Expression and localization of nitric oxide synthase isoforms during porcine oocyte growth and acquisition of meiotic competence

E. Chmelíková[1], M. Sedmíková[1], J. Petr[2], T. Kott[2], V. Lánská[1], L. Tůmová[1], H. Tichovská[1], M. Ješeta[3]

[1]Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic
[2]Institute of Animal Science, Prague-Uhříněves, Czech Republic
[3]Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: Reproduction biotechnologies depend on the use of fully meiotically competent oocytes. Growing oocytes without full meiotic competence are an interesting potential source due to their quantity, but the mechanisms regulating the processes of acquisition of meiotic competence have not been clarified to date. Nitric oxide synthase (NOS) and its product, nitric oxide (NO), may possibly play a role. Understanding the precise NO regulatory mechanism is therefore important for the development of *in vitro* growth methods. The objective of this work was to detect changes in the expression of NOS isoforms and their mRNA expression and changes in the intracellular localization of separate NOS isoforms during the growth period of the porcine oocyte, and also to determine whether these changes are related to the process of meiotic competence acquisition. mRNA for all NOS isoforms was already detected in oocytes at the beginning of their growth and was present in them until they completed their growth period. mRNA for iNOS and eNOS was also observed in granulosa and cumulus cells from these oocytes. But nNOS mRNA was not demonstrated in these types of cells. Pig oocytes and their surrounding cells contained all NOS proteins. Their amounts increased and localization changed with the acquisition of meiotic competence. nNOS was localized mainly in the cortex in meiotically incompetent oocytes, while meiotically competent oocytes contained nNOS in the nucleus as well. iNOS protein was distributed in the cytoplasm and nucleus in all oocytes, and meiotically incompetent oocytes contained iNOS in the nucleolus as well. eNOS protein was distributed in oocytes in the form of fine granules with a strong fluorescence signal. Protein was concentrated in the nuclear area in meiotically incompetent oocytes and also in the periphery in oocytes with partially and fully-developed meiotic competence. All these findings indicate that NOS isoforms may significantly influence the acquisition of meiotic competence in porcine oocytes.

Keywords: pig; oocyte; growth; meiotic competence; NO-synthase

The intensive development of biotechnologies such as *in vitro* embryo production and cloning by nuclear transfer requires a great number of oocytes with fully-developed meiotic competence. During *in vitro* culture these oocytes are able to reach the stage of second meiotic metaphase and complete their meiotic maturation. However, the number of these oocytes in the ovary is limited.

Supported by Czech Science Foundation (Grant No. 523/06/P291), Ministry of Agriculture of the Czech Republic (Grant No. QG 50052) and Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 6046070901).

Growing oocytes without meiotic competence are in numerical superiority in the ovary, and these growing oocytes are an interesting source for biotechnologies due to their quantity.

The meiotic competence of oocytes develops during the growth phase and depends on the oocyte size. Porcine oocytes with an internal diameter (size without *zona pellucida*) of 100 μm and less are meiotically incompetent and are not able to undergo germinal vesicle breakdown (GVBD) under *in vitro* conditions. Oocytes with an internal diameter of 110 μm are partially meiotically competent, they are able to undergo GVBD and reach the first meiotic metaphase. However, they are unable to exit from the first metaphase to reach the second meiotic metaphase stage, and to complete meiotic maturation. Full meiotic competence is acquired in pig oocytes reaching an internal diameter of 120 μm (Szybek, 1972; Sorensen and Wassarman, 1976; Motlík and Fulka, 1986; Petr et al., 1994, 1999, 2007a; Brevini et al., 2007).

The mechanisms controlling meiotic competence acquisition and meiotic maturation are not fully understood (Wierzchos, 2006; Wang et al., 2007; Szczerbik et al., 2008). The role of nitric oxide (NO), which is a cellular messenger arising from L-arginine oxidation by the NO-synthase (NOS) enzyme, is not clear yet.

NOS occurs in almost all types of cells in three different isoforms – neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Lamas et al., 1992; Marletta, 1994; Griffith and Stuehr, 1995). nNOS and eNOS are constitutively expressed and are activated by calcium-loaded calmodulin (Bredt and Snyder, 1990; Pollock et al., 1991). iNOS is expressed in response to stimuli by cytokines and endotoxins and once expressed, it is activated independently of calcium and calmodulin (Moncada et al., 1991; Lowenstein and Snyder, 1992). It is known that eNOS produces small amounts of NO (nM), while iNOS produces NO in μM amounts, and its NO production is constant (more than 20 hours) (Lamas et al., 1992; Xie et al., 1992; Nathan and Xie, 1994; Dixit and Parvizi, 2001). The amount of NO that is produced by the iNOS isoform is approximately 1 000× higher than NO production from the eNOS isoform (Tao et al., 2004). The exact role of NO during the growth and meiotic maturation of oocytes is not known in spite of numerous studies. However, works that concentrate on NOS isoform expression and localization contribute to its clarification.

eNOS and iNOS isoforms were detected in fully-grown rat oocytes (Van Voorhis et al., 1994, 1995; Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Jablonka-Shariff et al., 1999; Nakamura et al., 1999; Yamagata et al., 2002; Tao et al., 2004) and in mouse oocytes (Nishikimi et al., 2001; Mitchell et al., 2004). The nNOS isoform was not detected in oocytes. Only Abe et al. (1999) detected nNOS mRNA in mice oocytes. The eNOS isoform and mRNA for eNOS were detected in porcine fully meiotically competent oocytes and in their granulosa cells (Hattori et al., 2000; Grasselli et al., 2001; Takesue et al., 2001, 2003). The incidence of NOS isoforms in porcine meiotically incompetent growing oocytes is not known. The objective of this work was to monitor changes in NOS isoforms and their mRNA expression and changes in the intracellular localization of NOS isoforms during the growth phase of porcine oocytes and to determine if these changes are related to the process of acquisition of meiotic competence.

MATERIAL AND METHODS

Ovary collection

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrus cycle and transported to the laboratory within 1 hour in a saline solution (0.9% of sodium chloride) at 39°C.

Oocyte collection

Fully-grown oocytes were collected from follicles by aspiration of follicles measuring 2–5 mm in diameter with a 20-gauge needle (Sterican, Braun Melsungen, Germany).

Growing oocytes of different sizes were obtained from thin strips (10–15 mm long and 2–3 mm wide) dissected from the surface of the ovaries. The growing oocytes were released from their follicles by opening the follicular wall using the tip of a 25-gauge needle. The internal diameter of the oocytes (without *zona pellucida*) was measured using an ocular micrometer mounted on a microscope. The oocytes were washed three times in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of 7% solution per mil-

lilitre of medium), calcium lactate (0.06 mg/ml), sodium pyruvate (0.25 mg/ml), gentamicin (0.025 mg/ml), HEPES (1.5 mg/ml), 13.5 IU eCG:6.6 IU hCG/ml (PG 600 Intervet, Boxmeer, Holland) and 10% fetal calf serum (GibcoBRL, Life Technologies, Karlsruhe, Germany).

RT-PCR analysis

RNA was isolated using total RNA chemistry (ABI) of the Applied Biosystems 6100 PrepStation in accordance with the instruction manual. Total RNA was transcribed to cDNA with a High-Capacity cDNA Archive kit (Applied Biosystems, USA) in accordance with the producer's instructions and frozen at –20°C. cDNA was synthesized in a final volume of 10 µl. Sets of specific primers were synthesized in accordance with known sequences to amplify specific products for nNOS, iNOS and eNOS. nNOS sense (forward) primer 5'tca agg tca aga act ggg ag 3', antisense (reverse) primer 5'cct gca gct tgg acc act gg 3'(Solhaug et al., 2001), iNOS forward 5'gcc gac tgg att tgg ttg gt 3', reverse 5'gtt ggt gag ttc ttt cag cat 3' (Banning et al., 1999), eNOS forward 5´agc ggc tgc atg aca ttg ag 3', reverse 5'aaa agc tct ggg tgc gta tgc g 3' (Takesue et al., 2001). The PCR products were separated using 2% agarose gel electrophoresis and then visualized by ethidium bromide staining. The reactions took place in a 10 µl reaction mixture containing 0.1–5 ng RNA (cDNA), 5 pmol of primers and 1× concentrated PPP Master Mix – 150mM Tris-HCl, pH 8.8, 40mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5mM $MgCl_2$, 400µM dATP, 400µM dCTP, 400µM dGTP, 400µM dTTP, 100 U/ml Taq-Purple DNA polymerase, stabilizers and additives (Top Bio, Czech Republic). The PCR proceeded as follows: initial denaturation at 95°C for 3 min and 30 s followed by 35 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 30 s – nNOS; 35 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 30 s – iNOS; 40 cycles of 95°C for 30 s, 54°C for 45 s and 72°C for 30 s – eNOS. The final extension step lasted for 3 min at 72°C.

Western blotting analysis

The oocytes were mechanically denuded of their granulosa and cumulus cells. Granulosa cells from growing oocytes and cumulus cells from fully-grown oocytes represented separate samples. The oocytes, granulosa and cumulus cells were washed in phosphate buffer saline (PBS) and lysed in a lysis buffer supplemented with a protease inhibitor cocktail Complete Mini (Roche, Germany). The proteins were diluted in a 20 µl SDS sample buffer and then separated by SDS-PAGE with 4% stacking gel and 10% separating gel. An amount of 5 ng of purified protein (nNOS, iNOS and eNOS) (Alexis Biochemicals, USA) always ran in one well simultaneously as a positive control. Following the separation, the proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, USA). The successful transfer of the proteins was verified with pre-stained molecular weight standards (Bio-Rad Lab., Canada). After overnight blocking with low-fat milk (2%) in PBS containing 0.1% Tween 20, the membrane was incubated for 2 hours with anti-NOS antibodies (anti-nNOS, anti-iNOS and anti-eNOS, Alexis Biochemicals, USA) at a dilution of 1:2 000. The membrane was then washed and incubated with peroxidase-conjugated anti-rabbit IgG (Calbiochem, La Jolla, USA) at 1:120 000 dilutions. Proteins were detected using the ECL Advance Western Blotting Detection Kit (Amersham Pharmacia Biotech, UK).

The amount of NOS protein in each spot was measured with an image analysing system (L.U.C.I.A., version 4.71, Laboratory Imaging, Czech Republic) by performing densitometric analysis. The content of NOS isoform protein was measured on the basis of the integrated optical density (IOD) parameter (mean optical density × area of induction) (Vogel et al., 1997). For comparison of NOS quantities among the blots, the values of IOD were related to those of oocytes without meiotic competence (80–89 µm).

Immunohistochemical analysis

Granulosa and cumulus cells and z*ona pellucida* were removed from the oocytes in an acidified medium (0.1 N HCl) in 2.5% (w/v) paraformaldehyde in PBS. The fixed oocytes were permeabilized in 0.1% (v/v) Triton-X100 in PBS. The oocytes were then rinsed in PBS, placed in a blocking solution (0.5% (w/v) BSA and 0.1% (v/v) Tween 20 in PBS) and incubated overnight in a blocking solution with primary anti-NOS antibodies (Alexis Biochemicals, USA) at 1:100 dilution. The oocytes were incubated with a secondary antibody (anti-rabbit IgG con-

jugated with fluorescein isothiocyanate – FITC) (Jackson ImmunoResearch Laboratories, Europe, Ltd.) diluted in a blocking solution. They were then counterstained with Hoechst 33258 (Sigma Aldrich, Germany) to visualise chromatin. After repeated washing they were mounted on glass slides. The FITC fluorescence was visualized using a fluorescent (Nikon Eclipse E600, Japan) and laser scanning confocal microscope (Leica SPE, Germany). The control oocytes were incubated only in a secondary antibody. A blocking solution was used in lieu of a primary antibody.

EXPERIMENT DESIGN

Experiment 1: Verification of meiotic competence in growing porcine oocytes during their *in vitro* culture

In this experiment, the meiotic competence of porcine oocytes with different internal diameters was investigated under our laboratory conditions.

Growing oocytes (of internal diameter 80–89 µm, 90–99 µm and 100–110 µm) and fully-grown oocytes (of internal diameter 120 µm) were cultured *in vitro* for 48 hours. The oocytes were cultured in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3 ml of culture medium M199 at 39°C in a mixture of 5% CO_2 in the air. After their culture the oocytes were mechanically voided of their granulosa and cumulus cells by repeated pipetting through a narrow bore pipette. They were then mounted on slides, fixed with acetic alcohol (1:3, v:v) for at least 24 hours and stained with 1% orcein. The oocytes were examined under a phase contrast microscope, and the stages of meiotic maturation were determined in accordance with the criteria published by Motlík and Fulka (1986). Each group contained 50 oocytes of appropriate sizes.

Experiment 2: NOS mRNA RT-PCR analysis

The objective of this partial experiment was to ascertain if there is an NOS mRNA expression during the growth period of porcine oocytes and also to determine if there is a relationship between the NOS mRNA expression and the level of meiotic competence of oocytes. RNA was isolated from the oocytes and their granulosa or cumulus cells. Four groups of oocytes were analysed – 80–89 µm, 90–99 µm, 100–110 µm and 120 µm. Each sample of growing or fully-grown oocytes contained 50 oocytes; granulosa and cumulus cells were analysed separately from the oocytes.

Experiment 3: NOS Western blotting detection

The objective of this experiment was to establish which NOS protein isoforms are present in porcine oocytes during their growth phase and also to determine the relationship between the NOS protein expression and the level of meiotic competence. The expression of NOS was studied in growing porcine oocytes of internal diameter 80–89 µm, 90 to 99 µm, 100–110 µm and in fully-grown oocytes, at the germinal vesicle stage (120 µm). Samples from the granulosa and cumulus cells obtained from these 4 size groups of oocytes were prepared as well. Each sample of oocytes contained 50 oocytes. The cells surrounding these oocytes were sampled separately.

Table 1. Meiotic competence verification in growing porcine oocytes

Internal diameter	Stage of meiotic maturation reached after 48 h *in vitro* cultivation (%)			Oocytes (*n*)
	germinal vesicle	metaphase I	metaphase II	
80–89 µm	97.79 ± 2.20^{A}	1.48 ± 1.0^{A}	0.74 ± 0.70^{A}	200
90–99 µm	88.54 ± 6.03^{A}	9.93 ± 4.70^{A}	1.34 ± 1.20^{A}	200
100–110 µm	12.81 ± 5.35^{B}	87.19 ± 5.35^{B}	0 ± 0 ^{A}	200
120 µm	6.29 ± 6.14^{B}	6.86 ± 4.75^{A}	86.85 ± 5.67^{B}	200

oocytes of internal diameter of 80–89, 90–99, 100–110 and 120 µm were cultured *in vitro* for 48 hours, then fixed, and the stage of meiotic maturation was determined; the data are expressed as means ± standard deviation

statistically significant differences ($P < 0.05$) in the rates of respective stages of meiotic maturation between different size groups of oocytes (between different rows) are indicated by different superscripts (A and B)

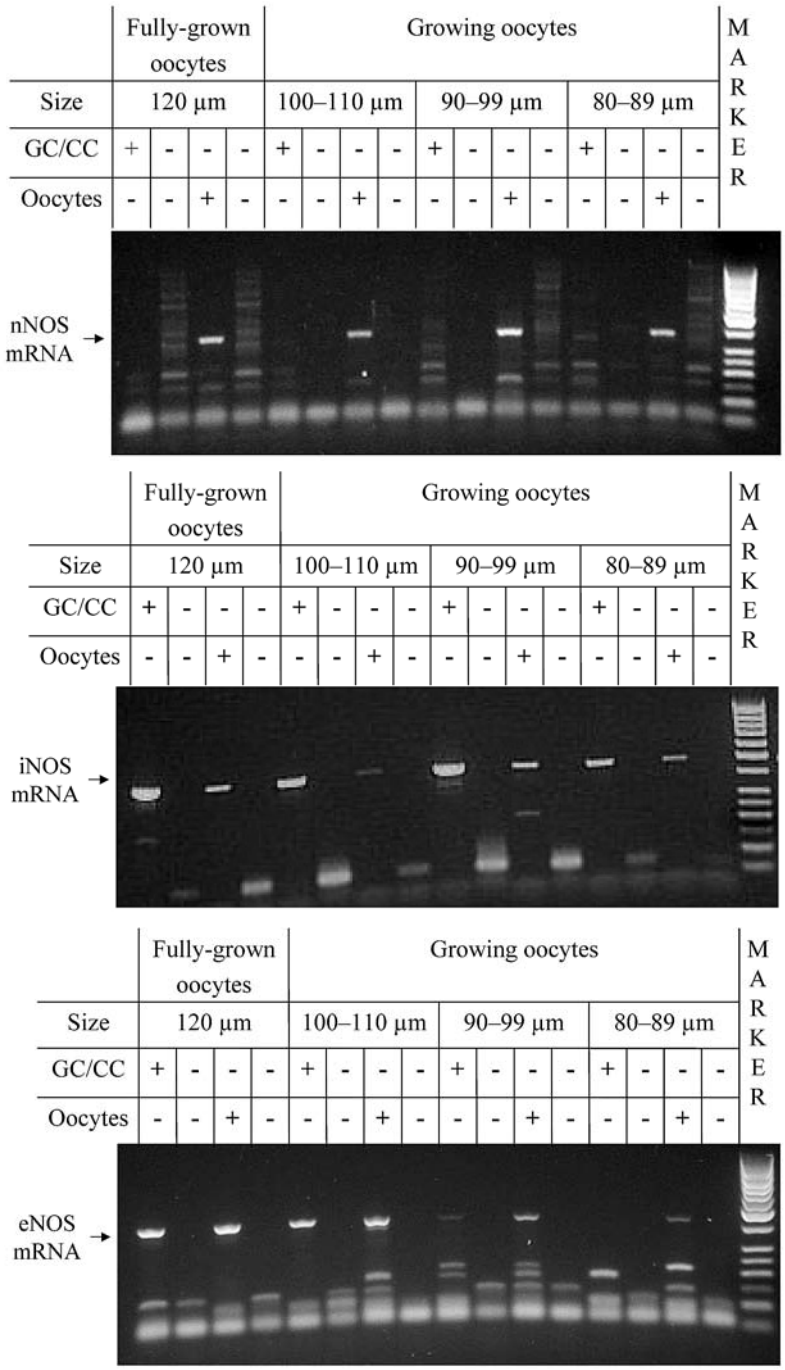

Figure 1. RT-PCR detection of NOS mRNA in porcine oocytes with different extents of meiotic competence and in the surrounding granulosa and cumulus cells; growing oocytes were prepared from the surface of the ovarian cortex; fully-grown oocytes were prepared from antral follicles (2–5 mm in diameter) as described in Materials and Methods. Total RNA was extracted from 50 oocytes of the appropriate size group and their granulosa (GC) and cumulus cells (CC) and subjected to RT-PCR. The type of sample (cumulus and granulosa cells or oocytes) is indicated by + sign in the table upper RT-PCR pictures. The data are representative of 4 independent experiments

Experiment 4: NOS immunohistochemical localization

The objective of this experiment was to study the localization of NOS isoforms during the growth period of porcine oocytes and to determine if there are any changes in NOS distribution during oocyte growth in relation to their acquisition of meiotic competence.

Oocytes of the four size groups described above were analysed (80–89 µm, 90–99 µm, 100–110 µm and 120 µm). Each experimental group contained 50 oocytes of a given size.

Statistical analysis

The experiments were repeated four times. The data were analysed by the least-squares analysis of variance using the program Statistica version 6.0. The differences among the experimental groups

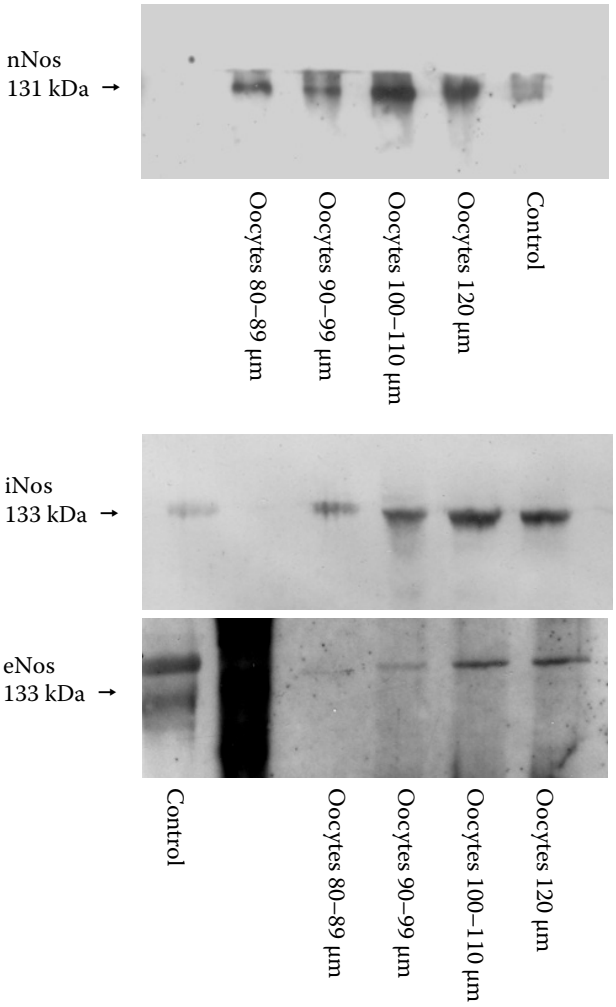

Figure 2. Western blotting of NOS from porcine growing and fully-grown oocytes; growing oocytes and fully-grown oocytes were prepared as described in Materials and Methods; proteins from 50 oocytes were separated by SDS-PAGE and analyzed by immunodetection using anti-specific NOS antibodies; the data are representative of 4 independent experiments. The control band represents 5 ng of purified NOS protein

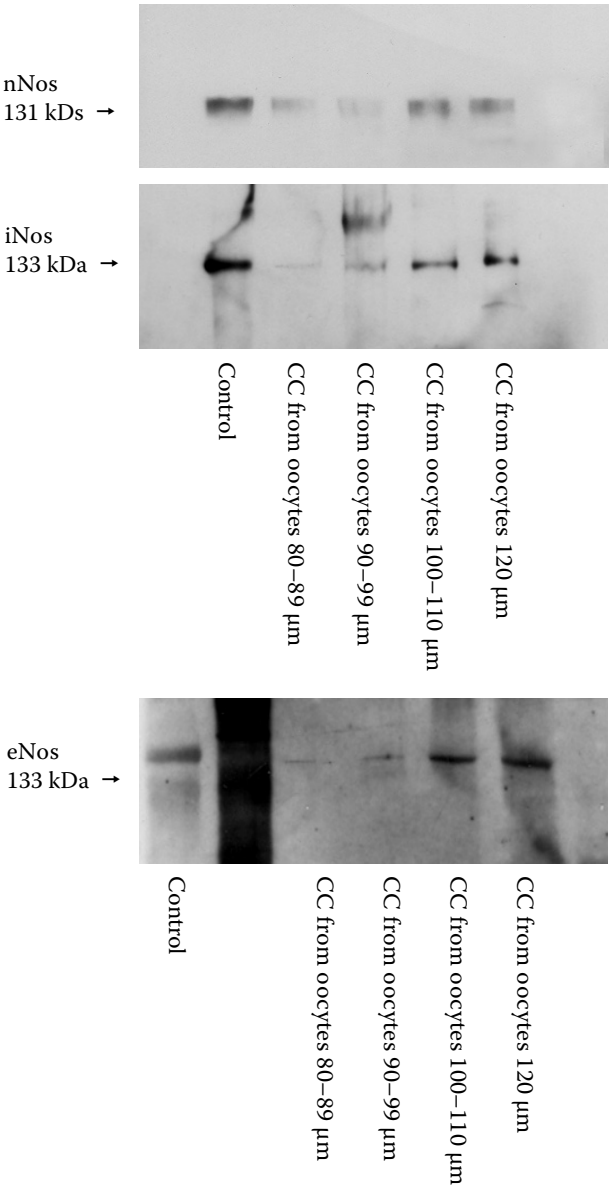

Figure 3. Western blotting of NOS in granulosa and cumulus cells obtained from porcine growing and fully-grown oocytes; granulosa cells (GC) and cumulus cells (CC) were prepared from growing and fully-grown oocytes as described in Materials and Methods; proteins from granulosa and cumulus cells from 50 oocytes were separated by SDS-PAGE and analyzed by immunodetection using an anti-specific NOS antibody; the data are representative of 4 independent experiments. The control band represents 5 ng of purified NOS protein.

of oocytes were determined by Tukey's HSD test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Verification of meiotic competence in growing porcine oocytes during their *in vitro* culture

Under our culture conditions, the groups of oocytes of internal diameters 80–89 and 90–99 µm were meiotically incompetent oocytes, those of internal diameter 100–110 µm had partially developed meiotic competence, and oocytes measuring 120 µm in diameter were fully meiotically competent (Table 1).

NOS mRNA RT-PCR analysis

Oocytes of internal diameter 80–89 μm, 90 to 99 μm, 100–110 μm, 120 μm and their granulosa and cumulus cells were analysed for the presence of nNOS, iNOS and eNOS mRNA.

mRNA for all NOS isoforms was detected in all four groups of oocytes studied (Figure 1). Granulosa and cumulus cells from these oocytes did not contain any nNOS mRNA. iNOS mRNA was observed in this type of somatic cells from all size groups of oocytes. For eNOS mRNA were positive only in the granulosa and cumulus cells obtained from oocytes larger than 100 μm. eNOS mRNA was absent in granulosa cells obtained from oocytes of internal diameter 80–89 and 90–99 μm (Figure 1).

NOS Western blotting detection

The Western blot analysis method demonstrated that porcine oocytes contained all three NOS isoforms. Proteins were detected in all four groups of analysed oocytes (Figure 2), and all three NOS isoforms were also present in the granulosa and cumulus cells from these oocytes (Figure 3). The densitometric analysis showed that the amount of proteins in oocytes and their surrounding cells increased during the growth phase and the process of acquisition of meiotic competence (Tables 2 and 3).

NOS immunohistochemical localization

The subcellular localization of nNOS, iNOS and eNOS proteins during porcine oocyte growth was studied. An immunohistochemical assessment showed the presence of all three NOS isoforms. The localization of individual proteins varied according to the oocyte size and their level of meiotic competence.

The nNOS isoform occurs in oocytes without meiotic competence and in oocytes with partially developed meiotic competence (internal diameter of 80–89 μm, 90–99 μm and 100–110 μm) mainly in the oocyte cortex area. In fully-grown oocytes, with fully developed meiotic competence, the protein can also be localized in the nuclear region in addition to the periphery. The protein did not accumulate in the nucleolus.

The iNOS protein isoform was similar to the nNOS detected on the periphery of the oocytes. All the groups of studied oocytes contained protein on the periphery; some protein also occurred in the nucleus, but the fluorescence intensity was weaker

Table 2. NOS expression in porcine oocytes

Monitored signs	Internal diameter of oocytes			
	80–89 μm	90–99 μm	100–110 μm	120 μm
nNOS				
IOD	1.09 ± 0.10^{A}	2.58 ± 0.09^{B}	3.28 ± 0.17^{C}	3.18 ± 0.11^{C}
RIOD	1.00^{A}	2.39 ± 0.16^{B}	3.04 ± 0.24^{C}	2.92 ± 0.21^{C}
iNOS				
IOD	1.32 ± 0.14^{A}	2.19 ± 0.24^{B}	3.21 ± 0.43^{C}	3.20 ± 0.02^{C}
RIOD	1.00^{A}	1.66 ± 0.05^{B}	2.43 ± 0.05^{C}	2.42 ± 0.05^{C}
eNOS				
IOD	0. 32 ± 0.10^{A}	0.73 ± 0.09^{B}	1.35 ± 0.17^{C}	1.45 ± 0.11^{C}
RIOD	1.00^{A}	2.28 ± 0.16^{B}	4.21 ± 0.24^{C}	4.54 ± 0.21^{C}

integrated optical density (IOD) and relative integrated optical density (RIOD) of NOS proteins (nNOS, iNOS and eNOS) in growing and fully-grown porcine oocytes were monitored; the NOS contents were determined according to their integrated optical density related to IOD of 80-89 μm oocytes; the data are expressed as means ± standard deviation; the different superscripts (A, B, C) denote statistically significant differences ($P < 0.05$) in the IOD and RIOD of appropriate NOS isoform expression (in rows)

Table 3. NOS expression in granulosa and cumulus cells of growing and fully-grown porcine oocytes

Monitored signs	Internal diameter of oocytes			
	80–89 μm	90–99 μm	100–110 μm	120 μm
nNOS				
IOD	0.88 ± 0.01^{A}	1.44 ± 0.01^{B}	1.98 ± 0.01^{C}	2.14 ± 0.01^{C}
RIOD	1.00^{A}	1.29 ± 0.16^{B}	2.25 ± 0.24^{C}	2.43± 0.21^{C}
iNOS				
IOD	1.49 ± 0.10^{A}	1.89 ± 0.09^{B}	2.46 ± 0.14^{C}	2.69 ± 0.13^{C}
RIOD	1.00^{A}	1.27 ± 0.16^{B}	1.56 ± 0.24^{C}	1.81 ± 0.22^{C}
eNOS				
IOD	0.99 ± 0.15^{A}	1.42 ± 0.07^{B}	2.22 ± 0.13^{C}	2.47 ± 0.11^{C}
RIOD	1.00^{A}	1.44 ± 0.14^{B}	2.25 ± 0.24^{C}	2.49 ± 0.21^{C}

integrated optical density (IOD) and relative integrated optical density (RIOD) of NOS proteins (nNOS, iNOS and eNOS) in granulosa and cumulus cells from growing and fully-grown porcine oocytes was measured; the NOS contents were determined according to their integrated optical density (IOD) related to the IOD of oocytes with internal diameter of 80–89 μm; the data are expressed as means ± standard deviation; the different superscripts denote statistically significant differences ($P < 0.05$) in the IOD and RIOD of the appropriate NOS isoform expression (in rows)

than on the periphery. Oocytes without meiotic competence also contained this isoform in their nucleolus. The nucleolus of fully-grown oocytes did not contain any iNOS protein.

eNOS occurred in the oocytes in fine granules with a strong fluorescence signal. Oocytes without meiotic competence contained these granules mainly in the nuclear area, and oocytes with partial and fully developed meiotic competence contained eNOS granules in the cytoplasm as well (Figure 4).

DISCUSSION

Our results demonstrated the expression of NOS isoforms during the growth period of porcine oocyte oogenesis. mRNA for nNOS, iNOS, eNOS, and also three NOS proteins were detected in all groups of studied porcine oocytes. The amounts of proteins increased and the localization underwent important changes during growth which can be related to the process of acquisition of meiotic competence. This presumption is also strengthened by the demonstrated significant role of NOS and NO in meiotic maturation, fertilization and pre-implantation embryo development (Jablonka-Shariff and Olson, 1998, 2000; Abe et al., 1999; Nishikimi et al., 2001; Tranguch and Huet-Hudson, 2003; Petr et al., 2005, 2007b; Tsutsui et al., 2006).

The expression of all three NOS proteins in one cell type is not surprising; it is known that the expression of several NOS isoforms occurs both in somatic cells (Mehta et al., 1995) and in mammalian oocytes (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Tao et al., 1997; Jablonka-Shariff et al., 1999). The expression of NOS mRNA was also observed in the cumulus and granulosa cells which enclose the oocyte during its growth period. We did not determine nNOS mRNA in this somatic follicular compartment. mRNA transcription for nNOS isoforms occurs courses in oocytes but not in the granulosa or cumulus cells which surround the oocyte. This indicates that nNOS could play a quite specific role in oocytes. The absence of eNOS mRNA in granulosa cells which surround oocytes without meiotic competence and the presence of eNOS mRNA in these oocytes also confirm the specific role of this NOS isoform in the early phases of acquisition of oocyte meiotic competence.

Differences in gene expression between the oocyte and the somatic compartment of follicles are not surprising in any case because of the fact that the oocyte and the somatic cells of the follicle comprise an interconnected functional complex. The existence of wide spectra of genes whose expres-

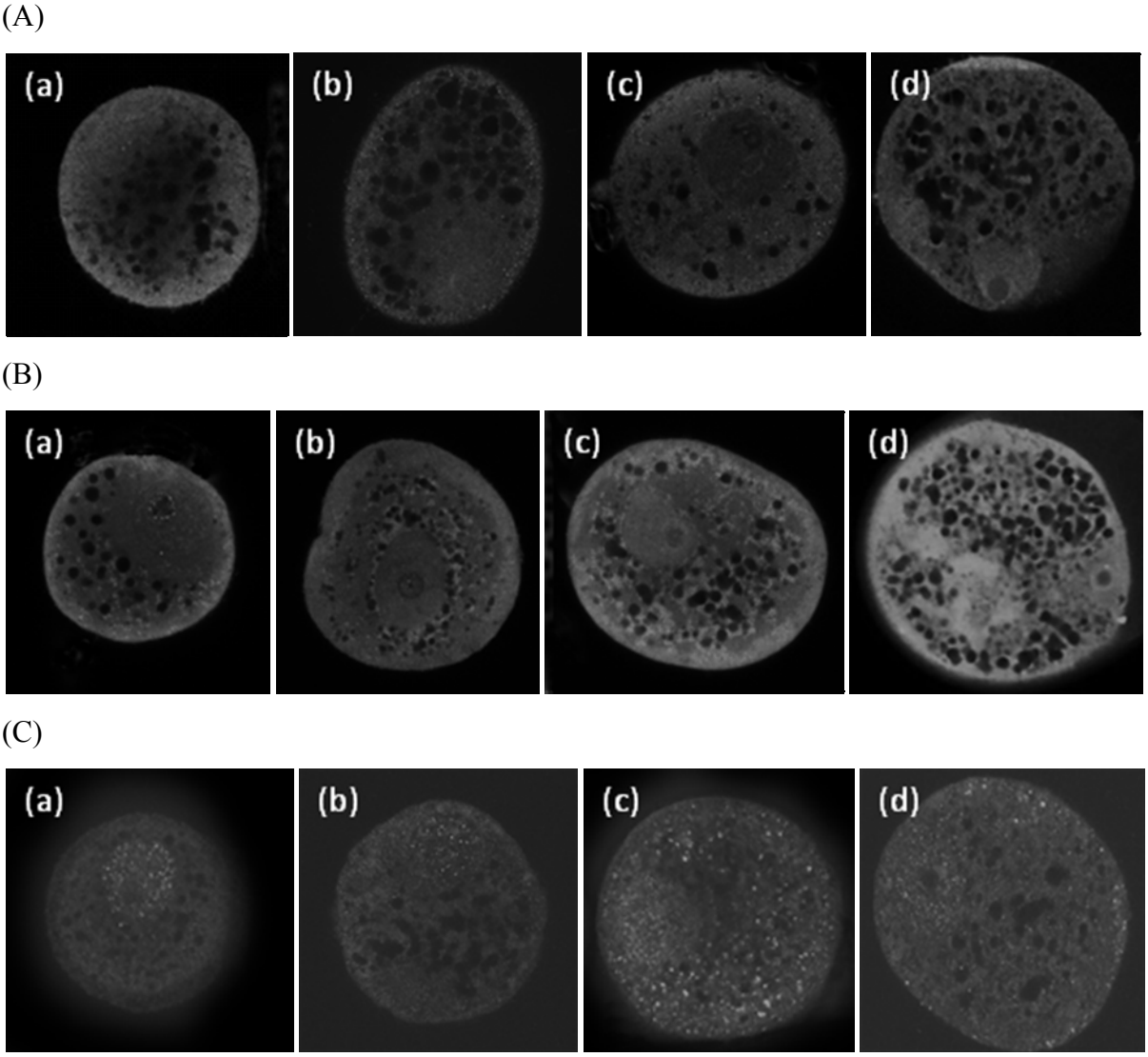

Figure 4. NOS localization in growing and fully-grown porcine oocytes
the oocytes were stained by immunofluorescence using anti-NOS specific antibodies. Blue: DNA; green: NOS protein – (A) nNOS, (B) iNOS, (C) eNOS, (a) 80–89 μm oocytes, (b) 90–99 μm oocytes, (c) 100–110 μm oocytes, (d) 120 μm oocytes, 50 oocytes in each group were analysed; the experiment was repeated 4 times and showed similar results on each occasion

sion is typical only of oocytes was demonstrated by Bonnet et al. (2008).

nNOS mRNA was detected in oocytes, but not in their granulosa or cumulus cells. On the other hand, protein was present in the oocytes and also in the cells surrounding them. These findings, e.g. the detection of nNOS protein in granulosa and cumulus cells, do not confirm an allegation of oocyte specific nNOS expression. It does not seem presumable that nNOS detected in granulosa and cumulus cells might be synthesized in other cells, e.g. in oocytes, and transported from the oocytes to these cells. The transport between oocyte and cumulus cells via gap junctions is reduced for small molecules with molecular weights up to 1 kDa. Another explanation offers alternative splicing of nNOS mRNA. RNA splicing allows the production of a large number of nNOS mRNA molecular species and the production of nNOS proteins that have different enzymatic activity and structure (Brenman et al., 1997; Wang et al., 1999; Gonzales-Cadavid et al., 2000). All transcripts do not influence the amino acid composition of nNOS (Wang et al., 1999). We can speculate that the alternative

splicing of nNOS mRNA does permit us to detect nNOS mRNA by the method we used, but synthesized protein was detected by our antibodies. We can presume that the absence of eNOS mRNA and the presence of eNOS protein in granulosa cells which enclose those oocytes without meiotic competence can be explained in a similar way, since alternative splicing was also described in the eNOS gene (Lorenz et al., 2007).

Our experiments also confirmed significant changes in protein amounts and their distribution in oocytes during oogenesis. The amount of proteins increased during growth. nNOS was localized mainly in the cortex in small and meiotically incompetent oocytes, while in meiotically fully-competent and fully-grown oocytes it also accumulated in the nucleus. This isoform is produced mainly by the neuronal tissue (Marletta, 1994), and many authors have not confirmed its presence in oocytes of different mammalian species (Jablonka-Shariff and Olson, 1997; Hattori and Tabata, 2006). On the other hand, Abe et al. (1999) confirmed the presence of nNOS mRNA in mouse oocytes.

The presence of iNOS on the periphery of oocytes is not surprising with regard to the well-known association of iNOS with sub-membranous cortical actin cytoskeleton in somatic cells (Webb et al., 2001) and the presence of cortical actin cytoskeleton in oocytes (Sun and Schatten, 2006). This peripheral localization is probably important for the propagation of NO-signalling in somatic cells (Webb et al., 2001), and we cannot exclude that it is also important for oocytes. Our observations are consistent with those made in oocytes from laboratory rodents (Van Voorhis et al., 1994, 1995; Jablonka-Shariff and Olson, 1997; Nakamura et al., 2002; Huo et al., 2005). There are very limited and inconsistent data concerning the expression and localization of iNOS in porcine oocytes. Hattori et al. (2000) did not find iNOS protein either in porcine oocytes or in their cumulus cells. Tao et al. (2004), on the contrary, confirmed the presence of iNOS in porcine oocytes. Our experiments confirmed the presence of iNOS also in the nucleolus of meiotically incompetent oocytes in which active RNA synthesis proceeds. On the other hand, iNOS was not detected in the nucleolus of any oocytes with a completed growth period which do not synthesize RNA any more (Bjerregaard and Maddox-Hyttel, 2004; Bjerregaard et al., 2004). This indicates that iNOS could be important for RNA synthesis during the growth period of the oocyte.

eNOS was distributed in the oocytes in fine granules with a strong fluorescence signal. Oocytes without meiotic competence contained these granules mainly in the nuclear area; those with partially and fully-developed meiotic competence contained eNOS granules in the cytoplasm as well. The increased amount of protein in the nucleus and cytoplasm in partially meiotically competent oocytes may be related to the acquisition of meiotic competence and the requirement of nitric oxide synthesis. Our results confirmed that eNOS is a frequently expressed isoform in mammalian oocytes (Van Voorhis et al., 1994; Jablonka-Shariff and Olson, 1997; Nishikimi et al., 2001; Mitchell et al., 2004) and is also abundant in the somatic compartments of the ovary (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Jablonka-Shariff et al., 1999; Nakamura et al., 1999; Yamagata et al., 2002; Mitchell et al., 2004). The expression and distribution of eNOS in porcine oocytes do not deviate from this pattern (Hattori et al., 2001; Takesue et al., 2003).

We can conclude on the basis of our results that the mRNA transcription of all three NOS isoforms proceeds during the growth phase of pig oocytes, and also the synthesis of all three NOS proteins takes place during the same period. The initiation of NOS transcription and proteosynthesis is evident even before the beginning of gradual acquisition of meiotic competence, and the amount of NOS protein increases with the growth phase of the oocyte. The NOS localization also changes with growth and with the acquisition of meiotic competence. All these signs indicate that NOS isoforms can significantly influence the process of acquisition of meiotic competence in porcine oocytes.

Acknowledgements

We thank Mrs. Lois Russell and Mrs. Marie Daskova for their editorial assistance with this manuscript.

REFERENCES

Abe K., Matsuoka K., Inoue N., Taga M., Kato T. (1999): Messenger RNA of neuronal nitric oxide synthase is expressed and possibly functions in mouse oocytes and embryos during preimplantation development. Biomedical Research-Tokyo, 20, 61–65.

Banning A.P., Groves P.H., Buttery L.D., Wharton J., Rutherford R.A., Black P., Winkler F., Polak J.M., Lewis M.J., Drexler H. (1999): Reciprocal changes in endothelial and inducible nitric oxide synthase expression following carotid angioplasty in the pig. Atherosclerosis, 145, 17–32.

Bjerregaard B., Maddox-Hyttel P. (2004): Regulation of ribosomal RNA gene expression in porcine oocytes. Animal Reproduction Science, 82–83, 605–616.

Bjerregaard B., Wrenzycki C., Philimonenko V.V., Hozak P., Laurincik J., Niemann H., Motlik J., Maddox-Hyttel P. (2004): Regulation of ribosomal RNA synthesis during the final phases of porcine oocyte growth. Biology of Reproduction, 70, 925–935.

Bonnet A., Iannuccelli E., Hugot K., Benne F., Bonaldo M.F., Soares M.B., Hatey F., Tosser-Klopp G. (2008): A pig multi-tissue normalised cDNA library: large-scale sequencing, cluster analysis and 9K micro-array resource generation. BMC Genomics, 9, 17.

Bredt D.S., Snyder S.H. (1990): Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. In: Proceedings of the National Academy of Sciences oft he USA. USA, 87, 682–685.

Brenman J.E., Xia H., Chao D.S., Black S.M., Bredt D.S. (1997): Regulation of neuronal nitric oxide synthase through alternative transcripts. Developmental Neuroscience, 19, 224–231.

Brevini T.A.L., Cillo F., Antonini S., Gandolfi F. (2007): Cytoplasmic remodelling and the acquisition of developmental competence in pig oocytes. Animal Reproduction Science, 98, 23–38.

Dixit V.D., Parvizi N. (2001): Nitric oxide and the control of reproduction. Animal Reproduction Science, 292, 1–16.

Gonzalez-Cadavid N.F., Burnett A.L., Magee T.R., Zeller C.B., Vernet D., Smith N., Gitter J., Rajfer J. (2000): Expression of penile neuronal nitric oxide synthase variants in the rat and mouse penile nerves. Biology of Reproduction, 63, 704–714.

Grasselli F., Ponderato N., Basini G., Tamanini C. (2001): Nitric oxide synthase expression and nitric oxide/cyclic GMP pathway in swine granulosa cells. Domestic Animal Endocrinology, 20, 241–252.

Griffith O.W., Stuehr D.J. (1995). Nitric oxide synthase: properties and catalytic mechanism. Annual Review of Physiology, 57, 707–736.

Hattori M., Tabata S. (2006): Nitric oxide and ovarian function. Animal Science Journal, 77, 275–284.

Hattori M.A., Nishida T., Takesue K., Kato Y., Fujihara N. (2000): FSH suppression of nitric oxide synthesis in porcine oocytes. Journal of Molecular Endocrinology, 24, 65–73.

Hattori M.A., Takesue K., Kato Y., Fujihara N. (2001): Expression of endothelial nitric oxide synthase in the porcine oocyte and its possible function. Molecular and Cellular Biochemistry, 219, 121–126.

Huo L.J., Liang C.G., Yu L.Z., Zhong Z.S., Yang Z.M., Fan H.Y., Chen D.Y., Sun Q.Y. (2005): Inducible nitric oxide synthase-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte. Reproduction, 129, 403–409.

Jablonka-Shariff A., Olson L.M. (1997): Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. Endocrinology, 138, 460–468.

Jablonka-Shariff A., Olson L.M. (1998): The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knockout mouse oocytes. Endocrinology, 138, 2944–2954.

Jablonka-Shariff A., Olson L.M. (2000): Nitric oxide is essential for optimal meiotic maturation of murine cumulus-oocyte complexes *in vitro*. Molecular Reproduction and Development, 55, 412–421.

Jablonka-Shariff A., Basuray R., Olson L.M. (1999): Inhibitors of nitric oxide synthase influence oocyte maturation in rats. Journal of the Society for Gynecologic Investigation, 6, 95–101.

Lamas S., Marsden P.A., Li G.K., Tempst P., Michel T. (1992): Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proceeding of the. National Academy of Sciencesof the USA, 89, 6348–6352.

Lorenz M., Hewing B., Hui J., Zepp A., Baumann G., Bindereif A., Stangl V., Stangl K. (2007): Alternative splicing in intron 13 of the human eNOS gene: a potential mechanism for regulating eNOS activity. The FASEB Journal, 21, 1556–1564.

Lowenstein C.J., Snyder S.H. (1992): Nitric oxide, a novel biologic messenger. Cell, 70, 705–707.

Marletta M.A. (1994): Nitric oxide synthase: aspects concerning structure and catalysis. Cell, 78, 927–930.

Mehta J.L., Chen C.Y., Kone B.C., Mehta P., Turner P. (1995): Identification of constitutive and inducible forms of nitric oxide synthase in human platelets. Journal of Laboratory and Clinical Medicine, 125, 370–377.

Mitchell L.M., Kennedy C.R., Hartshorne G.M. (2004): Expression of nitric oxide synthase and effect of substrate manipulation of nitric oxide pathway in mouse ovarian follicles. Human Reproduction, 19, 30–40.

Moncada S., Higgs E.A., Hodson H.F., Knowles R.G., Lopezjaramillo P., McCall T., Palmer R.M.J., Radomski M.W., Rees D.D., Schulz R. (1991): The L-arginine-ni-

tric-oxide pathway. Journal of Cardiovascular Pharmacology, 17, S1–S9.

Motlík J., Fulka J. (1986): Factors affecting meiotic competence in pig oocytes. Theriogenology, 25, 87–96.

Nakamura Y., Kashida S., Nakata M., Takiguchi S., Yamagata Y., Takayama H., Sugino N., Kato H. (1999): Changes in nitric oxide synthase activity in the ovary of gonadotropin-treated rats: the role of nitric oxide during ovulation. Endocrine Journal, 46, 529–538.

Nakamura Y., Yamagata Y., Sugino N., Takayama H., Kato H. (2002): Nitric oxide inhibits oocyte meiotic maturation. Biology of Reproduction, 67, 1588–1592.

Nathan C., Xie Q.W. (1994): Nitric oxide synthases: roles, tools and controls. Cell, 78, 915–918.

Nishikimi A., Matsukawa T., Hoshino K., Ikeda S., Kira Y., Sato E. F., Inoue M., Yamada M. (2001): Localization of nitric oxide synthase activity in unfertilized oocytes and fertilized embryos during preimplantation development in mice. Reproduction, 122, 957–963.

Petr J., Teplá O., Grocholová R., Jílek F. (1994): Inhibition of meiotic maturation in growing pig oocytes by factor(s) from cumulus cells. Reproduction Nutrition Development, 34, 149–156.

Petr J., Rozinek J., Vaňourková Z., Jílek F. (1999): Cyclopiazonic acid, an inhibitor of calcium-dependent ATPases, induces exit from metaphase I arrest in growing pig oocytes. Reproduction Fertility and Development, 34, 149–156.

Petr J., Rajmon R., Rozinek J., Sedmíková M., Ješeta M., Chmelíková E., Švestková D., Jílek F. (2005): Activation of pig oocytes using nitric oxide donors. Molecular Reproduction and Development, 71, 115–122.

Petr J., Chmelíková E., Dörflerová A., Ješeta M., Kuthanová Z. (2007b): Effects of protein kinase C on parthenogenetic activation of pig oocytes using calcium ionophore or nitric oxide-donor. Czech Journal of Animal Science, 52, 415–422.

Petr J., Chmelíková E., Tůmová L., Ješeta M. (2007a): The role of nitric oxide in parthenogenetic activation of pig oocytes: A review. Czech Journal of Animal Science, 52, 363–377.

Pollock J.S., Forstermann U., Mitchell J.A., Warner T.D., Schmidt H.H., Nakane M., Murad F. (1991): Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proceedings of National Academy of Science of the USA, 88, 10480–10484.

Solhaug M.J., Kullaprawithaya U., Dong X.Q., Dong K.W. (2001): Expression of endothelial nitric oxide synthase in the postnatal developing porcine kidney. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 280, R1269–R1275.

Sorensen R.A., Wassarman P.M. (1976): Relationship between growth and meiotic maturation of the mouse oocyte. Developmental Biology, 50, 531–536.

Sun Q.Y., Schatten H. (2006): Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. Reproduction, 131, 193–205.

Szczerbik P., Mikolajczyk T., Sokolowska-Mikolajczyk M., Socha M., Chyb J., Epler P. (2008): The influence of cadmium on Prussian carp oocyte maturation, development of eggs and hatching. Czech Journal of Animal Science, 53, 36–44.

Szybek K. (1972): *In-vitro* maturation of oocytes from sexually immature mice. Journal of Endocrinology, 54, 527–528.

Takesue K., Hattori M.A., Nishida N., Kato Y., Fujihara N. (2001): Expression of endothelial nitric oxide synthase gene in cultured porcine granulosa cells after FSH stimulation. Journal of Molecular Endocrinology, 26, 259–265.

Takesue K., Tabata S., Sato F., Hattori M.A. (2003): Expression of nitric oxide synthase-3 in porcine oocytes obtained at different follicular development. Journal of Reproduction and Development, 49, 135–140.

Tao M., Kodama H., Kagabu S., Fukuda J., Murata M., Shimizu A., Hirano H., Tanaka T. (1997): Possible contribution of follicular interleukin-1 beta to nitric oxide generation in human pre-ovulatory follicles. Human Reproduction, 12, 2220–2225.

Tao Y., Zhuo F., Zhang M., Xia G., Yang J., Xie H. (2004): Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Molecular and Cellular Endocrinology, 222, 93–103.

Tranguch S., Huet-Hudson Y. (2003): Decreased viability of nitric oxide synthase double knockout mice. Molecular Reproduction and Development, 65, 175–179.

Tsutsui M., Shimokawa H., Morishita T., Nakashima Y., Yanagihara N. (2006): Development of genetically engineered mice lacking all three nitric oxide synthases. Journal of Pharmacological Sciences, 102, 147–154.

Van Voorhis B.J., Dunn M.S., Snyder G.D., Weiner C.P. (1994): Nitric oxide: An autocrine regulator of human granulosa-luteal cell steroidogenesis. Endocrinology, 135, 1799–1806.

Van Voorhis B.J., Moore K., Strijbos P., Nelson S., Baylis S.A., Grzybicki D., Weiner C.P. (1995): Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary – effects of gonadotropin stimulation *in vivo*. Journal of Clinical Investigation, 96, 2719–2726.

Vogel P., Dux E., Wiessner C. (1997): Effect of heat shock on neuronal cultures: Importance of protein synthasis and HSP 72 induction for induced tolerance and survival. Metabolic Brain Disease, 12, 203–217.

Wang Y., Newton D.C., Marsden P.A. (1999): Neuronal NOS: gene structure, mRNA diversity, and functional relevance. Critical Reviews of Neurobiology, 13, 21–43.

Wang Z.G., Xu Z.R., Yu S.D. (2007): Effects of oocyte collection techniques and maturation media on *in vitro* maturation and subsequent embryo development in Boer goat. Czech Journal of Animal Science, 52, 21–25.

Webb J.L., Harvey M.W., Holden D.W., Evans T.J. (2001): Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. Infection and Immunity, 69, 6391–6400.

Wierzchos A. (2006): Parthenogenetic development of rabbit oocytes after electrical stimulation. Czech Journal of Animal Science, 51, 400–445.

Xie Q.W., Cho H.J., Calaycay J., Mumford R.A., Swiderek K.M., Lee T.D., Ding A.H., Troso T., Nathan C. (1992): Cloning and characterization of inducible nitric-oxide synthase from mouse macrophages. Science, 256, 225–228.

Yamagata Y., Nakamura Y., Sugino N., Harada A., Takayama H., Kashida S., Kato H. (2002): Alterations in nitrate/nitrite and nitric oxide synthase in preovulatory follicles in gonadotropin-primed immature rat. Endocrine Journal, 49, 219–226.

Zackrisson U., Mikuni M., Wallin A., Delbro D., Hedin L., Brännström M. (1996): Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. Human Reproduction, 11, 2667–2673.

Received: 2008–12–03
Accepted after corrections: 2009–01–27

Corresponding Author

Ing. Eva Chmelíková, Ph.D., Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6, Czech Republic
Tel. +420 224 382 952, fax +420 234 381 841, e-mail: chmelikova@af.czu.cz