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ACTIVITY OF ANTIOXIDANT ENZYMES AND CONCENTRATION OF MN, CU, ZN, AND PROTEIN IN PORCINE FOLLICULAR FLUID

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Abstract. Oxidative metabolism is essential for the gamete and the embryo energy production and is unavoidably associated with generation of reactive oxygen species (ROS). Enzymatic antioxidant defenses are present in the mammalian oocytes, embryos and follicular fluid (FF). An addition of porcine FF to maturation media have positive effects on the IVM and IVF results. The aim of this study was to study the CAT, SOD and GSH-Px activity, as well as Cu, Mn, and Zn concentration in porcine FF collected from the left and the right ovary. The ovaries were collected from 77 gilts at age 8 months. All the analyzed samples of FF revealed active enzymes ($24.2 \cdot 10^{-3}$ U · l^{-1}, $2.65 \cdot 10^{-3}$ U · l^{-1}, and 525 U · l^{-1} for CAT, GSH-Px, and SOD, respectively) and contained Zn, Cu and Mn ($13.8 \cdot 10^{3}$ mol · l^{-1}, $33.3 \cdot 10^{3}$ mol · l^{-1}, and $133 \cdot 10^{-9}$ mol · l^{-1}, respectively). In the pFF collected from the left ovary, SOD and GSH-Px activity was higher compared to pFF from the right ovary. On the contrary, the concentration of Cu and Mn was significantly lower in the left ovary pFF. The concentration of Cu ions was negatively correlated with SOD activity. The CAT activity in pFF form left or right ovary did not show any differences.

Keywords: catalase, glutathione peroxidase, microelements, porcine follicular fluid, superoxide dismutase

INTRODUCTION

Oxidative metabolism is essential for gamete and embryo energy production and is unavoidably associated with the generation of reactive oxygen species (ROS). An increase in their concentration may lead to an oxidative stress. Under such conditions, ROS are responsible for damages to macromolecules and cellular structures, with deleterious effects on the functions of the cell. Apart from non-enzymatic mechanisms, each organism has an enzymatic defence system to scavenge the oxidants, or repair damages caused by ROS.

Enzymatic antioxidant defences are present in mammalian oocytes, embryos and fol-

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licular fluid [Paszkowsky et al. 1995, Bisseling et al. 1997, Moautassim et al. 1999, Cetica et al. 2000, Tarin et al. 2000, Guérin et al. 2001, Sugino 2005]. Angelucci et al. [2006], who analysed the proteome of human follicular fluid, identified several important antioxidant enzymes, including catalase and superoxide dismutase.

Superoxide dismutase (Zn,Cu-SOD and Mn-SOD) belongs to the first step of the cellular enzymatic protection against toxic radicals [Suzuki et al. 1999]. SOD removes the superoxide anion in the dismutation reaction producing hydrogen peroxide and molecular oxygen. The removal of hydrogen peroxide is catalysed by either catalase (CAT) or GSH-Px. SOD activity is localized in developing follicles and in postovulatory follicles in the rat [Laloraya et al. 1989]. The studies on rat and human ovaries suggest that anion superoxide and SOD may play a role in the development of oocytes [Shiotani et al. 1991, Sato et al. 1992].

Investigations aimed to determine the optimal conditions for livestock animal follicular oocytes *in vitro* maturation have not brought satisfactory results so far. Porcine oocytes can develop to the stage of blastocyst following maturation and fertilization *in vitro*, but their developmental potential is lower than that of oocytes which matured *in vivo* [Beckmann et al. 1993, Petters and Wells 1993, Dobrinsky et al. 1996, Abeydeera and Day 1997, Wang et al. 1997, Kano et al. 1998, Marchal et al. 2003].

In vitro cultures are performed under higher concentrations of oxygen as compared to *in vivo* conditions, which results in enhanced production of ROS. The effects of adding follicular fluid to maturation media, in terms of the developmental competence after *in vitro* fertilization (IVF), have been investigated in domestic animals [Naito et al. 1988, Elmileik et al. 1995, Ikeda et al. 1999, Vatzias and Hagen 1999, Gallardo et al. 2001, Ito et al. 2008], and the findings show that an addition of porcine follicular fluid (pFF) to IVM medium have beneficial effects on IVM and IVF results. Ermilov et al. [1999] state that IVF media supplements, such as CAT, act as ROS scavengers and protect the DNA from damage.

The aim of this study was to investigate into the total SOD, CAT, and GSH-Px activity and Cu, Zn and Mn concentration in the porcine follicle fluid in gilts.

MATERIAL AND METHODS

Animals and collection of pFF

The ovaries were collected on 18 and 25 February from totally 77 gilts at the age of 8 month at the slaughter house and transported to the laboratory in 0.9% NaCl at 30°C. Within 2–3 hrs post slaughter, from 62 gilts pFF was aspirated separately from non cystic follicles of 4–7 mm in diameter from both the left and the right ovary, From 15 gilts the pFF was aspirated without division into left or right ovary. Aspirated pFF was centrifuged at 3000 G for 10 minutes to remove debris, blood, and granulosa cells. Thereafter, pFF supernatant was transferred to Eppendorf tubes and stored at –20°C until assayed. Follicular fluid samples with significant quantities of blood were not used for analyses.

Determination of catalase activity (*EC 1.11.1.6.*)

Catalase (CAT) activity was determined by the method described by Lück [1963].

For CAT activity measurement 0.04 cm^3 of the sample was pipetted into 3 cm^3 cuvette with $1.25 \cdot 10^{-2}$ M solution of H_2O_2 in 1/15 M phosphate buffer (pH 7.0) and mixed. The decrease in absorbance, caused by decomposition of H_2O_2, was monitored continuously at 240 nm for 90 s. Activity was calculated using extinction coefficient 0.036 $mM^{-1} \cdot cm^{-1}$. One unit of enzyme is the amount necessary to decompose 1 mmol of H_2O_2 per min. Specific activities were expressed as $U \cdot g^{-1}$ protein.

Determination of SOD activity (*EC 1.15.1.1, EC-SOD*)

To measure SOD activity, the Ransod kits (Randox Laboratories Ltd., London, UK) were used. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is measured by the degree of inhibition of this reaction. The absorbance was monitored continuously at 504 nm and 37ºC (modified spectrophotometer Specol, Carl Zeiss, Jena Germany). The SOD activity of each sample was determined based on the standard curve made as recommended in the manual of the kit. All standard rates and diluted sample rates were converted into the percentage sample dilution rate and subtracted from 100% to produce the percentage of inhibition. One unit of SOD activity expresses 50% inhibition of I.N.T. reduction. The SOD activity was expressed as units in 1 ml of pFF. Specific activities were expressed as $U \cdot g^{-1}$ protein.

Determination of GSH-Px (*EC 1.11.1.9.*) *activity*

GSH-Px activity in pFF was measured using the RANSEL kits (RANDOX Laboratories Ltd, UK), based on the method of Paglia and Valentine [1967], with hydroxycumene as substrate. The reaction was carried out in a spectrophotometer (EPOLL 20, Poll Ltd., Warsaw, Poland) at 37ºC. The method was based on an NADPH-coupled reaction, whereby oxidised glutathione (GSSG) produced by GSH-Px and hydroxyperoxide was reduced by exogenous glutathione reductase and NADPH. Enzyme activity was measured at 340 nm and expressed in units representing oxidation of 1 micromole NADPH per minute per ml pFF. Specific activities were expressed as $U \cdot g^{-1}$ protein.

Determination of protein

The protein concentration was measured based on the manual enclosed to the kit for protein determination (ALPHA DIAGNOSTICS sp. z o.o. Warsaw). The kit allows measurement of total protein in body fluids. In the alkaline environment, peptide bindings of protein react with copper ions and create blue-violet color complex. The color was measured at 37ºC and by the wave length of 540 nm with a spectrophotometer (EPOLL 20, Poll Ltd., Warsaw, Poland). The measured value of absorbance (trial and stabdard samples) is proportional to the total concentration of protein in the analyzed sample.

Determination of microelements (*Cu, Zn, and Mn*)

The concentration of microelements in pFF was determined with inductively coupled plasma optical emission spectrometry (ICP-OES), using the spectrometer Optima 2000 DV (PerkinElmer). In plasma emission spectroscopy (OES), a sample solution is intro-

duced into the core of inductively coupled argon plasma (ICP), which generates temperature of approximately 8000°C. At this temperature all elements become thermally excited and emit light at their characteristic wavelengths. This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its constituent wavelengths. Within the spectrometer, this diffracted light is then collected by wavelength and amplified to yield an intensity measurement that can be converted to an elemental concentration by comparison with calibration standards. The measuring was carried out on 150 µl follicular fluid which was diluted to 2 ml. To samples were added 0.2 ml inner standard. The content of microelements were expressed in mol per l.

Statistical analysis

The results were statistically analysed by means of the Statistica® software package (StatSoft Inc. Tulsa, OK, USA), using one-way ANOVA, multivariate analysis of variance (MANOVA), the Wilks-Rosenbaum tests, the Tukey test, and the correlation matrix. Also medical statistics software (MedCalc® 970217-4.11) was used for data analysis and visualisation.

RESULTS AND DISCUSSION

The results show that all of the studied enzymes are present in pFF (Table 1). The specific CAT activity ranged from 130 to 1210 · 10^6 U · g^{-1} protein, that of GSH-Px ranged between 5 and 227 U · g^{-1} protein, whereas the specific SOD activity remained within the range 2.1 to 28.7 U · g^{-1} protein.

Table 1. Summary statistics of analyzed enzymes and microelements
Tabela 1. Ogólna charakterystyka statystyczna badanych enzymów i mikroelementów

Parameter Parametr	Sample size Liczba prób	Arithmetic mean Średnia arytmetyczna	Standard deviation Odchylenie standardowe	Test for normality of distribution Test rozkładu normalnego	
				P =	P > 0.05
Total protein, g · l^{-1} Białko całkowite, g · l^{-1}	139	59.5	13.4	0.1822	+
SOD, U · l^{-1}	106	525	308	< 0.0000	–
CAT × 10^{-3} U · l^{-1}	141	24.2	10.63	< 0.0000	–
GSH-Px ×10^{-3} U · l^{-1}	131	2.65	1.04	0.1241	+
Zn × 10^{-3} mol · l^{-1}	86	13.8	3.34	0.8863	++
Cu × 10^{-6} mol · l^{-1}	55	33.3	5.54	0.9832	++
Mn × 10^{-9} mol · l^{-1}	86	133	69	< 0.0000	–

The concentration of antioxidant elements varied in the range 4–26 × 10^{-3} mol · l^{-1}, 21–42 × 10^{-6} mol · l^{-1} and 55–419 × 10^{-9} mol · l^{-1} for Zn, Cu and Mn, respectively.

In pFF collected from the left ovary, the SOD and GSH-Px activity, expressed in $U \cdot L^{-1}$ as well as in $U \cdot kg^{-1}$ protein, was higher than that in pFF of the right ovary (Tables 2 and 3). On the contrary, the concentration of Cu and Mn was significantly lower in the left ovary pFF (Table 4). The highest differences in enzyme activity or ions concentration in pFF between the left and right ovary were obtained for SOD and Cu ions, respectively. The concentration of Cu ions was significantly negatively correlated ($R^2 = 0.7857$) with SOD activity (Fig. 1). The CAT activity in pFF form left or right ovary didn't show any differences. All studied samples of porcine follicular fluid revealed active enzymes and contained Cu, Mn, and Zn.

Table 2. Total protein content and specific activity of antioxidant enzymes in pFF taken from left or right ovary

Tabela 2. Białko całkowite oraz właściwa aktywność enzymów antyoksydacyjnych w świńskim płynie pęcherzykowym pobranym z lewego lub prawego jajnika

Parameter Parametr	Side Strona	N	Specific activity Aktywność właściwa	
			mean średnia	standard deviation odchylenie standardowe
Total protein, $g \cdot l^{-1}$	left – lewa	61	62	14
Białko całkowite, $g \cdot l^{-1}$	right – prawa	59	61	12
CAT, $U \cdot g^{-1}$	left – lewa	61	410	178
Protein – Białka	right – prawa	59	410	208
GSH-Px, $U \cdot g^{-1}$	left – lewa	58	46	21
Protein – Białka	right – prawa	57	42	21
SOD, $U \cdot g^{-1}$	left – lewa	43	9	5
Protein – Białka	right – prawa	41	7	4

Table 3. Total activity of antioxidant enzymes in pFF taken from left or right ovary

Tabela 3. Aktywność całkowita enzymów antyoksydacyjnych w świńskim płynie pęcherzykowym pobranym z lewego lub prawego jajnika

Enzyme Enzym	Side Strona	N	Mean Średnia	Standard deviation Odchylenie standardowe
CAT × 10^3 $U \cdot l^{-1}$	left – lewa	62	24.7	9.8
	right – prawa	62	24.6	11.3
GSH-Px × 10^3 $U \cdot l^{-1}$	left – lewa	59	2.76	1.10
	right – prawa	60	4.48	0.98
SOD × $U \cdot l^{-1}$	left – lewa	43	555	376
	right – prawa	43	456	215

Follicular fluid SOD activity has been frequently studied in a number of organisms, including pigs [Tatemoto et al. 2004, Basini 2008], ruminants [Singh et al. 1998], rats [Tilly and Tilly 1995], as well as women [Carbone et al. 2003]. Data on the activity of antioxidant enzymes published by different authors vary very strongly. Tatemoto et al. [2004]

detected in porcine follicular fluid 16.0 U · ml^{-1} of SOD activity. In human follicular fluid, Carbone at al. [2003] observed the activity of catalase (20–40 mU · mg^{-1} protein), SOD (70–100 mU · mg^{-1} protein), as well as GSH-Px (0.7–0.8 mU · mg^{-1} protein). Singh et al. [1998], who studied SOD activity in small ruminants, found that specific as well as total SOD activity in the goat and the sheep was different (6.9 U · mg^{-1} protein or 107.9 U · g^{-1} tissue in sheep, respectively, and 3.5 U · mg^{-1} protein or 76.5 U·g^{-1} tissue in goat, respectively). The divergence between the cited data and those presented in this study is probably due to different collection method of follicular fluid and different, non standardized method of enzyme determination. The other factor which may differentiate the activity of antioxidant enzymes is the moment of the ovarian cycle. The above-mentioned results explicitly demonstrate that the activity of the antioxidant enzymes is species-dependent.

Table 4. Concentration of zinc, copper and manganese in pFF taken from left or right ovary
Tabela 4. Koncentracja Zn, Cu i Mn w świńskim płynie pęcherzykowym pobranym z lewego lub prawego jajnika

Parameter Parametr	Side Strona	N	Mean Średnia	Standard deviation Odchylenie standardowe
Zn × 10^{-3} mol · l^{-1}	left – lewa	44	13	3
	right – prawa	42	14	3
Cu × 10^{-6} mol · l^{-1}	left – lewa	40	32^{A}	5
	right – prawa	15	37^{A}	4
Mn × 10^{-9} mol · l^{-1}	left – lewa	43	110^{B}	60
	right – prawa	43	156^{B}	71

Basini et al. [2008] also detected the activity of CAT and GSH-Px in pFF. The authors state that oxidative stress does not affect follicle growth and that SOD and GSH-Px showed reduced activity during follicle development, but not CAT. They suggest that other factors could be involved in ROS detoxification during follicle development. On the other hand, other authors observed that supplementing the culture media with catalase or CuZn-SOD resulted in an increase in the rate of blastocysts formation in rabbit [Li et al. 1993], mouse [Nonogaki et al. 1992], and cattle [Lauria et al. 1994].

The differences in SOD and GSH-Px activity in pFF between the left and right ovary can eventually arise from different vascularization of the ovaries. Further research involving anatomical studies of gilts, especially the vascularity of the right and the left ovaries, are needed to ascertain whether the SOD and GSH-Px activity depends on the site pFF comes from. In women, the right ovarian artery is slightly shorter than the left one [Krechowiecki and Czerwiński 1991], which can lead probably to unequal supply of oxygen to the ovaries and to uneven production of ROS.

The interpretation of differences in Cu and Mn concentration in pFF in relation to the location side of the ovary is difficult. Szołtys [1992] states that follicular fluid is not only selected filtrate from blood serum, but also a product from the somatic cells of the follicle. Also the opinion of Wise [1987] is that follicular fluid is not simply filtrate of blood serum, but a complex of diverse substances synthesized by appropriate follicular cells.

The higher activity of SOD and lower concentration of microelements in pFF from the left ovary and a reverse pattern in follicular fluid from the right ovary can suggest that CuZn-SOD, Mn-SOD, and concentration of elements involved in these enzymes occur independently from each other. The correlation between SOD activity and total concentration of Cu in pFF deserves our special attention. The correlation is negative, non-linear, and statistically significant. It is of a two-phase character. In the medium with a low Cu concentration (20 to 30 · 10^{-6} mol · l^{-1}), SOD activity is high and depends on the Cu concentration. At a Cu concentration > 35 · 10^{-6} mol · l^{-1}, the correlation decreases, which implies that Cu-SOD activity correlation becomes stable in the medium with high SOD activity. This effect is difficult to explain without further research, since it can be due to, for example, different forms of SOD present in the medium and may consist in restricting one of SOD forms by free Cu ions or cuproproteins.

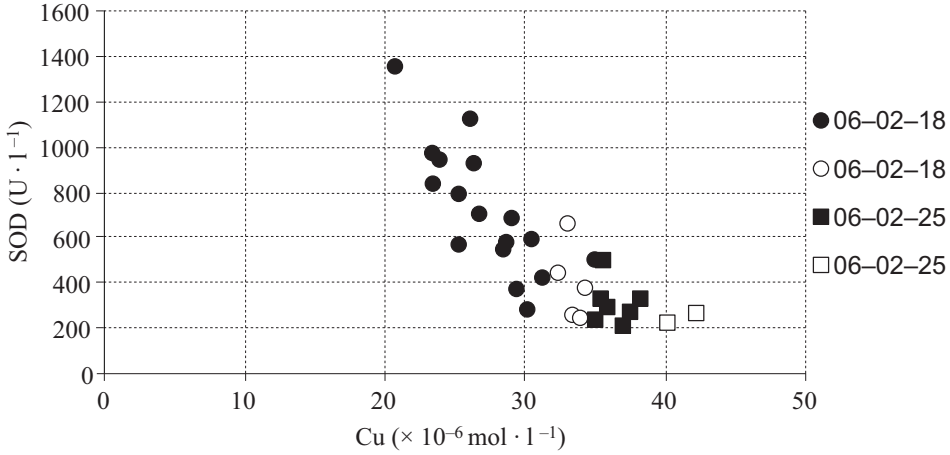

Fig. 1. Correlation between SOD activity and Cu concentration in pFF (black points – left ovary, transparent points – right ovary)

Rys. 1. Korelacja między aktywnością SOD a koncentracją Cu w pFF (punkty czarne – jajnik lewy; punkty białe – jajnik prawy)

The following conclusions can be drawn from the presented results:

– further investigations are need to find unequivocally whether the activity of antioxidant enzymes, SOD and GSH-Px, and concentration of Cu and Mn in the pFF depend on the side of the body the ovary is located;

– a significant negative correlation between Cu and SOD activity was observed, but other results show that antioxidant enzymes and microelements involved in these enzymes occur independently.

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AKTYWNOŚĆ ENZYMÓW ANTYOKSYDACYJNYCH ORAZ KONCENTRACJA BIAŁKA MN, CU I ZN W ŚWIŃSKIM PŁYNIE PĘCHERZYKOWYM

Streszczenie. Metabolizm oksydacyjny jest niezbędny do produkcji energii na potrzeby gamet i zarodków, co związane jest z wytwarzaniem reaktywnych form tlenu. Antyoksydacyjny mechanizm ochrony enzymatycznej znajduje się w oocytach i zarodkach ssaków, a także w płynie pęcherzykowym. Dodatek świńskiego płynu pęcherzykowego do odżywek hodowlanych korzystnie wpływa na wyniki zapłodnienia i dojrzewania *in vitro*. Celem badań było określenie aktywności katalazy (CAT) dysmutazy ponadtlenkowej (SOD) i persoksydazy glutationowej (GSH-Px) oraz koncentracji Cu, Mn i Zn w płynie pęcherzykowym świń pobranym z prawego i lewego jajnika. Ogółem pobrano jajniki od 77 loch w wieku 8 miesięcy. We wszystkich analizowanych próbkach badane enzymy wykazywały aktywność (24,2; 2,65 10^{-3} U · l^{-1} i 525 U · l^{-1} odpowiednio w przypadku CAT, GSH-Px i SOD) i zawierały jomy Zn, Cu i Mn (odpowiednio 13,8 · 10^3 mol · l^{-1}; 33,3 · 10^3 mol · l^{-1} i 133 · 10^{-9} mol · l^{-1}). W płynie pęcherzykowym pobranym z lewego jajnika aktywność SOD i GSH-Px była wyższa niż w płynie pęcherzykowym prawego jajnika. Natomiast koncentracja Cu i Mn była istotnie niższa w płynie z lewego jajnika. Koncentracja jonów Cu była skorelowana ujemnie z aktywnością SOD. Aktywność CAT w płynie pęcherzykowym z prawego lub lewego jajnika nie wykazała różnic.

Słowa kluczowe: dysmutaza ponadtlenkowa, katalaza, mikroelementy, peroksydaza glutationowa, świński płyn pęcherzykowy

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