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Determination of antioxidants in bovine oviduct epithelial cell culture isolated at different periods of the estrous cycle

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Abstract: Oxidative stress interferes with oviduct functions including oocyte maturation, capacitation, fertilization, and embryo and gamete transport. This study aimed to determine activity of the antioxidants glutathione peroxidase (GPX-I), superoxide dismutase (SOD), and catalase (CAT) in bovine oviduct epithelial cells (BOEC) isolated from the isthmus and ampulla of the oviduct at estral (n = 7) and luteal phases (n = 7) of the estrous cycle. The antioxidant activity was measured at the primary, first, and second passages of the cell culture, and was characterized by cytokeratin expression. The GPX activity increased over the passages in samples of the ampulla and the isthmus of each sexual phase without statistical significance. The SOD activity remained steady through the cell passages in both sexual phases. CAT activity at the primary culture was higher in the ampulla compared to the isthmus in both sexual phases with a significant difference for the estral phase ($P < 0.05$), and it decreased over the passages with no significant differences. In conclusion, the antioxidant enzyme activity profile of BOEC did not differ by region or sexual cycle except for that of CAT, which was higher in the ampulla. Further studies should focus on SOD, GPX, and CAT activity for the mechanism of BOEC adaptation to an in vitro environment.

Key words: Ampulla, bovine oviduct epithelial cell, catalase, glutathione peroxidase, isthmus, superoxide dismutase

1. Introduction

The oviduct creates optimal conditions for oocyte maturation, sperm capacitation, fertilization, and embryo and gamete transport (1). The different segments of the oviduct vary by means of effects on spermatozoon functions. The isthmus is the section of the oviduct which serves as a reservoir for sperm, while the ampulla is known as the fertilization area (2). The oviduct epithelial lining is composed of two kinds of cells: ciliated and secretory cells. The ciliated cells play a role in oocyte, spermatozoon, and embryo transport, while secretory cells secrete substances which are important in sperm function and early embryonal development (3). While the oviduct epithelium improves the vitality and motility of the sperm, it also fosters an environment for sperm capacitation (4). The secretions of oviduct epithelial cells vary over the cycle (2). Ovarian-derived steroids regulate oviduct physiology and reproductive events in the oviduct (5). Both estrogen and progesterone receptors are found in the bovine oviduct epithelium. Gamete maturation and breeding occur during estrogen-dominant phases of the cycle, while fertilization, early stages of clefts, and morphogenesis of morula occur in the phases of the cycle when progesterone levels increase (5). Hormonal changes in the mammalian reproductive cycle affect protein secretions, oviduct fluid volume, and electrolyte content of the oviduct lumen (6).

In vivo and in vitro studies have shown that the completion of physiological processes depends on the balance between reactive oxygen radicals and antioxidants in the oviduct which play a critical role in reproduction (1). In a healthy organism, reactive oxygen species (ROS) and antioxidants are in balance. Oxidative stress occurs when this balance deteriorates in the direction of an increase in ROS levels (7) or when the antioxidant defense mechanism decreases (8,9). Reactive oxygen species production is controlled by various antioxidants (1) that inhibit the formation of free radicals and damage to the body. Antioxidants can be classified as enzymes or nonenzymes (10). One of the best known of these enzymes is superoxide dismutase (SOD), which is responsible for the conversion of free oxygen radicals to H_2O_2, which is an extremely toxic entity and which must be removed immediately. Both glutathione peroxidase (GPX) and catalase (CAT) make H_2O_2 harmless by reducing it to water

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and oxygen (11). Glutathione peroxidase has at least 4 isoforms; the isoform GPX-1 is commonly found in many tissues (12). The superoxide dismutase enzyme catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen and protects cells from the harmful effects of free radicals of superoxide (10). Copper–zinc–SOD (CuZnSOD) in particular is expressed in cytosol (12). Catalase enzyme breaks up hydrogen peroxide into oxygen and water in peroxisomes pieces (10).

The balance between ROS and antioxidants positively contributes to sperm function, oocyte maturation, fertilization, and in vitro embryo development (13). Conversely, low-level lipid peroxidation, which reflects ROS activity, has been suggested to improve sperm metabolism and increase attachment of the zona pellucida in humans (14). The increase in oxidation causes insufficiency in the reproductive process. Antioxidant reactions play an important role in reducing the level of reactive oxygen species, with sustained protection and reproduction of gamete quality (15). Hydrogen peroxide has a toxic effect on sperm even at low concentrations. In addition to reduction in sperm viability, it also inhibits acrosome reaction, sperm oocyte binding, and oocyte penetration (16). An in vitro study determined that ROS concentrations, including those of superoxide anions and hydrogen peroxide, were critical for sperm capacitation and acrosome reaction in cattle, as low concentrations increased sperm binding to the zona pellucida (17). On the other hand, higher ROS concentrations including that of hydrogen peroxide reduced sperm motility in bulls and interfered with fertilization as well as embryo development (1). It has been reported that low levels of ROS are important in sperm capacitation, but peroxidative damage occurs in the sperm plasma membrane when ROS levels are excessive, and eventually causes DNA fragmentation in the nucleus (18–21). In addition, it has been reported that high concentrations of hydrogen peroxide reduce sperm motility in vitro (22), and attenuate fertilization and embryo development (1). Reactive oxygen species have been reported to have beneficial effects as well as harmful effects on reproduction (1). One of the most significant problems with reproduction in cattle is premature embryonic death, which constitutes up to 40% of reproductive losses (23). In this sense, oxidative stress has been reported to have a negative effect on blastocyst ratio, limiting in vitro embryo development (24)

Assisted breeding technologies, especially in cattle breeding, are inexpensive techniques compared to in vivo investigation. They also enable us to understand the relationship between gametes and mothers, as well as between mother and embryo to some degree. Thus, in vitro models of embryo–maternal communication are important, as they provide valuable information without use of any animals (25). Although many somatic cells have been used for embryo cultures, the most commonly used one is bovine oviduct epithelial cell (BOEC) culture, which is useful for the development of embryos during in vitro maturation and in vitro fertilization (26,27). An in vitro study by Cordova et al. (28) claimed that use of BOEC increased the blastocyst rate and quality. One of the mechanisms of action of BOEC to support embryo development is that BOEC fights against antioxidative enzymes including SOD, CAT, and GPX (28). Another mechanism is that BOEC synthesizes growth factors, embryothropic proteins such as OVGP1, and other factors (25,29,30). In addition to synthesis of such factors, BOEC cells also express a scavenging ability to remove toxic metabolites (31). Cultured BOEC cells exhibit a variety of secretory activities; it is reported that these secretory contents may affect sperm function and early embryonic development. Monolayer BOEC cultures are used as coculture for in vitro preimplantation of bovine embryos (3). BOEC is also used as coculture in cattle embryos as well as in embryos of other species (31).

To our knowledge, there have been no studies on determining antioxidant activity in primary and passaged BOEC. The aim of this study was to characterize BOEC cultures and determine antioxidant levels in primary and passaged BOEC cultures isolated from different regions of the oviduct during estral and luteal phases.

2. Materials and methods

2.1. BOEC isolation and primary cell culture

The oviduct samples were collected from a total of 14 cows, with 7 in estral and 7 in luteal phases. After ovaries were transported from the slaughterhouse to the laboratory, they were macroscopically examined for the presence of morphologies related to the sexual cycle (6,32). The respective oviducts were transferred to the laboratory in a sterile transport solution (penicillin–streptomycin and DMEM [Dulbecco's Modified Eagle Medium; Biological Industries, Cromwell, CT, USA, Lot no: 1734711]; Fetal Bovine Serum [FBS; Biological Industries, Cromwell, CT, USA, Lot no: 1624434]), and then the ampulla and the isthmus regions were collected separately. Upon opening the lumen of the oviduct sections, the mucosal epithelial lining was scraped mechanically, then homogenized with DMEM containing 10% FBS and transferred to culture dishes and incubated in a humidified chamber conditioned at 37 °C with 5% CO_2. The culture medium was changed every 3 days. Cells in culture dishes with approximately 90% confluence were passaged.

2.2. BOEC characterization by immunohistochemistry

The cell cultures were followed with an inverted microscope (Leica DMI600B, Wetzlar, Germany). Immunocytochemistry was conducted upon full

confluence of cells with cytokeratin-5, revealing that cells were of epithelial origin. Immunoperoxidase stains were performed using a commercial kit (Thermo Fisher Scientific, Waltham, MA, USA). All stages were performed in accordance with the kit protocol. Briefly, cells were seeded on coverslips and incubated with acetone for 2 min for fixation. The endogenous peroxidase activity was then quenched with 0.3% hydrogen peroxide solution in methanol. To prevent nonspecific bindings, fixed cells were incubated with normal goat serum. The cells were then treated with polyclonal cytokeratin-5 antibody (Abcam 194135; 1/200) and stained with HRP-labeled secondary antibody. After incubation with AEC chromogen (Thermo Fisher Scientific, Waltham, MA, USA), tissues were covered with a water-based adhesive. After each step, cells on coverslips were gently washed 3 times. Without considering the staining intensity, cytokeratin immunopositive cells were counted under a light microscope (Nikon Eclipse 50i, Tokyo, Japan), and an immunopositive cell ratio was determined. For positive control, bovine oviduct tissue was used. For negative control, the primary antibody was not omitted in a cell seeded coverslip.

2.3. Measurement of antioxidant enzymes activities

Commercial ELISA test kits for CAT (Cat. No. 707002, Cayman Chemical Co., Ann Arbor, MI, USA), for SOD (Catalog No. 706002, Cayman Chemical Co.) and for GPX (Catalog No. 703102, Cayman Chemical Co.) were used to determine their activities. Cells were centrifuged at 2000 rpm for 10 minutes at 4 °C. For adherent cells, a laboratory rubber policeman was used rather than proteolytic enzymes to harvest cells. Cell pellets were homogenized in cold buffer (PBS; Amresco, Solon, OH, USA). They were then centrifuged at 10,000 rpm for 15 minutes at 4 °C. Supernatant fluid was collected into 2-mL Eppendorf tubes and stored at –80 °C for antioxidant enzyme analysis. CAT, GPX, and SOD activity was determined using a microplate reader (Powerwave, Biotek, Winooski, VT, USA) and run in duplicate with the diagnostic commercial test kits mentioned above.

2.4. Statistical analysis

The biochemical data were compared based on the oviduct region and phase of the sexual cycle. Comparison was also made among passages. GPX, SOD, and CAT parameters were analyzed with the Shapiro–Wilk test for normal distribution. To reveal significance differences between the ampulla and isthmus, one-way ANOVA test was used. To compare estral and luteal phases, two independent sample t-tests (Student's t-test) were applied. Data for GPX did not exhibit a normal distribution; thus, the nonparametric Kruskall–Vallis test was used. A P-value of <0.05 was considered statistically significant. Data are given as mean ± standard mean of error.

3. Results

3.1. BOEC culture

The epithelial cells were observed on the cell culture in the flask with an inverted microscope. The secretory cells began to attach to the bottom of the flask at the third day of the culture (Figure 1A) and almost all of the cells had attached by the fifth day. In both the estral and luteal phases, ciliated cells were observed as they formed islets that were moving in all directions (Figures 1B–1C). Those movements were faster in the estral phases. Ciliary cell movement was much more pronounced in samples of the ampulla. When the samples were kept cold for a longer time with a prolonged transportation time, the movement by ciliary cells was negatively affected. In general, ciliary cell motility was observed until the eighth day of the culture. The secretory cells exhibited cytoplasmic granules, which were more pronounced in cells isolated from the ampulla (Figure 1D). Vacuoles became more apparent at the 16th day of the culture (Figures 1E–1F).

Cells in passaged cultures appeared with different morphologies, such as round and spindle shapes (Figures 2A and 2B). Cells with vacuoles were rarely found.

3.2. Cytokeratin-5 immunocytochemistry

Cytochemistry for cytokeratin-5 conducted on the 11th day of the primary BOEC culture revealed that approximately 90% of the cells were immunopositive without any significance for the oviduct regions or the phases. For instance, 85.33 ± 11.59% and 84 ± 14.84% of cells isolated from the ampulla expressed cytokeratin-5 for the estral and luteal phases, respectively (Figures 3A and 3B).

3.3. Antioxidant enzymes

All data measured are given in the Table. Glutathione peroxidase activity in the primary BOEC culture was very similar between the estral and luteal phases as well as between the oviduct regions. At the estral phase, GPX activity in BOEC isolated from the isthmus and the ampulla was 118.07 ± 30.55 nmol/min/mL and 117.70 ± 30.62 nmol/min/mL, respectively. At the luteal phase, GPX activity in BOEC isolated from the isthmus and the ampulla was 119.34 ± 34 nmol/min/mL and 122.25 ± 30.36 nmol/min/mL, respectively. As the number of cell passages increased, GPX activity increased, but the increases were not statistically significant.

Catalase activity in primary BOEC culture was very similar in both estral and luteal phases, but it was higher in the ampulla. The difference was significant in BOEC cells isolated at the luteal phase ($P < 0.01$). Catalase activity decreased throughout the passages, and the decline was significant in BOEC isolated from the ampulla at the estral phase ($P < 0.05$). At the estral phase, CAT activity in BOEC isolated from the isthmus and the ampulla was

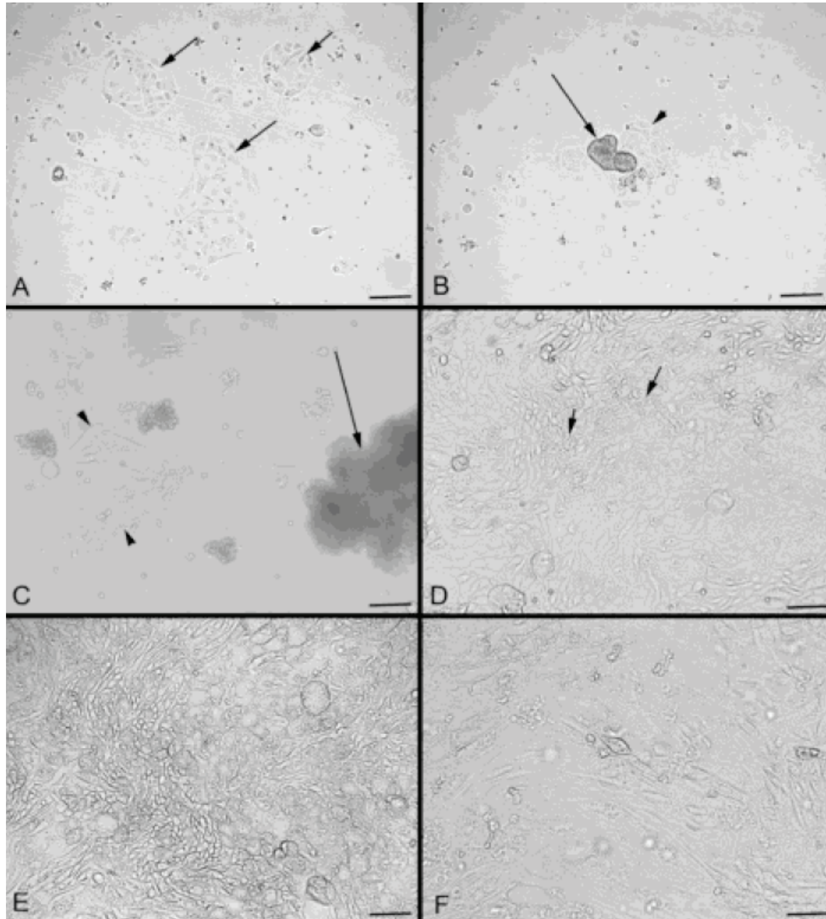

Figure 1. Microscopic pictures of the primary bovine oviduct epithelial cell (BOEC) culture. A) Secretory cells begins to attach at the third day of the culture (arrows). B) While secretory cells attach (arrowhead), ciliated cells form cell islets (arrows) and moves around. C) Ciliated cells (arrows) are still motile at the fifth day of culture. D) At the 9th day of culture, granules and vacuoles begins to be prominent. E) Vacuoles becomes much more prominent by the 16th day of culture. F) Similar observations are present in samples of the estral phase. Luteal phase (A, B, C, D, and E) and estral phase (F). Bar = 140 µm (A, B, D, and E) and 80 µm (C and F).

3.50 ± 0.57 nmol/min/mL and 6.79 ± 0.95 nmol/min/mL, respectively. At the luteal phase, CAT activity in BOEC isolated from the isthmus and the ampulla was 3.03 ± 0.71 nmol/min/mL and 5.76 ± 1.99 nmol/min/mL, respectively.

Superoxide dismutase enzyme activity in primary BOEC culture was similar in both estral and luteal phases as well as between the oviduct regions (Table). The SOD activity remained steady and comparable to those of the primary culture. At the estral phase, SOD activity in BOEC isolated from the isthmus and the ampulla was 9.40 ± 0.84 U/mL and 9.40 ± 0.50 U/mL, respectively. At the luteal phase, SOD activity in BOEC isolated from the isthmus and the ampulla was 9.73 ± 0.65 U/mL and 9.59 ± 0.44 U/mL, respectively.

4. Discussion

The oviduct provides a favorable environment for early embryonic development (33), fertilization, and sperm capacitation (34). It has been suggested that the different regions of the oviduct and the stages of the estrous cycle affect sperm physiology. It has been reported that in vitro fertilization rates of bovine oocytes with spermatozoa incubated with ampullar oviduct fluid of nonluteal phases are higher than luteal ampullar or nonluteal isthmic oviduct fluid (4). It has been observed that in vitro fertilization of bovine oocytes results in a species-specific developmental block in the 8–16 blastomeric stage (35). Antioxidant enzymes and other proteins minimize oxidative damage. The sources of antioxidants in the oviduct include embryo and oviduct epithelial cells (36,37). In the present study, BOEC exhibited activity of the well-known antioxidants GPX, SOD, and CAT. Not only the primary BOEC culture but also the first and second passages exhibited antioxidant activity to varying degrees. Thus, we think that BOEC has a strong antioxidant synthesis ability to fight ROS even in a very rigorous environment.

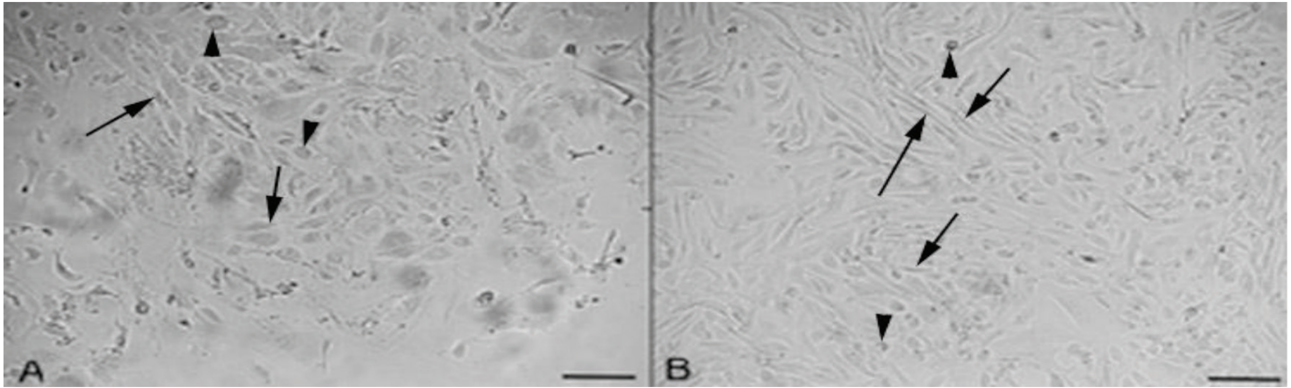

Figure 2. Microscopic pictures of bovine oviduct epithelial cell (BOEC) culture at the first passage. A and B) The passaged cells did not exhibited vacuoles. Pictures of BOEC in luteal phase at the 8th day of culture (A) and estral phase at the 22nd day. In cell passages of both phases, cells appeared in different morphologies, e.g., round (arrowheads) and spindle (arrows). Bar = 80 µm (A and B).

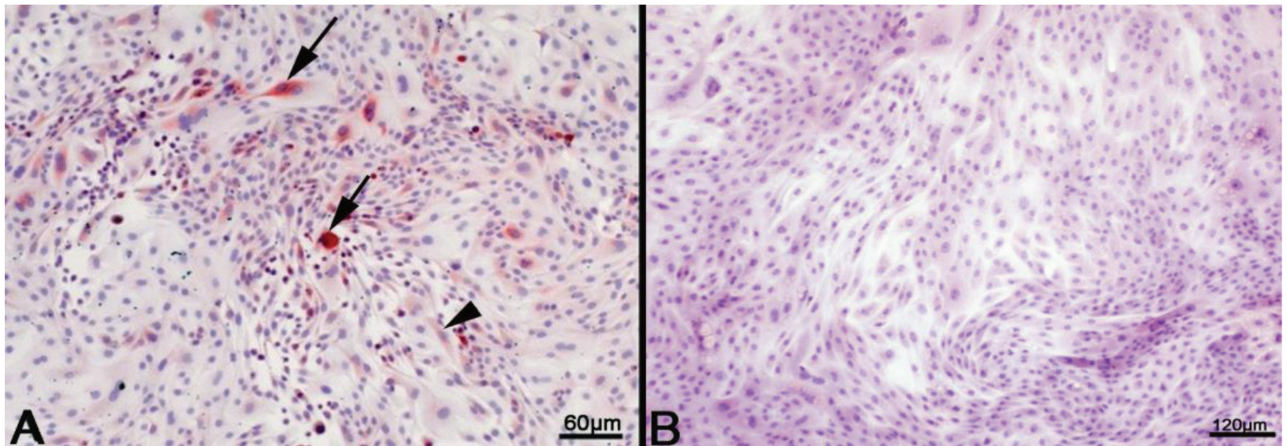

Figure 3. Cytokeratin immunoreactivity in bovine oviduct epithelial cell (BOEC) culture. A and B) Approximately 90% of BOEC expresses cytokeratin immunoreactivity of varies intensities. Some cells are intensely immunoreactive while the majority of cells are relatively less intensely immunoreactive.

The levels of various antioxidants may differ by region of the oviduct as well as by the phases of the sexual cycle. Lapointe and Bilodeau (13) have studied mRNA and enzymatic activity of antioxidants at different regions of the bovine oviduct throughout the estrous cycle. They observed regional differences for GPX while they found a homogeneous expression for CAT and SOD along the oviduct. GPX and CAT activity increased toward the end of the cycle (13). In our study, there was a fluctuation in antioxidant activities in BOEC cell cultures. The CAT activity was higher at the primary BOEC culture of the ampulla compared to that of the isthmus. La Pointe et al. (16) reported that CAT activity increased during the estrous cycle and reached maximum level before ovulation. They also argued that there was no significant difference between activity levels in the isthmus and ampulla (16). However, our study found that the CAT activity in the primary BOEC was higher in the ampulla compared to the isthmus. The difference was significant in the estral phases of the cycle, supporting the findings of La Pointe et al.'s study (16). In the meantime, the CAT activity was higher in BOEC of both regions at the estral phase compared to those of the luteal phase, but not significantly. The higher CAT activity in the ampulla, which is the fertilization site, suggests that CAT may play a greater role in physiological processes related to fertilization. In addition, the CAT activity decreased over BOEC passages and the decrease was significant in BOEC prepared from the ampulla of the estral phase. This suggests CAT activity should be investigated further with respect to BOEC adaptation to in vitro conditions.

Our study found that the SOD activity levels did not change either by regions of the oviduct or by phases of the sexual cycle. It did not even change with the passages. The molecular study by Lapointe and Bilodeau (13) claimed that SOD remained constant in BOEC. The lack of

Table. The antioxidant levels in primary and passaged bovine oviduct epithelial cell culture (BOEC).

Antioxidants	Phase	Oviduct region	Primary BOEC	1st passage	2nd passage	Level of significance
GPX (nmol/min/mL)	Estral	Isthmus	118.07 ± 30.55	110.25 ± 29.56	126.62 ± 32.85	P > 0.05
		Ampulla	117.70 ± 30.62	123.34 ± 28.68	128.80 ± 30.82	P > 0.05
		Level of significance	P > 0.05	P > 0.05	P > 0.05	
	Luteal	Isthmus	119.34 ± 30.34	121.89 ± 31.32	143.36 ± 37.89	P > 0.05
		Ampulla	122.25 ± 30.36	123.71 ± 29.65	134.44 ± 35.41	P > 0.05
		Level of significance	P > 0.05	P > 0.05	P > 0.05	
CAT (nmol/min/mL)	Estral	Isthmus	3.50 ± 0.57	2.46 ± 0.34	2.26 ± 0.57	P > 0.05
		Ampulla	6.79 ± 0.95	4.24 ± 0.93	2.55 ± 0.42	**P < 0.05
		Level of significance	*P < 0.01	P > 0.05	P > 0.05	
	Luteal	Isthmus	3.03 ± 0.71	2.76 ± 0.56	1.89 ± 0.24	P > 0.05
		Ampulla	5.76 ± 1.99	3.06 ± 0.70	2.72 ± 0.68	P > 0.05
		Level of significance	P > 0.05	P > 0.05	P > 0.05	
SOD (U/mL)	Estral	Isthmus	9.40 ± 0.84	8.61 ± 0.26	8.48 ± 0.41	P > 0.05
		Ampulla	9.40 ± 0.50	8.61 ± 0.22	9.00 ± 0.58	P > 0.05
		Level of significance	P > 0.05	P > 0.05	P > 0.05	
	Luteal	Isthmus	9.73 ± 0.65	8.67 ± 0.38	9.40 ± 0.24	P > 0.05
		Ampulla	9.59 ± 0.44	9.13 ± 0.38	9.00 ± 0.58	P > 0.05
		Level of significance	P > 0.05	P > 0.05	P > 0.05	

Note: * Indicates regional differences between the regions for the same sexual phase just for one antioxidant mentioned.
** Indicates differences in the same row. Data in the same row compare the differences among passages of BOEC isolated from the mentioned region of the sexual phase indicated.

variability suggests that SOD may have a limited effect on cellular adaptation to in vitro conditions or vice versa (13).

GPX is one of the major enzymes that protects mammalian cells, including sperm, ovum, and embryo. It is expressed not only by sperm and embryo but also by BOEC (37). Our study also found that GPX-1 activity was determined in BOEC cells isolated from the oviduct ampulla and isthmus of different phases of the sexual cycle. Unlike SOD, the increased GPX activity over the passages suggests that GPX-1 may have a powerful effect on BOEC adaptation to in vitro conditions or vice versa. Additionally, GPX-1 activity did not differ by region of the oviduct or phase of the sexual cycle. In a PCR study on oviduct tissues, Lapointe et al. (38) claimed that GPX-4 is limited in the bovine oviduct epithelial cells, but increases at the estral phase in response to estrogen. Thus, a future study should be designed to reveal expression of different GPX varieties including GPX-2, GPX-3, and GPX-4 in primary and passaged BOEC cultures.

The secretory cells of the oviduct mucosa contribute to reinstatement of optimal conditions for maturation of gametes, fertilization, and early embryonic development through synthesis and secretion of various molecules and substances (3). Presence or absence of spermatozoon or oocyte in the oviduct affects secretion modality of specific proteins, and the oviduct responds to spermatozoon and oocyte differently. For example, the presence of sperm and oocytes in oviducts has been implicated in regulation of SOD synthesis (39). Absence of sperm, oocyte, or embryo can be considered as one of the shortcomings of the present study. Our study did not evaluate antioxidant activity in the primary BOEC and passages in the presence of sperm, ovum, or embryo. Such a study may provide useful information regarding their mutual effects.

Cytokeratin is a well-known epithelial marker, and is used to characterize BOEC (40). In the present study, we also characterized BOEC by cytokeratin immunocytochemistry expression. While some cells showed intense staining, some of the cells expressed relatively weak staining. Without taking the staining intensity into consideration, we recognized that the majority of cells were cytokeratin immunoreactive in BOEC culture assayed at the 11th day of the culture. We also determined that there were no regional or sexual period effects on cytokeratin expression in BOEC. Some cells were intensely immunoreactive while most cells were

relatively less intense for cytokeratin expression. We think that this condition may be related to BOEC adaptation to 2-dimensional cultures.

The cell culture conditions, including cell culture medium and enzymes used, most likely affect ROS or antioxidant concentrations (41). Not only the cells but also other structural elements including basal membrane and connective tissue should be considered in the production of antioxidants. Another important factor that may significantly affect their activity is the presence of hormones. It has been known that treatment of BOEC with estradiol alone or in combination with progesterone increases the quality of embryos produced in vitro (42). Thus, further studies should be designed to mimic an in vivo environment with the presence of hormones at specific phases.

In the present study, the passaged BOEC appeared in various morphologies. This study did not specifically investigate which cell morphology contributes to which specific antioxidant concentrations. In the meantime, the presence of various cell morphologies might be related to cell attachment processes to the bottom of the flask and an increase in the number of cells due to cell division. Absence or rare appearance of cells with vacuoles in passaged BOEC is most likely related to cell death.

In conclusion, the antioxidant enzyme activity profile of BOEC, characterized by cytoketarin-5 immunoreactivity, does not differ by region or the phase of the sexual cycle except for CAT, which is higher in samples of the ampulla. Further studies should focus on SOD, GPX, and CAT activity for the mechanism of BOEC adaptation to an in vitro environment. In addition, cytokeratin expression in BOEC is not influenced by the region of the oviduct or phase of the cycle.

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