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The Effect of Nitric Oxide Inhibition and Temporal Expression Patterns of the mRNA and Protein Products of Nitric Oxide Synthase Genes During *In Vitro* Development of Bovine Pre-implantation Embryos

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Contents

This study was conducted to determine the effect of Nitric oxide (NO) inhibition in bovine *in vitro* development and expression analysis of the three Nitric oxide synthase (NOS) isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), mRNA and protein in bovine oocytes and embryos. Selective inhibitor of NOS, *N*-ω-nitro-L-arginine methyl ester (L-NAME) was applied at different doses (0, 0.1, 1 and 10 mM) in maturation (experiment 1A), culture medium (experiment 1B) and in both maturation and culture media (experiment 1C). No significant differences were observed in cleavage and blastocyst rates when oocytes were matured in the presence of L-NAME as long as the inhibitor was omitted during fertilization and culture. However, significantly lower blastocyst rates were observed when L-NAME was present at higher level (10 mM) in culture medium alone and in both maturation and culture media. In experiment 2, mRNA isolated from triplicate pools of oocytes and embryos (n = 15–20) was subjected to quantitative real time reverse transcription polymerase chain reaction to investigate the expression of eNOS, iNOS and nNOS mRNA in normal IVP bovine oocytes and embryos. While eNOS and iNOS transcripts were detected at higher level in oocytes (immature and mature), two-cell and four-cell stage embryos, the nNOS was detected only in immature oocyte, two-cell and morula stages. In experiment 3, eNOS and iNOS protein expression analysis was performed in IVP oocytes and embryos and both proteins were detected in the cytoplasm and the nuclei (weak) of oocytes and embryos. These data provide the first evidence for the role of NO production and the presence of mRNA and protein products of NOS isoforms during bovine embryogenesis.

Introduction

Nitric oxide (NO) is a free radical molecule that has been found to be involved in a wide range of reproductive processes including gonadal steroidogenesis, folliculogenesis, fertilization, implantation, pregnancy and labour (Rosselli et al. 1998). NO is generated from L-arginine by action of nitric oxide synthase (NOS), an enzyme existing in three isoforms: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS/bNOS) in almost all cell types (Griffith and Stuehr 1995). While the eNOS and nNOS are constitutive and Ca^{2+}-calmodulin-dependent isoforms, which are involved in cellular signalling, iNOS is an iNOS isoform generated in response to a certain stimulus and is Ca^{2+}-independent.

The NO is reported to play a role in early development in mammals such as oocyte activation in pigs (Petr et al. 2005) and sea urchins (Kuo et al. 2000), oocyte maturation, embryonic development and implantation in mouse (Gouge et al. 1998; Jablonka-Shariff and Olson 1998; Sengoku et al. 2001). The potential role of NO during early embryogenesis was evidenced by higher consumption of arginine in mouse and human pre-implantation embryos (Lamb and Leese 1994; Houghton et al. 2002). To elucidate the role of NO in early development, *N*-ω-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS (Rees et al. 1990), has been applied in embryo culture and found to inhibit mouse blastocyst development in a concentration dependent manner (Gouge et al. 1998; Chen et al. 2001; Nishikimi et al. 2001; Sengoku et al. 2001). Similarly, a dose dependent effect of L-NAME was reported on murine oocyte maturation and embryonic development (Sengoku et al. 2001) and in rat implantation (Biswas et al. 1998).

The NO is produced from L-argenine, which may be derived intracellularly by conversion from amino acids like glutamine via citrulline or from amino acids present in the culture medium. So far in the studies conducted in mouse no clear role of NO in pre-implantation development has been determined and this is largely due to various culture media being used in different studies (Manser et al. 2004). Data from various studies in the mouse have evidenced the importance of media constituents in the sensitivity of embryos to NO inhibition. The addition of L-NAME at a level of 0.25 mM to Whitten medium (which is free of amino acids) significantly reduced the proportion of mouse embryos beyond the two-cell stage (Tranguch et al. 2003). In similar study while 0.5 mM L-NAME in KSOM medium significantly reduced the day 4 blastocyst rate from 50% to 6%, whereas not even 1 mM L-NAME in KSOM supplemented with physiological level of amino acids (KSOMaa) significantly affected the day 4 balstocyst rate (Manser et al. 2004). In the present study we investigate for the first time the effect of NO inhibition by addition of different levels of L-NAME in our standard maturation [modified Parker medium (MPM)] and culture media (CR1aa) on bovine embryo development. Due to the sensitivity of the sperm cells for even lower level of L-NAME as observed in our preliminary study, the application of L-NAME was omitted in the fertilization medium.

On the other hand excess application of sodium nitroprusside (SNP), a NO donor, has been shown to

affect embryonic development in dose dependent manner (Chen et al. 2001; Tranguch et al. 2003). The inhibition of development due to lack of NO production could be reversed by addition of NO donor, SNP, and that NO production must be regulated within some specific limits. Experiments with lower concentration of L-NA (250 μM) with SNP (100 nM) did not rescue a significant number of embryos, suggesting that the concentration of NO produced must be controlled with limits (Tranguch et al. 2003). The data from various experiments in the mouse suggested that moderate amount of NO production is essential for successful embryonic development.

In order normal embryonic development to occur, the level of NO must be regulated and thus the mRNA and protein of the various isoforms of NOS should be expressed in mammalian pre-implantation development to support adequate amount of NO production. The expression of nNOS, eNOS and iNOS mRNA throughout the mouse pre-implantation period was recently investigated using the reverse transcription polymerase chain reaction (RT-PCR) (Tranguch et al. 2003). All the three isoforms were found to be expressed from the two-cell until late blastocyst stage, except for iNOS, which could not be detected at early blastocyst stage. The expression of iNOS and eNOS was found to increase during human endometriosis and adenomyosis and thus suggested to be correlated with early embryo loss (Ota et al. 1998). However, till now no studies have been carried out to investigate the role of NO in early bovine embryogenesis and it is not known whether the various NOS isoforms are expressed during bovine pre-implantation development or not. Therefore in the present study we aimed (i) to investigate the role of NO in bovine oocytes and pre-implantation embryos development by dose dependent application of L-NAME, a selective inhibitor of NOS, in maturation and culture media, (ii) to profile the temporal expression pattern of NOS genes in bovine pre-implantation developmental stages and (iii) to detect and localize the protein products of iNOS and eNOS in pre-implantation developmental stages using immunofluorescence staining.

Materials and Methods

Oocyte and embryo culture

Cattle ovaries were obtained from a local slaughterhouse and transported to the laboratory in the thermoflask containing 0.9% physiological salt solution at 37°C within 2–3 h after slaughter. The cumulus–oocyte complexes (COCs) aspirated from follicles of 2–8 mm in diameter were washed two times with MPM supplemented with 15% oestrus cow serum (OCS) and placed in groups of 50 in 400 μl maturation medium under mineral oil in four-well dishes (Nunc, Roskilde, Denmark). Only oocytes with evenly granulated cytoplasm and surrounded by multiple layers of cumulus cells were used for this experiment. The COCs were cultured in MPM as basic medium (Ponsuksili et al. 2002). Each millilitre of MPM was supplemented with 0.73 mg $NaHCO_3$, 1.27 mg HEPES (Sigma, St Louis, MO, USA), 0.23 mg sodium pyruvate, 10 mg gentamycin (Gibco BRL, Eggenstein, Germany), 0.6 mg hemicalcium lactate, 15% OCS and 10 μg follicle stimulating hormone (FSH) (Schering, Kenilworth, NJ, USA). Maturation was performed for 24 h at 39°C under a humidified atmosphere of 5% CO_2 in air.

Following *in vitro* maturation (IVM), *in vitro* fertilization (IVF) was performed in four-well dishes containing 400 μl fertilization medium (Fert-TALP) (Parrish et al. 1988), supplemented with 6 mg/ml BSA (Sigma), 2.2 mg/ml pyruvate (Sigma) and 1 mg/ml heparin (Sigma). An amount of 10 μl of penicillinamine, hypotaurine, epinephrine (PHE) was added to each well and covered with mineral oil. A swim-up procedure was applied to obtain motile sperm cells from frozen-thawed semen (Parrish et al. 1988). Fertilization was initiated during co-incubation of spermatozoa and the matured oocytes for 20 h in the same incubator under the same temperature and atmospheric CO_2 content as used for maturation.

After IVF, the presumptive zygotes were denuded of cumulus cells by gentle vortexing for 4 min. Cumulus-free zygotes were washed two times with CR1 culture medium supplemented with 10% OCS, 10 μl/ml BME (amino acids) and 10 μl/ml MEM (non-essential amino acids) (Gibco BRL) and transferred in groups of 50 in four-well dishes containing 400 μl CR1 culture medium (Rosenkrans and First 1994) covered with mineral oil and cultured at 39°C under humidified atmosphere of 5% CO_2 in air. Each millilitre of CR1 medium contained 0.6 mg hemicalcium lactate (Sigma), 6.4 mg NaCl, 0.2 mg KCl, 2.0 mg NaHCO3, 0.14 mg L-glutamine and 2 μl phenol red solution (5% in PBS) (Sigma) supplemented with 0.04 mg penicillin and 0.08 mg streptomycin sulphate (Sigma).

Experiment 1: nitric oxide inhibition and developmental study

In order to investigate the effect of NO inhibition in early embryo development various levels (0.1, 1 and 10 mM) of L-NAME (Sigma) were applied in either only maturation or culture medium and in both. Controls with no treatment (0 mM L-NAME) were run along with all treatment groups. All experiments were repeated four times each comprising 200 oocytes for maturation and subsequent culture *in vitro*. The time points of application of L-NAME during oocyte maturation and embryo culture as well as monitoring of embryo development during the experiment are shown in Fig. 1. As L-NAME was found to alter the pH of the culture, after each application the pH of the medium was checked and adjusted with the control maturation and culture media with out L-NAME (pH = 7.2).

As indicated in the Fig. 1 above, during all three experiments embryo development during *in vitro* culture was assessed by determining the cleavage rate at 48 hour post-insemination (hpi) and the blastocysts rate until day 9.

Application of l-NAME during maturation

Four groups of immature oocytes (50 oocytes in each group) were matured in the maturation media to which 0, 0.1, 1 and 10 mM L-NAME was added. However, subsequent IVF and culture of oocytes was performed

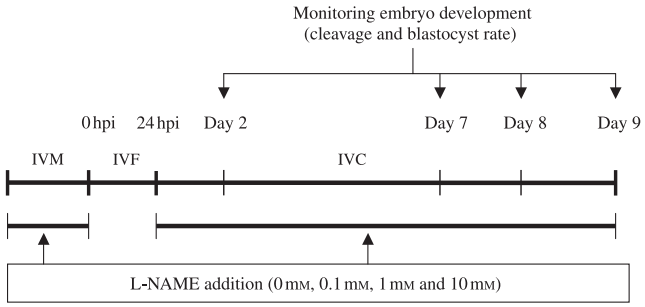

Fig. 1. Experimental design to assess the effect of addition of L-NAME (0, 0.1, 1 and 10 mM) either to the maturation or culture or both media on the cleavage and blastocyst rates of *in vitro* fertilized oocytes. hpi: hour post-insemination

without L-NAME addition. Matured oocytes were inseminated and presumptive zygotes were further cultured in the absence of L-NAME until day 9 post-insemination.

Application of L-NAME in culture medium

Four groups of immature oocytes (50 oocytes in each group) were allowed to mature in maturation medium in the absence of L-NAME. Following maturation, oocytes were inseminated in the fertilization medium in which L-NAME was omitted. After 20 h of co-incubation of oocytes and bull spermatozoa zygotes were transferred to wells containing culture medium to which 0, 0.1, 1 and 10 mM L-NAME were applied. The development of embryos was assessed until day 9 of post-insemination.

Application of L-NAME in both maturation and culture media

Equal groups and numbers of immature oocytes as used in experiments 1 and 2 were allowed to mature in the maturation media to which 0, 0.1, 1 and 10 mM of L-NAME was added and the resulting matured oocytes were inseminated *in vitro* in the absence of L-NAME. Presumptive zygotes were further cultured in the culture media, which was supplemented with 0, 0.1, 1 and 10 mM L-NAME.

Experiment 2: temporal expression analysis of eNOS, iNOS and nNOS mRNA in bovine oocytes and pre-implantation stage embryos

RNA isolation and reverse transcription

Poly(A) RNA was isolated from four independent pools of 15–20 immature oocytes, matured oocytes, two-cell, four-cell, eight-cell, morula and blastocysts stages of development. This was performed using oligo(dT) attached magnetic beads (Dynal, Oslo, Norway) following the manufacturer's instruction as described in Tesfaye et al. (2003) and 2004). In order to check for any possible genomic DNA contamination, 1 μl of the isolated mRNA was used as a template to perform a PCR using an intron spanning primer of GAPDH gene. The genomic DNA free mRNA samples from each pool and developmental stages was reverse transcribed in 20 μl total reaction containing 2.5 μM oligo(dT)$_{12}$ N (where N = G, A or C) primer, 4 μl of 5× first strand buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris–HCL pH 8.3), 2.5 mM of each dNTP, 10 U RNase inhibitor (Promega, Mannheim, Germany) and 100 U of Super-Script II reverse transcriptase (Invitrogen, Karlsruhe, Germany). In terms of the order of adding the reaction components, mRNA and oligo(dT)$_{12}$ primer were mixed first, heated to 70°C for 3 min, and placed on ice until the addition of the remaining reaction components. The reaction was incubated at 42°C for 90 min, and terminated by heat inactivation at 70°C for 15 min.

Quantitative real time PCR

Polymerase chain reactions were conducted in an ABI Prism® 7000 SDS instrument (Applied Biosystems, Foster City, CA, USA) using SYBR® Green as a double-strand DNA-specific fluorescent dye. Quantitative analyses of embryo cDNA were performed in comparison to histone H2a (endogenous control) (Robert et al. 2002), as applied in ours (El-Halawany et al. 2004; Tesfaye et al. 2004) and also others (Lonergan et al. 2003). PCR was performed by using 2 μl of each sample cDNA and specific primers, which amplify histone 2a, nNOS, iNOS and eNOS genes. Primer sequences, the size of amplified products and the GenBank accession number are shown in Table 1. Standard curves were generated for both target and internal control genes using serial dilutions of plasmid DNA (10^1–10^8 molecules/μl). PCRs were performed in 20 μl reaction volume containing 10 μl 2× SYBR Green JumpStart™Taq ReadyMix™ with internal reference dye (ROX) for quantitative PCR (Sigma), optimal levels of forward and reverse primers and 2 μl embryonic cDNA. The cDNA samples from four independent pools of 15–20 oocytes and embryos from each developmental stage (immature and mature oocytes, two-, four-, eight-, 16-cell, morula and blastocyst) were utilized to quantify all genes of interest. During each PCR reaction samples from the same cDNA source were run in duplicate to control the reproducibility of results.

Table 1. Sequence specific primers used for quantitative real time PCR amplification

Transcript	Primer sequence (5′–3′)	Amplicon length (bp)	GenBank accession no.
INOS	For-GGTGGAAGCAGTAACAAAGGA Rev-GACCTGATGTTGCCGTTGTTG	230	AF340236
eNOS	For-CCTCACCGCTACAATATCCT Rev-TGCTCGTTGTCCAGGTGCTTC	197	NM_181037
nNOS	For-CGTCTCTTCAAGCGCAAAGTT Rev-CGTTGACCGCAAGAATGATGT	151	HSNOS1S
H2a	For-CTCGTCACTTGCAACTTGCTATTC Rev-CCAGGCATCCTTTAGACAGTCTTC	148	NM_178409

A universal thermal cycling parameter (10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C) was used to quantify each transcript. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7-s interval until the temperature reached 95°C. Final quantification analysis was performed using the relative standard curve method (User bulletin no. 2 ABI PRISM 7700 SDS) and results were reported as the relative expression or n-fold difference to the calibrator cDNA (i.e. immature oocyte stage) after normalization of the transcript amount to the endogenous control (H2a gene).

Experiment 3: detection and localization of eNOS and iNOS proteins in bovine oocytes and embryos

Immunofluorescence staining of oocytes and embryos

In vitro produced bovine immature and mature oocytes, zygotes, two-cell, four-cell, eight-cell, morula and blastocyst stage embryos were washed three times in PBS, fixed in 4% (w/v) paraformaldehyde in PBS overnight at 4°C. The fixed specimens were permeabilized during 2.5 h incubation in 0.5% (v/v) Triton-X100 (Sigma) in PBS. In order to inhibit non-specific binding of the antibodies, samples were subsequently blocked in 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h. The oocytes and embryos were then incubated for 1 h at 39°C with 1:200 and 1:80 dilution of anti-eNOS monoclonal (Sigma-Aldrich) and anti-iNOS polyclonal primary antibody (Upstate, Charlottesville, VA, USA), respectively. After three consecutive washes with PBS, oocytes and embryos were further incubated for 1 h with 1:500 dilutions of secondary anti-rabbit IgG FITC conjugated antibody (Sigma). Negative controls were processed in the same manner except the primary antibody was omitted. In order to visualize the nucleus of the cells, oocytes and embryos were finally incubated with 0.5 μg/ml propidium iodide (Sigma). After an ultimate washing with PBS, oocytes and embryos were mounted on glass slides (Menzel GmbH$\leq$ 0.05 considered to be significant.

Results

Experiment 1: the effect of L-NAME application on bovine embryo development

The effect of L-NAME was examined to investigate the role of NO/NOS activity in the development of bovine embryos *in vitro* during the pre-implantation period. The *in vitro* development of bovine embryos was monitored at day 2 to assess the cleavage rate and blastocyst rate was monitored until day 9 during culture in the presence or absence of L-NAME at different concentrations either in maturation or culture medium or in both.

Effect of L-NAME addition in maturation medium alone

To determine if inhibition of NO by L-NAME during oocyte maturation stage affects embryo development, COCs were cultured for 24 h in either MPM alone (control) or with L-NAME at 0.1, 1 and 10 mM concentrations. No significant effect of inhibitor presence in maturation medium was observed both on cleavage and blastocyst rate when oocytes were further fertilized and cultured without the inhibitor as indicated in Fig. 2.

The effect of L-NAME addition in culture medium alone

To determine whether NO inhibition affects pre-implantation embryo development, oocytes matured and fertilized in medium devoid of L-NAME, were cultured in CR1aa medium either supplemented with L-NAME at 0.1, 1 and 10 mM or with out L-NAME as control (Fig. 3). When the presumptive zygotes were cultured in the presence of high level of L-NAME (10 mM), the blastocyst rate of these embryos was significantly affected ($p \leq 0.05$) compared to non-treated controls. Very low percentage of embryos developed to blastocyst at 10 mM L-NAME level (1 ± 2%) compared with those cultured at 0.1 mM (25 ± 3%), 1 mM (32 ± 8%) and untreated control group with (15 ± 3%). During culture monitoring it has been observed that most of the embryos cultured at high level of L-NAME (10 mM) arrested at eight-cell stage. The application of L-NAME

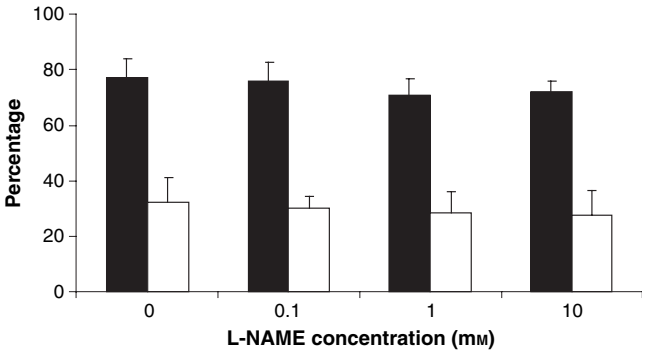

Fig. 2. Effect of NO inhibition by addition of L-NAME (0, 0.1, 1 and 10 mM) to maturation medium on the cleavage (black bars) and blastocyst rate (white bars) (Mean ± SD) of embryos fertilized and cultured in the absence of L-NAME

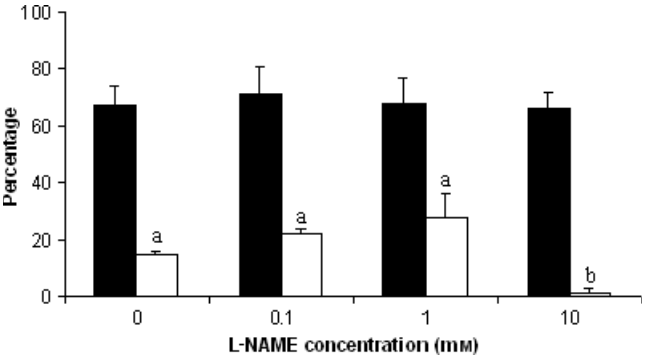

Fig. 3. Effects of addition of L-NAME (0, 0.1, 1 and 10 mM) to the culture medium alone on the cleavage (black bars) and blastocyst rate (white bars) of embryos. Similar bars (Mean ± SD) with different letters between treatment groups differ significantly ($p \leq 0.05$)

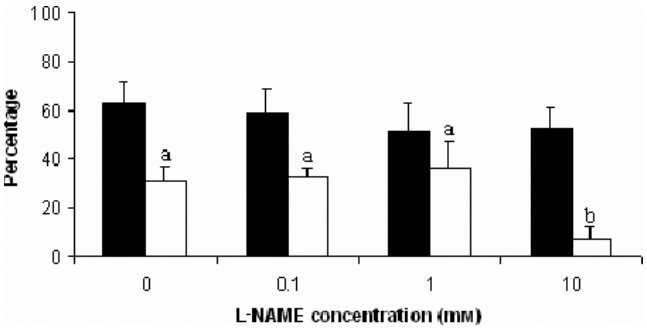

Fig. 4. Effects of addition of L-NAME (0, 0.1, 1 and 10 mM) to both maturation and culture media on the cleavage (black bars) and blastocyst (white bars) rate of embryos. Bars (Mean ± SD) with different letters between treatments differ significantly ($p \leq 0.05$)

at all concentrations in the culture medium had no effect on the first cleavage rate of zygotes.

The effect of L-NAME addition in both maturation and culture media

The effect of inhibition of NO on the developmental potential of embryos (with respect to cleavage and blastocyst rate) was assessed after addition of L-NAME in both maturation and culture medium. As shown in Fig. 4, significant effect of L-NAME application on embryo development was observed at a level of 10 mM. Blastocyst rate was found to reduce to 7 ± 1% by addition of L-NAME at a level of 10 mM compared with 31 ± 6% blastocyst rate in non-treated control group ($p < 0.05$). The blastocyst rate was not affected by L-NAME application at 0.1 mM (32 ± 4%) and 1 mM (36 ± 11%) concentrations compared with the untreated controls (31 ± 6%; $p > 0.05$). Moreover, no significant effect of L-NAME could be observed on the first cleavage rate of zygotes at all three concentration levels compared with the untreated controls ($p > 0.05$).

Experiment 2: quantitative expression profile of NOS genes

With the aim of studying the expression profiles of NOS genes throughout the pre-implantation developmental stages of normal *in vitro* produced bovine embryos, we investigated the transcriptional profile of eNOS, iNOS and nNOS isoforms using fluorescent monitored quantitative real time PCR.

As shown in Fig. 5 (a, b and c) differences in mRNA expression among the different embryonic developmental stages were found for eNOS, iNOS and nNOS genes. The eNOS transcript was found to be expressed at higher level ($p < 0.05$) in immature oocytes and down-regulated at matured oocyte stages. This transcript was found to be up-regulated again at the two- and four-cell developmental stages, after which no transcript could be detected. A comparable expression pattern has also been observed for iNOS. The iNOS transcript was abundant at higher level ($p < 0.05$) at early developmental stages namely: immature and matured oocytes and two- and four-cell developmental stages. Transcript abundance reduced significantly at eight-cell and morula stages with a slight increase at the blastocyst stage. A very different temporal expression pattern has been observed in the expression of nNOS. Significant level of nNOS transcript was detected at immature oocyte, four-cell and morula stages.

Experiment 3: immunohistological detection and localization of NOS proteins in bovine oocytes and embryos

Immunofluorescence staining procedure was employed to detect and localize expression of eNOS and iNOS proteins in *in vitro*-produced bovine oocytes and pre-implantation embryos. Both eNOS and iNOS proteins were detected in oocytes and embryos during the pre-implantation period (Figs 6 and 7). The pattern of immunostaining, yet, seemed to exhibit slight differences depending on the NOS isoform investigated. No fluorescence signal was detected in the negative controls ([Figs 6 and 7i), in which the primary antibodies are omitted, indicating the specificity of staining for the corresponding NOS protein.

The eNOS immunodetection (Fig. 6) has revealed a diffuse distribution of this protein throughout the cytoplasm of immature (a) and matured oocytes (b). From the zygotic (c) to the morula stage (g) eNOS was found concentrated in a patchy pattern underneath the

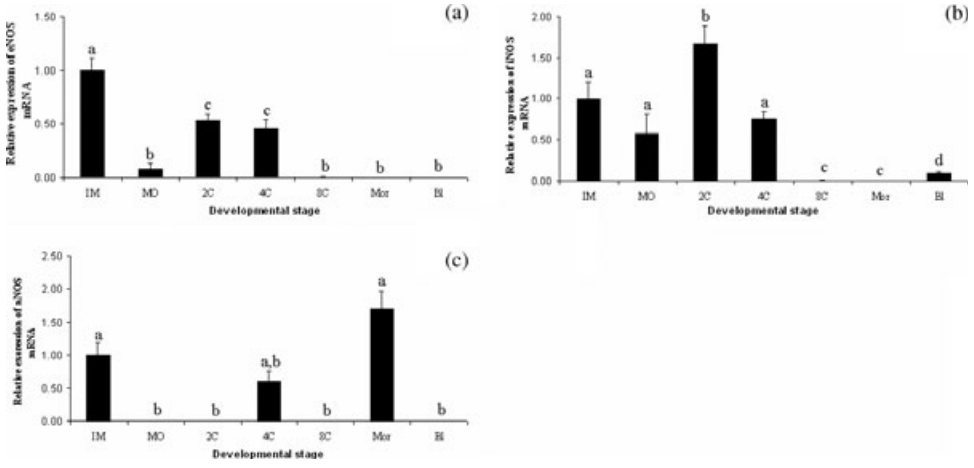

Fig. 5. The relative abundance of NOS transcripts, eNOS (a), iNOS (b) and nNOS (c) in *in vitro*-produced bovine pre-implantation stage embryos including, immature (IM) and mature (MO) oocytes then embryos at two-cell (2C), four-cell (4C), eight-cell (8C), morula (Mor) and blastocyst (Bl) stages. Similar bars with different letters differ significantly ($p \leq 0.05$)

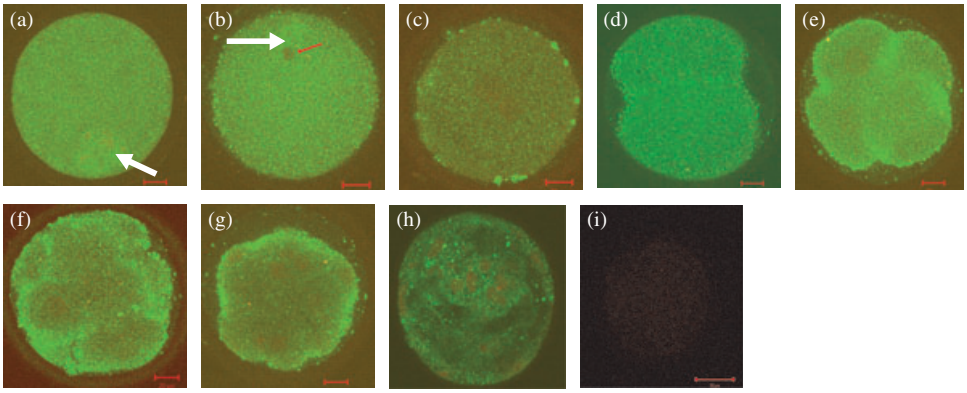

Fig. 6. Subcellular localization of endothelial nitric oxide synthase (eNOS) protein in bovine oocytes and in pre-implantation embryos [immature oocytes (a), matured oocytes (b), zygote (c), two-cell (d), four-cell (e), eight-cell (f), morula (g) and blastocyst (h) stages]. Controls (i) was stained without primary anti-eNOS antibody. White arrows indicate concentrated localization of eNOS protein. This figure is representative of 10 oocytes or embryos stained in the experiment. Nuclei are stained by propidium iodide (red). Scale bars represent 20 μm

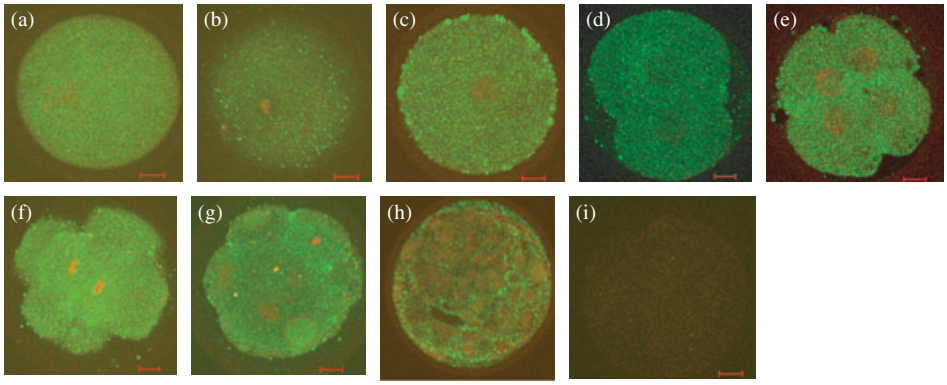

Fig. 7. Subcellular localization of iNOS protein in bovine oocytes and in pre-implantation embryos [immature oocytes (a), matured oocytes (b), zygote (c), two-cell (d), four-cell (e), eight-cell (f), morula (g) and blastocyst (h) stages]. In the control (i) primary anti-iNOS antibody was omitted during staining. This figure is representative of 10 oocytes or embryos stained in the experiment. Nuclei are stained by propidium iodide (red). Scale bars represent 20 μm

plasma membrane showing marked accumulation at the sites of cell–cell contacts. At the blastocyst stage (h), the staining was apparently more concentrated in the inner cell mass compared with the trophectoderm cells. Yet, some accumulation of eNOS protein could also be detected in the trophoblast cells.

As shown in Fig. 7 the iNOS protein was uniformly distributed throughout the cytoplasm of immature oocytes (a), whereas matured oocytes (b) showed a more granular pattern of staining. This granular pattern was also observed from the zygotic (c) to the morula stage (g). In the blastocyst the granular staining appeared to be concentrated in the trophectoderm cells as compared with the inner cell mass (h). Yet, there is some detectable level of iNOS protein in the inner cell mass of the blastocyst.

Discussion

In light of studies focused on NO production and emphasizing its crucial importance during the early embryogenesis in mice (Jablonka-Shariff and Olson 1998; Kuo et al. 2000; Chen et al. 2001; Nishikimi et al. 2001; Nakamura et al. 2002; Tranguch et al. 2003; Manser et al. 2004) we investigated the effect of inhibition of NO in bovine oocytes and embryo development to get insight into the role of NO in *in vitro* development of bovine pre-implantation embryos. Moreover, in this study we have for the first time evidenced the expression of NOS genes at mRNA and protein level in bovine oocytes and pre-implantation embryos.

In order to understand the role of NO in embryogenesis so far various investigations have been carried out in mouse and murine species using inhibitors (L-NAME, L-NA and L-N^6-monomethyl-L-argenine (L-NMMA)), scavengers (methylene blue and haemoglobin) and donors (SNP) of NO. Most of the NO inhibitors are known to differ in their affinity for the three isoforms of NOS (Rosselli et al. 1998). L-NAME is one of the most widely used argenine substrate analogue and its effect could be reversed by addition of L-argenine (Manser et al. 2004) or NO donor, SNP (Chen et al. 2001; Sengoku et al. 2001; Tranguch et al. 2003) in the culture. In the present study we have used L-NAME as selective inhibitor of NO synthesis and we have demonstrated that its addition in the maturation medium alone has no effect on the further development of the embryos as long as the inhibitor was omitted in the fertilization

and culture medium. This may be attributed to the ability of the embryo to regenerate the production of NO using the substrate available in media during fertilization and culture in the absence of the inhibitor (Chen et al. 2001). In similar studies in murine, where NOS inhibitor affected oocyte maturation in dose-dependent manner, its effect could be reversed by the concomitant addition of NO donor, SNP (Sengoku et al. 2001).

Even though the assessment of maturation status of oocytes treated with NO inhibitor was not the focus of this study, dose-dependent function of NO in oocyte maturation has been observed in mouse (Bu et al. 2003). In that study examination of nitrite/nitrate in the culture medium revealed that NO stimulated the meiotic maturation of mouse oocytes at concentration of 31 ± 5 μmol/l, while it inhibited the maturation at 60 ± 8 μmol/l. Furthermore, by using both NO donor and NO inhibitor, it has been proven that the NO pathway plays important roles in ovarian function and follicle development (Sengoku et al. 2001). Ovarian defects observed in eNOS knock-out mice suggest that eNOS-derived NO is a modulator of oocyte meiotic maturation (Jablonka-Shariff and Olson 2000). Moreover, the iNOS was found to be concentrated in the mouse germinal vesicle suggesting that iNOS-derived NO affects the meiotic resumption of oocytes and signals within the oocytes (Huo et al. 2005).

In experiments 1B and 1C of this study, the application of NO inhibitor in either culture medium alone or in both maturation and culture media had no effect on the first cleavage rate of the embryos. However, the presence L-NAME at higher concentration (10 mM) in the culture medium either in the presence or in the absence of the inhibitor in maturation medium had a significant effect on the number of zygotes developed to the blastocyst stage. In both cases most of the zygotes cleaved normally but arrested at the eight-cell stage in the culture and failed to reach to the blastocyst stage. Similar observations were also made in the mouse where no significant difference in the percentage of embryonic development was observed within six- to eight-cell stage when L-NAME was used at the concentrations of 0.1–10 μM (Chen et al. 2001). The study by Manser et al. (2004) has also shown that the application of L-NAME at levels of 0.5 mM and 1 mM in the KSOM culture (without amino acids) had no significant effect on the proportion mouse embryos between zygote and eight-cell stages compared with the untreated control groups. However, significant number of embryos have been found to arrest beyond the compacted eight-cell stage after treatment with L-NAME. These all data evidenced the stage specific sensitivity of pre-implantation embryos to the application of L-NAME thereby to the balance in the NO in the culture.

The various studies in the past have shown that NO is not only required for normal pre-implantation embryo development but that it also must be produced within a limited range of concentration (Chen et al. 2001; Tranguch et al. 2003). In the present study even though differences are not significant, a slight increase in blastocyst rate was observed in the presence of L-NAME at 0.1 and 1 mM concentrations in the maturation and culture media compared with the untreated controls. Similarly, increased proportion of 16-cell and morula stage embryos developing to blastocyst stage were obtained by application of NO scavenger, hemoglobin, in the culture, which may limit diffusion of toxic amounts of NO into the developing embryos (Lim and Hansel 1998). This may show the need to revise the *in vitro* culture condition under practise with respect to the level of substrates for NO production in the culture. Moreover, there is a possibility to optimize the pre-implantation development through the application of NO inhibitors or scavengers to attain physiologically optimal NO level in the culture.

Because of the different culture media used in various studies, no clear effect of NO on pre-implantation development has emerged so far. Media constituents, mainly amino acids, play an important role in the sensitivity of embryos to inhibition of NO. In the study conducted in mouse, while the presence of 0.5 mM L-NAME in KSOM significantly reduces the day 4 blastocyst rate from 50.2 to 6%, the same L-NAME level in KSOM-glut (KSOM with no glutamine) prevents all development beyond the compacted eight-cell stage (Manser et al. 2004). However, in KSOMaa not even 1 mM L-NAME significantly affected the day 4 blastocyst rate. In contrast, the addition of 0.25 mM L-NAME to Whitten medium (which is free of amino acid) significantly reduced the number of mouse embryos developing beyond the two-cell stage (Tranguch et al. 2003). Similarly, in the present study significant effect of the L-NAME has been observed at 10 mM level but not at a level of 0.1 and 1 mM. The amount of amino acids available in the culture medium of the present study seemed to facilitate the production of enough amount of NO, which can even resist the application of L-NAME at up to a level of 1 mM without any effect on development. Comparison between laboratories on the effect of L-NAME will be very difficult due to the various culture systems utilized specially with respect to specific substrate of NO production. Therefore, at any specific culture condition it is important to ensure a physiological level of NO is for a better oocyte/embryo survival and subsequent development.

In the present study, we showed for the first time the expression of mRNA for all three NOS isoforms (eNOS, iNOS and nNOS) in bovine oocytes and pre-implantation embryos. Analysis of temporal quantitative expression patterns revealed the potential complex interactions between these isoforms during early bovine embryo development. The presence of multiple NOS isoforms in bovine pre-implantation period indicates that they assure the production of NO during development. The compensatory role of NOS isoforms was evidenced in mice as animals deficient in one isoform of NOS are viable (Huang et al. 1993; Irikura et al. 1995; MacMicking et al. 1995). As eNOS derived NO is the key modulator of oocyte meiotic maturation (Jablonka-Shariff and Olson 2000), the reduction in eNOS mRNA after maturation in our study may support the hypothesis that a reduction of NO is necessary to stimulate GVBD by decreasing cGMP during oocyte maturation (Nakamura et al. 2002). Moreover, Nakamura et al. (2002) proposed that the iNOS-NO may play an

important role in oocyte meiotic maturation through cGMP pathway. According to a bovine cDNA microarray anaylsis performed in our laboratory between matured oocytes and blastocysts stage embryos, the eNOS was clustered with genes involved in cell growth and differentiation, while iNOS was similar to signal transduction genes (Mamo 2004).

To further elucidate the expression and the potential role of NOS genes, we used immunocytochemistry to detect and localize eNOS and iNOS proteins in bovine oocytes and embryos. The eNOS protein was predominantly detected in the peripherical areas of the cytoplasm and the staining was most intensive towards the junctions between blastomeres (two-, four-, eight cell and morula). These predominant granules may represent eNOS in some organelles such as Golgi apparatus, mitochondria or plasma membrane since this isoform is known to be membrane-bound (Pollock et al. 1993; Jablonka-Shariff and Olson 1998). At the blastocyst stage the protein from this isoform were found to be detected more in the inner cell mass than in trophoblast cells. However, the iNOS protein was detected more in the trophoblast cells than in the inner cell mass cells. Immunocytochemistry analysis for the presence of iNOS and eNOS in mouse blastocysts recovered 1 h after exposure to oestrogen showed that both protein products were detected in trophoblast cells, suggesting their role in regulating vascular events at the site of implantation (Gouge et al. 1998). Therefore, the mRNA and protein expression of multiple isoforms in pre-implantation embryos further indicate the production and importance of NO during early bovine embryogenesis.

In conclusion the present study has demonstrated the stage specific and dose dependent sensitivity of bovine pre-implantation embryos for inhibition of NO through L-NAME. Moreover, we have for the first time demonstrated the presence of the mRNA and protein products of the multiple NOS isoforms in bovine oocytes and embryos, which ensure the production of NO during pre-implantation development.

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