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Adding of ascorbic acid to the culture medium influences the antioxidant status and some biochemical parameters in the hen granulosa cells

[1]Capcarova M, [1]Kolesarova A, [1]Kalafova A, [1]Bulla J, [2]Sirotkin AV

[1]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Nitra, Slovak Republic, [2]National Agricultural and Food Centre, Research Institute of Animal Production, Department of Genetics and Reproduction, Nitra, Slovak Republic
E-mail: marcela.capcarova@uniag.sk

Objectives. The aim of the present study was to determine the activity of superoxide dismutase (SOD), total antioxidant status (TAS) of the hen granulosa cells, and selected biochemical parameters, including calcium, phosphorus, sodium, potassium, glucose, cholesterol, proteins, in the culture medium of granulosa cells after exposing them to ascorbic acid *in vitro* conditions.

Methods. Ovarian granulosa cells of hens were incubated with various doses of ascorbic acid (E1 0.09 mg/ml, E2 0.13 mg/ml, E3 0.17 mg/ml, E4 0.33 mg/ml, E5 0.5 mg/ml).

Results. Ascorbic acid did not manifest antioxidant potential and higher doses of ascorbic acid (0.17; 0.33 and 0.5 mg/ml) decreased the activity of SOD in granulosa cells. Vitamin application resulted in a significantly ($p<0.05$) higher accumulation of Na^+ and K^+ in culture media of granulosa cells and decreased the concentration of glucose and proteins.

Conclusion. These results indicate that ascorbic acid might be involved in the regulation of selected biochemical and physiological processes in ovarian granulosa cells.

Key words: antioxidants, ascorbic acid, biochemistry, granulosa cells, hen

Vitamins are important nutrients involved in a variety of cell functions, including mammalian reproduction, not only as cellular antioxidants, but also as modulators of many intracellular or extracellular biochemical processes (Tao et al. 2004). As an important antioxidant in extracellular fluids, L-ascorbic acid (vitamin C) plays a role in many biological processes such as oocyte maturation (Tatemono et al. 2001), biosynthesis of collagen and other components of the extracellular matrix, and is the most important antioxidant in the extracellular fluids (Warren et al. 2000). It is widely distributed in animal tissues, whereas the highest concentrations have been described in the pituitary, adrenal gland, and gonads (Luck et al. 1995). The ovarian follicular microenvironment is an important background for reproduction and development (Smolikova et al. 2012). Granulosa cells represent a major endocrine compartment of the ovary producing sex steroid hormones and are known to play a key role in the ovarian physiology (Yeh et al. 2006). L-ascorbic acid facilitated the *in vitro* maturation of porcine cumulus-free oocytes and improved the development potential (Tao et al. 2010). Some studies have documented that L-ascorbic acid may prevent follicular cells from apoptosis (Murray et al. 2001; Tao et al. 2004). The presence of high concentrations of ascorbic acid in endocrine tissues is thought to be important for the production of steroid hormones (Tsuji et al. 1989). This vitamin might also be involved in the glucose metabolism (Tao et al. 2010).

Corresponding author: Marcela Capcarova, PhD., Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Nitra, Slovak Republic; phone:+421 37 641 4343; e-mail: marcela.capcarova@uniag.sk.

Reproductive health of female could be affected by many endogenous and exogenous factors (Mlynarcikova et al. 2005). Growing interest is focused on the role of reactive oxygen species (ROS) in female reproduction rises. ROS are involved in the modulation of physiological reproductive functions (Agarwal et al. 2005) and serve as key signals in the initiation of apoptosis in antral follicles and granulosa cells by diverse stimuli and antioxidants protect against these stimuli (Devine et al. 2012). When ROS are overproduced, oxidative stress may develop in the body (Jones 2008). Superoxide dismutase (SOD) serves as front-line antioxidant defense (Scandalios 2005), reacting with superoxide anion radicals to form oxygen and H_2O_2 (Ho et al. 1998). SODs were also found in normal cycling human ovaries (Suzuki et al. 1999). SOD activity has been measured in human follicular fluids (Carbone et al. 2003; Pasqualotto et al. 2009), human granulosa luteinized cells (Dineva et al. 2011), rat (Capcarova et al. 2013; 2014), hen (Capcarova et al. 2012), and porcine granulosa cells (Capcarova et al. 2009; 2015).

Many chemically defined media used for *in vitro* cultivation of various cells contain vitamins (Tao et al. 2004). It has been published that ascorbic acid improved the quality of bovine follicles and their survival in a serum-free culture system (Thomas et al. 2001). We have previously demonstrated that ascorbic acid given to the culture cells *in vitro* in different concentrations, i.e., 0.09 mg/ml, 0.17 mg/ml, 0.33 mg/ml, 0.50 mg/ml, and 1 mg/ml, may significantly stimulate the secretion of progesterone in the hen ovarian granulosa cells (Kolesarova et al. 2014). In the same experiment, an anti-apoptotic marker Bcl-2 was also significantly stimulated, while a marker of apoptosis (caspase-3) was significantly reduced in ascorbic acid-treated groups. In this work, we are continuing in the examining the effect of ascorbic acid on the granulosa cells in hens with aim to broaden our previous data. Therefore, the attempt of the present study was to determine the response of hen granulosa cells to ascorbic acid treatments, antioxidant status of cells, and possible changes in biochemical representation of basic nutrients in the cell culture medium.

Material and Methods

Preparation, culture and processing of granulosa cells from ovaries. White Leghorn hens (n=12) about 500 days old, with an egg laying rate with more than 75%, were kept under standard conditions at the Experimental Station of the Department of Poultry and Small Animal Husbandry of the Slovak University of Agriculture in Nitra. Conditions of their care, manipulations and use did correspond to the instruction of EC no. 178/2002 and related EC documents, and they were approved by local ethic commission. Birds were decapitated between 9:00 and 11:00 and ovaries were collected and transported to the laboratory in a thermal container containing sterile saline at 37°C within 1 h post-slaughter. The ovaries were rinsed two times with ethanol and immediately with the saline for four times. The largest (F1-F2) follicles were isolated from the ovary. The stage of folliculogenesis was determined by recording the time of the last oviposit and by the size and position of the next ovarian follicle. Granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™) and 1% antibiotic/antimycotic solution (Sigma, St. Louis, Mo, USA) at a final concentration of 10^6 cells/ml of medium. Portions of the cell suspension were dispensed to 24-well culture plates (Nunc™, Roskilde, Denmark, 1 ml/well). The well plates were incubated at 38.5°C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (4 days). At this point, the medium (1 ml/well) was renewed.

Vitamin C treatment. Ascorbic acid was purchased from Sigma Chemical Co. (St Louis, MO, USA). The doses of ascorbic acid were chosen according to the literature, e.g. experiments with ascorbic acid were done on porcine oocytes (doses 0, 50, 100, 250, 500, and 750 μmol/l; Tao et al. 2010) and experience in our laboratories with some other substances given to the culture medium of granulosa cells with the reference to examined topic. Ovarian granulosa cells were incubated with the 1% antibiotic-antimycotic solution and with ascorbic acid administrations as follows: group E1 (0.09 mg/ml), group E2 (0.13 mg/ml), group E3 (0.17 mg/ml), group E4 (0.33 mg/ml), group E5 (0.5 mg/ml). Group without any supplementation served as the control (C group). Incubation was done for 18 h. The culture media from well plates were aspirated and cells were manually smashed to obtain lysate solution.

SOD, TAS and biochemical analysis. The activity of antioxidant enzyme SOD was measured in cells lysate using spectrophotometer Genesys 10 (Thermo Fisher Scientific Inc, USA) and antioxidant RANDOX kits (Randox Labs., Crumlin, UK) according to manufacturer instructions. Total antioxidant status (TAS)

was assayed in medium by ELISA using commercial kits (RANDOX, Randox Labs., Crumlin, UK). The concentrations of calcium, phosphorus, magnesium, sodium, potassium, total lipids, total proteins, glucose, cholesterol and triglycerides in culture medium of hen ovarian granulosa cells after ascorbic acid treatment were measured by semi–automated clinical chemistry analyzer Microlab 300 (Vilat Scientific, Dieren, The Netherlands), microprocessor-controlled analyzer EasyLite (Medica, Bedford, USA) and spectrophotometer Genesys 10 (Thermo Fisher Scientific Inc, USA).

Statistical analysis. Each group was represented by four culture wells of cultured granulosa cells obtained from three experiments. Assay of substances in incubation medium were performed in duplicate. The data are expressed as mean ± SEM, and analyzed by one-way analysis of variance (ANOVA) followed by Turkey´s post hoc test using Sigma Plot statistical program. The level of significance was accepted as $p<0.05$ (95% confidence interval).

Results

Effect of ascorbic acid on activity of antioxidant enzyme and TAS of hen granulosa cells. The activity of antioxidant enzyme SOD reflects the intracellular antioxidant status (Fig. 1). Incubation of hen ovarian granulosa cells with ascorbic acid resulted in slight changes ($p>0.05$) of SOD activity only in E1 and E2 groups with low doses of ascorbic acid (doses: 0.09 and 0.13 mg/ml) in comparison with the controls. Higher doses of ascorbic acid (0.17; 0.33 and 0.5 mg/ml) treatment had opposite effect. The activity of SOD decreased significantly ($p<0.05$) in E5 group when compared to the controls.

Treatment with ascorbic acid caused only slight changes ($p>0.05$) of TAS in the hen granulosa cells in comparison with the controls (Fig. 2).

Effect of ascorbic acid on selected biochemical parameters of the hen granulosa cells. Ascorbic acid application resulted in a higher accumulation of Na^+ and K^+ in culture media of granulosa cells (Table 1). Significant differences ($p<0.05$) were found in E1, E3, E4, and E5 group when compared with the controls. Slight changes were found also in E2 group in comparison with the controls, however, the differences were not significant ($p>0.05$). Increased values of Cl^- in medium of experimental groups against the controls were observed after ascorbic acid exposure. Significant differences ($p<0.05$) were found in E1 and E4 groups when compared to the controls. The content of proteins in culture medium was the highest in the control group. The adding of ascorbic acid to the culture medium caused decrease of protein accumulation in medium in all experimental groups in comparison with the controls. All results were significant ($p<0.05$). Similar results were found also in the case of the content of glucose in the culture medium of granulosa cells. In experimental groups E1, E3, E4, and E5 the values of this parameter were significantly ($p<0.05$) lower in comparison with the controls.

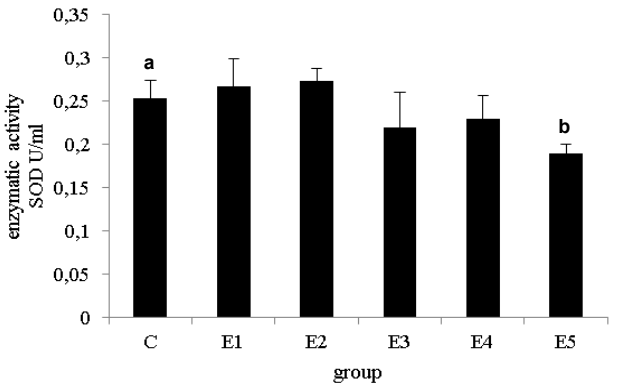

Fig. 1. Effect of different doses of ascorbic acid on SOD activity in hen granulosa cells. Cells were incubated in absence (C group) or presence of vitamin (E1 0.09 mg/ml, group E2 0.13 mg/ml, group E3 0.17 mg/ml, group E4 0.33 mg/ml, group E5 0.5 mg/ml). Results are expressed as mean ± SEM, a-b means significant differences among the groups ($p<0.05$, one-way ANOVA).

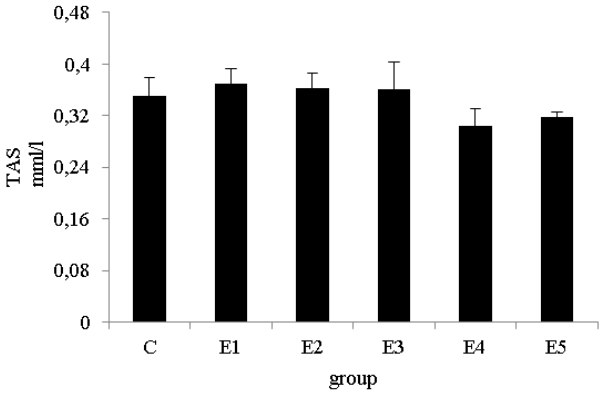

Fig. 2. Effect of different doses of ascorbic acid on TAS of hen granulosa cells culture medium. Cells were incubated in absence (C group) or presence of vitamin (E1 0.09 mg/ml, group E2 0.13 mg/ml, group E3 0.17 mg/ml, group E4 0.33 mg/ml, group E5 0.5 mg/ml). Results are expressed as mean ± SEM, the results were not significant ($p>0.05$, one-way ANOVA).

Table 1

Effect of different doses of ascorbic acid on biochemical parameters in culture medium of hen granulosa cells

Parameter (mmol/l)	C	E1	E2	E3	E4	E5
Na^+	160.65±2.39[a]	188.13±5.60[b]	204.88±5.44	188.78±9.14[b]	173.20±3.34[b]	204.78±3.86[b]
K^+	4.71±0.07[a]	5.16±0.11[b]	5.49±0.09	5.20±0.16[b]	4.90±0.06[b]	5.44±0.06[b]
Cl^-	139.56±1.77[a]	157.56±4.66[b]	171.70±4.15	158.05±7.04	146.53±2.59[b]	173.18±3.44
TP	11.04±0.18[a]	9.50±0.14[b]	9.16±0.17[b]	9.75±0.28[b]	9.99±0.15[b]	9.04±0.13[b]
GLU	14.3±0.37[a]	12.15±0.66[b]	11.68±0.26	11.45±0.36[b]	12.75±0.25[b]	11.23±0.45[b]

Na+ – sodium, K+ – potassium, Cl- – chlorides, TP – total proteins, GLU – glucose
Cells were incubated in absence (C group) or presence of vitamin (E1 0.09 mg/ml, group E2 0.13 mg/ml, group E3 0.17 mg/ml, group E4 0.33 mg/ml, group E5 0.5 mg/ml). Results are expressed as mean ± SEM; a-b means significant differences among the groups ($p<0.05$, one-way ANOVA).

The adding of ascorbic acid had no effect on the other biochemical parameters and the values between the groups remains insignificant ($p>0.05$, data not presented).

Discussion

Antioxidant vitamins are notable constituents of the ovary (Zreik et al. 1999). The hen ovulatory cycle is characterized by alterations in plasma levels of ovarian steroid hormones and luteinizing hormone (Yu et al. 1996). Our previous studies performed on the hen granulosa cells (Kolesarova et al. 2014) have revealed that ascorbic acid may significantly stimulate the secretion of steroid hormones by ovarian granulosa cells of hens. Based on these results, our study aimed to investigate the changes in the antioxidant status and accumulation of some biochemical elements in culture medium of the hen granulosa cells after exposing them to the ascorbic acid *in vitro*.

SOD is an important antioxidant enzyme responsible for the elimination of superoxide radical (Hu et al. 2005). In our study, significant decrease in SOD activity was found in groups with the highest dose of ascorbic acid (0.5 mg/ml) against the control. Significant increase in SOD activity in porcine granulosa cells was measured also in our previous studies with natural antioxidants, quercetin (Capcarova et al. 2015) and resveratrol (Petruska et al. 2012). Antioxidant potential of ascorbic acid was manifested only in lower doses. Increase of ascorbic acid doses had opposite effect. Thus, higher doses of vitamin can contribute to the elevating of oxidative stress and overproduction of ROS. It seems that the balanced dose of natural antioxidants as well as vitamins after giving to the organism must be precisely set due to reason that an excessive dose increase might change the expected results and the treatment with those substances became ineffective or harmful for the organism. It has been observed that each organism needs produce only a certain amount of free radicals that are used as signal molecules and have many beneficial physiological functions (Watson 2014). Therefore, targeted elimination of ROS without deep understanding of physiological status can lead to a disturbance in internal balance in the organism.

The antioxidant properties of various biological fluids are evaluated either by quantification of individual antioxidants or by assessing their aggregate, cumulative action, and synergic effect. This latter concept is known as the total antioxidant capacity or status (TAC or TAS) (Fingerova et al. 2007). TAS represents the level of cumulative antioxidant reserve of the body and enables evaluation of the average antioxidant potential. Thus, the overall antioxidant status will give more relevant biological information compared to that obtained by the measurement of individual components (Miller et al. 1993). In our study, ascorbic acid had no effect on TAS of hen granulosa cells.

In this study, significantly higher content of Na^+ and K^+ was found in the culture medium in ascorbic acid-treated groups against the control. An energy and Na^+-dependent ascorbic acid transporter in rat luteal and granulosa cells and in human granulosa-lutein cells has been found (Behrman et al. 1996; Musicki et al. 1996; Zreik et al. 1999). Incubation of granulosa-lutein cells in a low Na^+ medium significantly inhibited ascorbic acid uptake. Transport of ascorbic acid by human granulosa-lutein cells involves an active process that depends on a sodium gradient established by the Na^+/K^+ ATPase system (Zreik et al. 1999). Our study suggests that Na^+ and K^+ are involved in the homeostatic

and biochemical processes modulated by ascorbic acid addition to the culture medium of the hen granulosa cells. The detailed mechanism of action has not been described yet. Dramatic decrease in the intracellular K^+ concentration has been found during apoptosis of granulosa cells (Gross et al 2001).

Glucose is a critical energy providing substrate for many cellular processes through its catabolism to produce ATP (Munoz-Gutierrez et al. 2004). It has been found as a preferred energy substrate to support the gonadotropin-induced differentiation of ovine granulosa cells *in vitro* (Campbell et al. 2010). Our results revealed decrease in the glucose and proteins contents in the culture medium of the hen granulosa cells after ascorbic acid treatment what is probably also connected with the granulosa cell physiological processes during differentiation and maturation. Significant increase in the glucose and protein contents in buffalo and sheep follicles have been found as follicles became larger (Nandi and Kumar 2008). The requirement for glucose to induce cellular differentiation of granulosa cells was shown in *in vitro* study on sheep granulosa cells (Campbell et al. 2010).

In conclusion, ascorbic acid is an important vitamin affecting many physiological processes. However, the dose given to the organism or cells must be designed precisely. Excessive increase of the doses can have opposite effect, i.e. distinct from the expected one. In our study, higher doses caused significant decrease in the SOD activity. Lower doses had no effect on the hen granulosa cells. In this case, ascorbic acid did not display antioxidant potential in culture granulosa cells. Adding of ascorbic acid caused an increase in Na^+ and K^+ concentration in the culture medium. On the contrary, concentration of glucose and proteins in the medium decreased after ascorbic acid treatment. The results correlated with biochemical and physiological processes in granulosa cells and with the decrease in the apoptotic cell occurrence, as it has been revealed by the previous data of our laboratory.

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