epicatechin gallate against induced mitochondrial dysfunction were explored in intestinal epithelial Caco-2 cells and even in this case no protection was observed **(Carrasco-Pozo *et al.*, 2011)**.

On the contrary to the above mentioned studies our results revealed stimulating effects of all EPI concentrations on the motility behavior following its supplementation to rabbit spermatozoa. Such data are in agreement with **Purdy *et al.* (2004)** who aimed to determine whether supplementing diluents with catechin, would aid in maintaining the viability of caprine sperm during cooling and storage at 5 °C. The study revealed significant differences ($P<0.05$) in motility between the control (34%) and the 25, 50, 75 and 100 µmol/L concentrations of catechin (57, 53, 55 and 64% motile cells, respectively) at 96h. These results demonstrated that catechin may aid in maintaining the motility of cooled goat sperm in a dose dependent manner.

Isoquercitrin (ISO)

According to the CASA analysis the initial MOT (Time 0h) was insignificantly increased in experimental groups subjected to treatment with 1, 5 and 50 µmol/L ISO when compared to the Control (Table 5). This non-significant but motility-promoting effect of 1, 5, 50 and 100 µmol/L ISO remained notable after 2h of *in vitro* culture. After 4h, a significantly higher MOT was recorded in the group exposed to 5 µmol/L ISO when compared to the Control ($P<0.01$). At 6h the spectrum of ISO concentrations exhibiting beneficial effects on the sperm motility expanded, as a significantly higher MOT was recorded in groups administered with 1, 5, 10 ($P<0.05$) and 50 µmol/L ISO ($P<0.001$). Nevertheless, at the end of the *in vitro* culture, a significantly higher MOT was detected in the experimental group supplemented with 10 µmol/L ISO ($P<0.05$) when compared to the Control. Interestingly, a notble however non-significant ($P>0.05$) decrase of sperm MOT was recorded following exposure to the highest ISO concentration (100 µmol/L ISO). Administration of the rest of the selected concentrations led to an insignificantly higher rabbit sperm MOT ($P>0.05$) in comparison with the Control (Table 5).

Table 5 Time- and dose-dependent effects of isoquercitrin (ISO) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L ISO	50 µmol/L ISO	10 µmol/L ISO	5 µmol/L ISO	1 µmol/L ISO
0h	80.55±2.33	79.89±6.67	81.98±7.99	79.56±4.90	84.89±9.02	81.99±7.02
2h	69.03±4.02	76.77±4.42	76.56±6.01	67.97±5.09	77.87±7.02	76.54±4.01
4h	59.12±3.33	63.54±5.09	67.87±5.55	66.67±6.09	76.87±6.98**	67.22±5.98
6h	37.55±2.99	44.87±3.99	48.35±4.78*	73.78±8.00***	73.77±4.78***	65.56±3.33***
8h	20.04±1.88	11.09±1.89	20.09±1.98	30.99±4.09*	21.34±2.06	17.97±2.11

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Isoquercitrin is found in foods such as apple and onion, as well as in a variety of medicinal plants, likely contributing to the pharmacological qualities of a large number of botanical medicines. Some of the most commonly used plants containing these flavonoids include Horse chestnut, Ginkgo, Horsetail, Fennel, Rooibos, Hops, Sundew, Bilberry and Mulberry (**Dok-Go *et al.*, 2003; Appleton, 2010**). ISO is one of the naturally occurring glucosides of quercetin. Isoquercitrin is also sometimes called isoquercetin, a nearly identical quercetin-3-monoglucoside. Technically the two are different (isoquercetin has a pyranose ring whereas isoquercitrin has a furanose ring), but functionally the two molecules are indistinguishable. The literature often considers them as one and uses the names interchangeably (**Appleton, 2010**). To our knowledge, no study is currently available on the impact of isoquerctrin on male reproduction. According to our data, although being structurally similar to QUE, the molecule did not exhibit significant negative effects on the sperm MOT when compared to QUE. On the other hand, potential beneficial effects of lower concentrations of ISO were less notable and significant in comparison with QUE. Although experiments based on the administration of pure ISO have not been done yet, numerous animal studies emphasize on potential ameliorative and antioxidant effect of plant extracts containing ISO on the testicular structure and function, as well as sperm concentration, motility and morphology in sickness and health (**Awoniyi *et al.*, 2011; Ayeleso *et al.*, 2014; Ďuračka *et al.*, 2016**). As such, we may suggest that more specific experiments on the roles of ISO are to be done in the future in order to elucidate its beneficial and/or harmful roles in male reproduction.

CONCLUSION

A relatively broad spectrum of positive as well as negative effects has been linked to the treatment of individual biologically active compounds in experimental in vitro and in vivo models. Their mechanisms of action have only recently started to be uncovered, particularly in relation to male reproduction. Our study has unraveled a specific behavior of each biomolecule, providing evidence on their toxic and beneficial concentrations as well as exposure periods adequate for the exhibition of their protective or harmful impact on rabbit sperm survival.

Identification of a normal physiological range of biologically active compounds and their metabolites in mammalian tissues is of utmost importance if researchers aim to determine if the effects observed with respect to a certain dose are physiologically relevant. Furthermore, determining the clinical relevance of results obtained from animal or in vitro studies may be difficult as these studies often use doses which may exceed physiological concentrations. As such, these aspects must be taken into account in the design of future experimental studies, irrespective of whether they are aimed at evaluating beneficial or adverse effects of natural biomolecules.

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