The *in vitro* Effect of Quercetin on the Oxidative Properties of Rat Testicular Tissue

Filip Benko[1], Patrik Hrnčiar[1], Norbert Lukáč[1], Róbert Kirchner[2], Eva Tvrdá[1*]

[1]*Department of Animal Physiology, Slovak University of Agriculture, 949 76 Nitra, Tr. A. Hlinku, 2, Slovakia*
[2]*University Vivarium, Slovak University of Agriculture, 949 76 Nitra, Tr. A. Hlinku, 2, Slovakia*

Abstract
In the reproductive tissues, free radicals are formed as a result of exogenous and endogenous effects. Their excessive production leads to a state of imbalance with the body's antioxidant system, leading to oxidative stress. Oxidative stress is an important factor that affects the condition of male fertility. Preservation and handling of reproductive structures in vitro are accompanied by oxidative damage. Antioxidants are substances that alleviate the effects of oxidative stress and are responsible for slowing the oxidation of biomolecules. The flavonoid quercetin is a bioactive substance exhibiting free radical-quenching activities. This antioxidant is present in a wide variety of fruits and vegetables. In this study we analyzed the effect of various concentrations of quercetin (1 μmol/L; 10 μmol/L; 100 μmol/L) on the oxidative properties of testicular tissue in rats. Testes from adult Wistar rats were used. Quercetin-enriched medium was added to the testicular fragments, except for the control group, and the samples were cultured at 37 °C (5% CO_2) for 24 hours. Lysates were obtained from the samples and used to determine the production of reactive oxygen species (ROS), total antioxidant capacity (TAC), protein carbonyl production, malondialdehyde production (MDA). In the groups supplemented with quercetin, a significant decrease in ROS ($P<0.01$), MDA ($P<0.01$) and protein carbonyls ($P<0.01$) was observed in comparison to the control group. The total antioxidant capacity was significantly increased ($P<0.01$) in all experimental samples when compared to the control group. We may conclude that quercetin exhibits antioxidant and antiradical properties by reducing the production of reactive oxygen species and subsequent oxidative damage, as well as by increasing the antioxidant profile of testicular tissue.

Keywords: oxidative stress, free radicals, antioxidants, quercetin, testicular tissue, rats

1. Introduction

Oxidative stress (OS) is one of the main factors contributing to the development of male infertility. Testicular tissue is particularly susceptible to OS because of higher levels of unsaturated fatty acids in comparison to others. High generation of reactive oxygen species (ROS) may cause serious damage to the male reproductive cells and defects in spermatogenesis [1]. It is important to create a balance between ROS and antioxidants production. The main role of antioxidants is to detoxify and repair the negative effects of ROS. Antioxidants work as ROS scavengers, preventing their formation in the testicular tissue [2]. Nowadays, an increasing interest has been detected in the field of natural compounds as a source of antioxidants. Several studies in experimental animals support the positive effects of quercetin against OS [3-5]. Quercetin (Figure 1.), (3,3',4',5,7-pentahydroxyflavone; QE) is a plant flavonol from the flavonoid group of polyphenols. It is found in diverse fruits, berries, vegetables, tea leaves, seeds and grains. Red onions and kale represent the most common foods containing appreciable amounts of quercetin. Quercetin has been reported to inhibit the oxidation of other molecules and it is classified as an antioxidant. Its polyphenolic chemical substructure stops oxidation by acting as a scavenger of free radicals that are responsible for

*Corresponding author: MSc. Eva Tvrdá, PhD., Tel: +421-37-641-4918, Email: evina.tvrda@gmail.com

oxidative chain reactions. The application of QE is associated with numerous health benefits due to its antioxidant, anti-inflammatory, antiviral and anticancer properties [6-8]. The objective of our experiment was to evaluate the effects of quercetin on the oxidative properties of rat testicular tissue under *in vitro* conditions.

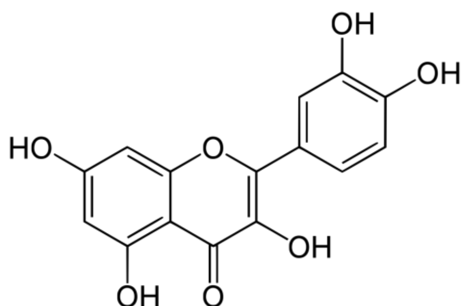

Figure 1. Chemical structure of quercetin

2. Materials and methods

Animals

In this study, 9 sexually mature Wistar rats (age of 120 days) were used. The animals were obtained from the Institute of Experimental Pharmacology (Slovak Academy of Sciences, Slovakia) and raised in Slovak University of Agriculture (Nitra), kept in plastic cages at 24 +/- 1°C and 12 h light/12 h dark photoperiod. All animals were provided standard food and water *ad libitum*. Institutional and national guidelines for the care and use of laboratory animals were followed, and all procedures were approved by the State Veterinary and Food Institute of the Slovak Republic (no. 3398/11-221/3) and Ethic Committee.

Testes collection and culture

After anesthesia, all animals were ethically euthanized. Testes were collected by opening and pulling out of scrotum. Epididymes and excess tissue were separated from testes by using scalpel. Cleaned testes were washed in saline solution (Imuna Pharm, a.s., Šarišské Michaľany, Slovak Republic) and subsequently store at -20°C in the freezer for later analysis. Prior the cultivation, the testes were cut into smaller fragments and cultured with different concentrations of quercetin (1; 10 and 100 µmol/L) at 37°C, 5% CO_2 concentration for 24 hours. The culture medium comprised D-MEM (Dulbecco's modified Eagle Medium, Sigma-Aldrich, St. Louis, USA), 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic and antimycotic (Sigma- Aldrich).

Medium collection and preparation of lysates

After cultivation, the testicular fragments and medium were moved into tubes and centrifuged at 1200 RPM for 10 minutes. Medium was separated into new test tubes and testicular fragments were used for testicular tissue lysates. Lysates were prepared by using a lysis RIPA solution and sonicator (Branson Digital Sonifier, Branson/Emerson, St. Louis, USA) on ice. All lysates were centrifuged at 4°C, 5000 RPM for 10 minutes.

Reactive oxygen species (ROS) generation

Evaluation of reactive oxygen species (ROS) was performed by the chemiluminescence assay and luminol was used as a probe (5-amino-2, 3-dihydro-1, 4-phthalazinedione). The chemiluminescence signal was measured on 96-weel plates using Glomax Multi+ Combined spectro-fluoro luminometer (Promega Corporation, Madison, WI, USA) [9].

Total antioxidant capacity (TAC)

The study of total antioxidant capacity was performed with an improved chemiluminescence antioxidant assay using horseradish peroxidase conjugate, luminol and Trolox as standard. The measurement of chemiluminescence was done in the Glomax Multi^{+} Combined Spectro-Fluoro Luminometer using (Promega Corporation) 96-well plates. The results are calculated as µmol Trolox Eq./g protein [9].

Protein oxidation

Determination of carbonyl groups was assessed by 2,4-dinitrophenylhydrazine (DNPH) method. The values of absorbance were measured with spectrophotometer (UV-vis spectrophotometer Cary, Pittsburg, USA) at 360 nm. The concentration of carbonyl groups was expressed as nmol/mg B [10].

Lipid peroxidation (LPO)

For the assignment of lipid peroxidation were applied TBARS (thiobarbituric acid reactive substances) assay, modified for a 96-well plate. In this method malondialdehyde (MDA) as a product

of lipid peroxidation, create a pink-coloured complex which was measured through the Glomax Multi+ Combined spectro-fluoro luminometer (Promega Corporation, Madison, WI, USA) at specific wavelength 530-540 nm. The values of MDA were expressed as µmol/g protein [10].

Total proteins quantification

Analysis of total proteins was evaluated by using the commercial kit for Total protein (DiaSys, Holzheim, Germany) and concentration of total proteins was measured with RX Monza (Randox, Crumlin, United Kingdom) at 540 nm [9].

Data analysis

Data were processed using the GraphPad Prism program (version 6.02 for Windows; GraphPad Software, La Jolla, California, USA, www.graphpad.com). For the advanced statistical analysis One-way ANOVA and Dunnet's test was used. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

3. Results and discussion

The results indicate that generation of ROS (Table 1) was significantly decreased (P<0.01) in the group supplemented with 10 µmol/L QE when compared to the control group. The effect of QE was studied in testis of rats, which had induced diabetes with streptozotocin. After QE treatment the inhibited activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) were significantly increased, which was accompanied by a decrease of ROS generation and testicular damage [11]. Flavonoids like QE can prevent oxidative damage as a result of their capacity to scavenge ROS. Furthermore, it has been reported that QE has potential protective effects on the metabolism of antioxidant enzymes, which provide protection against OS [12,13]. Under *in vivo* conditions QE was reported to be a potent antioxidant, nevertheless it also acts as pro-oxidant. As such, the antioxidant activity of QE depends on its dose. Higher doses of QE may cause an increase of ROS generation and disruption of endocrine control in the testicular tissue [14]. Moreover, QE ameliorated rotenone-induced testicular toxicity and oxidative damage in rats [15]. In the presented study, lower doses of QE decreased ROS generation and improved the enzymatic and antioxidant activity of testicular tissue under *in vitro* conditions.

The total antioxidant capacity (Table 1) was significantly higher (P<0.05; P<0.01) in tissue supplemented with 1 and 10 µmol/L QE than in the control tissue without QE. Previous findings confirmed the ability of QE to decrease the TAC consumption and to increase the antioxidant potential of testicular tissue [16]. It was noticed that QE addition significantly increased (P<0.05) the level of TAC and reduced MDA production in male Albino rats with atrazine-induced reproductive toxicity [17]. Some differences were recorded between *in vivo* and *in vitro* QE administration. Testicular values of TAC did not alter significantly after gastric tube application of QE. Following dietary ingestion, QE undergoes rapid and extensive metabolism that makes differences between *in vitro* and *in vivo* studies. Poor water solubility, chemical instability and low bioavailability partially limited QE applications under *in vivo* conditions [18-20].

In the case of protein oxidation, tissues exposed to 1 and 10 µmol/L QE showed a significant decrease of protein oxidation (P<0.05; P<0.01) in comparison to the control group. QE was capable to significantly increase (P<0.05) the enzymatic activity of catalase (CAT), superoxide dismutase (SOD) and glutathione-depend enzymes (GPx, GS, GSH) and reverse the testicular toxicity caused by insecticides [21]. However, higher doses of QE were responsible for a significant decrease in the activity of CAT, SOD, GSH in testicular tissue of mice under *in vivo* conditions [14].

The level of lipid peroxidation (LPO) and concentration of MDA (Table 1) were significantly decreased (P<0.01) in the group supplemented with 10 µmol/L QE when compared to the control group. MDA is commonly used as a marker of LPO in tissues and cells [22]. Quercetin supplementation (50 mg/kg) in rats, which had OS induced with arsenic (50 ppm in drinking water) caused that the levels of LPO were significantly suppressed and weakened, while the antioxidant defense mechanisms were restored [5]. Protective benefits of QE were confirmed against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testicular tissue. QE treatment showed a significant decrease in LPO when compared to the diabetic group [23]. It has been confirmed that QE is capable to reduce the toxic effects of arsenic

on a significant level and decreased oxidative stress, LPO and apoptosis in rat testis [3]. Oral administration of QE inhibited OS induced with tetrachloride methane and decreased the level of testicular apoptosis and LPO. The authors conclude that QE had antiperoxidative effects. QE effectively reduced the elevation in TBAR substances and restored the activity of antioxidant enzymes in testicular tissue [24,25].

Table 1. The effects of different concentrations of quercetin on the oxidative properties of rat testicular tissue

	Control	1 µmol/L	10 µmol/L	100 µmol/L
ROS	11.03±0.42	7.85±1.31	5.02±1.67**	10.18±0.83
TAC	29.34±5.99	49.28±8.29*	62.31±18.23**	41.49±15.14
PC	4.70±2.30	3.02±0.33*	1.14±0.41**	4.68±1.99
MDA	3.04±0.37	2.20±0.33*	1.54±0.57**	2.35±0.21

Mean±SEM; ***(P<0.001); **(P<0.01); *(P<0.05)

4. Conclusions

The addition of QE during the *in vitro* cultivation of rat testicular tissue could improve the oxidative properties and decrease the risk of development of oxidative stress. Nevertheless, the use of QE as an antioxidant depends on dosage and conditions.

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