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COMPARATIVE ANALYSIS OF THE EFFECTS OF CURCUMIN AND EPICATECHIN ON THE VITALITY OF ROOSTER SPERMATOZOA

Filip Benko[*1], *Michal Ďuračka*[1], *Ján Kováč*[1], *Norbert Lukáč*[1], *Eva Tvrdá*[1]

Address(es): Ing. Filip Benko,
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.

*Corresponding author: filip.benko276@gmail.com

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ABSTRACT

The objective of our study was to assess the effects of two selected antioxidants (curcumin - CUR and epicatechin - EPI) on the vitality of rooster spermatozoa. We used semen samples from 10 sexually mature Lohmann light roosters, which were collected from a local farm, diluted in PBS and cultured with different concentrations of CUR and EPI (1, 5, 10, 25, 50 and 100 µmol/L). Motility, membrane and acrosome integrity were analysed after 0, 2 and 24 h of *in vitro* culture. Sperm motility was evaluated by the computer-assisted sperm analysis (CASA). The membrane integrity was determined by a combination of eosin/nigrosin dyes, and the acrosome integrity was detected by fast green/rose bengal dyes. The data were statistically evaluated using One-way ANOVA and the Dunnet's test. The results indicate that after 0 h of cultivation all selected parameters stay unchanged. After 2 h significant differences ($P<0.0001$) were observed in case of the motility and membrane integrity. Following 24 h a significant ($P<0.0001$) preservation of motility was recorded. The level of membrane damage and acrosome integrity was significantly decreased ($P<0.01$). Based on our results we may conclude that protective effects of CUR and EPI on the vitality of rooster spermatozoa were observed already after 2 h. This impact was even more dominant after 24 h. CUR showed better effects at lower concentrations (1, 5, and 10 µmol/L) while EPI at higher doses (25 and 50 µmol/L). Our findings could contribute to the potential application of these antioxidants in avian reproduction technologies.

Keywords: curcumin, epicatechin, rooster, spermatozoa, oxidative stress

INTRODUCTION

Worldwide poultry farming is focused mainly to produce high-quality eggs and meat. The economic and nutritional demands of meat production require many poultry breeds in confinement during a short period of time. Poultry and especially chicken do not require any special conditions for their breeding. Another advantage is their short reproductive cycle and incubation time of fertilized eggs. Nowadays chickens are breed mostly for meat. There are a lot of varieties and breeds which are classified as heavy meat-type chickens. However, an increase of body mass could cause problems with natural breeding because roosters are too heavy and thus are not able to reproduce naturally (**Burton *et al.*, 2016)**. This is the reason why artificial insemination (AI) is so necessary in poultry reproduction **(Iqbal *et al.*, 2002)**. AI process in birds includes the transportation of semen into female reproductive tract manually. It starts from the collection of the semen from the male and its evaluation in terms of motility, viability and concentration. The main advantage of AI in poultry is that one sexually mature male could cover up 20 females, which is important for an increased reproduction speed **(Karayat *et al.*, 2016)**. An important aspect of successful AI is the highest possible quality of fresh or cryopreserved sample. Nevertheless, commonly used cryoprotectants or semen extenders have a contraceptive or toxic effect and increase the risk of oxidative stress (OS) development associated with the overproduction of reactive oxygen species (ROS), **(Çifti and Aygün, 2018)**. OS is one of the prime mediators responsible for reduction of ejaculate quality leading to malfunction of cell-egg interactions and male sub-fertility or infertility. OS is characterized as physiological imbalance between prooxidants and antioxidants. Under physiological conditions ROS are necessary for the sperm maturation, hyperactivation, capacitation, acrosome reaction as well as fertilization **(Hubert *et al.*, 2007; Mora *et al.*, 2017)**. Avian spermatozoa are susceptible to oxidative damage due to high proportions of polyunsaturated fatty acids (PUFAs), a unique sperm head shape and membrane fluidity. PUFAs can be easily oxidized by ROS and make sperm cells more vulnerable to OS. In the intensive poultry production, animals are often exposed to negative conditions such as inadequate diet supplementation, fast growth, antibiotics or the other environmental stressors like atmospheric temperature, humidity, radiation, ammonia, bacterial and viral infections. All these conditions can stimulate ROS generation and enhance OS **(Panda and Cherian, 2014; Surai, 2020)**. Although ROS production is involved in many biological functions of spermatozoa and excess of ROS could cause wide spectrum of negative effects such as decrease of sperm motility or membrane and acrosome damage. For the treatment of this condition compounds of natural origin with antioxidant activity are often used **(Khan, 2011; Dutta *et al.*, 2019)**. The addition of antioxidant compounds into fresh semen could defeat the deleterious effects of ROS on the sperm quality and fertility potential. Many antioxidants of natural origin provided positive effects on some sperm parameters such as motility and membrane integrity **(Mehdipour *et al.*, 2019)**. Curcumin (CUR) is a natural phenol produced by *Curcuma longa* characterized by bright yellow pigment and is commonly used as a spice or food-coloring agent. Therapeutic properties of curcumin are often associated with antioxidant and anti-inflammatory activity. Epicatechin (EPI) is a type of flavonoid compound very abundant in green tea, grape, cocoa bean and berries to which are ascribed a potential antioxidant activity **(Omur and Coyan, 2016; Bernatoniene and Kopustinskiene, 2018)**. In our study were selected CUR and EPI because of high levels of their antioxidant activity. Previous reports studying the ROS-scavenging potential effects of CUR and EPI on mammalian spermatozoa **(Tvrdá *et al.*, 2016; Ďuračka *et al.*, 2017; Greifová *et al.*, 2017)** showed promising results, however data in the field of avian reproduction are still missing. Therefore, it is necessary to find solutions for the potential application and use of CUR and EPI for the improvement of the quality of avian spermatozoa which are used for AI. The objective of our study was to assess the effects of two selected antioxidants (CUR and EPI) on the selected quality parameters (motility, membrane and acrosome integrity) of rooster spermatozoa.

MATERIAL AND METHODS

Sample collection and cultivation

Sexually mature (61-67 weeks old) and clinically healthy Lohmann Light breeder males (n=10) reared in a private breeding facility (Liaharenský podnik Nitra Ltd., Slovak Republic) were used in the experiments. The roosters were housed in individual cages, under a constant photoperiod of 14 h of light day and were feed

a commercial standard diet with water given *ad libitum*. Semen samples were obtained by massaging of cloaca, diluted in phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, USA) and cultured with different concentrations of CUR and EPI (1, 5, 10, 25, 50 and 100 µmol/L). Selected quality parameters (motility, membrane and acrosome integrity) were analyzed after 0, 2 and 24 h of *in vitro* culture at 4°C.

Analysis of sperm motility

For the evaluation of the sperm motility rate (%, MOT), we used the computer-aided sperm analysis (CASA, Version 14.0 TOX IVOS II., Hamilton-Thorne Biosciences, Beverly, MA, USA). All samples (10 µl) were applied in Makler's counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and analyzed immediately.

Membrane integrity

Condition of the cell membrane was detected using a combination of eosin and nigrosin dye. Eosin is mainly used to stain the cytoplasm and cytoplasmatic membrane while nigrosin is used as a contrast dye for a better differentiation of the background. The slides for the determination of the cell membrane integrity were prepared as follows: we applied a drop of sperm suspension on the slide and stained it first with 4 µl of eosin solution (Sigma-Aldrich, St. Louis, USA). Subsequently we added 4 µl of nigrosin solution (Sigma-Aldrich, St. Louis, USA). After the application of both dyes, we used another slide glass to prepare a smear. The samples were air-dried at a laboratory temperature and analyzed using a light microscope (Olympus, Tokyo, Japan) at a magnification of 40 x. On each slide we observed 100 cells to assess the percentage of live and dead cells.

Acrosome integrity

For the assessment of the acrosome integrity status, we used a double fast green-rose bengal stain technique. First of all, we applied 10 µl of sample on a glass slide and we added the same volume of fast green-rose bengal mixture (Sigma-Aldrich, St. Louis, USA). The stained samples were incubated for 60 seconds and following incubation we used another glass slide to prepare a smear which was air-dried at laboratory temperature. All slides were analyzed using a light microscope (Olympus, Tokyo, Japan), evaluating the integrity and compactness of the sperm acrosome. Damaged acrosome was characterized by a disruption of the membrane and cluster stain present in the sperm head. At least 100 cells were observed in each slide and we calculated the percentage of cells with a damaged or normal acrosome.

Statistical analysis

All data were statistically evaluated using GraphPad Prism (version 6.0 for Windows, GraphPad Software incorporated San Diego, California, USA, http://www.graphpad.com/). Differences between control and experimental groups were statistically evaluated using one-way ANOVA analysis of variance followed by the Dunnet comparison test, comparing all groups amongst each other. The results obtained from the experimental groups are expressed as percentage of the control (± S.D.). The statistical significance was set at the levels ****(P<0.0001); ***(P<0.001); **(P<0.01); *(P<0.05).

RESULTS AND DISCUSSION

Evaluation of the sperm motility

The results displayed in Figure 1 indicate that at time 0 h, the sperm motility remained without any changes. Significant changes (P<0.001; P<0.0001) were observed in the case of 50 and 100 µmol/L of CUR after 2 h of *in vitro* culture. The highest CUR concentrations decreased the sperm motility when compared to the control group. In contrast with CUR, in the case of EPI no differences were observed after 2 h of *in vitro* culture. Following 24 h of *in vitro* culture were recorded a significant (P<0.0001) preservation of the sperm motility in the case of both antioxidants at 1, 5, 10 µmol/L of CUR and 5, 10, 25, 50 µmol/L of EPI. Contrary to our results **Marouei *et al.* (2018)** observed beneficial effects of higher CUR concentrations (200 and 300 µmol/L) on rooster sperm motility parameters, viability and membrane integrity. However, the differences maybe explained by a different sample processing. **Marouei *et al.* (2018)** used post-thawed semen samples, we used fresh samples of rooster sperm. In the study of **Tvrdá *et al.* (2016)** authors used CUR against oxidative stress developed using FeAA (ferrous ascorbate) in bull spermatozoa. The doses of CUR in a concentration range of 25-50 µmol/L lead to a preservation of sperm motility (P<0.001) when compared to the control group. We may hypothesize that the application of CUR and its effects depend on the species. In our study the CUR concentration of 50 µmol/L had the opposite effect and decreased the motility of rooster spermatozoa in comparison to the control group. These findings could support the theory that lower doses between 1-10 µmol/L of CUR could exhibit higher preservation effects on fresh avian ejaculates. **Kazemizadeh *et al.* (2019)** focused on the effects of CUR under *in vivo* conditions. CUR was supplemented to the feed of twenty-eight broiler roosters at a concentration range of 10-30 mg/rooster per day from 61 weeks of age. Their study highlights the beneficial effects of increasing dietary supplementation of CUR on the sperm motility and plasma membrane integrity. Moreover, the authors used semen samples for artificial insemination and their results showed an improved (P<0.01) fertility rate in the groups supplemented with CUR in comparison with the control group. **Al-Daraji (2012)** studied the beneficial effects of grape flavonoids after *in vitro* supplementation on diluted rooster semen with 4 ml of grape juice concentrate as a source of EPI. Their results revealed that following 1, 7 and 14 days of *in vitro* storage in the refrigerator (temperature 4-6 °C) the presence of EPI significantly preserved the sperm motility (P<0.05) and improved percentage of live spermatozoa against the control group. Positive effects of EPI were also confirmed by **Abdo *et al.* (2010).** They added 1, 3 and 5% green tea extract as a source of EPI into the diet of 21 Inshas roosters. The results of this study showed an improvement of the sperm concentration and a decreased percentage of damaged and dead sperm. Taking advantage of rabbit spermatozoa **Ďuračka *et al.* (2017)** recorded positive and beneficial effects of CUR on one hand but also toxic and negative effect of this molecule on the motion behaviour. Similarly, to our report, higher doses (50 and 100 µmol/L) of CUR significantly decreased (P<0.001) the percentage (%) of rabbit sperm motility when compared to the control group. On the other hand, higher doses (100 µmol/L) of EPI exhibited significant (P<0.001) preservation effects on the motility of rabbit spermatozoa.

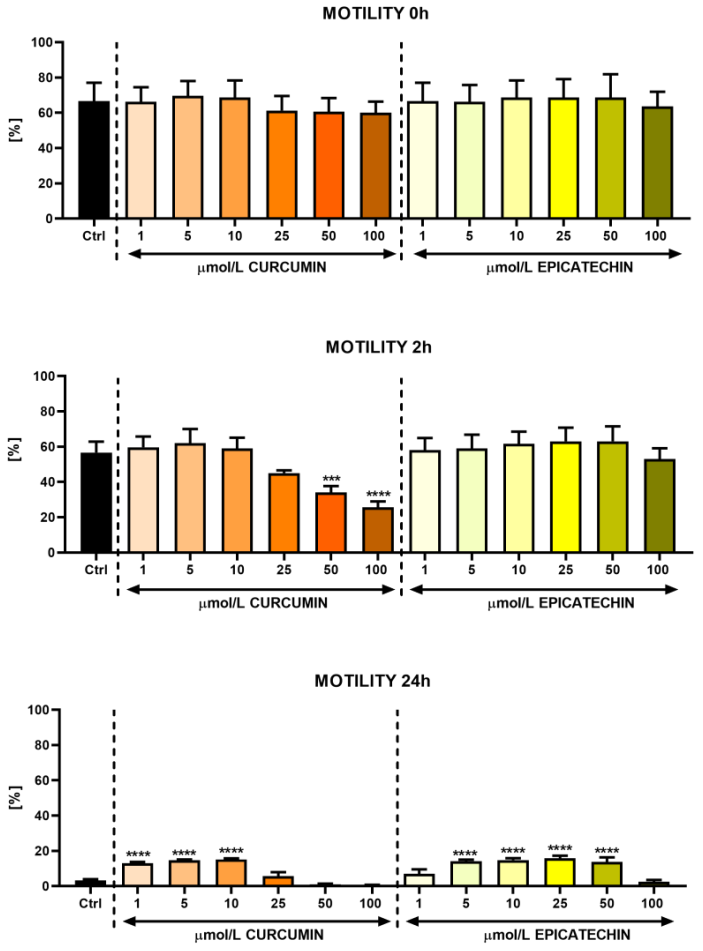

Figure 1 The effects of different concentrations of curcumin - CUR and epicatechin - EPI (1, 5, 10, 25, 50 and 100 µmol/L) on the motility of rooster spermatozoa after 0, 2 and 24 h of *in vitro* culture (***P<0.001; ****P<0.0001).

Membrane integrity

In the case of the membrane integrity (Figure 2) no changes were noticed at time 0 h of the *in vitro* culture. After 2 h of incubation, the percentage of cells with damaged membrane was significantly decreased (P<0.01; P<0.001) in the

experimental groups exposed to 5 and 10 µmol/L CUR respectively. However, in the case of 50 and 100 µmol/L CUR were observed that the percentage of cells with damaged membranes was significantly higher (P<0.0001) when compared to the control group. This finding could support our hypothesis that high doses of CUR could exhibit toxic effects on the cells instead of antioxidant actions and damage the cell membrane even more. This phenomenon was even more pronounced after 24 h of *in vitro* culture. Inversely lower doses (5 and 10 µmol/L) of CUR showed protective effects while higher CUR concentrations (50 and 100 µmol/L) had a negative impact on the cell membrane integrity. On the other hand, EPI exhibited positive effects up to 24 h of *in vitro* culture. There was a significant decrease (P<0.05; P<0.01) of cells with damaged membrane in case of 25 and 50 µmol/L EPI. According to **Yan *et al.* (2017)** *in vivo* CUR supplementation exhibited positive effects and decreased the percentage of cells with damaged membranes while at the same time it increased the viability of rooster spermatozoa. **Jalili *et al.* (2020)** observed beneficial effects of CUR on the sperm quality parameters and fertility of broiler roosters. After 5 weeks of feeding roosters with different concentrations (0-30 mg/rooster/day) of CUR the function and integrity of the sperm plasma membrane was significantly increased (P<0.05) in all CUR treated groups. These observations could be compared with different effects of CUR under *in vivo* and *in vitro* conditions. It appears that higher doses of CUR exhibited better functions under *in vivo* conditions and during long term supplementation as a food additive.

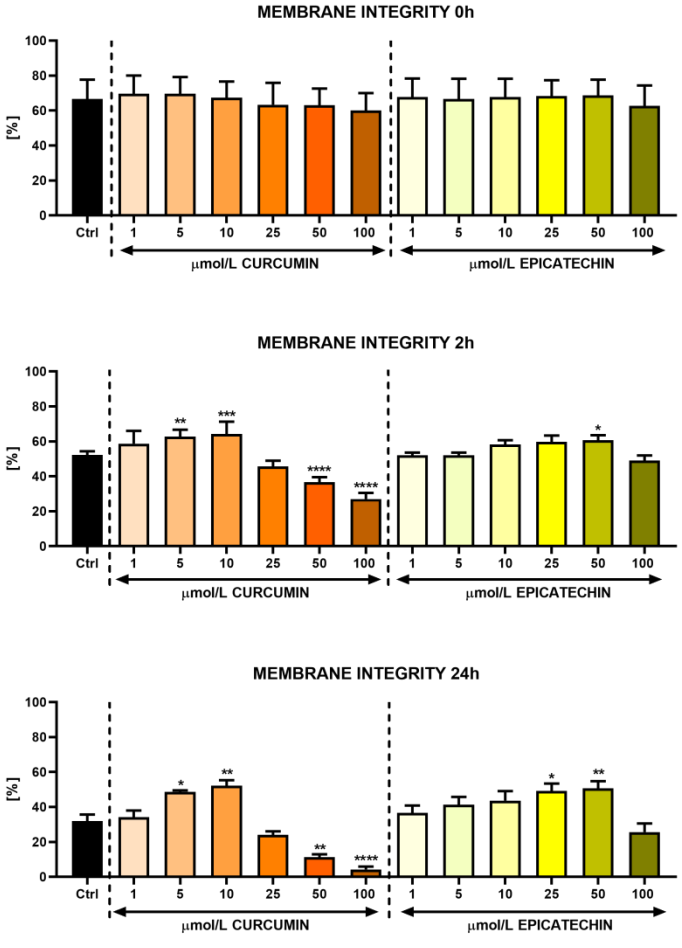

Figure 2 Membrane integrity of rooster spermatozoa after 0, 2 and 24 h *in vitro* culture with different concentrations of curcumin - CUR and epicatechin - EPI (1, 5, 10, 25, 50 and 100 µmol/L), (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In the already mentioned research of **Kazemizadeh *et al.* (2019)** CUR administration helped to increase the percentage of cells with a functional plasma membrane and to decrease the cell membrane damage in all groups with CUR supplementation. **Greifová *et al.* (2017)** evaluated the dose and time-dependent effects of EPI on bovine spermatozoa after 0, 2, 6 and 24 h of *in vitro* culture. Similarly, to our research, the concentration of 50 µmol/L of EPI showed significant beneficial changes (P<0.001) in the motility and viability of cells after 24 h of *in vitro* culture. If we would compare the doses of CUR and EPI, we could see the differences between them. In most cases CUR exhibited better effects in lower doses but under *in vitro* conditions. Under *in vivo* conditions CUR displayed better antioxidant effects in higher doses. On the other hand, EPI functioned better in higher doses and after a longer exposure time.

Acrosome integrity

According to the assessment of the acrosome integrity (Figure 3) no significant differences were observed after 0 and 2 h of *in vitro* culture. Changes were visible after 24 h of incubation. As in the previous parameters CUR showed dual effects on the integrity of the acrosome. In case of 50 and 100 µmol/L CUR were noticed a significant decrease (P<0.0001) of acrosome integrity when compared to the control group without CUR. Only 10 µmol/L CUR led to a significant increase (P<0.01) of the acrosome integrity and exhibited protective effects against oxidative stress-associated damage to the acrosome structures. Similarly, EPI displayed dichotomic effects on the acrosome integrity, as 50 µmol/L EPI significantly increased (P<0.05) the level of acrosome integrity while 100 µmol/L significantly decreased (P<0.01) the percentage of cells with a compact acrosome. **Raheja *et al.* (2018)** used 1.5 mM CUR extract as a supplement to the semen extender for the protection of bovine spermatozoa. Addition of CUR increased the progressive motility, plasma membrane integrity and percentage of cells with intact acrosomes. **Chanapiwat and Kaeoket (2015)** confirmed that lower doses of CUR exhibited better effects and increased the progressive motility, viability and acrosome integrity of cryopreserved boar semen. **Omur *et al.* (2016)** found out that antioxidants could be used as additives to freezing extenders and improve the quality of freeze thawed spermatozoa.

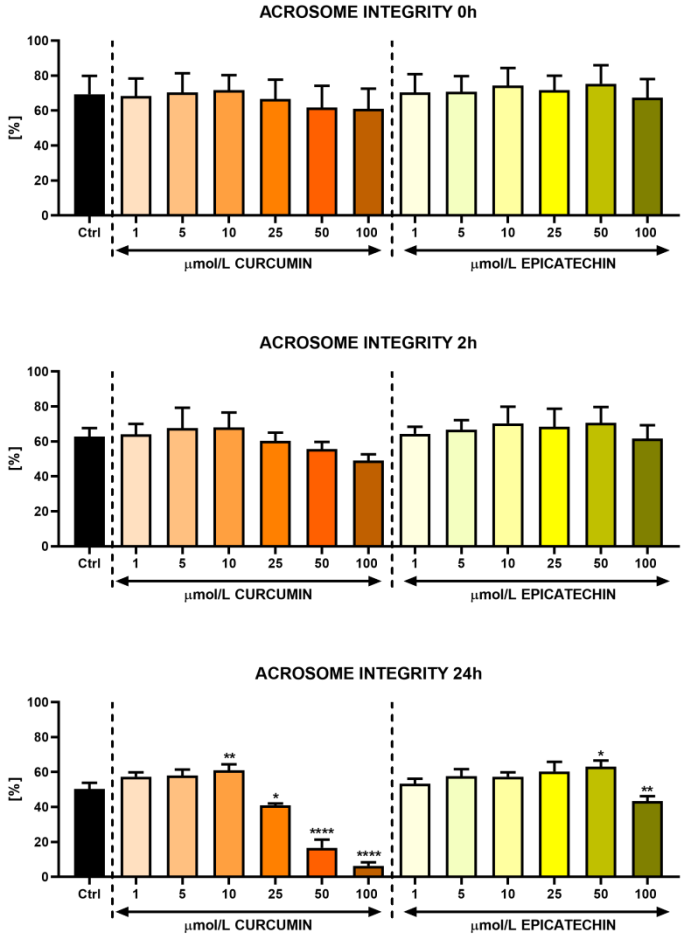

Figure 3 Acrosome integrity of rooster spermatozoa after 0, 2 and 24 h of *in vitro* culture with different concentrations of curcumin - CUR and epicatechin - EPI (1, 5, 10, 25, 50 and 100 µmol/L), (*P<0.05; **P<0.01; ****P<0.0001).

They compared all groups supplemented with 1- and 2-mM CUR to the control group. Their results indicate a significant increase (P<0.05) of the percentage of sperm acrosome integrity. The addition of catechin to an extender for boar spermatozoa preserved the sperm motility, viability and decreased the acrosome damage caused by oxidative stress **(Boonsorn *et al.*, 2010)**. The use of higher doses of catechin (50 and 100 µmol/L) provided outstanding results in all quality parameters of goat spermatozoa including the improvement of acrosome integrity

of sperm cells exposed to catechin in comparison to the control group **(Silva *et al.*, 2019)**.

CONCLUSION

Based on our results the addition of antioxidants such as CUR and EPI could have positive as well as negative effects on rooster spermatozoa. The mechanism of their effects depends on numerous factors like dosage, animal species, *in vitro/in vivo* conditions and time interval of cultivation. Our experiments confirmed the beneficial and protective effects of both antioxidants against oxidative stress and defined their optimal doses for future experimental studies on avian spermatozoa.

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