The role of nitric oxide synthase isoforms in aged porcine oocytes

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ABSTRACT: In the sphere of reproductive biotechnologies, the demand for sufficient numbers of high-quality oocytes is still increasing. In some cases, this obstacle is overcome by *in vitro* prolonged cultivation. However, a prolonged oocyte culture is accompanied by changes called ageing. Ageing is manifested by spontaneous parthenogenetic activation, programmed cell death or lysis. Various substances, such as caffeine or dithiothreitol, have been tested for ageing suppression. In this respect, research into gasotransmitters (hydrogen sulphide, carbon monoxide, and nitric oxide) has currently been intensified. The objectives of the present study were to localize nitric oxide synthases (NOS) and to evaluate NOS inhibition of aged porcine oocytes. We demonstrated the presence of NOS isoforms in oocyte cultivation prolonged by 24, 48, and 72 h. After 72 h of prolonged cultivation, NOS inhibition by the non-specific inhibitor L-NAME or the specific inhibitor aminoguanidine caused suppression both of programmed cell death and lysis. Although NOS amount rapidly decreased after the 72-h cultivation, changes induced by NOS inhibition were statistically significant. We can presume that NOS play an important physiological role in porcine oocyte ageing.

Keywords: nitric oxide; L-NAME; aminoguanidine; oocyte ageing; pig

Fully grown oocytes undergo meiotic resumption during mammal oogenesis. Meiotic maturation starts with germinal vesicle breakdown, passing through metaphase I, anaphase I, and telophase I to metaphase II (Motlik and Fulka, 1986; Wassarman, 1988). After reaching metaphase II (MII), oocytes are spontaneously arrested until fertilization and activation (Yanagimachi, 1988). Reproductive biotechnologies use MII oocytes matured *in vitro* conditions for *in vitro* fertilization, transgenesis or nuclear transfer cloning.

Sufficient numbers of high-quality MII oocytes are necessary for successful use in biotechnology. To meet this requirement, MII oocytes exposed to prolonged cultivation are frequently used. During prolonged cultivation, oocytes exhibit a series of complex changes called ageing (Petrová et al., 2004, 2009). Ageing is based on biochemical changes of key factor activities as well as on damage to the ultrastructural cytoskeleton and various organels (Kikuchi et al., 2000; Suzuki et al., 2002). These changes result in the following morphological manifestations: spontaneous parthenogenetic activation, programmed cell death (apoptosis, fragmentation), and lysis (Petrová et al., 2004; Miao et al., 2009).

Research into biochemical and structural changes during oocyte prolonged cultivation in order to postpone the onset of undesirable changes in aged oocytes is needed. Various substances were tested

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to suppress the ageing, such as dithiotreitol (Tarín et al., 1998), caffeine (Kikuchi et al., 2000), growth factors (Petrová et al., 2005), or trichostatin (Ješeta et al., 2008). Recently, the role of gasotransmitters (hydrogen sulphide (H_2S), carbon monoxide (CO), and nitric oxide (NO)) in the reproductive system has been observed (e.g. Šmelcová and Tichovská, 2011). The gastrotransmitters are expected to affect the ageing process.

As concerns NO, its function as a cell messenger has been examined in the cardiovascular system (Gómez-Fernández et al., 2004), nerve tissues (Muramatsu et al., 2000), and in reproductive processes, particularly in folliculogenesis, meiotic maturation, and ovulation (Jablonka-Shariff and Olson, 1998, 2000). NO is necessary for germinal vesicle breakdown and metaphase I to metaphase II transition (Chmelíková et al., 2010). Moreover, NOS inhibition supresses cumulus expansion of sheep oocytes (Amale et al., 2011). The role of NO in oocyte activation and early embryonic development of fertilized or parthenogenetically activated oocytes was also studied (Goud et al., 2008; Krejčová et al., 2009). Although not essential for mouse oocyte fertilization (Hyslop et al., 2001), NO is able to activate the oocytes of pigs and amphibians (Petr et al., 2005, 2010; Jeseta et al., 2012), presumably throughout cGMP and PKG signalling pathways (Petr et al., 2006).

In vivo NO production is carried out by nitric oxide synthase (NOS), an enzyme catalyzing L-arginine conversion to citrulin and NO (Kwon et al., 1990; Lamas et al., 1992). NOS exists in three isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Whereas eNOS and nNOS are Ca^{2+}-dependent and produce a small amount of NO in a short time (Bredt et al., 1991; Lamas et al., 1992), iNOS is Ca^{2+}-independent and steadily generates 100 to 1000 times higher levels of NO for hours at a time (Nathan, 1992; Xie et al., 1992). All of the three NOS isoforms and NOS mRNA were detected in mammal oocytes and cumulus cells (Van Voorhis et al., 1995; Hattori et al., 2000).

The role of NOS and NO in physiological processes has been studied by various methods including genetic knock-out (Jablonka-Shariff and Olson, 1998) and NOS inhibitor use (Goud et al., 2008). For NOS inhibition in oocytes, a non-specific inhibitor, N^{ω}–nitro-L-arginine methylester (L-NAME) (Abavisani et al., 2011) or a specific iNOS inhibitor (aminoguanidine) were used (Chmelíková et al., 2010).

The involvement of NO in programmed cell death of neurons was demonstrated by Kang et al. (2004). Moreover, the influence of NOS was described in early embryonic development (Saugandhika et al., 2010). The NOS isoforms effects could be expected in the case of prolonged mammal oocyte cultivation, too.

The present study has two objectives: (1) to demonstrate the presence of NOS isoforms in aged porcine oocytes and describe their dynamics during prolonged cultivation and (2) to evaluate NOS inhibition influence on ageing processes after 72 h of prolonged cultivation.

MATERIAL AND METHODS

Isolation and cultivation of porcine oocytes

Porcine ovaries were obtained from non-cycling gilts at a slaughterhouse. Ovaries were transported to the laboratory in a saline solution (0.9% NaCl) at 39°C. Oocytes were collected from ovarian follicles (2–5 mm) with a 20-gauge aspirating needle. Only fully grown oocytes with intact cytoplasm and compact cumuli were used in further experiments.

The oocytes were maturating in a modified M199 medium (Sigma-Aldrich, St. Louis, USA) containing sodium bicarbonate (32.5mM), calcium L-lactate (2.75mM), gentamicin (0.025 mg/ml), HEPES (6.3mM), 13.5 IU eCG: 6.6 IU hCG/ml (P.G. 600) (Intervet International B.V., Boxmeer, the Netherlands) and 10% (v/v) foetal calf serum (GibcoBRL) (Life Technologies, Darmstadt, Germany). The oocytes were getting matured in 3.5 cm Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of culture medium at 39°C in a mixture of 5.0% CO_2 in air.

After *in vitro* maturation, the cumuli were removed by pipetting through a narrow glass capillary. The denuded oocytes were exposed to prolonged cultivation for 24, 48, and 72 h, and under the same conditions as by the oocyte maturation.

NOS subcellular localization in oocytes after prolonged cultivation

The zonae pellucidae of oocytes cultured for 24, 48 or 72 h were removed by a 0.1% (w/v) pronase treatment (Sigma-Aldrich). The oocytes were then washed three times in 0.01M phosphate-buffered saline (PBS) with 0.1% bovine serum albumin

(BSA), and fixed in 2.5% (w/v) paraformaldehyde in PBS for 1 h. After treatment in PBS – 0.5% BSA – 0.5% Triton X-100 (Sigma-Aldrich) for 2 h, the oocytes were incubated overnight at 4°C with mouse polyclonal anti-eNOS, anti-nNOS or anti-iNOS (1 : 100; Sigma-Aldrich) diluted in PBS – 0.1% BSA – 0.1% Tween 20 (Sigma-Aldrich). After being washed in PBS – 0.1% Tween 20, the oocytes were incubated at room temperature for 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 : 100; Sigma-Aldrich). Chromatin was stained with 0.5% (w/v) Hoechst 33258 (Sigma-Aldrich). The oocytes were mounted on slides with a SlowFade antifade kit (Invitrogen, Carlsbad, USA) used according to protocol. Images were obtained using a laser scanning confocal microscope Leica TCP SPE (Leica Microsystems, Wetzlar, Germany), and signal intensity was measured through imaging analysis by NIS Elements 3.00 (Laboratory Imaging s.r.o., Prague, Czech Republic). Measured values were related to MII oocytes and expressed relatively.

NOS inhibition during prolonged oocyte cultivation

In vitro matured and denuded oocytes were for 24, 48 and 72 h exposed to prolonged cultivation with a non-specific inhibitor, namely N^{ω} –nitro-L-arginine methylester L-NAME (2.5mM, 5mM, 7.5mM, 10mM; Sigma-Aldrich), or with a specific iNOS inhibitor, namely aminoguanidine (2.5mM, 5mM, 7.5mM, 10mM; Sigma-Aldrich). The oocytes were later mounted on slides and fixed in acetic alcohol (1 : 3, v/v) for 48 h. Morphological changes of oocytes were observed after staining with 1.0% orcein under a phase contrast light microscope. The oocites were classified into four groups: intact MII oocytes, parthenogenetically activated, fragmented (apoptotic), and lytic oocytes.

Statistical analysis

All the experiments were repeated four times and subjected to statistical analysis using SAS software (Statistical Analysis System, Version 9.0, 2008). The differences of signal intensity and percentages of morphological stages between the experimental groups and control were evaluated by the Analysis of Variance (Sheffé's test) and the non-parametric *F*-test, respectively. *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Subcellular localization of NOS isoforms in aged oocytes

The aim of the experiment was to localize and quantify NOS isoforms in porcine oocytes during prolonged *in vitro* cultivation.

All three NOS isoforms were localized in matured and aged oocytes after 24, 48, and 72 h of prolonged cultivation (Figure 1). There were subcellular localization differences between the individual isoforms. Endothelial NOS frequently occurred in small cytoplasmic foci in aged oocytes. Neuronal NOS was observed close by the cytoplasmic membrane and parthenote pronucleus. Inducible NOS was detected especially near the cytoplasmic membrane.

Isoform dynamics during prolonged cultivation was analyzed. After 24 h of prolonged cultivation the signal intensity of nNOS and iNOS decreased by 63 and 88%, respectively, while after 48 and 72 h the signal intensity was invariable. In the case of eNOS, the signal intensity decrease after 24 h was not statistically significant; its dramatic decrease came after 72 h of prolonged cultivation (Table 1).

NOS inhibition during oocytes prolonged cultivation

The aim of the second experiment was to evaluate the influence of NOS inhibitors on oocytes aged for 72 h. The non-selective inhibitor L-NAME

Table 1. Signal intensity of nitric oxide synthases (NOS) in porcine oocytes during prolonged cultivation

	MII	24 h	48 h	72 h
eNOS	1 ± 0.32^{a}	0.79 ± 0.19^{a}	0.76 ± 0.13^{a}	0.50 ± 0.21^{b}
nNOS	1 ± 0.17^{a}	0.37 ± 0.04^{b}	0.36 ± 0.02^{b}	0.34 ± 0.03^{b}
iNOS	1 ± 0.22^{a}	0.12 ± 0.04^{b}	0.15 ± 0.04^{b}	0.10 ± 0.02^{b}

eNOS = endothelial isoform, nNOS = neuronal isoform, iNOS = inducible isoform

signal intensity is related to metaphase II (MII) oocytes; statistically significant differences during prolonged cultivation for the same isoform (i.e. within the rows) are indicated by different superscripts ($P < 0.05$)

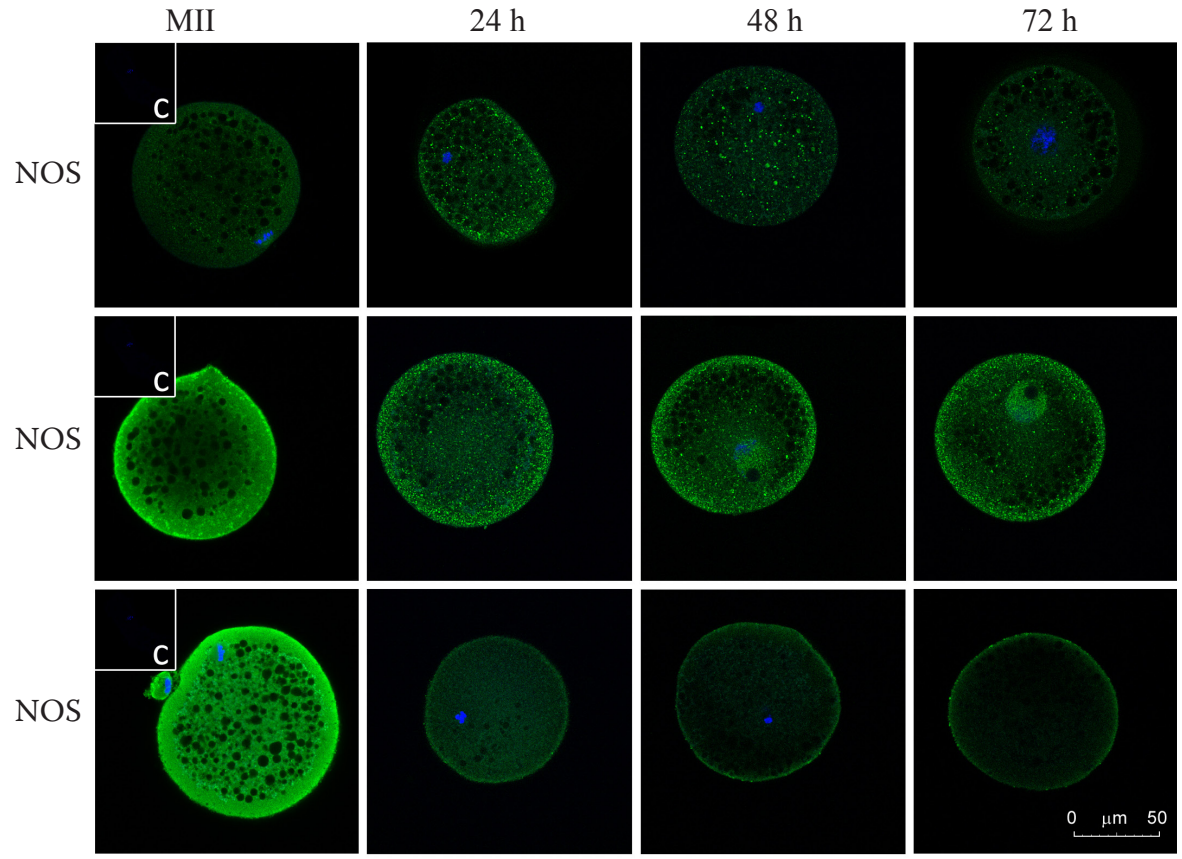

Figure 1. Subcellular localization and distribution of nitric oxide synthase (NOS) isoforms in MII and aged porcine oocytes. Oocytes were stained with mouse anti-eNOS, anti-nNOS or anti-iNOS and anti-mouse IgG-FITC. DNA was stained with Hoechst 33258. Magnification 400×

MII = matured MII oocytes without prolonged cultivation, 24 h, 48 h, 72 h = hours of prolonged cultivation, C = control oocytes exposed only to anti-mouse IgG

and selective inhibitor aminoguanidin were used. Morphological changes in oocytes ageing for 72 h under NOS inhibition were evaluated.

Both inhibitors suppressed cell death (apoptosis and lysis) in a dose-dependent manner. After 72 h of prolonged cultivation in 10mM L-NAME and 10mM aminoguanidin, only 1 and 8% of aged oocytes, respectively, succumbed to cell death. On the contrary, more oocytes remained intact or parthenogenetically activated (Figure 2). The highest rate of intact MII oocytes was observed in the culture of 10mM L-NAME.

DISCUSSION

In the study, the presence of all three NOS isoforms (eNOS, nNOS, iNOS) and their role in ageing porcine oocytes *in vitro* was demonstrated. The NOS isoforms occurred in matured and ageing oocytes. According to Hattori et al. (2000), mRNA NOS is present in matured mammal oocytes.

Although mRNA NOS and NOS are detected during oocyte growth and meiotic maturation in mice (Jablonka-Sharif and Olson, 1997; Abe et al., 1999), cattle (Tesfaye et al., 2006), and pigs (Chmelíková et al., 2010), NOS de novo proteosynthesis (according to the mRNA pattern) or the persistence of proteins synthesized in earlier oogenesis stages is uncertain in aged oocytes. However, significant NOS isoform relocalizations observed during oocyte ageing indicate the important role this enzyme plays in the ageing process.

The foci of eNOS were localized in the cytoplasm of aged oocytes. The findings are in agreement with eNOS localization in maturing porcine oocytes (Chmelíková et al., 2010). The fact that nNOS and iNOS are enclosed by the cytoplasmic membrane

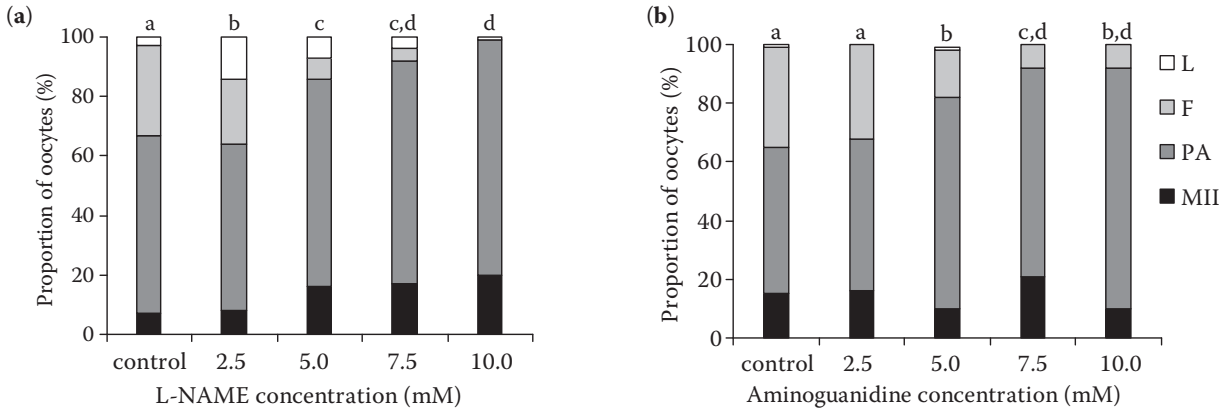

Figure 2. Influence of (**a**) L-NAME and (**b**) aminoguanidine on porcine oocytes aged for 72 h

MII = intact MII oocytes, PA = parthenogenetic activation, F = fragmentation, L = lysis
bars with different superscripts indicate significant differences between the ratios of aged oocyte stages ($P < 0.05$)

could be due to the known affinity of the actin cytoskeleton component for these two isoforms (Brophy et al., 2000; Zeng and Morrison, 2001). In spontaneously parthenogenetically activated oocytes, nNOS occurred mainly in the pronucleus. The nNOS localization in the cell nucleus is also known in somatic cells, and it is associated with transcription regulation (Yuan et al., 2004). For the genome reactivation of porcine embryos occurring in later embryonic development, nNOS localization may be either a form of parthenogenetic zygote preparation for embryonic genome reactivation or a reflection of early transcription of the same genes prior to complete genome reactivation (Latham and Schultz, 2001; Barnetová et al., 2012).

The signal intensity decrease of NOS isoforms in aged porcine oocytes was observed. After 24 h of prolonged cultivation, nNOS and iNOS levels decreased drastically. The decrease in eNOS level was less abrupt, and significant differences were determined after 72 h of prolonged cultivation. The reason for the various rates of isoform decrease is uncertain. Due to the fact that nNOS and iNOS levels in oocytes were relatively low and eNOS indicated a higher-intensity signal, the differences in the signal decrease rate may have resulted from diverse levels of specific isoforms (Kim et al., 2005; Chmelíková et al., 2010). Accordingly, detectable iNOS and nNOS signals in oocytes have yet to be observed (Kim et al., 2005; Hattori and Tabata, 2006). Moreover, the different localizations of NOS isoforms in oocytes and/or the effects of other substances with the ability to influence the half-life of NOS can play important roles in oocyte ageing (Ramet et al., 2003).

NOS isoforms play an essential part in meiotic maturation (Tao et al., 2005; Chmelíková et al., 2010). Our experiments highlight the role of NOS during prolonged cultivation of porcine oocytes. The ageing process is influenced by inhibitors, L-NAME, and the utilization of aminoguanidin. After 72 h of prolonged cultivation, most of aged oocytes remained either intact in MII phase or parthenogenetically activated. Presumably, the effect of NOS inhibition consisted in programmed cell death and lysis suppression. A similar impact was described for aminoguanidine in pancreatic β-cells of the islets of Langerhans (Corbett and McDaniel, 1996). Therefore, the higher percentage of parthenotes was not a result of NOS inhibition and NO absence, but rather of apoptosis or lysis inhibition. Conversely, recent studies have established the activation ability of NO donors (Petr et al., 2010).

Futher experiments are necessary to elucidate the physiological effects of NOS and to better understand the roles of NO in oocytes and somatic cells.

CONCLUSION

All three NOS isoforms are present in aged porcine oocytes, although their levels decrease during prolonged cultivation. NOS has the potential to play a role in physiological functions of porcine oocytes during *in vitro* ageing. Further experiments are necessary to definitely confirm this role.

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