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BENEFICIALL EFFECT OF THE EUROPEAN RASPBERRY (*RUBUS IADEUS*) EXTRACT ON THE ACTIVITY AND OXIDATIVE PROFILE OF BOVINE SPERMATOZOA

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ABSTRACT

Berries belong to the best dietary sources of bioactive compounds. These fruits contains bioactive compounds such as antioxidants (phenolic compounds and fruit colorants), vitamins (ascorbic acids) and minerals. This study aimed to estimate the effect of three concentrations (75 µg/mL, 150 µg/mL and 300 µg/mL) of the European raspberry (*Rubus iadeus*) on the selected quality parameters of bovine spermatozoa after 0h, 2h, and 24h of *in vitro* culture. Sperm motility was evaluated using the Computer-assisted sperm analysis (CASA) system. The mitochondrial activity was detected by the metabolic (MTT) assay. The determination of ROS (Reactive Oxygen Species) quantity was observed through the luminol method. The protein and lipid oxidation were evaluated using spectrophotometric assays. The experiment showed that the highest applied concentration of European raspberry extract at 2h ($p<0.05$) and all concentration after 24h ($p<0.05$; $p<0.001$) exhibited motility-promoting effects. We observed the highest increase of mitochondrial activity using 300 µg/mL by 2h ($p<0.05$) and using 150 µg/mL ($p<0.05$) and 300 µg/mL ($p<0.01$) after 24h. Exposure to this extract led to decreasing levels of ROS generation at 2h of incubation ($p<0.05$) with the highest dose as well as after 24h using 150 µg/mL ($p<0.01$) and 300 µg/mL ($p<0.001$) applied doses. The protein oxidation levels showed decreasing rates at 2h ($p<0.05$; 300 µg/mL) and at time of 24h ($p<0.01$; 150 µg/mL and $p<0.001$; 300 µg/mL). All selected raspberry concentration prevented oxidative degeneration of lipids during 2h ($p<0.05$) and after 24h ($p<0.05$; $p<0.001$). Our results indicate, that European raspberry extract has beneficial effect on the measured parameters of the bovine spermatozoa during *in vitro* incubation but depending on time and applied dose.

Keywords: European raspberry, bull, spermatozoa, reactive oxygen species

INTRODUCTION

Raspberry plant belongs to the *Rosaceae* family. *Rubus idaeus* is a small fruit which grows on different places in the world (**Gordon and Woodford, 2000**). Raspberries as the most popular berry fruit in Europe is produced in Poland, as well as in Serbia and Spain (**Ponder and Hallmann, 2019**).
The increased consumption of fruits and vegetables for their antioxidant phytochemical contents is recommended by dietary guidelines to prevent chronic diseases. Secondary metabolites produced by the plant are characterized by a relatively strong biological activity (**Bobinaité *et al.*, 2012**). Fruits, especially berries contain sources of natural antioxidants and are an excellent component of a healthy diet (**Baby *et al.*, 2017**). The major health benefits of berry fruits are referred to the phenolic compounds, like flavonoids, phenolic acids and tannins (**Baranowska *et al.*, 2014**). Polyphenolic compounds such as anthocyanins and ellagitannins are the most common antioxidant phytochemicals present in raspberries (**Rao and Snyder, 2010**). The anthocyanins as a natural organic compound are known for their preventive effects against diseases, such as tumor, senile and cardiovascular diseases. Anthocyanins can protect cells and body from oxidation by scavenging free radicals (**Teng *et al.*, 2017**). Biological activities and higher anthocyanin content of the raspberry indicate that their consumption would be useful for health, and that they could be used during production of functional foods with effective dose of anthocyanins (**Bowen-Forbes *et al.*, 2010**). Phenolic compounds composition, concentration and related antioxidant activity in berries depend on the maturity of fruit, agro – environmental conditions and post – harvest technologies (**Veljković *et al.*, 2019**).
Bioactive components in berries exhibit antimicrobial, anticancer, antidiabetic, anti-inflammatory, antioxidant and cardioprotective properties (**Baby *et al.*, 2017; Veljković *et al.*, 2019; Noratto *et al.* 2017**). The fruit of *Rubus idaeus* is the most common herbal drug in folk medicine, but the shoots of this plant can be also used as a treatment to cold, fever or flu – like infections (**Baranowska *et al.*, 2014**). Raspberry leaves are also used for tea, and in folk medicine has been used to treat wounds, colic pain, diarrhoea and as a uterine relaxant for centuries (**Vera *et al.*, 2002**).
Despite its importance, the effect of European raspberry on the male reproductive system is still unclear. The aim of this *in vitro* study was to evaulate the chemical composition and the effects of raspberry on the male reproductive cells.

MATERIAL AND METHODS

Plant material collection and processing

Raspberry fruits were collected at the Botanical Garden of the Slovak University of Agriculture in Nitra, Slovakia at the end of July 2016. After the drying process, the fruits were crushed, weighed and soaked in ethanol (96 %, Centralchem, Bratislava, Slovakia) during 14 days at room temperature in the dark in order to avoid the degradation of active biomolecules. The ethanolic extracts were subjected to evaporation under reduced pressure at 40 °C (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany) to eliminate any residual ethanol. Crude extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) and adjusted to 1000 mg/mL as stock solution.
For the following chemical analysis, the fruits were freeze dried and milled. Methanol extracts were produced by adding 25 mL 80 % aqueous methanol (HPLC grade; Sigma-Aldrich) to 1 g of each sample. The mixtures were shaken at room temperature for 8 h on a horizontal shaker (250 rpm). The samples were then filtered through filter paper (84 g/m2; Munktell, Germany) and kept at 5 °C for further analysis.

HPLC-DAD analysis

Standards, methanol (HPLC grade), acetonitrile (gradient HPLC grade) and phosphoric acid (ACS grade) were purchased from Sigma-Aldrich. Double

deionized water (ddH2O) was adjusted (0.054 mS/cm[1]) in a Simplicity 185 purification system (Millipore SAS, Molsheim, France). Standard solutions were prepared by dissolving 0.5 mg each of them with methanol in 10 ml. Following homogenization the lyophilized samples (2 g) were extracted with 20 ml of 80% methanol at laboratory temperature for 8 h by horizontal shaker (Unimax 2010; Heidolph Instrument GmbH, Germany). The extract was filtered through munktell no 390 paper (Munktell & Filtrac, Germany) and stored in closed 20 ml vial tubes. Prior to injection the standard solutions and extracts were filtered through the q-max syringe filter (0.22 mm, 25 mm; Frisenette Aps, Knebel, Denmark) (**Bajčan *et al*., 2016**).

For the determination of the chemical composition of the European raspberry extract the agilent 1260 infinity high performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) was used with quaternary solvent manager coupled with degasser (G1311B), sample manager (G1329B), column manager (G1316A) and dad detector (G1315C). All HPLC analyses were performed on a Purosphere reverse phase C18 column (4 mm x 250 mm x 5 mm) (Merck, KGaA, Darmstadt, Germany). The mobile phase consisted of acetonitrile (gradient) (A) and 0.1% phosphoric acid in ddh2o (B). The gradient elution was as follows: 0-1 min isocratic elution (20% A and 80% B), 1-5 min linear gradient elution (25% A and 75% B), 5-15 min (30% A and 70% B) and 20-25 min (40% A and 60% B). The initial flow rate was 1 ml/min1 and the injection volume was 1 ml. Column oven temperature was set up to 30 °C and the samples were kept at 4 °C in the sample manager. The data were obtained and processed using the agilent Openlab ChemStation software for LC 3D Systems (**Luksic *et a*l., 2016**).

Semen sample collection and processing

Ejaculates (n=20) were collected from four adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). Semen samples were obtained regularly (once a week for five consecutive weeks) from each animal using an artificial vagina. Right after the collection, semen concetration and motility were estimated by phase-contrast microscopy (200 x). Samples with required quality (min. 70 % motility and concetration of 1 x 10^9 sperm/mL) were chosen for further experiments. Institutional nad national guidelines for the care and use of animals were followed, and all experimental processes were confirmed by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.

Ejaculates were diluted in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma Aldrich) containing different concentrations of the European raspberry extract (300; 150; 75 µg/mL) using a dilution ratio of 1:40. Each semen samples were cultured at laboratory temperature (22-25°C). After culture periods of 0, 2, 24h, spermatozoa motility, mitochondrial activity reactive oxygen species (ROS) production, protein and lipid peroxidation were evaluated in each group.

Spermatozoa motility analysis

Sperm motion parameters were measured using the computer-aided sperm analysis CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). The system was set up as follow: freame rate – 60 Hz; minimum contrast – 20; static head size – 0.25 – 5.00; static head intensity – 0.40 – 2.00; static elongation – 20 – 100; default cell size - 4 pixels; default cell intensity – 40. Ten microliters of each semen sample were placed into the Makler counting chamber (depth 10 µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and spermatozoa motility (MOT; percentage of motile spermatozoa; motility > 5 m/s; %) was assessed immediately. Ten fields of the microscope were subjected to each analysis to contain at least 300 cells (**Tvrdá *et al*., 2018**).

Mitochondrial activity (MTT test)

Mitochondrial activity of the spermatozoa was assessed using the colorimetric metabolic activity (MTT) test. This process is based on the transformation of a yellow tetrazolium salt (3 – (4,5 – dimetylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide; MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. The tetrazolium salt (Sigma – Aldrich) was dissolved in PBS (Dulbecco's Phopshate Buffer Saline without calcium chloride and magnesium chloride; Sigma – Aldrich) at 5 mg/mL. Each semen suspension was mixed with 10 µL of the tetrazolium solution. Following 2 hours of incubation (shaker, 37 °C, 95 % air atmosphere, 5 % CO_2), the formazan crystals were dissolved in 80 µL of acidified (0.08 mol/L HCl; Centralchem) isopropanol (Centralchem). Optical density was established at a wavelength of 570 nm against 620 nm as reference using a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are expressed as percentage of the control set to 100 % (**Kňažická *et al*., 2012).**

Reactive Oxygen Species (ROS) generation

ROS production was analysed in all groups by the chemiluminescence assay using luminol (5 – amino – 2,3 – dihydro – 1,4 phthalazinedione; Sigma – Aldrich) as the probe (**Kashou *et al.*, 2013**). The blank contained 400 µL of PBS, negative control consisted of 400 µL of PBS, luminol (10 µL, 5 mM) and positive control was added by extra 25 µL hydrogen peroxide (30 %; 8.8 M; Sigma – Aldrich). The experimental samples and tested control contained 400 µL of luminol and sample. Chemiluminescence was assessed by Glomax Multi$^+$ Combined Spectro – Fluoro Luminometer (Promega Corporation, Madison, WI, USA) on 48 – well plates in 15 cycles of 1 min. The results are expressed as relative light units (RLU)/s/10^6 spermatozoa.

Protein oxidation

The protein carbonyl content was quantificated using the standard 2,4 – dinitrophenylhydrazine (DNPH) method. One mL of the pre – treated sample was mixed with one mL DNPH (10 nM in 2 N HCl; Sigma Aldrich) and incubated at 37 °C in the dark place for one hour. After 1 mL trichloro acetic acid (20 % w/v; Sigma Aldrich) addition, the compound was incubated at 4 °C for 10 min and afterwards centrifuged at 11 828 x *g* for 15 min. The supernatant was removed without interfering the pellet which was subsequently washed three times with one mL ethanol/ethyl acetate (1/1; v/v) to discard any free DNPH reagent. Lastly, the pellet was resuspended in 1 mL 6 M guanidine – HCl (Sigma – Aldrich) before absorbance measurement at 360 nm, using 6 M guanidine – HCl as a blank solution. The molar absobtion coeficient of 22 000 M/cm was used to determine the concetration of protein carbonyls. The measurements are formulated as nmol/mg protein (**Weber *et al*., 2015**)

Lipid peroxidation

The extent of lipid peroxidation which was formulated as malondialdehyde (MDA) concetration was determined with the help of the TBARS assay, modified for a 96 – well plate and ELISA reader. The final product generated by the reaction of MDA and thiobarbituric acid (Sigma - Aldrich) under high temperature (90 – 100 °C) and acidic conditions was measured at 530 – 540 nm (**Tvrdá *et al*., 2016**). MDA is expressed as µmol/g protein.

Protein quantification

The amount of protein was evaluated using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the Randox RX Monza clinical chemistry analyzer (Randox Laboratories, Crumlin, Great Britain). The measurement is based on the Biuret reaction in which protein is detected using a mixture of sodium hydroxide solutions and copper sulphate to form a violet blue colour complex if the protein was detected. The intesity of the colour is directly proportional to the concetration of the protein when assessed at 540 nm

Statistical analysis

The obtained data were statisticaly analysed using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One – way Anova was used for specific statistical evaluations. Dunnett's test was applied as a follow – test to ANOVA, based on a comparisonn of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** $P < 0.001$, ** - $P < 0.01$, * - $P < 0.05$.

RESULTS AND DISCUSSION

For the identification and quantification of the main chemical compounds found in the European raspberry extract the high preformance liquid chromatography was used. External standard method was used for quantitative determination and the identified compounds with their concentration are showed in Table 1. The major detected compounds found in the extract were myricetin 1200.66 mg/kg and cynarozid 1176.48 mg/kg. From the analyzed phenolic acids, chlorogenic acid, neochlorogenic acid, caffeic acid, trans - caffeic acid, elagic acid, trans – sinapinic acid, trans – ferulic acid, rosmarinic acid and trans - coumaric acid were quantified with the first being the most abundant (621.08 mg/kg). Eight flavonoid glycosides, myricetin, cynarosid, resveratrol, quercetin, kaempferol, vitexin, rutin, apigenin were found in the European raspberry extract (Table 1).

Table 1 Major chemical compounds identified and quantified [mg/kg] in the *Rubus iadeus* extract

European raspberry extract	
Cynarosid	1176.48
Rutin	10.88
Apigenin	5.55
Kaempferol	18.97
Vitexin	9.15
Quercetin	35.44
Myricetin	1200.66
Resveratrol	55.33
Chlorogenic acid	621.08
Neochlorogenic acid	520.43
Trans – Coumaric acid	5.19
Trans – Caffeic acid	87.53
Trans – Sinapinic acid	26.55
Trans Ferulic acid	17.27
Rosmarinic acid	8.85
Elagic acid	35.34
Caffeic acid	88.89

The next part of our study was focused on the *in vitro* effects of *Rubus iadeus* on five quality parameters of bovine sperm. All assessments we performed at 0, 2, 24 hours using working solutions with 300, 150, 75 µg/mL plant extract.
The first measured parameter was the sperm motility evaluated by CASA system. The 150 µg/mL addition of the raspberry extract exhibited positive effect during the second hour of the analysis. The highest concetration (300 µg/mL) significantly increased (P<0.05) the sperm motion when compared to the control group. Following 24 h the lowest and the middle raspberry extract doses showed statistically significant (P<0.05) increased motility of bovine spermatozoa as well as the highest concetration showed (P<0.001) (Figure 1).

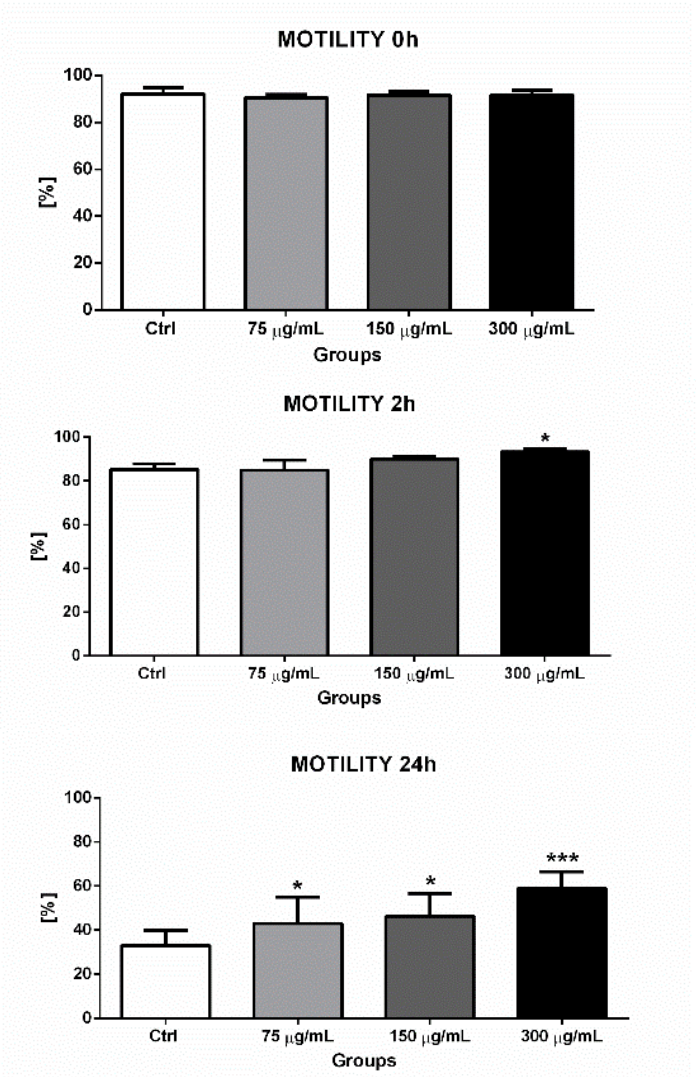

Figure 1 The effect of various concentrations of the Euroean raspberry extract on the motility of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM). The level of significance was set at * P < 0.05; *** P < 0.001.

Mitochondria supply the sperm cells with fuel they need to support important functions. Mitochondria provide energy for the sperm movement, so the activity of mitochondria is closely related to the motility. Therefore, we decided to analyze the effect of European raspberry extract on the mitochodria viability. Figure 2 shows the results of MTT test. At the beginning of the experiment, all concetration of the extract showed an immediate positive effect on sperm viability, but with no statistically significant difference. We observed a significant increase (P<0.05) of the mitochondrial viability following the highest dose of extract (300 µg/mL) at 2 hours. After 24h of incubation wiht European raspberry extract, the middle and highest concetration (150; 300 µg/mL) significantly increased the mitochondrial viability (P<0.01; P<0.001). We can say, that sperm vitality can be positively affected by European raspberry extract (Figure 2).

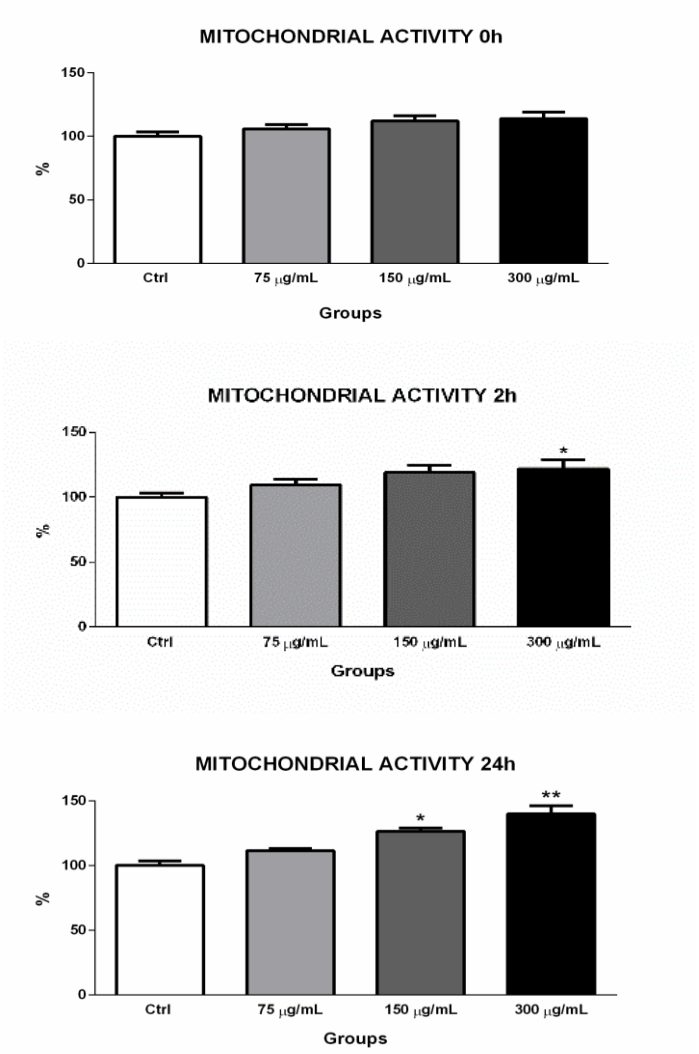

Figure 2 The effect of various concentrations of the Euroean raspberry extract on the viability of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The level of significance was set at * P < 0.05; ** P < 0.01.

ROS quantification was performed as high ROS production can negatively influence the plasma membrane of the sperm followed by the functional integrity of the cell. Motility and vitality can be reduced by increasing ROS levels. First measurements at 0h were without any significant change. Administration of the European raspberry extract at the highest added dose (300 µg/mL) significantly reduced (P<0.05) the ROS overgeneration by male reproductive cells after 2h as well as the reduction of ROS levels was significant after 24h at the middle (150 µg/mL; P<0.01) and by the highest European raspberry extract administration (300 µg/mL; P<0.001). Therefore, we suppose that the European raspberry extract may have positive effect by reduction of reactive oxygen species production by sperm cells (Figure 3).

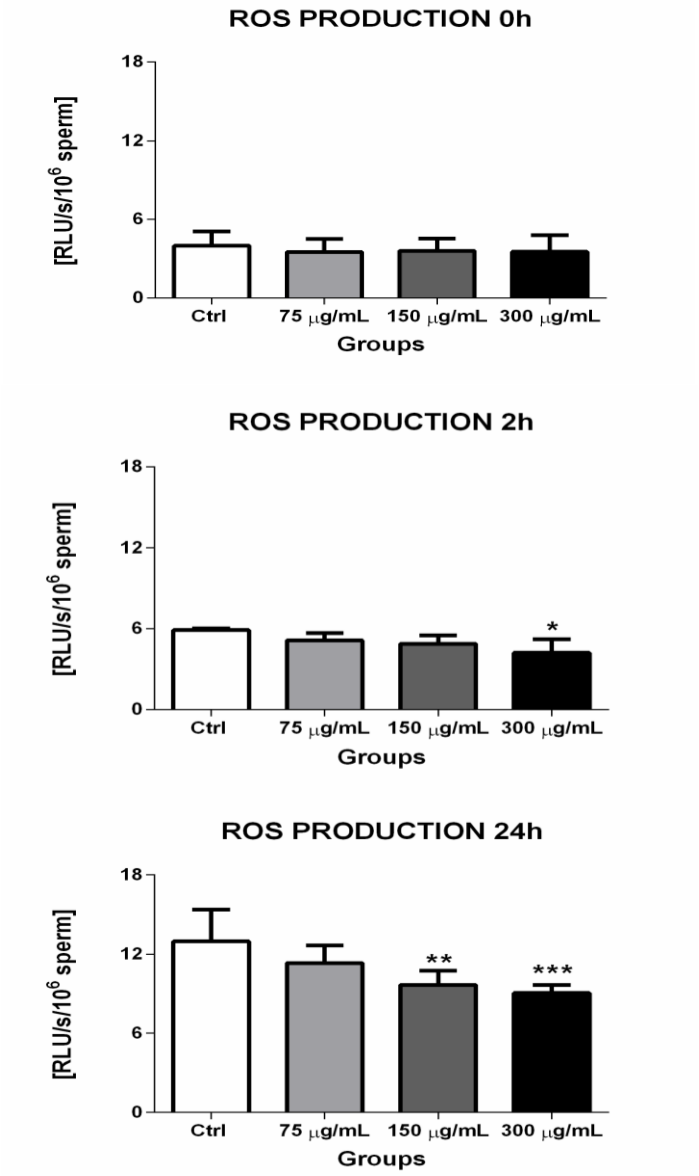

Figure 3 The effect of various concentrations of the Euroean raspberry extract on the ROS production by bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM). The level of significance was set at * P < 0.05; ** P < 0.01, *** P < 0.001.

Oxidation can influence the conformation or activity of proteins in male reproductive cells. Therefore, we decided to analyze the effect of the European raspberry extract on the protein modifications. Initially, no significant differences were observed. After 2h, the results indicated a significant difference (P<0.05) following the highest dose (300 μg/mL). Following 24h, we experienced that all groups exhibited decreasing levels but only groups treated with 150 μg/mL showed singnificant decreasing levels (P<0.01) of protein oxidation after European raspberry extract additon, as well as by the highest added dose of this extract (300 μg/mL; P<0.001). These facts may suggest, that sperm cells can be treated by this extract to decrease protein oxidation (Figure 4).

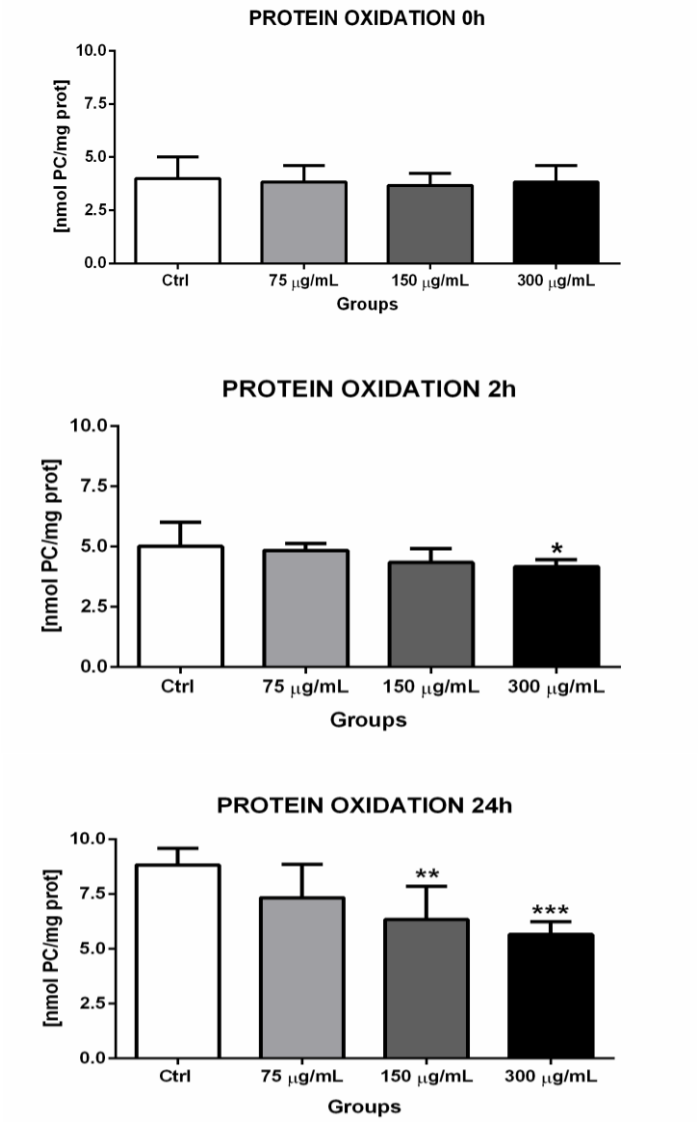

Figure 4 The effect of various concentrations of the Euroean raspberry extract on the protein oxidation of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM). The level of significance was set at * P < 0.05; ** P < 0.01, *** P < 0.001.

Male reproductive cells have high levels of polyunsaturated fatty acids in their plasma membrane, which are uncovered to oxidative attacks. Our first measurements at 0h did not indicate any change after raspberry extract addition. After 2h of incubation, the all observed groups showed statistically significant (P<0.05) effect of European raspberry extract by decreasing levels of lipid peroxidation. The lowest concetration
(75 μg/mL) suggested significantly decreasing lipid damage (P<0.05), similarly, the other doses, 150 μg/mL and 300 μg/mL showed a significant decrease of lipid peroxidation after 24h of *in vitro* culture. Addition of this substance can be able to protect bovine germ cells before oxidative damages of lipids (Figure 5).

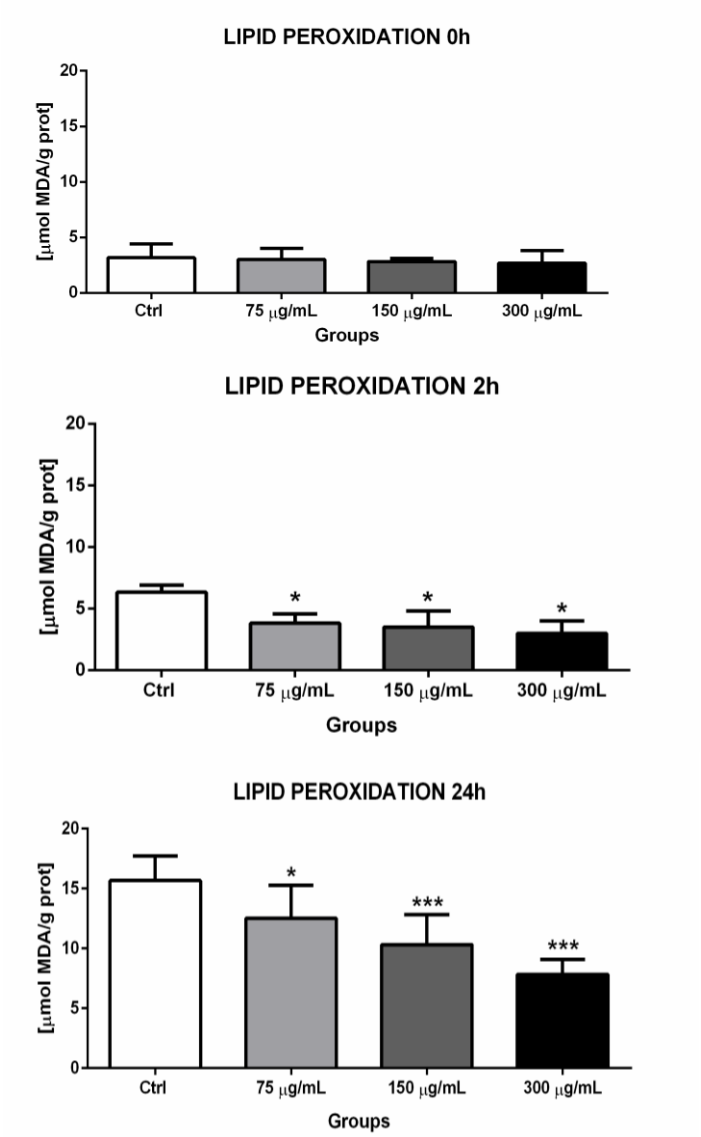

Figure 5 The effect of various concentrations of the Euroean raspberry extract on the lipid peroxidation of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (±SEM). The level of significance was set at * P < 0.05; *** P < 0.001.

Yoon *et al.* (2011) determined that after *in vivo* raspberry administration extract to 8 – week old male Wistar rats, this extract intesively increased the weight of the rat testes, epididymal sperm count, sperm motion, and testosterone level compared to the control group. Their results implyed, that raspberry extract has a protective effect to spermatogonial cells against spermatogenic effect and oxidative stress on male reproductive system what is corresponding with our data.

The positive effect of raspberries were verifyed by **Jeon *et al.*** (2008). They investigated the effects of fermentation filtrates from *Rubus coreanus* on the selected functions of the male reproductive system. They found, that addition of this substance relatively increase the function of the male reproductive sytem by triggering penile erection, improving testosterone levels in serum and enhancing epididymal sperm counts.

The addition of *Rubus coreanus* leaf and stem extract can help to increase sperm viability and motility during boar semen preservation (**Yi *et al*., 2017**).

Noratto *et al.* (2016) investigated the effects of red raspberry consumption on obese diabetic mice. They detected, that red raspberry consumption can decrease the oxidative and inflammatory stress levels. Similarly, our results showed oxidative termination by significantly decreasing reactive oxygen species, protein oxidation and lipid peroxidation levels. **Noratto *et al.*** (2017) demonstrated, that *in vivo* intake of red raspberry by mice for 8 weeks, showed a protective effect against diabetes-induced oxidative stress.

Another protective effects of raspberry was followed by **Garcia *et al.*** (2017). These authors claimed, that raspberry polyphenols can figure as a dietary route to the retardation or amelioration neurodegenerative – related dysfunctions.

Zhang *et al.* (2011) reported, that orraly administered *Rubus idaeus L*. fruits to the experimental rats showed diuretic effect, what means, that raspberry fruits might have protetctive effect against renal diseases.

Ellagic acid is one of the major chemical compound of European raspberry. **Ceribasi *et al.*** (2012) demonstrated the effect of ellagic acid on adriamycin induced testicular and spermatozoal toxicity associated with the oxidative stress in male rats. After 8 weeks of intraperitoneal treated period they found, that ellagic acis has a protective effect on adriamycin induced testicular lipid peroxidation and apoptosis.

CONCLUSION

This study was aimed to investigate the chemical composition of the *Rubus iadeus* extract and its *in vitro* effects on male reproductive cells. The following markers of the bovine spermatozoa were monitored: motility, mitochondrial activity, quantification of ROS as a marker of oxidative stress, protein oxidation and lipid peroxidation. To describe main chemical components of the European raspberry extract the HPLC method was applied. After measuring of the selected paramethers, it can be concluded, that the highest applied concetration of *Rubus idaeus* extract (300 µg/mL) significantly increased the motility, mitochondrial activity as well as decreased the ROS production, protein oxidation and lipid peroxidation at 2h and 24h. The other two lower doses exhibited positive effects too. Lipid peroxidation was significantly decreased by the lower doses after 2h and 24h. At 24h, the motility (75 µg/mL; 150 µg/mL), mitochondrial activity were significantly increased and the ROS production and protein oxidation were decreased after the additon of 150 µg/mL extract. We can conclude, that European raspberry extract addition in different concetrations caused a significant increase of the sellected parameters, which finally points to the positive and protective effect of this extract on the bovine reproductive cells. At the same time, further data validation for more complex sperm analyses and *in vivo* experiments are highly recommended.

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