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Nitric oxide synthase (NOS) genes expression in catlle in vitro produced embryos

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Nitric oxide synthase (NOS) genes expression in cattle in vitro produced embryos

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Untersuchung der Expression von Nitric Oxid Synthase (NOS) Genen in in vitro erzeugten Rinderembryonen

Diese Untersuchung wurde durchgeführt, um die Rolle von NO in bovinen Oozyten und während der Entwicklung der Embryonen im präimplantativen Stadium zu untersuchen, das temporale Expressionsmuster der NOS Gene in den präimplantativen Entwicklungsstadien zu identifizieren und die Protein Produkte von iNOS und eNOS während dieser Zeit zu finden und lokalisieren. Kumulus-Oozyten Komplexe und Einzell- Embryonen wurden in Maturations- und/oder Kulturmedium ohne/mit unterschiedlichen Mengen von L-NAME, einem NO Inhibitor, inkubiert. Nach der Inkubation wurden die Blastozystenrate und ebenfalls die Teilungsrate festgestellt. Es wurden keine signifikanten Effekte des Inhibitors bei der Maturation der Oozyten mit L-NAME im Medium festgestellt. Bei der Kultivierung unterdrückt L-NAME die Embryoentwicklung bei 10 mM signifikant ($p \leq 0,05$) verglichen mit der Kontrollgruppe (Blastozystenraten von 1 ± 2 % bei 10 mM verglichen mit 15 ± 10 % bei 0 mM). Dieser Effekt wurde verkleinert, wenn L-NAME auf 0,1 oder 1 mM reduziert wurde. Ähnliche Ergebnisse wurden nach der Zugabe des Inhibitors in Maturations- und Kulturmedium beobachtet. Die Zugabe des Inhibitors hatte keinen Effekt auf die Teilungsrate weder bei der Maturation, Kultur oder beiden. Bei der Maturation wurde eine Teilungsrate von 71 ± 11 %, 63 ± 13 % bis 63 ± 11 % bei jeweils 0, 1 und 10 mM L-NAME beobachtet. Mit der Real-Time RT PCR Technik wurde festgestellt, dass die iNOS und eNOS Gene in Oozyten, 2-Zell und 4-Zell Embryonen gut exprimiert waren, jedoch weniger stark in anderen Stadien mit der Ausnahme von iNOS im Blastozystenstadium. Das Expressionsniveau war in maturierten Oozyten verglichen zu immaturen Oozyten niedrig. nNOS war in Oozyten, 4-Zelenl und Morulaes Embryonen exprimiert. NOS Proteine wurden im Cytoplasma der Oozyten bis zu den Blastozystenstadien mit einer schwachen Färbung im Zellkern lokalisiert.

Unsere Ergebnisse zeigen zum ersten Mal alle drei NOS Expressionsprofile in preimplantativen Rinderembryonen, was bestätigt, dass NO aus drei verschiedenen Isoformen synthetisiert wird, welche die bovine präimplantative Entwicklung beeinflussen.

Nitric oxide synthase (NOS) genes expression in cattle in vitro produced embryos

This study was conducted to determine whether NOS-NO affects oocytes meiotic maturation and embryo development *in vitro* in bovine, to profile the temporal expression pattern of NOS genes, then to detect and localize the protein products of iNOS and eNOS in preimplantation developmental stages. Cumulus-oocyte complexes and one-cell embryo were incubated in maturation or/and culture media without or with different doses of L-NAME, an inhibitor of NO. End point was blastocyst rate but cleavage rate was examined also. No significant effect of the inhibitor was obtained when oocytes were matured in presence of L-NAME as indicated by a blastocyst rate of 28 ± 14 %, 19 ± 15 % and 22 ± 11 % at 0, 1 and 10 mM respectively. However, high levels of arrested blastocyst rates were indicative of an impairment of normal development. At culture, L-NAME inhibited embryo development at 10 mM, significantly ($p \leq 0.05$) compare to control group (blastocyst rates of 1 ± 2 % at 10 mM against 15 ± 10 % at 0 mM). The inhibitory effect was reduced when L-NAME was decreased to 0.1 or 1 mM. Similar result was observed after application of the inhibitor in maturation and culture media. The application of the inhibitor had no effect on the cleavage rates either at maturation, culture or both at any concentration compared to the control group. In maturation, 71 ± 11 %, 63 ± 13 % and 63 ± 11 % cleavage rates have been obtained at 0, 1 and 10 mM L-NAME respectively. Using real-time RT PCR, the iNOS and eNOS genes were found well expressed in oocytes, 2C and 4C embryos but were far less prevalent in other developmental stages, except iNOS at blastocyst stage. Their expression levels were low in mature oocytes compare to immature oocytes. The absence of quantifiable eNOS or iNOS at 8C and morulae was in contradiction to the qualitative identification. Neuronal NOS mRNA was measurable only in immature oocytes, 4C and morulae stages. NOS proteins detected by immunohistochemistry were localized in cytoplasm of oocytes to blastocyst stages with a weak staining in the nuclei. In germinal vesicle-stage oocytes, the immunoreactivity was localized in the ooplasm but after GVBD, it mostly accumulated around the condensed chromosomes. Our results show for the first time all three NOS profiles, confirming that NO is synthesized from different isoforms to ensure bovine preimplantation development, and also their proteins are detectable by using immunofluorescence staining.

List of abbreviations

A	: Adenosine
Acc. N°	: Accession number
AI	: Artificial insemination
ANOVA	: Analysis of variance
APS	: Ammonium peroxydisulphate
Arg	: Arginine
BH_4	: Tetrahydrobiopterin
bp	: Base pair
BSA	: Bovine serum albumin
C	: Cytosine
°C	: Degree Celcius
CaMPII	: Calmodulin-dependant protein kinase II
cADPR	: Cyclic adenosine diphosphate ribose
cAMP	: cyclic adenosine monophosphate
CaM	: Calmodulin
CAT	: Cationic anionic transporter
cDNA	: Complementary deoxy ribonucleic acid
cGMP	: Cyclic guanosine monophosphate
CNG	: Cyclic nucleotide gated chanel
COC	: Cumulus oocyte complex
cpm	: Count per minute
C_T	: Threshould cycle
DEPC	: Diethyl pyrocarbonate
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
D-PBS	: Dubbleco-phosphate buffer saline
DTT	: 1, 4, Dithio theritol
eCG	: Equine chorionic gonadotrophin
E.coli	: Escherichia coli

EDTA : Ethylenediaminetetraacetic acid
EGTA : Ethyleneglycol-bis-(b-amino-ethyl) N,N´-tetra-acetic acid
ENAP-1 : Endothelial nitric oxide synthase associated protein-1
EPO : Erythropoietin
Erk : Extracellular signal-related kinase
EST : Expressed sequenced tag
FCS : Fetal calf serum
FGF : Fibroblast growth factor
FSH : Follicle stimulating hormone
FSHRH : Follicle stimulating hormone-releasing hormone
G : Guanine
g : Gram
GVBD : Germinal vesicle break down
h : Hour
hCG : Human chorionic gonadotrophin
HCl : Hydrochloric acid
hpi : Hour post insemination
Hsp : Heat shock protein
ICM : Inner cell mass
IFN-γ : Interferon-gamma
IL : Interleukine
IP_3R : Inositol 1, 4, 5-triphosphate receptor
IPTG : Isopropyl β-D- thiogalactopyranoside
IVC : In vitro culture
IVF : In vitro fertilization
IVEP : In vitro embryo production
IVP : In vitro production
IU : International unit
Kb : Kilobase
kDa : Kilodalton
LH : Luteinizing hormone
LHRH : Luteinizing hormone-releasing hormone

L-NA	: N^{G}-nitro-L-arginine
L-NIL	: L-N^{6}-(1-iminoethyl)lysine hydrochloride
L-NIO	: L-N^{5}-(1-iminoethyl)ornithine
L-NMMA	: N^{G}-monomethyl-L-arginine
L-PA	: $N^{\omega\omega}$-propyl-L-arginine
L-VNIO	: L-N^{5}-(1-imino-3-butenyl)-ornithine
L-NAME	: N^{ω}-L-nitro arginine methyl ester
LPS	: Lipopolysaccharide
MAPK	: Mitogen-activated protein kinase
mg	: Milligram
min	: Minute
ml	: Milliliter
MMP	: Matrix metalloproteinase
MOET	: Multiple ovulation embryo transfer
MPF	: Maturation-promoting factor
mRNA	: Messenger RNA
mtNOS	: Mitochondrial nitric oxide synthase
mV	: Millivolt
Mw	: Molecular weight
NADPH	: Nicotinamide diphosphate
NaOH	: Sodium hydroxide
NF-*k*B	: Nuclear transcription factor kappa B
NiAm	: Nicotiamide
NO	: Nitric oxide
NOS	: Nitric oxide synthase
NOSIP	: Nitric oxide synthase interacting protein
ns	: Not significant difference
OCS	: Oestrus cow serum
OD	: Optical density
OPU	: Ovum pick up
PAF	: Platelet activating factor
PBST	: Phosphate buffered saline tween

PCR : Polymerase chain reaction
PDE : Phosphodiesterase
PDGF : Platelet derived growth factor
PDTC : Pyrrolidine dithiocarbonate
RE : Relative expression
RNA : Ribonucleic acid
RNS : Reactive nitrogen speces
ROS : Reactive oxygen speces
RyR : Ryaodine Receptor
rpm : Rotations per minute
SAS : Statistical Analysis System software
SD : Standard deviation of mean
SDS : Sodium dodecyl sulfate
sGC : soluble guanylyl cyclase
S-me-TC : *S*-methyl-L-thiocitrulline
T : Thymidine
TAE : Tris-Acetate acid-EDTA buffer
TBE : Tris-Boric acid-EDTA buffer
TE : Tris-EDTA buffer
TEMED : N, N, N´, N´-Tetramethylendiamine
TNF-α : Tumor necrosis factor-alpha
TGF : Transforming growth factor
UTR : Untranslated region
VEGF : Vascular endothelial growth factor
W : Watt
w/v : Weight by volume
X-gal : 5-Bromo 4-chloro-3-indolyl-β-D-galactoside
µg : Microgram
µl : Microliter

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1 Introduction

In vitro production systems are required to produce large numbers of viable embryos for biotechnical manipulations or commercial purposes in farm animals. The integration of *in vitro* produced embryos into production systems as a means of producing inexpensive embryos or salvaging embryos from injured, old, or infertile cows that no longer respond to super-ovulation is dependent on having viable embryos at the time of transfer (Kawarsky et al. 1999). During preimplantation development, 15-50% of the embryos die as a result of factors involving genes that control both rate of development and degree of fragmentation (Warner et al. 1998). The rapid expansion of in vitro production has not been without problems, mostly on embryo quality and viability (abortion, neonatal mortality) where nitric oxide (NO) is involved in the impairment of normal development. Nitric oxide is produced from L-arginine by a family of enzymes, the nitric oxide synthases (NOS). Three main distinct genes encoding mammalian NOS isoenzymes have been identified. Constitutive neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) are highly regulated by calcium and calmodulin. The third inducible form of NOS (iNOS or NOS II) binds calmodulin tightly and is thus relatively calcium-independent. However, recent findings suggest that eNOS and nNOS expression can also be induced and that in some tissues iNOS appears to be present at all times. The NO/NOS system is involved in reproduction and development of organisms at many levels such as follicular development, ovulation, embryo development and implantation, and spermatogenesis (Van Voorhis et al. 1994; Shukovski and Tsasfriri 1994; Bonello et al. 1996). Nitric oxide plays a biphasic role in reproduction: a narrow range of NO concentrations, usually low, will stimulate or enhance these early events in reproduction, but either a lack of NO or too much NO has negative consequences (Barroso et al. 1998, Hanafy et al. 2001). The biosynthesis of NO is significantly increased in uterus and cervix as demonstrated by Ali et al. 1997, in the rat during pregnancy as a consequence of an increased expression of iNOS. Nitric oxide was supposed to be necessary and sufficient for egg activation and fertilization (Kuo et al. 2000). It has been demonstrated that nitric oxide inhibits oocytes meiotic maturation and embryo development in rodents or that excessive generation of nitric oxide can induce oxidative stress and apoptosis (Barroso et al. 1998, Jablonka-Shariff et

al. 2000; Nakamura et al. 2002, Yoon et al. 2002). The deleterious effects of NO on embryo development and implantation may be partly responsible for low *in vitro* fertilization (IVF) success rates. Various investigators have sought to identify and determine the cellular localization of NOS isoforms in mammalian tissues. Using immunohistochemistry, Gouge et al. (1998), Jablonka-Shariff and Olson (1998) and Nishikimi et al. (2001) identified eNOS and iNOS in the mouse oocytes and early embryos. The three NOS were reported by Trangusch et al. (2003) in mouse oocytes and embryos by real time PCR. However, thesse authors studies, failed to quantify NOS-specific mRNA in different embryonic developmental stages. One of the current difficulties of the field is the lack of molecular details concerning the mechanism of NO action, due in part to lack of technology for effective detection of NO and its molecular targets (Thaler and Epel 2003). While considerable progress has been made in elucidating nitric oxide regulatory mechanisms in rodents, much less is known about its synthesis and role in early embryo development in farm animals. Understanding the physiological roles of nitric oxide synthase in oocyte maturation, fertilization and embryo development is of great importance in reproduction improvement. Given the growing importance of NO as a messenger in the oocyte or embryo and the minimal information regarding its production and role in cattle, the present study was undertaken with the following objectives:

I. Investigation of the physiological role of NOS/NO system on bovine embryo development, using dosage dependent application of N-omega L-nitro arginine methyl ester, (L-NAME) a selective inhibitor of NO production at maturation or/and culture medium.

II. Identification of nitric oxide synthase gene isoforms in bovine oocytes and/or preimplantation embryos with help of polymerase chain reaction (PCR)

III. Quantitative expression profiling of the NOS genes in bovine ooctes and preimplantation embryos using real-time PCR technology

IV. Detection and localization of NOS protein in bovine oocytes and preimplantation embryos using immunohistological analysis

2 Literature review

2.1 Cattle embryo production and development

In vitro production (IVP) and multiple ovulation and embryo transfer (MOET) progress through scientific research to field testing and end up in commercial application. Immature oocytes recovered, matured, fertilized and cultured *in vitro* to blastocyst stage are either transferred to recipients immediately or frozen for transfer at a later date. Oocytes can originate from the ovaries of live intact animals or recovered from ovaries after slaughter.

2.1.1 Developmental competence

Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization. The developmental competence of the oocyte is acquired within the ovary during the stages that precede ovulation or in case of *in vitro* maturation, precede the isolation of the oocyte from its follicle (Mayes 2002). It is a difficult parameter to assess since embryonic development may fail due to reasons independent of oocyte quality. Developmental competence is usually expressed as the percentage of oocytes that can develop to the blastocyst stage (Gandolfi 1997). However, development to the blastocyst stage does not guarantee that the embryo will develop to term. Other aspects used to evaluate developmental competence include morphological evaluations, such as number of blastomeres or the ratio between inner cell mass and trophoectoderm cell numbers and metabolic rates (Crosier et al. 2001). The size and the quality of the follicle of origin (Hazeleger et al. 1995) influence the developmental capacity of bovine oocytes. It appears that the oocyte requires an additional "prematuration" to express their competence (Hendriksen et al. 2000). If *in vivo*, this pre-maturation occurs during preovulatory growth before the lutenising hormone (LH) surge, the ovarian morphology, the number and size of the follicles present in the ovary at the time of aspiration, the composition of the follicular fluid (Hazeleger et al. 1995, Madison et al. 1992, Lonergan et al. 2003a) may be critical for the oocyte to acquire developmental competence. The developmental competence of the

oocyte may also be lost during *in vitro* maturation since the number and quality of cumulus cells surrounding the oocyte are important in this process (Blondin and Sirard 1995, Gandolfi et al. 1997).

The absence of reliable markers for the identification of viable embryos for transfer at the early cleavage stage is likely to contribute to the generally low implantation rates in *IVF* treatment (Fenwick et al. 2002). Early cleavage is indicative of increased developmental potential in embryos and may be useful as a criterion in the selection of embryos for transfer. To improve the selection of the embryo with the highest implantation potential, Van Montfoort et al. (2004) suggested that selection for transfer should not be based on cell number and morphology on the day of transfer alone, but also on early cleavage status.

2.1.2 Oocyte maturation

Oocyte maturation is a complex phenomenon during which the oocyte progresses from the diplotene to the metaphase II stage (nuclear maturation). The oocyte resumes meiosis in response to the ovulatory LH surge or removal from the follicle. In cattle, germinal vesicle breakdown (GVBD) occurs within hours after removal from the follicle or the ovulatory LH signal. The oocyte remains arrested at the metaphase II stage until fertilization takes place and the oocyte completes meiosis and forms the pronucleus. However the completion of nuclear maturation alone does not guarantee subsequent embryo development (Yang et al. 1998). Oocyte maturation also involves transformations at the cytoplasmic level that prepare the cell to support fertilization and early embryonic development (cytoplasmic maturation).

Oocytes matured *in vitro* or *in vivo* have similar rates of nuclear maturation, fertilization and cleavage, but clearly differ in their developmental potential (Sirard and Blondin 1996). Differences in development between *in vivo* and *in vitro* cultured bovine oocytes are expressed at the morula-blastocyst stage (Farin and Farin 1995). Important factors either in the form of proteins or stable mRNAs are stored during oocyte growth and final follicular maturation after the growth has been completed (Blondin and Sirard 1995). The ability of the oocyte to complete meiosis is known as meiotic competence, which is acquired gradually during follicular growth. It is closely correlated with oocyte

size, which in turn is correlated with follicle size (Armstrong 2001) and the size of the antral follicle at which the oocyte acquires meiotic competence is species-specific (Mayes 2002). Cleavage and blastocyst rates increased in parallel with meiotic competence and significantly higher developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 μm (Hazeleger et al. 1995). Once the oocyte becomes meiotically competent, inhibitory factors are necessary to maintain the oocyte in meiotic arrest. The nature of meiotic arrestor in bovine (follicles) is poorly understood. Nitric oxide synthase derived nitric oxide system seemed to be implicated in this process (Nakamura et al. 2002). The regulation by the interplay of cyclic adenosine monophosphate (cAMP) of cell-cell interactions between granulosa and cumulus cells and the oocyte in mediating maintenance of meiotic arrest was hypothesized by Aktas et al. 2003. Cumulus expansion and oocyte developmental competence are induced by different cAMP (Luciano et al. 2004).

2.1.3 Nitric oxide in embryo growth and development

Growth and development rates are important indicators of embryo viability (Morris et al. 2000). Cattle embryo growth and development is characterized by cell proliferation and differentiation, gene expression and protein synthesis. The signal transduction systems, cAMP and cGMP are an integral part of these processes. Many hormones, neurotransmitters and other `first messengers' like nitric oxide act by regulating the synthesis or breakdown of cAMP or cGMP. It has been established that from day 13 to day 16 intracellular and extracellular concentrations of cAMP and cGMP decreased, consistently with the decrease in protein synthesis, phosphorylation and metabolic activity (Morris et al. 2001). The pathway by which NO will affect transduction depends on its concentration and the molecular environment (Hanafy et al. 2001). Specifically, high concentrations of NO can result in autooxidation or nitrosylation. Besides the concentration of NO, the existence of strong oxidants in the environment, such as superoxide radical can also modify NO, forming peroxynitrite, which reacts with the phenol moiety in tyrosine resulting in nitrotyrosine. A summary of the low and high concentration effects of NO is provided in figure 1. Guanylate cyclase catalyzes the formation of cGMP, which is utilized as an intracellular amplifier and second

messenger in modulating the function of protein kinases, phosphodiesterases, ion channels, smooth muscle tone regulation and the inhibition of platelet adhesion (Dröge 2002).

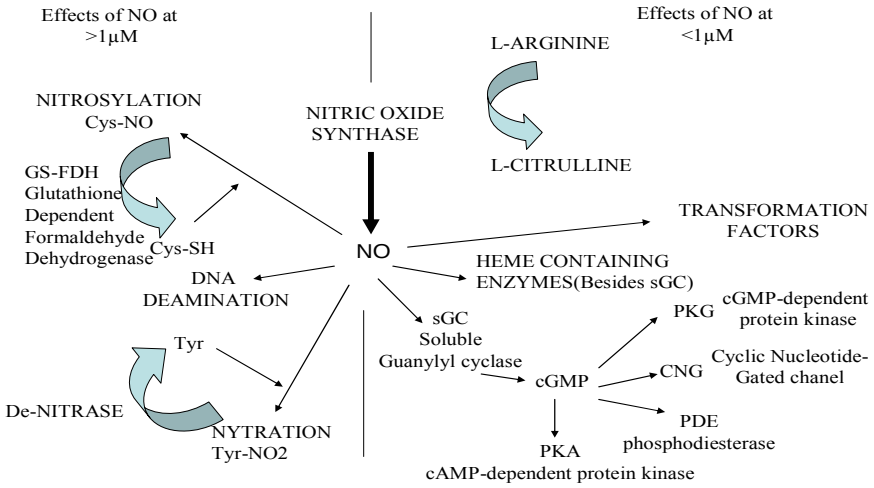

Figure 1: Summary of nitric oxide transduction (Hanafy et al. 2001)

2.1.4 Genetic control of early embryonic development

Mammalian embryogenesis is not totally explored, may be due to currently lack of appropriate technique and the difficulty in obtaining sufficient amounts of timed embryos.

Maternal gene expression

Oogenetic mRNAs and proteins are required to confer normal or full developmental competence. During early stage of development accumulated transcript, macromolecules and proteins are of importance as mentioned by De Sousa et al. (1998a) and Trounson et al. (2001). Variations in level of transcript abundance (relative mRNA) between groups (*in vitro/in vivo*, immature/matured, follicle size) of some genes related to developmental competence in bovine oocytes have been reported (Lonergan et al. 2003b). Transcription activity in bovine oocytes during folliculogenesis begins as early as the secondary follicle stage (Fair et al. 1997). Bovine germinal vesicle

stage demonstrated transcriptional activity significantly higher than mature oocyte (Memili et al. 1998). Among gene transcripts required for oocyte development identified by De Sousa et al. 1998a in mouse like c-mos proto-oncogene, connexin 37, growth differentiation factor-9 (GDF-9), zona pellucida glycoproteins (ZP1, ZP3 and ZP3), we can include NOS.

Embryonic gene activation

If gene expression of maternal origin is necessary, it is not sufficient to insure further development. Embryonic gene activation (EGA) where embryo begins to synthesize its own mRNA and protein (taking place of the inherited one from the mother in the egg) is required (Schultz 1993). The transition from the maternal to embryonic control of early embryonic development in mammals takes place at different periods, depending on the species (reviewed by Kanka 2003) and starts as early as the one- to 2-cell stage in mice, at the four- to eight- cell stage in humans. Although the bovine maternal-zygotic transition occurs at the 8- to 16-cell stage and is characterized by a major onset of transcription, minor transcription is observed as early as the one-cell embryo (Memili and First 1999). Many genes of potential role in early transcription during bovine embryo development are reported by Vignault et al. 2004.
At which level of embryonic development are nitric oxide synthase genes involved still to be investigated. NOS mRNA aspect, their protein patterns after NO synthesis inhibition by L-NAME will allow us to determine their role before fertilization and at early bovine stage of development. NOS genes structure, regulation and NO role plus mechanism of action in other tissues are reviewed in the following section.

2.2 Nitric oxide synthase genes

Nitric oxide is ubiquitous in that it is found in virtually all tissues; however, NOS types appear to be tissue-specific (Boucher et al. 1999).

2.2.1 Nitric oxide synthase structure

Three NOS isoforms, sharing a common basic structural organization and requirement for substrate cofactors to enable enzymatic activity, have been described in mammals as illustrated in figure 2. Two of these genes, neuronal nitric oxide synthase (nNOS or NOSI) and endothelial nitric oxide synthase (eNOS or NOSIII) were supposed constitutively expressed, calcium-calmodulin dependent and produced small amount of nitric oxide (NO) in response to transient elevation in intracellular calcium. It is becoming clear now that they are modulated by a lot of factors in tissue specific ways (Boucher et al. 1999). Inducible nitric oxide synthase (iNOS or NOSII) functions independently of a rise of intracellular calcium. The three isoforms of NOS, although products of distinct genes, share 50-60 % homology at the nucleotide and amino acid levels and are functional only as homodimers (head-to-head) (Murphy 2000). Each NOS isoform has similar catalytic domains (Mayer and Andrew 1998): a reductase (C-terminal) exhibiting binding sites for flavins and NADPH, and an oxygenase (N-terminal) domain that contains heme and the site for tetrahydrobiopterin (BH4) binding. Till date only bovine eNOS is totally characterized whereas iNOS is partially sequenced. The bovine eNOS gene spans 20 kb and contains 26 exons and 25 introns plus 2.9 kb of 5'-flanking sequence (Venema et al. 1994). Evolutionary conservation of transcriptional regulation is suggested by these authors since high sequence homology of the promoter region to the human eNOS gene promoter was found (75 % nucleotide identity in 1.6 kb of 5'-flanking sequence).

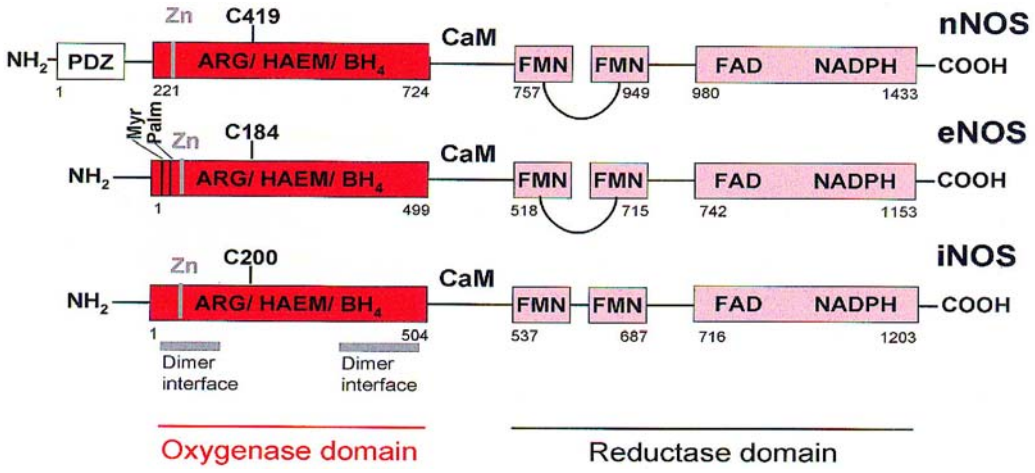

Figure 2: Domain structure of human nNOS, eNOS and iNOS (Alderton et al. 2001)

2.2.2 Other isoform: mitochondrial NOS

The existence of a nitric oxide synthase that is localized in the mitochondria (mtNOS) was described but this finding was supposed to be one of the recognized NOS isoforms targeted to the mitochondria after protein synthesis in the cytosol (Kanai et al. 2001). It was reported that the eNOS isoform was localized to the inner mitochondrial membrane in all tissues that were tested, including brain, kidney, liver, skeletal and cardiac muscle. More in-depth studies using a variety of NO detection techniques adding further support for the existence of an mtNOS were unable to determine whether the enzyme was novel or related to the nNOS, iNOS and eNOS isoforms. Only extended studies to the investigation of the functional implications of mtNOS and use of a NO-sensitive dye to stain the mitochondria in intact cells demonstrated the presence of NO within these organelles (López-Figueroa 2000). Despite a number of positive reports, scepticism remains regarding the existence of an mtNOS. In fact high altitude significantly increased heart mtNOS activity (Gonzales et al. 2005). But they observed using western blot analysis that heart mitochondria reacted only with anti-iNOS antibody, whereas the postmitochondrial fraction reacted with anti-iNOS and anti-eNOS antibodies.

2.2.3 Regulation of nitric oxide synthase

Nitric oxide is now known to be synthesized in a large number of different tissues and playing a wide range of physiological roles. Due to its potential cytotoxicity, the unregulated production of NO may be detrimental to tissues (Park et al. 1997). The regulation of NOS activity in order for NO to perform this variety of roles is complex. Cellular and tissue specific localization of the NOS isoforms by transcriptional (Alderton et al. 2001), translational and posttranscriptional (Kone et al. 2003) regulation have been reported.

2.2.3.1 Regulation of nitric oxide synthase activity

Elucidation of the mechanisms and factors determining transcription of the NOS genes under different physiological/pathophysiological conditions is crucial to understand the

alterations in vascular NO production (Fleming and Busse 2003). They investigated also the stimuli associated with the effects on NOS protein levels (oestrogen, TNF and shear stress) to regulate posttranscriptional processes that mainly determine NOS mRNA stability after studies on the activity of the NOS promoter and changes in NOS mRNA expression.

Regulation of NOS mRNA

Steady-state mRNA levels represent the balance between gene transcription and mRNA degradation. The half-lives of mRNAs (modulated in response to extra- and/or intracellular signals) in mammalian cells varies from minutes to many hours and the translation has a critical role in the regulation of mRNA stability (Bloch 1999). There is a marked discrepancy among amounts of eNOS mRNA, protein, and activity, demonstrating complex regulatory mechanisms after transcription and translation. Messenger RNA stability is regulated by protein/mRNA interactions (Searles et al. 1999). Posttranscriptional mechanisms have emerged as important regulatory pathways in the decrease of eNOS expression with mRNA destabilization playing a significant role in the rapid downregulation of eNOS mRNA levels (Tai et al. 2004).

Nitric oxide synthase dimer formation

Nitric oxide synthase activity requires not only binding of calomodulin and tetrahydrobiopterin (BH4) but also the formation of a homodimer. Homodimers of NOS differ markedly in the association strength of their monomers, their full interfaces, and the influence of L-arginine and BH4 on their formation and stability (Kone et al. 2003). Homodimerization depends on the binding of L-arginine, stoichiometric amounts of heme, and BH4 (Stuehr 1996). In turn, dimerization potentiates NOS activity by creating high-affinity binding sites for L-arginine and BH4, removing heme from the solvent phase, and facilitating electron flow from the reductase domain to the oxygenase domain (Crane et al. 1999, Li et al. 1999). Studies have indicated that only the oxygenase domain is involved in iNOS dimer formation, whereas interactions between the reductase domains and between the reductase and oxygenase domains are critical for

dimerization of nNOS and eNOS (Venema et al. 1997). Using complementation analysis, Xie et al. (1996) found that binding of one molecule of BH_4 was sufficient for dimer formation and iNOS activity. In contrast, the binding of one molecule of heme sustained dimerization but not enzymatic activity. The active site requires two hemes whereas; the BH_4 could be supplied by the same chain that took in electrons from NADPH, or by the other chain.

Degradation/proteolysis of nitric oxide synthase

Selective proteolytic degradation of NOS is one of the mechanisms for regulation of NOS enzyme (Osawa et al. 2003). NOS degradation is influenced by diverse agents, including glucocorticoids, caveolin-1, heat shock protein (Hsp) 90, neurotoxic molecules and certain NOS inhibitors (Kone et al. 2003). The calpain and ubiquitin-proteasome pathways, dexamethasone promotes proteolysis of iNOS in rat glomerular mesangial cells (Kunz et al. 1996) whereas neutral cysteine protease calpain I act on iNOS monomer as a direct substrate for degradation (Walker et al. 2001). L-arginine and tetrahydropterin by facilitating dimerization of the monomers protect iNOSox and nNOSox dimers against trypsin proteolysis, whereas the eNOSox dimer is resistant to proteolysis under all conditions (Panda et al. 2002). Kolodziejski et al. (2003) showed that intracellular iNOS forms dimers that are "undisruptable" by boiling, denaturants, or reducing agents.

Calcium-calmodulin

Calmodulin interacts with NOS and is necessary for the enzymic activity of all three isoforms. The calcium-dependence of NO synthesis distinguishes the NOS isoforms, with nNOS and eNOS having a much higher calcium requirement than iNOS. Calmodulin binding increases the rate of electron transfer from NADPH to the reductase domain (Boucher et al. 1999) and also triggers electron transfer from the reductase domain to the heme centre. Neuronal NOS and eNOS differ in their primary structure from iNOS, having inserts in the middle of the FMN-binding subdomain, which has been described as an auto-inhibitory loop, and acts by destabilizing

calmodulin binding at low calcium and by inhibiting electron transfer from FMN to the heme in the absence of calcium -calmodulin (Alderton et al. 2001).

Tetrahydrobiopterin (BH_4) and phosphorylation

Tetrahydrobiopterin plays a key role in dimmer formation from monomers, stability of dimeric NOSs, and NO formation (Griscavage et al. 1994). Crystallographic data show that dimerization results in strong modifications of the monomeric protein, including interactions between tetrahydrobiopterin and the heme carboxylate group, the a-NH2 group of L-arginine, and residues involved in the dimer interface (Boucher et al. 1999). Phosphorylation of the nNOS and eNOS isoforms has an effect on their enzyme activity. Serine (Ser) at active site of eNOS is phosphorylated by protein kinase Akt which results in an increase in NO production (Michell et al. 2002). In contrast, the phosphorylation of nNOS at Ser by calmodulin-dependent kinases leads to a decrease in its activity (Alderton et al. 2001). In unstimulated, cultured endothelial cells, Ser^{1177} is not phosphorylated but is rapidly phosphorylated after the application of fluid shear stress, estrogen and VEGF, insulin or bradykinin in human endothelial cells (Fleming and Busse 2003). The negative regulatory site Thr^{495}, is constitutively phosphorylated in all of the endothelial cells: its phosphorylation is associated with a decrease in enzyme activity (Fleming et al. 2001). Changes in Thr^{495} phosphorylation are generally associated with bradykinin and histamine that elevate endothelial intracellular calcium and increase eNOS activity.

Other protein interaction

Endothelial NOS-associated protein-1 (ENAP-1) is tyrosine phosphorylated in response to bradykinin stimulation of eNOS in bovine endothelial cells. Kalirin appears to inhibit iNOS by preventing the formation of iNOS dimers and may play a neuroprotective role during inflammation. Dynamin 2 is supposed to associate with endothelial NOS, in the Golgi apparatus (Cao et al. 2001) and an increase in dynamin levels results in enhanced eNOS catalysis by means of direct protein interactions (Dessy et al. 2000).

2.2.3.2 Regulation of nitric oxide synthase localization

NO production is not simply dependant on the expression of the (endothelial) NOS enzyme but is determined by an (endothelial) NOS signaling complex that consists of an enzyme and a conglomerate of adaptor proteins, structural proteins, kinases, phosphatases, that affect complex association and determine intracellular localization (Fleming and Busse 2003). The main localization of NOS isoforms have been reported by Boucher et al. (1999) and summarized in table 2.1 below.

Table 2.1: Main localizations of the three NOS isoforms in rat (Boucher et al. 1999)

Isoform	nNOS	iNOS	eNOS
Type	constitutive	inducible	constitutive (but regulated)
Subcellular localization	cytosol	cytosol	membrane and cytosol
Cell, tissue	brain	liver	endothelium
	neuron	hepatocyte	epithelial cell
	lung	chondrocyte	
	kidney	macrophage	
		smooth muscle	

Myristoylation and palmitoylation

Endothelial NOS is acylated by both myristate and palmitate and thus can associate with intracellular membranes. This membrane association is required for the phosphorylation and activation of eNOS in response to stimuli such as VEGF (Fulton et al. 2002). Functional eNOS was detected in three membrane compartments, the plasma membrane (Hecker et al. 1994), plasmalemmal caveolae (Liu et al. 1996) and the Golgi apparatus (Fulton et al. 2002). Busconi and Michel (1993) suggested that N-terminal myristoylation of the endothelial NOS may determine subcellular localization. Palmitoylation is dynamically regulated by agonist and is necessary for efficient

localization of eNOS to the plasmalemmal caveolae of endothelial cells. The main pathway leading to a functional eNOS enzyme is depicted in the figure 3 showing some transcriptional regulation, (de)stabilization of eNOS mRNA, and protein-protein interactions. Once the enzyme is functional, the presence of substrate arginine and cofactor BH4 determines whether eNOS is producing nitric oxide (NO) or superoxide (Govers and Rabelink 2001).

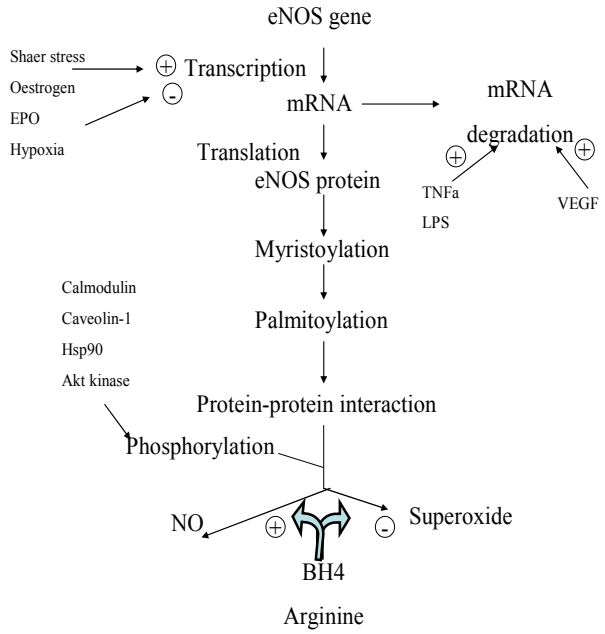

Figure 3: Cellular events involved in the regulation of endothelial NOS activity (Govers and Rabelink 2001)

Regulation of inducible nitric oxide synthase

Numerous cytokines and microbial products, acting in synergy, stimulate expression of iNOS but the effective agents and combinations depend on cell type and species. It is difficult to tell whether iNOS in epithelium is expressed "constitutively" or is continually "induced" (MacMicking et al. 1997). Indeed certain cells in the normal rat kidney and ovarian follicles during certain phases of the cycle express iNOS (Van Voorhis et al. 1995). Calcium ionophores enhance induction of iNOS mRNA by LPS in macrophages but increased intracellular calcium suppresses interleukin-1 (IL-1)-induced nitric oxide production in human chondrocytes by reducing the stability of iNOS mRNA (Geng and Lotz 1995). In mouse it has been demonstrated that iNOS

induction by IFNγ and LPS was transcriptional (Xie 1997), although there may be an additional effect on mRNA stability. Multiple transcriptional initiation sites have been observed in human iNOS at the 5' UTR (Chu et al. 1995). Interleukin 4 exerts a delayed suppressive effect on transcription of iNOS (Bogdan et al. 1994) whereas interleukin 10's suppressive effect is exerted indirectly via its suppression of TNF production. At posttranscriptional regulation, the most potent suppressors of iNOS expression in mouse macrophages are TGFβ1-3 (Vodovotz et al. 1994) which destabilized iNOS mRNA, retarded the synthesis of iNOS protein, and accelerated its degradation. Posttranslational regulation through availability of heme or BH_4 has been reported (Albakri and Stuehr 1996). Heme depletion is implicated for the loss of iNOS enzyme activity in macrophages (Vodovotz et al. 1994, Nathan and Xie 1994).

Regulation of neuronal nitric oxide synthase

The majority of nNOS immunoreactivity in neurons is associated with endoplasmic reticulum and within specialized electron-dense synaptic membrane structures and postsynaptic densities. Although nNOS was first characterized as a soluble (cytosolic) protein, this protein undergoes an important association with the sarcolemma in skeletal muscle (Michel and Feron 1997). Neuronal NOS is expressed as catalytically active N-terminally truncated forms that are required for dimer assembly and catalysis (Panda et al. 2003). Sagami et al. (2001) confirmed that the mechanism of activation of nNOS by calmodulin is not solely dependent on the activation of electron transfer to the hemes but may align adjacent reductase and the oxygenase domains as proposed by Panda et al. (2001). Tetrahydrobiopterin is a critical element in the nNOS metabolism of L-arginine to L-citrulline and NO or superoxide (Rosen et al. 2002).

2.2.3.3 Nitric oxide synthase inhibition

If NOS enzymes are regulated in part by post-translational proteolysis, then certain types of NOS inhibitors can enhance the degradation of NOS protein as reported by Wei et al. (1998) and Vuletich et al. (2002). Most NOS inhibitors vary in their affinities for the isoforms of NOS (Rosselli et al. 1998), and analogs of L-arginine act as competitive

inhibitors reversible by addition of excess L-arginine, although prolonged exposure may cause irreversible inhibition. The most commonly used inhibitors have been aminoguanidine, N-mono-methyl L-arginine (L-NMMA), N-nitro-L-arginine (L-NNA) and its methyl ester: N^G-nitro-L-arginine methyl ester (L-NAME)(Rees et al. 1990 and Reif et al. 1995). Some NOS inhibitors, NO donors and scavengers with their activity sites are illustrated in figure 4 below.

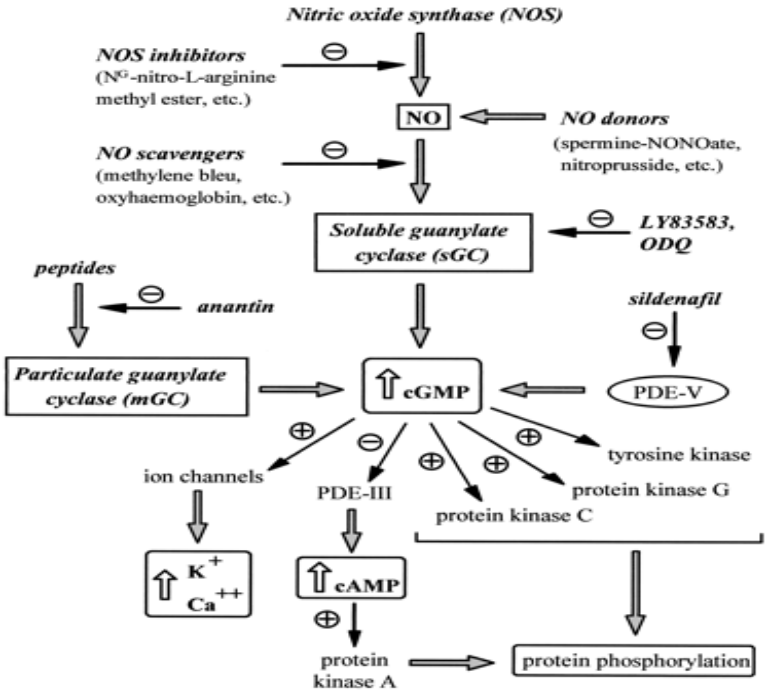

Figure 4: Schematic presentation of the NO/cGMP pathway, its inhibitors and activators (Revelli et al. 2002)

Inhibitors of nitric oxide synthase: arginine analogs

Competitive inhibitors bind with about tenfold higher affinity than L-arginine to the substrate site of NOS. N-nitro-L-arginine (L-NNA) is the active NOS inhibitor formed by hydrolysis from the inactive prodrug N-nitro-L-arginine methyl ester (L-NAME) and exhibits a fairly pronounced selectivity towards the constitutive isoforms (Mayer and Andrew 1998). Although the binding affinity of L-NNA is similar to that of N-methyl-L-arginine (L-NMA), it triggers a rapid, turnover-independent enzyme inactivation that is accompanied by a complete blockade of NADPH-dependent heme reduction. Dissociation of the L-NNA-NOS complex is slow, rendering L-NNA a highly potent tight-binding inhibitor of the constitutive isoforms (Mayer and Andrew 1998). Potent

and selective inhibitors of iNOS were shown to prevent iNOS dimerization in cells (McMillan et al. 2000). The inhibitors bind to a heme-containing iNOS monomer species to form an inactive iNOS monomer-heme-inhibitor complex in a BH_4- and L-arginine-independent manner by allosterically disrupting protein-protein interactions at the dimer interface. The energetics and kinetics of monomer-dimer equilibria are substantially different for the mammalian NOS isoforms (Blasko et al. 2002). Affinity/selectivity or potency/selectivity of NOS inhibitors, particularly substrate analogs in human are given in Table 2.2a and 2.2b. Selective substrate analogs with 10- to 30-fold selectivity for the constitutive isoforms (neuronal and endothelial NOS) are L-NA and L-NAME but L-NAME is 30- to 100-fold less potent than L-NA (Boer et al. 2000). In contrast Abu-Soud et al. (1994) showed that L-NAME inhibits NOS NADPH uptake through binding within the substrate binding site in iNOS and nNOS. L-NAME abolished the increased nitrite production due to up-regulation of iNOS-eNOS mRNA and protein in activated mouse macrophages or bovine pulmonary artery endothelial cells (Zuo and Johns 1997).

Table 2.2a: Affinity and selectivity of NOS inhibitors of human isoenzymes (Boer et al. 2000)

Compound	Affinity			Selectivity		
	iNOS	nNOS	eNOS	i-/eNOS	i-/nNOS	n-/eNOS
Substrate analogs		pIC_{50} value ± SD			ratio	
L-NA	5.5 ± 0.1	7.33 ± 0.02	7.04 ± 0.05	0.03	0.02	2
L-NAME	4.5 ± 0.1	6.12 ± 0.02	5.82 ± 0.02	0.05	0.02	2
L-PA	4.0 ± 0.1	5.40 ± 0.1	5.06 ± 0.03	0.1	0.04	2
L-NMMA	5.58 ± 0.04	6.60 ± 0.1	6.50 ± 0.10	0.1	0.1	1
L-NIO	5.8 ± 0.1	6.02 ± 0.03	6.00 ± 0.01	1	1	1
L-VNIO	6.1 ± 0.1	6.40 ± 0.1	5.85 ± 0.04	2	0.5	4
L-NIL	5.7 ± 0.2	5.4 ± 0.2	5.11 ± 0.04	4	2	2
S-me-TC	6.7 ± 0.1	7.4 ± 0.1	6.94 ± 0.03	1	0.2	3

Table 2.2b: Potency and selectivity of NOS inhibitors at human isoenzymes (Boer et al. 2000)

Compound	Potency ($logIC^{50}$-value)			Selectivity		
	iNOS	nNOS	eNOS	i-/eNOS	i-/nNOS	n-/eNOS
Substrate analogs		pIC_{50} value ± SD			ratio	
L-NA	5.8 ± 0.1	7.5 ± 0.2	7.1 ± 0.2	0.05	0.02	3
L-NAME	4.3 ± 0.1	5.7 ± 0.2	5.6 ± 0.1	0.05	0.05	1
L-PA	4.6 ± 0.2	6.3 ± 0.2	5.7 ± 0.1	0.08	0.02	4
L-NMMA	5.9 ± 0.2	6.0 ± 0.2	6.5 ± 0.2	0.3	0.8	0.3
LCM;ONIO	6.6 ± 0.1	6.3 ± 0.2	6.2 ± 0.2	3	2	1
L-VNIO	6.15 ± 0.02	6.3 ± 0.1	5.84 ± 0.06	2	0.7	3
L-NIL	6.2 ± 0.1	5.3 ± 0.2	5.06 ± 0.05	14	8	2
S-me-TC	7.2 ± 0.1	8.1 ± 0.6	7.27 ± 0.04	0.9	0.1	7

Possible feedback inhibition of nitric oxide synthase by nitric oxide

Enzymes that form a reduced-ferrous-heme intermediate have the potential to be inhibited by nitric oxide due to the stability of ferrous nitrosyl complexes (Alderton et al. 2001). Both iNOS and nNOS can form inhibitory nitrosyl species during turnover (Abu-Soud et al. 1995). Perez-Sala et al. (2001) found that inhibition of iNOS activity could be reversed by NO donors but with the time a subsequent inhibitory effect of NO donors on iNOS protein and mRNA levels is reached suggesting that NO may contribute to iNOS induction and downregulation. NO can modulate its own production by interfering with NF_k-B interaction with the promoter region of the iNOS gene (Park et al. 1997). Nitric oxide interacts with enzyme-bound ferric heme to inhibit neuronal NOS activity and this inhibition was reversed by BH4 (Griscavage et al. 1994). In 1995 Ravichandran et al. who showed reversible inhibition of semi purified eNOS enzymatic activity by NO and its donors confirmed that interaction of NO with the heme prosthetic component of NOS may lead to inhibition of their activity. Nitric oxide as a negative-feedback regulator of endothelial isoform expression (via cGMP) was proposed by

Vaziri and Wang (1999). A negative feedback effect may be necessary for limiting nitric oxide production.

2.3 Nitric oxide synthase expression in female reproductive tract and gonad tissue

Nitric oxide synthase genes are expressed in a variety of mammalian reproductive tissues including ovary, uterus, vagina, oocyte in mouse suggesting regulatory roles of the NO in oocyte meiotic maturation, fertilization and even embryo development and implantation (Novaro et al. 1997, Gouge et al. 1998, Biswas et al. 1998, Purcell et al. 1999, Jablonka-Shariff and Olson 2000, Sengoku et al. 2001, Tranguch et al. 2003).

2.3.1 Uterus and vagina

The presence of NOS has been shown by various studies in mammals. In rat uterus tissue (nonpregnant and at gestation), eNOS and iNOS have been reported whereas nNOS was not detectable (Farina et al. 2001). In early embryonic loss, the cytotoxic mechanisms involving natural killer cells and mononuclear cells in resorbing compared to non-resorbing embryos showed the involvement of nitric oxide in mice (Haddad et al. 1995). In rabbit, Al-Hijji et al. (2000) reported that NOS activity was downregulated by estrogen and progesterone in uterus while the relatively high NOS activity was down regulated by estrogen and up regulated by progesterone in vagina. At uterine level, throughout gestation myometrial NO-production is upregulated and contributes to achieve uterine quiescence. Close to term, its production decreases promoting effective contractions resulting in labor (Ali et al. 1997). For cervical ripening, in contrast to the myometrium, NO-production in the cervix is low during pregnancy and becomes upregulated once pregnancy advances to term in mouse (Maul et al. 2003); in human at delivering (Väisänen-Tommiska et al. 2004) and postpartum (Tschugguel et al. 1999).

2.3.2. Ovary

Nitric oxide synthase have been implicated in several aspects of ovarian functions. Nitric oxide has emerged as one of several important intraovarian regulatory factors in

particular, in the processes of ovulation and atresia-related apoptosis. In the ovary nitric oxide is expressed not only by neurons and vasculature but also by other cell types like granulosa and theca at the follicle level, and luteal cells after ovulation (Zackrisson et al. 1996, Basini et al. 1998, Tamanini et al. 2003). Both eNOS and iNOS are expressed depending on animal species and different ovarian processes. In fact, eNOS presence has been reported in thecal, granulosa, luteal cells in human, rat, and pig by Van Voorhis et al. (1994), Jablonka-Shariff et al. (1997) and Takesue et al. (2001) respectively. On the other hand, iNOS has not been found in granulosa cells in rat (Jablonka-Shariff et al. 1997) whereas this isoform has been indicated by Van Voohris et al. (1995) and Zackrisson et al. (1996) respectively in rat granulosa cells from preovulatory follicles; stroma, thecal and luteal cells. In 2000 Matsumi et al. have demonstrated that inducible nitric oxide synthase is predominantly localized in granulosa cells of healthy immature follicles in the rat ovary whereas, granulosa cells of either healthy mature follicles or atretic follicles are devoid of iNOS. In bovine granulosa cells NO production by endothelial and inducible NOS, which are regulated by gonadotropins has been proposed by Schoenfelder et al. (2003) after Basini et al. (2000) who did not define which isoform was implicated in nitric oxide synthesis. Skarzynski et al. (2003) suggested that NO is not only produced in the bovine corpus luteum but it inhibits luteal steroidogenesis and it may be one of the components of an autocrine/paracrine luteolytic cascade induced by prostaglandin ($PGF_{2\alpha}$). Regulation of nitric oxide synthase isoforms and role of nitric oxide during $PGF_{2\alpha}$-induced luteolysis in rabbits is reported by Boiti et al. 2003 in rabbit.

Locally produced nitric oxide is important for the maintenance and increase of rat ovarian blood flow during the preovulatory period (Mitsube et al. 2002). Nitric oxide influences apoptotic cell death of follicular cells as a follicle survival factor through hsp70 (Yoon et al. 2002, Takesue et al. 2003) and may inhibit oocyte meiotic maturation at ovulation, Nakamura et al. (2002) as illustrated in figure 5 where cumulus cell seem to play an important role (Tanghe et al. 2002). Nitric oxide seemed to be essential for optimal meiotic maturation of murine (Jablonka-Shariff and Olson 2000) and necessary for follicle rupture in rat (Shukovski and Tsafriri 1994, Hess et al. 2003). Endothelin-1 (ET-1) and NO (from iNOS) may modulate the production of each other in luteal microenvironment (Klipper et al. 2004). Nitric oxide was found to be involved in

the formation of hCG-induced murine follicular cysts since complications associated with these cysts were ameliorated by the NOS inhibitor L-NAME (Nemade et al. 2002). Many studies on NO have depended on the use of pharmacological inhibitors of NOS. However, the three NOS isoforms share L-arginine as a substrate and have similar enzymatic mechanisms.

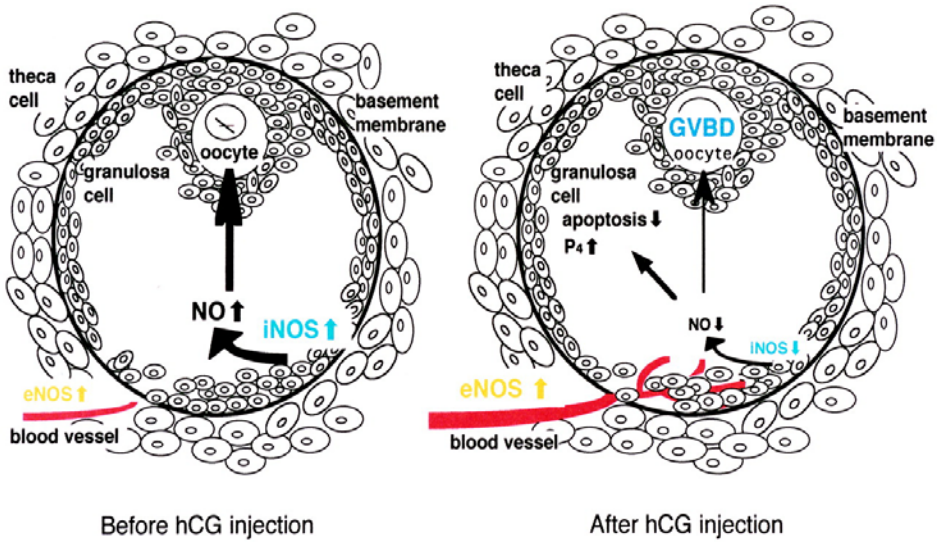

Figure 5: A scheme showing the different roles of the iNOS-NO and eNOS-NO systems in preovulatory follicles before and after hCG injection (Nakamura et al. 2002)

Many NOS inhibitors cannot distinguish between the NOS isoforms, and there are no known inhibitors that are absolutely selective. Genetic approach circumventing the lack of specificity of NOS inhibitors is necessary to complement pharmacological approaches by making targeted deletions in the NOS genes as shown in the table 2.3 below.

2.4 NO/NOS expression in embryos

Embryos have the necessary genes and proteins needed for protection against death and the development of strategies to reduce apoptosis in these embryos would be beneficial. It is therefore important to determine and if possible, prevent extrinsic factors that reduce embryo viability. The development of bovine embryos is supported by inorganic substrates, amino acids, carbohydrates, growth factors and antioxidants (Van Winkel

2001, Lim and Hansel 1998). Essential amino acids may support preimplantation blastocyst growth not only as nutrients but also as signaling molecules that influence protein kinases involved in protein synthesis regulation. Arginine is among the most effective essential amino acid signaling molecules, used as a substrate for the synthesis of polyamines and nitric oxide (Van Winkle 2001).

Table 2.3: Ovarian responsiveness in NOS deficient mice
(Adapted from Gregg 2003)

Authors	Mouse strain background	Knock-out isoform	Oocyte retrieved	Litter size	Methods
Jablonka-shariff and Olson (1998)	Mixed 129Sv/C57BL6 WT: 129Sv	eNOS	Reduced	Reduced	Super ovulation
Jablonka-shariff et al. (1999b)	Mixed C57BL6/129Sv/Ev Mixed C57BL6/129Sv/Ev WT:129Sv/Ev	eNOS iNOS	Reduced Same		Natural ovulation
Tempfer et al. (2000)	C57BL6/J	eNOS	Reduced		Super ovulation
Hefler and Gregg (2002)	C57BL/6J 129Sv C57BL/6J 129Sv	eNOS eNOS iNOS iNOS	Reduced Same Same Same		Super ovulation
Tranguch and Huet-Hudson (2003)	C57BL6/J C57BL6/J B6129SF2/J101045 Double knockout	eNOS iNOS nNOS e/iNOS		Same Same Same Same	Natural ovulation

Immunocytochemistry method (Gouge et al. 1998, Nishikimi et al. 2001) for NOS localization, incubation with NO donors and/or NOS inhibitors (Barroso et al. 1998, Jablonka-Shariff et al. 1999a) was used in mouse or rate. NOS genes disruption (Jablonka-Shariff et al. 1999b, Hefler and Gregg 2002) for NO physiological role and now polymerase chain reaction (Tranguch et al. 2003) for NOS isoforms identification, have been used to study NOS expression in embryos in rodents. It is clear now that in mouse oocytes and preimplantation embryos all three NOS genes are expressed (Tranguch et al. 2003). According to Nishikimi et al. (2001) eNOS protein is localized in nucleus and cytoplasm, whereas iNOS is only found in cytoplasm. How NOS/NO production is modulated is still to be investigated. Whether nitric oxide is involved in early embryo normal development or/and death; embryotoxic stimuli: IFNγ, TNFα, LPS in mice and their ability to induce NO production by 2-cell stage embryos were examined by Athanassakis et al. (2000). Their results confirm the possible embryotoxic character of NO (more than 2 μM/embryo treated against undetectable in the control), leading to the hypothesis that NO plays a potential role in early embryo death in mice. The NO may control programmed cell death by up-regulation of the tumor suppressor p53, or by changes in the expression of Bcl-2 family members, cytochrome c relocation, activation of caspases, chromatin condensation, and DNA damage as shown in figure 6 (Chen et al. 2001). According to these authors embryos cultured in vitro may be exposed to oxidative stress for which defence mechanisms are insufficient to protect delicate cellular structures from oxidative damage.

Manser et al. (2004) demonstrated that mouse preimplantation embryos produce NO. Consumption of oxygen was correlated to the medium with or without addition of L-NAME confirming that L-NAME is a specific inhibitor of NO production in embryo even if it is a non-selective NOS inhibitor. They hypothesized that NO may limit oxygen consumption at the blastocyst stage and by doing so participate to embryo developmental competence. Nitric oxide production at oocyte maturation, ovulation, fertilization, cleavage and implantation is reviewd by Thaler and Epel (2003).

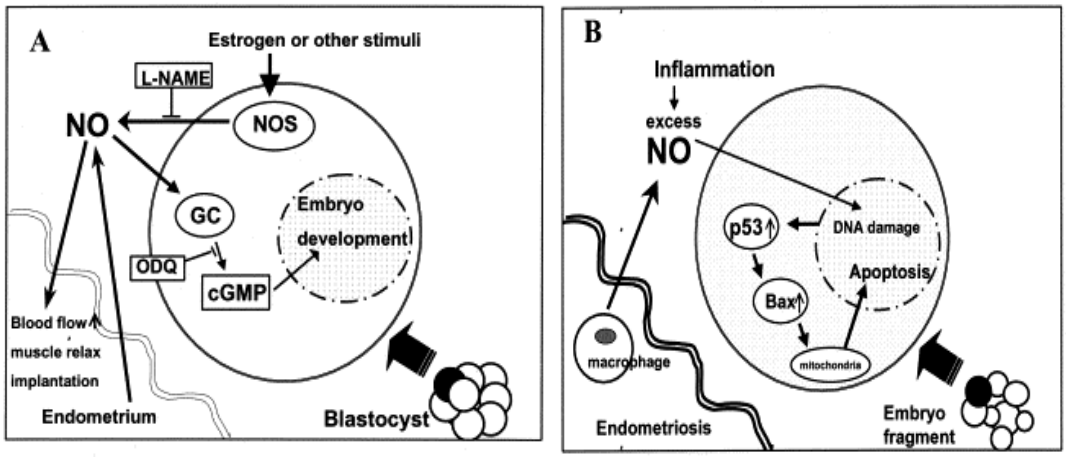

Figure 6: Hypothetical model for the role of NO in embryo implantation in which NO/cGMP pathway may increase blood supply and muscle relaxation in the endometrium and also be involved in the embryo development (A). In the patients with endometriosis, the inflammatory cells may produce excess NO to induce embryo apoptosis through the p53/Bax partway (B) (Chen et al. 2001)

2.5 The role of nitric oxide

Nitric oxide is a free radical gas, which plays a role in mammalian signaling and immunology (table 2.4). It plays a role in the brain as a neurotransmitter and in the control of blood flow (Huang and Fishman 1996). Nitric oxide causes vasodilation of blood vessels and by binding the heme of guanylate cyclase it exerts its effects at low concentration. It can be broken down by reaction with oxygen and/or haemoglobin. As a cytotoxin, in response to infection, macrophages synthesize NO, which is capable of cytotoxic and cytostatic effects on the invading organisms (Murphy 2000). Nitric oxide reacts with key metabolic enzymes, thus preventing continued metabolism, and is capable of combining with superoxide, generated in macrophages, to form peroxynitrite a potent agent of oxidative damage (Alderton et al. 2001). Since nitric oxide is diffusible, it may be able to exert its toxic effects outside the cell itself. Nitric oxide differs from other neurotransmitters and hormones in that it is not regulated by storage, release or targeted degradation, but solely by synthesis. Circulating nitric oxide products are increased during follicle development then decreased right after ovulation and iNOS-inhibition results in a 50 % reduction of ovulation (Maul et al. 2003). Nitric oxide

plays an important role before (sea urchin eggs activation: Kuo et al. 2000) and during embryonic development such as cell proliferation and differentiation in Drosophila, (Kuzin et al. 1996); at early development in mammals as reported by Jablonka-Shariff and Olson (1997), Gagioti et al. (2000), Nishikimi et al. (2001), Nakamura et al. (2002), Tranguch et al. (2003), Tranguch and Huet-Hudson (2003) by affecting embryos viability. For Sengoku et al. (2001), murine oocyte maturation, embryo development, and trophoblast outgrowth require nitric oxide in vitro.

Table 2.4: Possible role of nitric oxide (Huang and Fishman 1996)

Biological tissue	Function
Central nervous system	Neural signaling molecule
	Retrograde messenger in long-term potentiation
	Mediator of excitatory amino acid neurotransmission
	Mediator of excitotoxicity
	Coupler of cerebrovascular blood flow to metabolism
Peripheral nervous system	Neurotransmitter, smooth muscle relaxation
	Vasodilator, regulator of vascular tone
Vasculature	Inhibitor of smooth muscle proliferation
	Inhibitor of platelet aggregation
	Inhibitor of leukocyte adhesion and activation
	Paracrine modulator of contractility and heart rate
Heart	Defense mechanism against pathogens, tumor cells
Immune system	Mediator of homodynamic changes in sepsis

It is still unclear by which mechanism NO plays its role at the embryonic level. It has been hypothesized that embryos with a lower amino acid turnover (a low metabolism probably mediated by NO), are developmentally more competent in human (Houghton et al. 2002). Other authors suggested that NOS activity during nidation is modulated by oestradiol and that NO production is necessary for successful embryo implantation (Novaro et al. 1996, Biswas et al. 1998, Ota et al. 2000). But Zhang et al. (2002) observed that reduction in the number of implanted embryos in L-NAME treated group

was associated with decreased matrix metalloproteinase 9 (MMP-9) activity and concluded that NO facilitates embryo implantation in mouse.

2.5.1 Nitric oxide and infertility

Early embryo loss is supposed to be associated to local production of NO from iNOS by decidual cells in mouse (Haddad et al. 1995). Recently it has been confirmed by Ogando and colleagues (2003) at the increased production of NO in LPS-induced embryonic resorption. Previously, iNOS expression by peritoneal macrophages in endometriosis was found to be associated with infertility in human (Osborn et al. 2002). Not only inducible isoform (Burnett et al. 2002) is implicated since, deletion of exon 6 of the neuronal NOS gene in mice results in hypogonadism and infertility (Gyurko et al. 2002). NOS deficient mice give more insight in the role of NO in ovulation (table 2.3).

2.5.2 Nitric oxide and hypothalamic-pituitary-gonadal axis

Nitric oxide plays a crucial role at every level of reproduction in the organism. It activates the release of luteinizing hormone-releasing hormone (LHRH) at hypothalamic level, which activates the release of gonadotropins by activating nNOS in the pituitary gland. Follicle stimulating hormone-releasing hormone (FSHRH) selectively releases follicle stimulating hormone (FSH) also by activating NOS (Rosselli et al. 1994). In the gonad, NO induces ovulation and causes luteolysis (Yang et al. 2003), whereas in the reproductive tract, it relaxes uterine muscles via cGMP and constricts it by prostaglandins (McCann et al. 2003).

2.5.3 Nitric oxide in oocyte maturation, fertilization and embryo development

During the brief period (12-24 h) of oocyte maturation in mammalian species, significant qualitative changes in protein synthesis occur to initiate and regulate resumption of meiosis (Fissore et al. 2002). Activation of maturation-promoting factor (MPF) [responsible for inducing germinal vesicle break down] and mitogen-activated protein kinase (MAPK) [plays a role in regulating the transition from meiosis I to

meiosis II or oocyte arrest at metaphase II] are of importance in cytoplasmic maturation. Bradykinin regulates interactions of the mitogen-activated protein kinase pathway with the endothelial nitric-oxide synthase (Bernier et al. 2000). The ability for cell to release calcium determines their viability or arrest in this process. The inositol 1, 4, 5-triphosphate receptor (IP_3R) is the channel of choice in calcium oscillations at maturation and fertilization (Fissore et al. 2002). Cyclic adenosine diphosphate ribose (cADPR) is a potent endogenous calcium-mobilizing agent (Willmott et al. 1996). In sea urchin eggs, nitric oxide activates a soluble guanylate cyclase resulting in the conversion of GTP to cGMP. The resulting cGMP elevation activates a cGMP-dependent protein kinase leading to an increase in cADPR levels, which by binding to its receptor contributes to the opening of Ryanodine Receptor (RyR)-like calcium channel in the endoplasmic reticulum, resulting in a rise in calcium (figure 7). This hypothesis seemed to be possible in mammals since in human, calcium-binding proteins and calcium release channels in maturing oocytes, zygotes and early preimplantation embryos have been identified by Balakier et al. (2002). Comparative biology of calcium signaling during fertilization and egg activation in animals has been review in detail by Stricker 1999. Kuo et al. (2000) suggested after microinjection of NO donors or recombinant NOS that NO was not only necessary but sufficient for successful fertilization in sea urchin. Simultaneous measurement of intracellular nitric oxide and free calcium levels in mouse eggs demonstrate that nitric oxide plays no role in fertilization (Hyslop et al. 2001). Nakamura et al. (2002) hypothesized that NOS-NO system plays different roles in the outside and inside basement membrane of follicles before ovulation. According to these authors through cGMP, NO (especially from iNOS) inhibits oocytes maturation before ovulation and therafter by inhibition of MAPK. In 2003, Luckie et al. demonstrated that NO is unlikely to be the primary egg activator but, rather acts after the initiation of calcium wave to regulate the duration of fertilization in sea urchin. But phospholipase C zeta (PLCζ) that triggers calcium oscillations similar to those caused by sperm seemed to maintain the cell cycle-dependent regulation of calcium oscillations following fertilization (Larman et al. 2004). It remains to be determined if an increase in PLC activity modulated by tyrosine kinases or other regulators necessarily requires receptor stimulation or if it may also occur in response to soluble sperm factors (Stricker 1999). Moreover, receptor-mediated and

sperm-factor-based pathways could function in concert during fertilization. Higher concentrations of nitric oxide (0.1 or 1 mM) inhibit embryo development (no development beyong 2-cell stage) in vitro and at implantation (no implantation sites) in vivo in mice as demonstrated by Barroso et al. (1998).

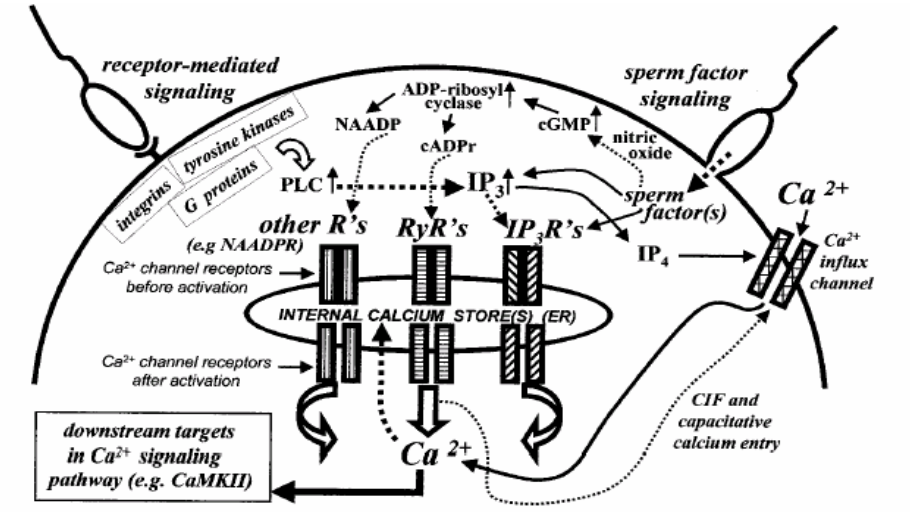

Figure 7: Diagram of some possible components of fertilization-induced calcium signaling, showing receptor-mediated and sperm-factor based pathways as well as contributions from internal calcium release and external calcium influx (Stricker 1999).

Recently, Manser et al. (2004) hypothesized that blastocysts with a higher developmental potential have reduced oxygen consumption, a characteristic that may be mediated by NO. The physiological role for NO in mouse preimplantation metabolism and development may be to limit oxygen consumption at the blastocyst stage at the level of mitochondrial cytochrome c oxidase. The existence of any hypothetical mitochondrial NOS could be easily explained by this assessment.

2.5.4 Nitric oxide and apoptosis in embryos

Preimplantation embryo depends on maternal mRNAs and proteins deposited and stored in the oocyte prior to its ovulation. If the oocyte is not sufficiently equipped with maternally stored products, or if zygotic gene activation does not commence at the correct time, the embryo will die (Jurisicova et al. 1998). Apoptosis is an evolutionarily

conserved mechanism for eliminating cells containing genetic damage from the developing organism. Therefore, activation of apoptosis in preimplantation embryos could act to restrict transmission of genetic damage; but, failure to activate apoptosis could result in the accumulation of damage in the foetus (Metcalfe et al. 2004). It is apparent that excess NO leads to degeneration of embryos while insufficient amounts of NO arrests development (Tranguch et al. 2003).

In many tissues, NO mediates its effects through the cGMP pathway (Chen et al. 2001) as shown in preimplantation embryo development however, NO worked not exclusively through the cGMP pathway. It can promote apoptosis in some cells, whereas it inhibits apoptosis in other cells a consequence of the rate of nitric oxide production and the interaction with biological molecules such as iron, thiols, proteins, and reactive oxygen species (Chung et al. 2001). These authors demonstrated that long-lasting production of nitric oxide acts as a pro-apoptotic modulator by activating caspase family proteases through the release of mitochondrial cytochrome c into the cytosol, upregulation of p53, and altering the expression of apoptosis-associated proteins including Bcl-2 family.

The ratio of Bcl-2 to Bax may be used to gauge the tendency of oocytes and embryos towards either survival or death (Yang and Rajamahendran 2002). They demonstrated that the expression of Bcl-2 was high in bovine good quality oocytes and embryos, low in fragmented embryos, and hardly detectable in denuded oocytes in contrast to Bax found in all types of oocytes and embryos with the highest expression in the denuded oocytes. However, low or physiological concentrations of NO prevent cells from apoptosis induced by trophic factor withdrawal, Fas, TNFα and LPS showing the double-edged role of nitric oxide suggested in preimplantation embryo apoptosis (Esfandiari et al. 2002).

3 Materials and methods:

3.1 Materials

In the present study bovine *in vitro* produced (IVP) embryos were used. Oocytes aspirated from ovaries obtained from nearby slaughter house were used in the IVP embryos.

3.1.1 Media used for IVP

Modified Parker Medium		
(MPM-110 ml)	Sodium hydrogen carbonate	0.080 g
	Hepes	0.140 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.010 g
	Streptomycin phosphate	0.003 g
	Penicillin	0.002 g
	Medium 199	99.0 ml
	Hemicalcium lactate	0.060 g
	Water added to	110.0 ml
Capacitation medium (50 ml)	Sodium chloride	0.2900 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0017 g
	Hepes	0.1190 g
	Magnesium chloride & H2O	0.0155 g
	Calcium chloride	0.0145 g
	Sodium lactate solution (60 %)	184 µl
	Phenol red solution (5 % in D-PBS)	100 µl
	Water added to	50 ml

Fertilization medium (FM-50 ml)		
	Sodium chloride	0.3300 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin G	0.0032 g
	Magnesium chloride & H2O	0.0050 g
	Calcium chloride	0.0150 g
	Sodium lactate solution	93 µl
	Phenol red solution (5 % in D-PBS)	100 µl
	Water added to	50 ml
Culture medium (CR1-50 ml)	Hemicalcium lactate	0.0273 g
	Potassium chloride	0.0112 g
	Podium chloride	0.3156 g
	Sodium pyruvate	0.0021 g
	Penicillin G	0.0032 g
	Magnesium chloride & H2O	0.0050 g
	Calcium chloride	0.0150 g
	L-Glutamin	0.0073 g
	Phenol red solution (5 % in D-PBS)	100 µl
	Water added to	50 ml

Bovine embryo production *in vitro*

Maturation medium: Modified Parker Medium (MPM):

The above chemicals were dissolved in 100 ml TCM-199 in order to reach 728 µg/ml sodium hydrogen carbonate, 1.28 mg/ml hepes, 227 µg/ml sodium pyruvate, 91 µg L-Glutamin, 55 µg/ml gentamycin and 545 µg/ml hemicalcium lactate already dissolved in 10 ml water. The solutions are mixed thoroughly, sterile filtered and stored at 4 °C. Before use 12 % heat inactivated oestrus cow serum and FSH (10 µg/ml) were added.

Fertilization medium: The fertilization medium is a modified Tyrode-Solution (Parrish et al. 1988). Prior to fertilisation the medium is supplemented with 6 mg/ml BSA, 2.2 µg/ml sodium pyruvate, 2 µg/ml heparin, 12 µM hypotaurin and 1.2 µM epinephrin.

Penicillin, hypotaurin, epinephrine (PHE) preparation:
PHE is a combination of 3 different solutions. Solution 1 contains 0.9 % NaCl; in solution 2 1.1 mg hypotaurin is dissolved in 10 ml 0.9 % NaCl-solution. Solution 3 contains 40 mg sodium dihydrogen sulphate, 100 µl 60 % sodium lactate solution and 1.8 mg epinephrin, dissolved in 40 ml water. The three solutions are mixed, sterile filtered and store at – 20 °C.

Capacitation medium: The capacitation medium is also a modified Tyrode-Solution (Parrish et al. 1988). Before use it is supplemented with 6mg/ml BSA, 110µg/ml sodium pyruvate and 2µg/ml heparin.

Culture medium: The culture medium called CR1aa (Rosenkrans and First 1994) is supplemented with 10 % oestrus cow serum, 10 µl/ml minimal essential medium (MEM) and 10 µl/ml basal medium eagle (BME) prior to use.

3.1.2 Reagents and other media

All solutions used in this investigation were prepared with deionized and demineralized (Millipore) water and pH was adjusted with Sodium hydroxide or Hydrochloric acid.
The chemicals, reagents, kits and buffers used in this study are given below.

Amersham, Biosciences (Freiburg, Germany): ECL Plus Western blot reagents, donkey anti-rabbit antibody

Biomol (Hambourg, Germany): Phenol, Lambda DNA *Eco*91I (BstE II) and Lambda DNA *Hind*III Anti-iNOS/NOS II (rabbit polyclonal IgG)

Biozym Diagnostik (Hessisch-Oldendorf, Germany): Sequagel XR sequencing gel (National Diagnostics) and SequiTherm Excel^{TM}II DNA sequencing kit (Epicentre Technologies)

Dynal (Oslo, Norway): Dynabeads

Gibco/BRL, Life Technologies (Karlsruhe, Germany): Trizol™ reagent and SuperScript™II reverse transcriptase

MWG Biotech (Ebersberg, Germany): Oligonucleotide primers

Promega (Mannheim, Germany): DTT, pGEM®-T vector, Pfu DNA polymerase and 10x reaction buffer, 10 x BSA and 10 x buffer, Rnase free-Dnase, Rnasin Ribo-nuclease inhibitor, RQ1 Rnase-free Dnase and 10 x buffer, T4 DNA ligase and 2x rapid ligation buffer

Qiagen (Hilden, Germany): RNeasy Mini Kit

Roth (Karlsruhe, Germany): Acetic acid, Agar-Agar, Ampicilin, Ammonium peroxodisulpahte (APS), Boric acid, Bromophenol blue, Chlorofrom, Dimethyl sulfoxide (DMSO), dNTP, EDTA, Ethanol, Ethidiumbromide, Formaldehyde, Formamide, Glycerin, Hydrochloric acid, Hydrogen peroxide (30%), IPTG, N,N´-dimethylformamide, Nitric acid, Peptone, Proteinase K, SDS, Silver nitrate, Sodium carbonate, Sodium chloride, Sodium hydroxide, TEMED, Tris, X-gal, Xylencyanol, Yeast-extract and petridish plate (Ø = 9 mm. and 12 x 12 mm.)

Schleicher-Schuell GmbH (Dassel, Germany): Nitrocellulose Transfer Membrane, Whatman paper

Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany): Albumin bovine, 2,2´Azino bis (3-ethylbenzthiazo-line-6-sulphonic acid), Agarose, Anti-rabbit IgG (whole molecule) FITC conjugated, Blue dextran, Calcium chloride, Dulbecco's phosphate buffered saline (DPBS), Ethylene glycol-bis (2-amino- ethylether)-N,N,N´,N´-tetraacetic acid (EGTA), Polyvinylpyrolidone (PVP), Fetal calf serum (FCS), Glutamin, Isopropanol, Magnesium chloride, Oligonucleotide primers Penicillin, Rabbit anti-nitric oxide synthase-endothelial (eNOS) (569-609), Streptomycin G, SYBR®Green JumpStart Taq ReadyMix, *Taq* polymerase, Triton X-100 (t-octylphenoxypolyethoxyethanol) and Tween-20.

Stratagene (Amsterdam, Holland): 5α DH *Escherichia coli* competent cell and Lambda DASH II

StressGen Biotechnologies Corp (Victoria, Germany): Rabbit Anti-NOS I polyclonal antibody

3.1.3 Buffers and solutions

All solutions used in this investigation were prepared with deionized millipore water and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

Binding buffer:	Lithium chloride (5 M)	1000 µl
	Tris HCl (1 M pH 7.5)	10 ml
	EDTA (0.005 M pH 8)	20 ml
	Water added to sterile filtered	50 ml and
Blotting buffers: Anode buffer I	Tris-HCl pH 10.4	300 mM
	Methanol	10 %
Anode buffer II	Tris-HCl pH 10.4	25 mM
	Methanol	10 %
Cathode buffer	Tris-HCl pH 9.4	25 mM
	Methanol	10 %
	6-aminohexanoic acid	60 mM
Cytoskeleton buffer (CB buffer)	Sodium chloride (150 mM/l)	0.438 g
	Magnesium chloride (5 mM/l)	0.051 g
	EGTA(5mM/l)	0.095 g
	Glucose(5mM/l)	0.049 g
	MES (10mM/l)pH 6.1	0.098 g
	Water added to	50 ml
DEPC-treated water	DEPC: 1 ml/L water incubate at 37 °C	
	overnight and inactivated by autoclaving	
Epinephrin solution	Sodium disulphate	0.0400 g
	Epinephrin	0.0018 g
	Water added to	40 ml

10 x FA buffer	MOPS	200 mM
	Sodium acetate	50 mM
	EDTA	10 mM
	adjust pH to 7.0	
1.2 % FA buffer	Agarose	1.2 g
	10 x FA buffer	10 ml
	37 % formaldehyde	1.8 ml
	Ethidium bromide	1 µl
	fill up to 90 ml with depc water and boil	
IPTG (0.5 M):	IPTG	1.2 g
	Water added to	10 ml
LB-agar plate:	Sodium chloride	8 g
	Pepton	8 g
	Yeast extract	4 g
	Agar-Agar	12 g
	Sodium hydroxide, 1N	480 µl
	Water added to	800
LB-broth:	Sodium chloride	8 g
	Pepton	8 g
	Yeast extract	4 g
	Sodium hydroxide, 1N	480 µl
	Water added to	800 ml
Lysis buffer:	Igepal	8 µl
	Rnasin	5 µl
	DTT	5 µl
	Water	82 µl

Physiological solution:	Sodium chloride	9 g
	Water added to	1000 ml
PHE medium:	0.9 % sodium chloride solution	16 ml
	Hypotaurin solution	10 ml
	Epinephrin solution	4 ml
Propidium iodide(0.5µg/ml): stock solution	Propidium iodide	1 mg
	Added water to	5 m1
Running buffer (10 x):	Tris base (0.25M)	30.3 g
	Glycin (1.92M)	144 g
	SDS	10.0 g
	Water added to	1000 ml
Sample buffer (4 x):	Tris 1M pH 6.8	13 ml
	SDS	6 g
	2-Mercaptoethanol	10 ml
	Glycerine	20 ml
	Bromophenol blue	10 mg
	Water added to	50 ml
Separating gel (10 %):	Water	4.25 ml
	Acrymlamide bis 30/0.8 %	5 ml
	Tris 1M pH 8.8	5.6 ml
	SDS 10 %	0.15 ml
	TEMED	0.010 ml
	APS 20 %	0.030 ml
Stacking gel (4 %):	Water	7.15 ml

	Acrymlamide bis 30/0.8 %	1.5 ml
	Tris, 1M, pH 6.8	1.3 ml
	SDS, 10 %	0.15 ml
	TEMED	0.010 ml
	APS 20 %	0.030 ml
Solutions for immunohistochemistry:		
Paraformaldehyde (16 %) stock solution	PFA	1.6 g
	added water to	10 ml
PBS + polyvinyl alcohol (PVA)	PVA	300 mg
	added PBS to	50 ml
Glycine-PBS (30 mmol/ml)	Glycine	0.022521 g
	added PBS + PVA solution	10 ml
Permeabilization solution:	Triton x-100	5 µl
	added Glycine-PBS	10 ml
Blocking buffer (3% BSA)	BSA	0.15 g
	added PBS + PVA	5 ml
TAE (50x) buffer, pH 8:	Tris	242 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100 ml
	Water added to	1000 ml
TBE (10x) buffer:	Tris	108 g
	Boric acid	55 g
	EDTA (0.5 M)	40 ml
	Water added to	1000 ml
TE buffer:	Tris (1 M)	10 ml
	EDTA (0.5 M)	2 ml
	Water added to	1000 ml

Washing buffer:	Lithium chloride (5 M)	1500 µl
	Tris-HCl (1 M pH 7.5)	500 µl
	EDTA (0.005 M pH 8)	1000 µl
	Water added to	50 ml and filtered
X-gal:	X-gal	50 mg
	N, N´-dimethyl-formamide	1 ml
	store at – 20 °C	

3.1.4 Equipments

ABI Prism Sequence Detection System Instrument: ABI, Forster City, CA, USA
Automated sequencer (LI-COR 4200): MWG Biotech, Ebersberg, Germany
Centrifuge: Hermle, Wehingen, Germany
Carbon dioxide incubator (BB16): Heraeus, Hanau, Germany
Carbon dioxide incubator (MCO-17AI): Sanyo, Japan
CLSM LSM 510: Carl Zeiss, Germany
Electrophoresis (for agarose gels): BioRad, München, Germany
Fluorescence microscope (DM-IRB): Leica, Bensheim, Germany
PCR thermocycler (PTC100): MJ Research, USA / BioRad, Germany
Power Supply PAC 3000: BioRad, München, Germany
Power Supply Mini-Protan^{R}: BioRad, Italy
Spectrophotometer (DU-62): Beckman
UV Transilluminator (Uvi-tec): Uni Equip, Martinsried, Germany
Videocamera (TK-C1306BE): JVC, Japan

3.1.5 Software programms

BLAST programm: http://www.ncbi.nlm.nih.gov/blast/
Multiple sequence alignment: http://prodes.toulouse.inra.fr/multalin/multalin.html
Automated sequencer: Li-COR, MWG-Biotechnology, USA
Primer design: Primer Express Software -ABI prism version 2.0

SAS version 8.0: SAS Institute Inc., Cary, NC

Image analysis: Bio-Rad Laser- Sharp MRC-1024 CLS software

3.1.6 Biological material

Bovine ovary, follicle, granulosa cells and *in vitro* produced oocytes and embryos were used in this work.

3.2 Methods

The overview of the methods used in the present study is given in figure 8 below.

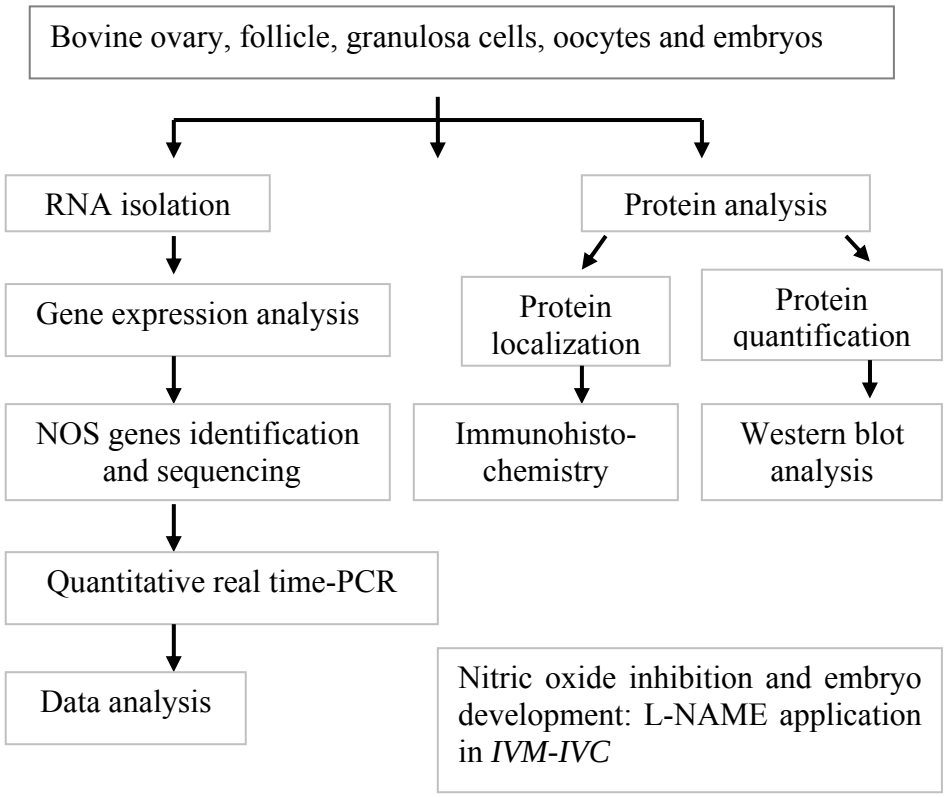

Figure 8: Overview of the methods used in the present study

3.2.1 Biotechnology methods

All techniques used in bovine *in vitro* embryo production will be described in this section.

3.2.1.1 Oocyte collection and maturation

The ovaries obtained from local slaughterhouse were washed with 70 % ethanol and two times with 0.9 % physiological saline solution (both solutions warmed at 30 °C). The follicles with a diameter of 1 to 8 mm were punctured using a sterilised 5 ml syringe attached to a 20 G needle (0.9x40). The follicular liquid was collected in sterilized 15 ml tube warmed at 30°C and left stand for 15 minutes to sediment or centrifuged. The oocytes surrounded by cumulus cells were collected under the microscope in the modified parker medium. After three times of washing counted oocytes (50 oocytes/well) were transferred in 400 µl maturation medium smeared with mineral oil (Sigma) in four well dishes (Nunc, Roskilde, Denmark). Maturation was performed in an incubator at a temperature of 39 °C and an atmosphere of 5 % CO_2 for 22-24 h.

3.2.1.2 *In vitro* fertilization

Two sperm straws were thawed at a temperature of 39 °C for 8 seconds in a water bath, and separated by means of the "swim up" technique in capacitation medium in the incubator for 50 minutes (Parrish et al. 1988). The supernatant was carefully discarded away and the sperms were resuspended in 3.5 ml capacitation medium, centrifuged at 250 g for ten minutes with discarding the supernatant. The sperm were diluted and counted in a haemocytometer. The matured oocytes in group of 50 were transferred in fertilization medium: Fert-TALP (Parrish et al. 1988) fertilized at $1x10^6$ spermatozoa/ml and incubated for 18 h.

3.2.1.3 *In vitro* culture

Following fertilization, zygotes were treated with 0.1 % (w/v) hyaluronidase and vortexing for approximately 5 minutes, to remove the cumulus cells. Cumulus free zygotes were washed and transferred to CR1aa media (Rosenkrans and First 1994) supplemented with essential and non essential amino acids. Further culture in cuture medium was performed in the same incubator in the same conditions as used for maturation and fertilization.

3.2.1.4 Nitric oxide inhibition and embryo development study

In order to investigate the effect of NO inhibition in early development three doses of L-NAME (0.1 mM, 1 mM and 10 mM) were applied in either only maturation or culture medium and in both maturation and culture media. Controls with no L-NAME addition were run along with all treatment groups. All experiments were repeated at least three times each comprising 200 oocytes for maturation and subsequent culture.

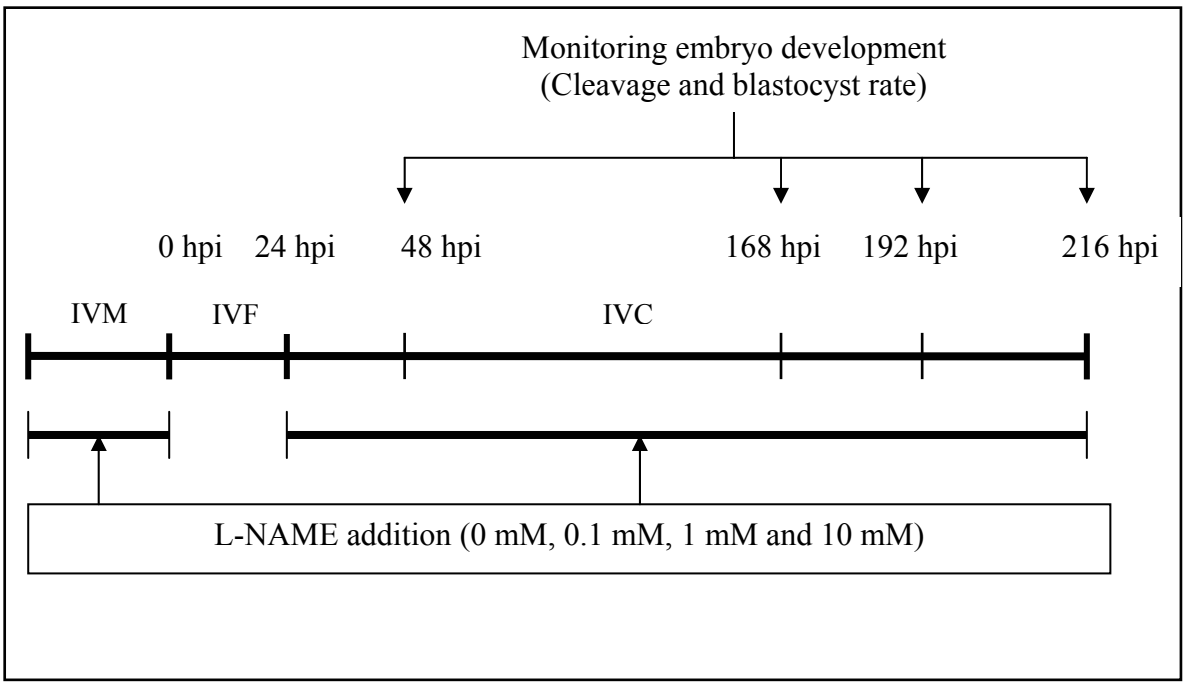

Figure 9: Experimental design to assess the effect of addition of L-NAME (0 mM, 0.1 mM, 1 mM and 10 mM) either to the maturation or culture or both media on the cleavage and blastocyst rates in vitro fertilized oocytes.

Experiment 1. Four groups of immature oocytes (50 oocytes in each group) were incubated in the maturation media to which 0, 0.1, 1 and 10 mM L-NAME was added then omitted in the further fertilization and culture of the oocytes.

Experiment 2. Here also four groups of immature oocytes were allowed to mature in the absence of L-NAME. Following maturation, oocytes were co-incubated with bull spermatozoa zygotes for 20 h and transferred to wells containing culture medium to which 0, 0.1, 1 and 10 mM L-NAME were added.

Experiment 3. Equal groups and numbers of immature oocytes as used in experiment 1 and 2, were allowed to mature in the maturation media to which 0, 0.1, 1 and 10 mM L-NAME was added and fertilized in the absence of the inhibitor. Presumptive zygotes were further cultured in media which was supplemented with the corresponding dosages of L-NAME.

As indicated in the figure 9 above, during this experiment embryo development *in vitro* culture was assessed by determining the cleavage rate at 48 hpi and the blastocyst rate at days 7 (168 hpi), 8 (192 hpi) and 9 (216 hpi).

3.2.1.5 Statistical analysis

Data from a minimum of three repeats were analysed using one-way ANOVA and the Tukey test. The statistical model includes glm and the Tukey test was used to appreciate the difference in proportions of embryos at the appropriate stage of development in the control embryos versus those cultured in NOS inhibitor (L-NAME). Differences were considered significant if $p \leq 0.05$.

3.2.2 Molecular genetics methods

General methods of molecular genetic analysis as used in this study are described in the following section

3.2.2.1 RNA isolation

Messenger RNA was isolated from pooled frozen bovine embryos of immature oocytes, mature oocytes, 2-cell stage (2C), 4-cell stage (4C), 8-cell stage (8C), morulae (Mor) and blastocyst (Bl) using oligo $(dT)_{25}$-attached magnet beads Dynal® according to the manufacturer indications. It is based on the pairing between the polyA tail of most mRNA and the oligo dT sequences bound to Dynabeads surface. Embryos were lysed in 30 µl binding buffer at 70 °C for 5 minutes and cells lysat was mixed with a prewashed Dynabeads. The lysate mixed with Dynabeads, was kept for 30 min to anneal at room temperature. The mRNA attached to Dynabead was washed twice with 150 µl washing buffer, 12 µl ddH_2O added and denatured at 90 °C for 5 minutes. After short centrifuge the eluate (mRNA) was taken in new sterile labelled tubes and store at – 20 °C for further use. The RNA samples were checked for DNA contamination if there is any by PCR amplification with β-actin gene primers: (forward: 5′-GAGAAGCTCTGCTACGTCGA-3′ and reverse: 5′-CAGACAGCATGTTGGC-3′) using standard protocols before cDNA synthesis.

Total RNA was extracted from frozen bovine ovary, follicle and granulosa cell using TRIzol reagent. Some milligrams (30-50) of tissue were ground into powder in a prechilled mortar, resuspended in digestion buffer: TRIzol® reagent (Life Technologies) in a volume of 1 ml homogenised with syringe and needle and incubated 5 min at RT. The samples were mixed with 0.2 ml chloroform vigorously shaken with hand for 15 sec and left 2-3 min at RT. The mix is centrifuged at 1200 x g for 15 min at 2-8 °C. The upper aqueous phase containing RNA was transferred in new 2 ml tube mixed with 0.5 ml isopropanol and allowed to precipitate at RT for 10 min. After centrifugation, the RNA precipitate visible as white pellet was collected by draining the supernatant off. The pellet was once again washed with cold ethanol (75 %), then removed and RNA briefly dried at air for 10 min. The RNA was dissolved in 20 µl RNase-free water.
To remove eventual residual DNA, the RNA was submitted to DNase. The RNA was mixed with 1µl RNasin, 4µl of RQ1 buffer, 7.5 µl of RQ DNase I, made up to 40 µl final volume with RNase-free water then incubated at 37 °C for 1h. Once DNA digested the RNA was purified with RNeasy Mini kit for RNA clean up (Qiagen) according to the manufacture's instruction. The integrity of the RNA was checked by electrophoresis

on 1.2 % FA gel at 120 volts for 15 min. The concentration (ng/µl) was determined by measuring the OD at 260 and 280 for the ratio $OD_{260/280}$ and calculated as 40 x OD_{260} x dilution factor. The stock RNA solution was stored at – 80 °C until use.

3.2.2.2 cDNA synthesis

The first strand cDNA was synthesized from both the messenger and total RNA. Eleven (11) µl of mRNA was mixed with 100 µM Oligo dT (11) N (5′-TTTTTTTTTTTN-3′) primer in 12.4 µl volume. This mixture was heated at 70 ^{o}C for 3 minutes and cooled to 0 ^{o}C for 2 minutes. Then 4 µl of 5 x First-Strand Buffer (GibcoBRL), 2 µl of DTT (0.1 M), 1 µl of 10 mM dNTP, 0.1µl Rnasin and 0.5 µl of SuperScriptII RNase H^- reverse transcriptase (200 units/µl, GibcoBRL) were added. This reaction was incubated at 42 ^{o}C for 90 minutes and the reverse transcriptase was deactivated by heating at 72 ^{o}C for 15 minutes. The first strand cDNA reaction was diluted or not and kept at -20 ^{o}C for the subsequent use.
The synthetized cDNA was tested by amplifying a portion of histone 2a gene using standard PCR conditions. Amplification of 2 µl of cDNA was performed with Taq polymerase (Sigma) in a final reaction volume of 20 µl, containing 0.5 µl of dNTPs (10 mM), 0.5 µl of each of primers at 10 µM (forward: 5′-CTCGTCACTTGCAACTTGCTATTC-3′ and reverse: 5′-CCAGGCATCCTTTAGACAGTCTTC-3′) in 1 x PCR buffer in thermal cycler for 35 cycles at 95 ^{o}C denaturation, 60 ^{o}C annealing and 72 ^{o}C extension followed by 5 minutes extension at 72 ^{o}C. Finally, products were visualized on a 2 % agarose gel.

3.2.2.3 Nitric oxide synthase genes primer design

The PCR primers were based on the bovine nitric oxide synthase (eNOS and iNOS) or human (nNOS) EST sequences using Primer Express Software (version 2.0, ABI) and were as shown in table 3.1. The specific primers were obtained by exclusion of conserved sequence using multiple sequence alignment programm: http://prodes.toulouse.inra.fr/multalin/multalin.html

Table 3.1: List of specific primers used in this study

Gene	GeneBank Acc. No	Primer sequence (5´- 3´)	Product size (bp)	Annealing temperature (°C)
	Qualitative	PCR		
eNOS	M95674	For-TTCCCAGAGACTGTTGGATTCC Rev-ACCTAAACACCAAAGGGCTGAC	137	60
iNOS	AF340236	For-ATCTTCGCCACCAAGCAG Rev-ACCTGATGTTGCCGTTGTTG	167	60
GAPDH	BTU85042	For-ACCCAGAAGACTGTGGATGG Rev-ACGCCTGCTTCACCACCTTC	247	62
	Quantitative	real time PCR		
iNOS	AF 340236	For-GGTGGAAGCAGTAACAAAGGA Rev-GACCTGATGTTGCCGTTGTTG	230	50-56
eNOS	NM_181037	For-CCTCACCGCTACAATATCCT Rev-TGCTCGTTGTCCAGGTGCTTC	197	50-56
nNOS	HSNOS1S1	For-CGTCTCTTCAAGCGCAAAGTT Rev-CGTTGACCGCAAGAATGATGT	151	43-49
H2a	NM_178409	For-CTCGTCACTTGCAACTTGCTATTC Rev-CAGGCATCCTTTAGACAGTCTTC	148	60

3.2.2.4 Polymerase chain reaction (PCR)

PCR amplification was performed in 20 µl reaction volume in 1 x PCR buffer (Pfu DNA polymerase, Promega), containing 2 µl of embryo cDNA templates or 50 ng/µl genomic DNA, 0.5 µl of each primer (10 µM), 0.5 µl of dNTP (50 µM), and 0.5 U of *Taq* polymerase (Pharmacia or Sigma). The PCR reactions were carried out in a PT-100 Thermocycler (MJ Reasearch) and the thermal cycling program was: denaturation at 94 ^{o}C for 4 min, followed by 35 cycles at 94 ^{o}C for 30 sec, annealing at the corresponding temperature (as shown in table 3.1) for 30 sec and extension at 72 ^{o}C for 1 min, final extension step at 72 ^{o}C for 5 min. The PCR product was subjected to electrophoresis with a lambda DNA *Bst*EII marker as a reference for fragment size. The gel was photographed under UV- transilluminator.

3.2.2.5 DNA extraction from agarose gel

The amplified products were electrophorosed in 0.7-1 % agarose gel having large combed well. The product was visualized under UV transilluminator and the gel having the DNA fragment of interest was cut and DNA isolated by phenol-chloroform extraction method. The gel was homogenized in 0.5 ml of 1 x TE buffer, mixed with 0.5 ml of phenol-chloroform and centrifuged at 10,000 rpm for 20 minutes. The supernatant was transfered to a new tube and 0.5 ml of chloroform added, mixed thoroughly and centrifuged at 10,000 rpm for 20 min. The DNA was precipitated with double the volume of chilled absolute alcohol after adding $1/20^{th}$ volume of 3 M sodium acetate (60 µl) and stored at –20 ^{o}C overnight. The extracted DNA was washed with 70 % alcohol, dried at air and dissolved in 6 - 8 µl of distilled water for further used.

3.2.2.6 Cloning and sequencing of PCR fragments

Ligation

The ligation reaction was setup in a PCR tube as follows: 2.5 µl of 2 x Rapid ligation buffer, 0.5 µl of pGEM®–T vector (50 ng / µl) (Promega), 0.5 µl of T4 DNA ligase (3 Units / µl) and 1.5 µl of template DNA were added and mixed by pipetting. The reaction was incubated overnight at 4 ^{o}C.

Transformation

The tubes containing the ligation reaction were spinned briefly to collect the contents at the bottom of the tube. About 3.5 µl of each ligation mixture was added to a sterile 15 ml tube, kept in ice. The competent cells (JM109 Strain or DH5α strain of *E. coli*) for transformation were removed from -80 °C just prior to adding and thawed by placing in ice. After gently flicking the tube, sixty (60) µl of competent cells was transferred into the 15 ml tube and incubated along with 3.5 µl of ligation mix on ice for 30 minutes. Then, the competent cells were heat shocked at 42 ^{o}C for 90 seconds in a water-bath and immediately cooled on ice for 2 minutes. Finally 650 µl of LB broth was added and incubated at 37 ^{o}C in shaking incubator for 2 h.

Cloning and screening of the transformants

Two LB/ ampicillin/ IPTG/ X-gal plates for each ligation reation waere prepared by adding 20 µl of 0.1 M IPTG and 20 µl of X-gal (50 mg/ml) to the LB plates with ampicillin and spread with a glass spreader (20 minutes prior to use). The transformation culture was plated to LB/ ampicillin/ IPTG/ X-gal plates in duplicate and incubated at 37 ^{o}C overnight until colonies are visible.

Successful cloning of an insert in the pGEM-T vectors interrupts the coding sequence of β-galactosidase, hence the vectors with insert produce white colonies in plate against blue colony which doesn't have inserts. Two white colonies and a blue colony (negative control) are picked in 30 µl of 1 x PCR buffer, from each plate. The bacteria were lysed by heating at 95 ^{o}C for 15 minutes. The colonies were screened for the insert by performing a PCR with primers designed in M13 promoter region of the vector.

M13 PCR has been done to confirm transformation success and identify the clones with insert. 20 µl of reaction volume containing 10 µl of lysate, 0.5 µl dNTPs (10 mM), 0.5 µl of each of primers (forward: 5′-TTGAAAACGACGGCCAGT-3′, reverse: 5′-CAGGAAACAGCTATGACC-3′), 0.5 U of Taq polymerase (Sigma) in 1 x PCR buffer were amplified in PTC 100 (MJ Research) thermal cycler for 35 cycles at 95 ^{o}C denaturation, 60 ^{o}C annealing and 72 ^{o}C extension followed by 10 minutes of final extension at 72 ^{o}C. The products were electrophoresed in 2 % agarose gel. Clones having insert would be having higher molecular weight fragments than the blue clones.

Sequencing

The positive clones were sequenced by using LI-COR automated DNA sequencer. The sequencing is carried out by dye termination method using a SequiTherm Excel ^{TM} II DNA sequencing Kit (Epicentre Technologies, Biozyme). For each sample, 4 reactions were performed, each reaction having one dideoxy nucleotide termination mix (ddATP, ddTTP, ddCTP and ddGTP) which will terminate the elongation reaction once the nucleotide binds to the elongating strand. The primers used were designed from Sp6 (5′-TAAATCCATGTGTATCTTATG-3′) and T7 (5′-ATTATGCTGCTGAGTGATATATCCCGCT-3′)

promoter regions of the pGEM-T vector and is flourescent labelled which can be laser read by LI-COR automated DNA sequencer. Each reaction consists of 1µl of termination mix and 2 µl of premix solution (3.6 µl of sequencing buffer, 0.8 pM of 700 IRD-labeled SP6 primer, 0.8 pM of 800 IRD-labeled T7 primer, 1.5 µl of M13 PCR product and 2.5 U Taq polymerase). PCR was performed in a thermal cycler (MJ Research) for 29 cycles with 95 ^{o}C denaturation, 59 ^{o}C annealing and 72 ^{o}C extension, followed by 5 minutes last extension at 72 ^{o}C. Immediately after PCR, 1.5 µl of stop buffer was added and the product was stored at –20 ^{o}C till loading. Before loading, the sequencing reactions were denatured at 95 ^{o}C for 5 minutes and loaded onto 41 cm 6 % Sequagel-XR (National Diagnostics, Biozyme). Electrophoresis was performed on a LI-COR model 4200 automated DNA sequencer in 1x TBE buffer at 50 ^{o}C, 50 W and 1500 V. Sequence data was analysed by using Image Analysis programm, version 4.10 (LI-COR Biotechnology).

3.2.3.7 Plasmid isolation

Plasmid DNA containing the insert of each of the isoform should be isolated to be used as standard for real-time PCR assay. The PCR products were amplified using the above mentioned primers covering the isoform specific site and cloned in pGEM-T vector. The positive colonies were cultured overnight at 37 ^{o}C in a 5 ml LB broth containing ampicillin. The recombinant E. coli cultures were pelleted by centrifuging at 12,000 rpm for 1 minute. Plasmid DNA was isolated by using the GenElute plasmid Miniprep Kit (Sigma), following the manufactures instructions. Briefly, the cells were resuspended with 200 µl of the resuspension solution, mixed thoroughly by vortexing and the cells were lysed by incubating with 200 µl of lysis solution for 4 minutes. The cell debris were precipitated by adding 350 µl of Neutralization or binding buffer and centrifuged at 12,000 rpm for 10 minutes. In the mean time, columns were prepared by using column preparation solution. The cleared lysate were added to the prepared column and centrifuged at 12,000 rpm for 1 minute and the flow through was discarded. The column were again washed with 750 µl of wash solution and centrifuged at 12,000 rpm for 1 minute and the flow through was discarded. Finally the plasmid DNA was eluted by

adding 100 µl of distilled water and eluted by centrifugation. The isolated plasmid DNA was sequenced to confirm the presence of the specific isoform.

3.2.3.8 Plasmid serial dilutions

The concentration of the plasmid DNA was estimated by reading the absorbance at 260 nm (A_{260}) in a spectrophotometer UV/visible light (Beckman Du^R 62). The plasmid concentration (ng/µg) = A_{260} x dilution factor x 50, was converted in number of copies (molecules) using the following program: www.molbiol.ru. The plasmid DNA was diluted to be similar to the concentration of the target in the embryos. Then serial dilutions were prepared for each EST from 10^1 up to 10^8 copy number in 45 µl volume.

3.2.3 Real-time quantitative PCR

In order to monitor gene expression, we used real-time RT-PCR analysis. Real time PCR was performed using mRNA, isolated from pooled oocytes and embryos, with SYBR® Green (Sigma) in an ABI prism 7000 SDS instrument (Applied Biosystems, USA). The amount of transcript in a sample is estimated as a function of how a fluorescent signal is observed above threshold (C_T value) during the process of sequence amplification. The threshold cycle (C_T) is the point at which the fluorescence signal is first recorded as statistically significant above the background. During every cycle the fluorescence values are recorded and represent the amount of the product amplified to a precise time. The correlation is the more a template present at the beginning; the fewer number of cycles are needed to reach the C_T point.

3.2.3.1 Optimization of the PCR conditions

SYBR Green binding dye was used to generate fluorescence as a function of temperature to perform a melting or dissociation curve of the amplicon which is sequence specific. A dissociation curve is produced by monitoring fluorescence continuously while heating the sample in order to observe the loss of fluorescence at the denaturation temperature. Single strong pick represent single PCR product whereas two

or more peaks indicate multiple PCR products. As the product specificity is determine by its primer, a prerequisite optimizing step was done.

SYBR®Green JumpStart™ Taq ReadyMix™ with internal reference dye for quantitative PCR (Sigma) combining non-specific product formation prevention to reaction normalization was used. PCR was performed in ABI prism 7000 sequence detection system. PCR condition is 50 °C for 1 sec, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min.

The specificity of the target quantification is reached by determining the minimum primers concentrations which give the lowest threshold value and minimizing non-specific amplification. Analysis of the dissociation curve to check the absence of non-specific product was confirmed by agarose gel electrophoresis. The optimum combination was selected for use as standard primer concentration for further steps.

3.2.3.2 Quantification: precision/reproductibility of the replicates

A standard curve was generated for each isoform by amplifying the serial dilutions of the known quantities of plasmid DNA (10^1-10^8 molecules). Precision and reproducibility are important in any quantification system and can be measured through the replicates (technical and biological replicates). In this work the primers were tested in identical duplicates using the same pool preparation with the same PCR reagents for each fragment.

Each reaction was performed in 20 µl containing 2 µl cDNA, optimal up and down stream primers, SYBR®Green JumpStart™ Taq ReadyMix™ (Sigma) and water.

To measure expression levels accurately, normalization by multiple housekeeping genes instead of one is required (Vandesompele et al. 2002); but it is generally accepted that gene-expression levels could be normalized by a carefully selected stable internal control gene. Indeed the internal control should not change in relative abundance during the course of treatments or investigation (Suzuki et al. 2000). Over eight housekeeping genes RNA levels quantified using real time PCR, histone 2a was the best internal standard in preimplantation period of bovine embryo (Robert et al. 2002). Final quantification analysis was done using the relative standard curve method (User bulletin # 2 ABI PRISM 7700 SDS) and results were reported as the relative expression (RE) or

n-fold difference to the calibrator cDNA (i.e. the sample with the lowest normalized quantity) after normalization of the transcript amount to the endogenous control.

3.2.3.3 Statistical analysis

Data of mRNA expression were analysed using one-way ANOVA followed by the Tukey test. Differences were considered significant if $p \leq 0.05$.

3.3 NOS proteins detection and localization in bovine oocytes and embryos

The elucidation of specific temporal and spatial protein localisation in early embryos is a key prerequisite for understanding development (Kurth 2003). However it is not easy to obtain protein localisation data from whole embryos with adequate resolution. In this section, NOS proteins localization using immunofluorescence will be described.

3.3.1 Immunohistochemistry

Immunohistochemistry is the localization of antigens in tissue by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, and radioactive element.

Direct or indirect method

Direct method is one step staining method, and involves a labelled antibody reacting directly with the antigen in tissue. Indirect method involves an unlabelled primary antibody which reacts with tissue antigen and a labelled secondary antibody reacts with primary antibody. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second antibody may be labelled with a fluorescent dye such as FITC, Rhodamine or Texas red and is called indirect immunofluorescence method. Indirect immunoenzyme method design the second antibody labelled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase.

Fixation and blocking

Fixation is the critical step in the preparation of histological specimens (Kurth 2003). For immunocytochemistry, fixation has to yield preservation of the structure and antigenicity. To ensure the preservation of cell morphology, adequate fixation is essential. In fact, inappropriate or prolonged fixation may significantly diminish the antibody capability. Background staining may be specific or non-specific. Inadequate or delayed fixation may give rise to false positive results due to the diffusion of the antigen. Antibodies, (especially polycolonal antibodies) are sometimes contaminated with other antibodies and this will lead to the background staining in cell or tissue sections. Using higher antibody dilution might be helpful to reduce the background.

Confocal laser scanning microscope principle

Observing thickbiological specimens using a conventional light microscope is often an unrewarding experience: structures above and below the plane of focus being examined usually badly obscure the image (White and Dixon 2003). The last decade confocal imaging has gained favour as a method for fluorescence microscopy, allowing direct visualisation within thick, fluorescently labelled tissue. The big advantage of confocal microscopy is the possibility to collect light exclusively from a single plane. The laser scanning microscope scans the sample sequentially point by point, line by line and assembles the pixel information to one image with high contrast and resolution.

3.3.2 Immunofluorescence

3.3.2.1 Pre-treatment of samples

Oocytes and embryos were washed three times in PBS, fixed in freshly prepared 4 % (w/v) paraformaldehyde in PBS overnight at 4 °C. The fixed specimens were washed twice in (0.3 mmol/l) glycine-PBS supplemented with PVA then permeabilized by incubation in PBS containing 0.5 % (v/v) Triton-X100 for 2h30min at RT. Non-specific

immunoreactions were avoid by incubating oocytes and embryos in 3 % (w/v) BSA freshly added in PBS plus PVA for 1h and then washed three times in PBS solution.

Table 3.2: Daily working solutions

Paraformaldehyde 4 %	250µl stock solution +750 µl CB buffer
Glycine-PBS 0.3 mmol/l	100µl stock solution + 9.9 ml PBS/PVA
Propidium iodide 0.5 µg/ml	1µl stock solution + 399 µl PBS/PVA
Antibody	Dilution
Endothelial NOS	1 : 200
Inducible NOS	1 : 80
Anti-rabbit FITC conjugate	1 : 500

3.3.2.2 Incubation with specific NOS antibodies

Aliquoted antibody was removed from -20 °C, thawed and diluted (table 3.2) at the corresponding factor (determined after a serial dilution) in BSA solution shortly before use. Indeed BSA treatment reduces non-specific binding reactions. In drops (100 µl) of prepared primary (NOS) antibody in a 96 well petri-dish smeared with mineral oil the embryos were kept at 39 °C in incubator without CO_2 for 1h. The specimens were washed three times 10 min each in PBS. Omitting primary antibody treatment before incubation with secondary antibody was used to determine the specificity of the first antibody.

3.3.2.3 Identification of antigen-antibody complex by FITC secondary antibody

Antigen-antibody recognition was made possible with help of fluoroscein isothiocyanate (FITC) conjugated to anti-rabbit antibody. Fluorescein is a small organic molecule, conjugated to proteins via primary amines (lysines), excited by the 488 nm line of an argon laser, and emission is collected at 530 nm. As for the primary antibody, prior to use the aliquot of secondary antibody (anti-rabbit immunoglobulin antibody conjugated to FITC) was diluted in BSA solution (table 3.2). The embryos are incubated

in 100 µl of prepared antibody smeared with mineral oil at 39 °C for 1h in dark. They were washed three times 10 min. each in PBS again.

3.3.2.4 Propidium iodide staining

In order to visualize cell nucleus propidium iodide was use in this section. Propidium iodide stain is an intercalating dye that appears red at 488 nm and used in flow cytometry to analyze cellular DNA content. The samples were incubated in well containing propidium iodide (0.5 µg/ml) for 25 min in dark at RT.
After an ultimate three times wash in PBS the embryos were mounted on slides (SuperFrost^{R}Plus, Braunschweig, Germany) in a drop of Vectashield mounting medium (Vector^{R}) protected by cover slip sealed with nail polish, stored at 4 °C till microscopic analysis as fast as possible to reduce any fluorescence damage.

3.3.2.5 Image capture and analysis

Fluorescence of FITC was visualized by excitation at 488 nm with the argon laser on a confocal laser scanning microscope (CLSM LSM-510; Carl Zeiss, Oberkochen) equipped with Bio-Rad Laser-Sharp MRC-1024 confocal laser scanning software.
Immunohistochemistry approach to analyse protein localization, in order to gain a comprehensive understanding of complex cellular processes and pathways could be confirmed by western blot analysis which is described in the following section.

3.4 Nitric oxide synthase proteins immunoblotting

Western blot analysis can detect one protein in a mixture of any number of proteins while giving information about the size of the protein. It does not matter whether the protein has been synthesized *in vivo* (native) or *in vitro* (recombinant). This method is however, dependent on the use of a high-quality antibody directed against a desired protein. Western blotting tells how much protein has accumulated in cells but if a protein is degraded quickly, western blotting won't detect it well.
3.4.1 Protein isolation

Sample buffer with loading buffer (26 % of Tris 1M pH 6.8, 12 % of SDS, 20 % of 2-Mercaptoehtanol and 40 % of Glycerol) was used to lyse cells (causes cell membranes to break and proteins to be released into buffer). Protease inhibitor cocktail (Sigma) was used to stop protease enzymes from denaturing proteins. Protease, if not inhibited, would be released as the cell was lysed and catalyzes the splitting of interior peptide bonds of proteins. Tissues or embryos samples were homogenized in sample buffer then centrifuged 10 minutes at 4 °C. The supernatant was collected after heating the homogenate at 95 °C for 5 min and stored at – 80 °C to avoid any denaturation till use.

3.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone, confers a negative charge to the polypeptide in proportion to its length. The SDS-PAGE was performed according to Laemmli (1970): 4 % stacking gel plus 10 % separating gel. Separating gel is used to separate proteins into their respective sizes allowing sharp bands to be seen whereas the stacking gel organize proteins before they enter the separating one.

To prepare a gel properly two gel glasses were cleaned thoroughly using soap and distilled water, assembled with space maker and water was added to test the porosity then dried. The separating gel (3.6 ml) was poured into the gel glass; the remainder of the space was filled with isopropanol to ensure that no air bubbles would form at the surface of the gel during gel polymerisation (5-10 minutes). The isopropanol was poured out, the gel dried with filter paper before adding the stacking gel on top of the separating one. A comb was inserted between gel plates and the gel polymerised at RT. In between the heater was set at 95 °C and proteins were warmed 5 minutes.

A standard vertical gel electrophoresis apparatus was used to enable the loading of protein homogenate by adding 5 to 10µl of sample to the wells of the gel. One lane was reserved for 5 µl of a molecular marker solution (to compare with proteins in gel to determine their size). The gel was placed in an electrophoresis unit that was filled with one time running buffer and run at 200 mA until the bromophenol blue reach the bottom of the gel.

3.4.3 Semi dry blotting and blocking

Nitrocellulose transfer membrane poresize 0.45 µm (Protan®, Schleicher and Schuell,), nine pieces of filter paper Whatman (Schleicher and Schuell) by sample in size of 6.5/8.5cm were soaked in water and blotting buffers (I, II, III) respectively for approximately 20 min before use. The transfer "sandwich" was assembled in the Trans-Blot® SD Semi-Dry-Transfer Cell (Bio-Rad, USA). Time of transfer was 1h at 1.75 mA/cm^2 of gel. After the transfer was complete, the nitrocellulose blot was washed shortly in water and subsequently stained with 2 % Ponceau in 10 % acetic acid for approximately 5 min or until molecular molecular marker: PageRuler Prestained Protein Ladder (Fermentas, Life Sciences, France) bands appeared. The nitrocellulose blot was washed in TBST shortly and incubated in 1% polyvinyl pyrolidone (PVP) diluted in TBST at RT for 30 min to 1h on an orbital shaker to prevent the non-specific adsorption of the immunological reagents.

3.4.4 Immunodetection (antibody affinity)

The nitrocellulose blot was incubated in specific (anti-NOS) primary antibody diluted (1: 10000) in TBS supplemented with 0.1 % PVP overnight in the refrigerator at 4 °C. To prevent non-specific binding of primary and secondary antibody and reduce background of the blot, the nitrocellulose membrane was washed with 0.05 % (v/v) Tween-20 Tris buffer saline at room temperature (six times for 10 min each) on an orbital shaker. The second antibody anti-rabbit horsreddish peroxidase conjugated was diluted in the 0.1 % PVP (1:10000) and incubated with the blot for 1h at room temperature with agitation. At the end the membrane was washed six times 10 min each.

3.4.5 Bands identificafion

The enhanced chemilumnescence (ECL) prepared solution for detection consists of ECL plus western blotting detection reagent A and B (40:1) from Amersham (Biosciences). After removing excess buffer from the membrane (by draining the membrane over a piece of folded paper and briefly touching the edge of the membrane to the paper) mixed ECL solution was added for 5 minutes incubation time. Once the solution removed, the membrane was taped to the inside autoradiography film cassette. In the dark, a sheet of x-ray film was added to the cassette then exposed to the membrane for impregnation and developed, then washed and fixed. In fact horsereddish peroxidase reacts with the substrate acridinium, resulting in a light emission by exposure to autoradiographic film (Kodack^{R} Biomax XAR film, Kodak, Japan).

4 Results

4.1 Nitric oxide and bovine embryo development *in vitro*

The effects of the NOS inhibitor L-NAME, was examined to investigate the role of NO/NOS activity in the development of bovine embryos *in vitro* during the preimplantation period. The *in vitro* development of bovine embryos was monitored daily at day 2 for cleavage rate, day 7 and day 9 during culture for blastocyst rate in medium with or without L-NAME at different concentrations.

Experiment 1: L-NAME addition in maturation medium

To determine if inhibition of embryo development by L-NAME can be achieved at oocytes level, COC were cultured for 24 h in either MPM alone (control) or with L-NAME at 1 and 10 mM. No significant effect of the inhibitor was obtained when oocytes were matured in presence of L-NAME, fertilized and then cultured without the inhibitor as indicated by a blastocyst rate of 28 % and 22 % at 0 mM and 10 mM respectively. It seemed that the application of the NOS inhibitor had no effect on the maturation of oocytes and further embryo development when added at MPM. However high arrested rate (81 % and 88 % at 1 and 10 mM respectively) observed in treated group compared to 72 % in control, are indicative of impairement of normal development (table 4.1).

Table 4.1: Effects of the addition of L-NAME (0, 1 and 10 mM) to maturation medium on development of embryos derived from *in vitro* system. The results are given as mean ± SD

L-NAME concentration	Number oocytes	Cleavage rate (%)	Blastocyst rate (%)	Arrested rate (%)
0 mM	908	71 ± 11	28 ± 14	72 ± 14
1 mM	455	63 ± 13	19 ± 15	81 ± 15
10 mM	605	63 ± 11	22 ± 11	78 ± 11

Since high concentration (10 mM) at maturation did not affect either the cleavage or the blastocyst rates, the treatment with lowest dose 0.1 mM was omitted.

Experiment 2: L-NAME application in culture medium

To determine whether NO affects preimplantation embryo development, fertilized oocytes were cultured in either CR1 medium alone (control) or with L-NAME at 0.1, 1 and 10 mM level (table 4.2). When one-cell embryos (presumptive zygotes) were treated with high concentration (10 mM) L-NAME, the development of most of these embryos was significantly affected ($p \leq 0.05$) compared to non treated group. Very low percentage of embryos developed to blastocyst at 10 mM (1 %) compared to the control group (0 mM) with (15 %). L-NAME inhibited normal embryo development past the eight-cell stage at 10 mM (data not shown). The inhibitory effect of L-NAME was lost when its concentration was decreased to 0.1 or 1 mM (most of embryos developed to the blastocyst stage). The blastocyst rate seemed to be stimulated at 0.1 and 1 mM compared to the control: almost two times more blastocyst rate at 1 mM (28 %) compared to the control (15 %).

Table 4.2: Effects of the addition of L-NAME (0, 0.1, 1 and 10 mM) to culture medium on development of one-cell embryos *in vitro* system. The results are given as mean ± SD. Values with different superscripts within a column differ significantly ($p \leq 0.05$)

L-NAME concentration	Number oocytes	Cleavage rate (%)	Blastocyst rate (%)	Arrested rate (%)
0 mM	640	67 ± 7	15 ± 10^{a}	85 ± 10^{a}
0.1 mM	218	71 ± 10	22 ± 12^{a}	78 ± 12^{a}
1 mM	457	68 ± 9	28 ± 18^{a}	72 ± 18^{a}
10 mM	412	66 ± 6	1 ± 2^{b}	99 ± 2^{b}

Experiment 3: L-NAME addition in maturation and culture media

To determine whether embryos that remained unaffected after L-NAME applications in MPM were further susceptible or not to nitric oxide reduction, COC were matured in presence of L-NAME, fertilized without then culture with. L-NAME inhibited normal embryo development (past the eight-cell stage) at 10 mM. The result was similar to the inhibitor addition in culture only as shown in table 4.3 with signifcative effect at high level: 7 % at 10 mM against 31 % blastocyst rate in not treated group. The blastocyst rate seemed to be stimulated at 0.1 and 1 mM compared to the control: from 32 % to 36 % blastocyst rate at 0.1 and 1 mM respectively compared to 31 % in the control group.

Table 4.3: Effects of the addition of L-NAME (0, 0.1, 1 and 10 mM) to maturation and culture media on development of embryos derived from *in vitro* system. The results are given as mean ± SD. Values with different superscripts within a column differ significantly ($p \leq 0.05$)

L-NAME concentration	Number oocytes	Cleavage rate (%)	Blastocyst rate (%)	Arrested rate (%)
0 mM	203	63 ± 8	31 ± 6[a]	69 ± 6[a]
0.1 mM	256	59 ± 10	32 ± 4[a]	68 ± 4[a]
1 mM	210	51 ± 12	36 ± 11[a]	64 ± 11[a]
10 mM	215	53 ± 8	7 ± 15[b]	93 ± 15[b]

In general the application of the inhibitor had no effect on the cleavage rates either at maturation (table 4.1), culture (table 4.2) or both (table 4.3) at any concentration level when compared to the control group. A great percentage of oocytes incubated with L-NAME cleaved regardless of the dose applied in maturation medium: 71 %, 63 % and 63 % cleavage rates at 0, 1 and 10 mM respectively. A cleavage rate of 67, 71, 68 and 66 % were obtained at 0, 0.1, 1 and 10 mM L-NAME application in the culture medium respectively. These data demonstrated that L-NAME inhibited the embryo development after cleavage to blastocyst stages in a concentration-dependent manner at certain conditions or stage.

** Spermatozoa and NO-inhibitor test

Viability and motility of sperm ($2x10^6$/ml) in fertilization medium without oocytes was tested after L-NAME addition. At high level (20-50 mM) of L-NAME, sperm cells die between 1 and 3 h, the minimum required for the fertilization to begin. At low concentration (1-10 mM), after 5 h they still alive and conserve their motility comparable to the control.

Concentration	1h	2h30	5h	24h
Control	+++	+++	++	-
1 mM	+++	+++	++	-
10 mM	+++	++	+ slower	death
20 mM	+++	death		
50 mM	death			

+++ = viable spermatozoa with high motility, ++ = viable spermatozoa with motility, + = viable spermatozoa with low motility, - = viable spermatozoa without motility

Due to the toxicity of L-NAME on the spermatozoa after some hours only as shown on the viability and motility, the application of this inhibitor at fertilization was avoided.

4.2 Qualitative identification of endothelial and inducible NOS genes

To determine which of the NOS isoforms are expressed in embryos at different stages of preimplantation development, embryonic mRNAs were amplified using RT-PCR. Amplification products were identified by agarose gel electrophoresis visualised under UV light after confirmation of the sequence. In addition, to determine the specificity of changes in eNOS and iNOS expression, RT-PCR for GAPDH was carried out. We expected the level of GAPDH, often considered as housekeeping gene, to remain constant during embryogenesis. All pooled embryo samples as well as positive controls (granulosa cells, follicle and ovary) expressed the highly abundant GAPDH gene. Amplification of a 137-bp fragment of the eNOS gene was evident in all pooled oocyte and embryo samples at different level of transcript. This was also true for expression of a 167-bp fragment of the iNOS gene. The pattern expression of these genes changed

through development: eNOS was higher in immature oocyte and lower in mature oocyte and 8-cell embryos contrasting with iNOS with remarkable transcript in oocytes, two and four cell embryos than further stages (figure 10). Their transcripts were identical and highest in the positive controls.

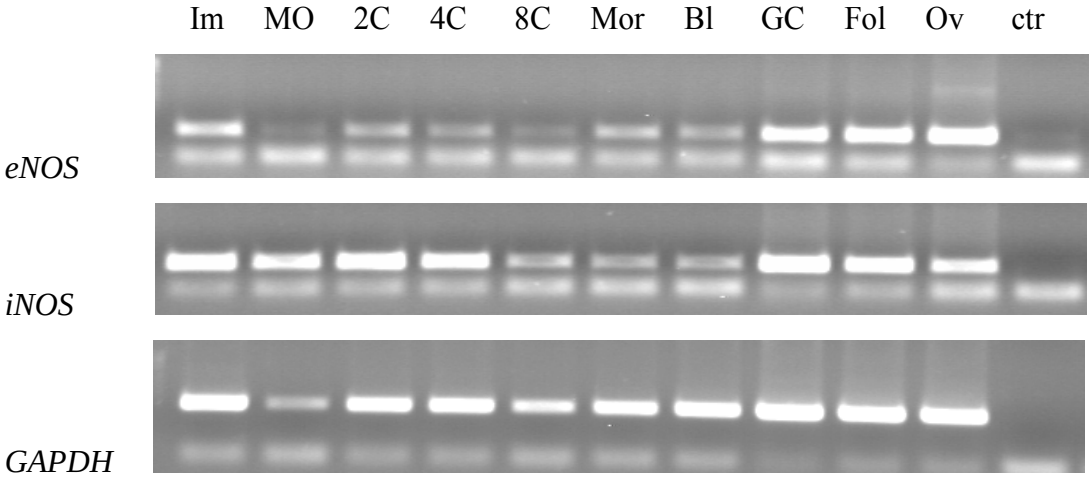

Figure 10: RT-PCR amplification of endothelial and inducible NOS genes from cDNA amplified from bovine immature (Im) and mature (MO) oocytes, two-cell (2C), four-cell (4C), eight-cell (8C), morulae (Mor) and blastocyst (Bl) with the negative control (no template). Granulosa cells (GC), follicle (Fol) and ovary (Ov) are indicated as positive control. GAPDH amplified as an endogenous control.

4.3 Quantitative expression profile of NOS isoforms in oocytes and embryos

With the aim of studying the quantitative expression profiles of NOS genes in different embryonic stages, eNOS, nNOS and iNOS were investigated. Embryonic mRNAs were subjected to fluorescence-monitored real-time quantitative PCR using sequence specific primers. Since the investigated genes were all expressed at immature oocyte, this stage has been selected as a calibrator and its values are set as 1 to present the relative abundance of each transcript in the preimplantation development stages after normalization with the endogenous control (histone 2a gene). The relative expression for each gene was calculated using the relative standard curve method.

As illustrated in figures 11, 12 and 13 below, differences in mRNA expression among the different embryonic developmental stages were found for eNOS, iNOS and nNOS genes.

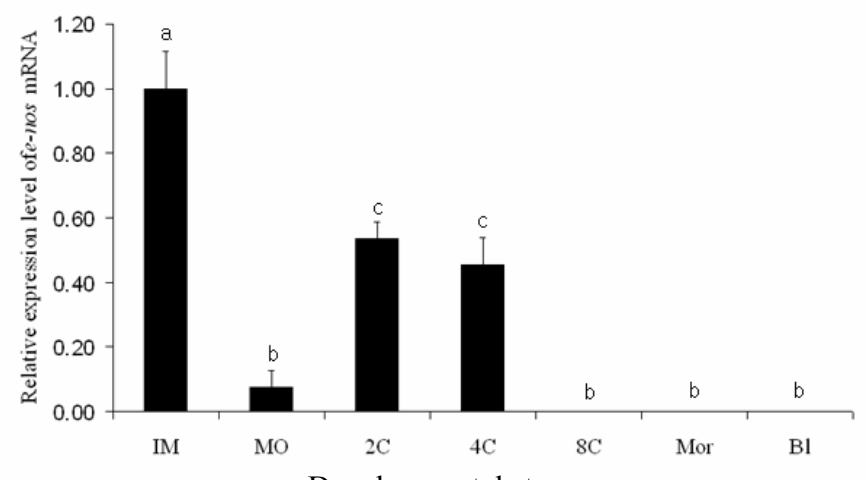

Figure 11: The relative distribution of eNOS isoform mRNA expression within oocytes and early embryonic developmental stages: immature (IM) and mature (MO) oocytes then embryos at two-cell (2C), four-cell (4C), eight-cell (8C), morula (Mor) and blastocyst (Bl) stages. Values with different letters differ significantly ($p \leq 0.05$)

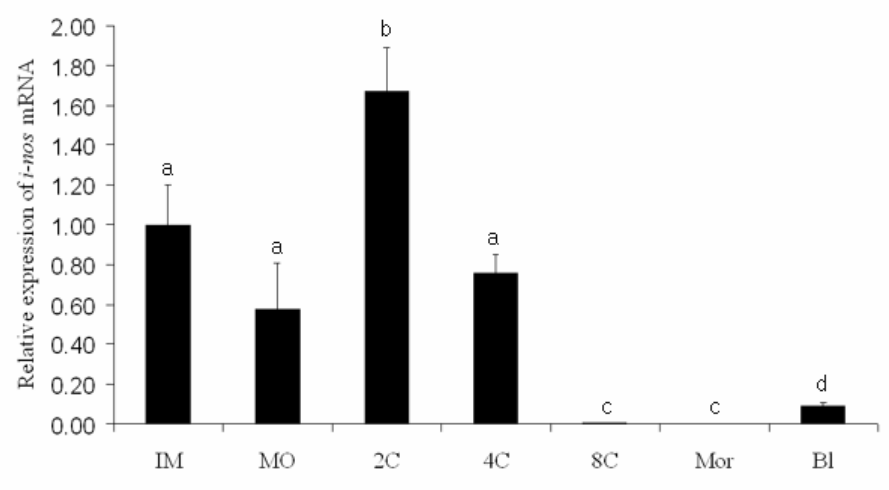

Figure 12: The relative distribution of iNOS isoform mRNA expression within oocytes and early embryonic developmental stages: immature (IM) and mature (MO) oocytes then embryos at two-cell (2C), four-cell (4C), eight-cell (8C), morula (Mor) and blastocyst (Bl) stages. Values with different letters differ significantly ($p \leq 0.05$)

The iNOS and eNOS genes were strongly expressed in oocytes, 2-cell and 4-cell embryos but were far less prevalent in other developmental stages, except iNOS at blastocyst stage. This graph is comparable to typical accumulation transcript from maternal followed by progressive degradation after fertilization. The pattern of eNOS and iNOS expression was different in relation to oocytes steady state which shows low level of expression of both NOS mRNA in mature oocyte compare to immature oocyte probably in relation with their role at maturation. Nitric oxide synthase and nitrite production in human spermatozoa were evidenced and it was suggested that endogenous nitric oxide is beneficial to sperm motility (Lewis et al. 1996, Herrero et al. 2003). The significant ($p \leq 0.05$) increased transcript at two-cell embryo stage compare to mature oocyte may be due to the contribution of NOS from spermatozoa. A significantly expression of iNOS was observed at blastocyst compare to earlier stage (eight-cell, morulae).

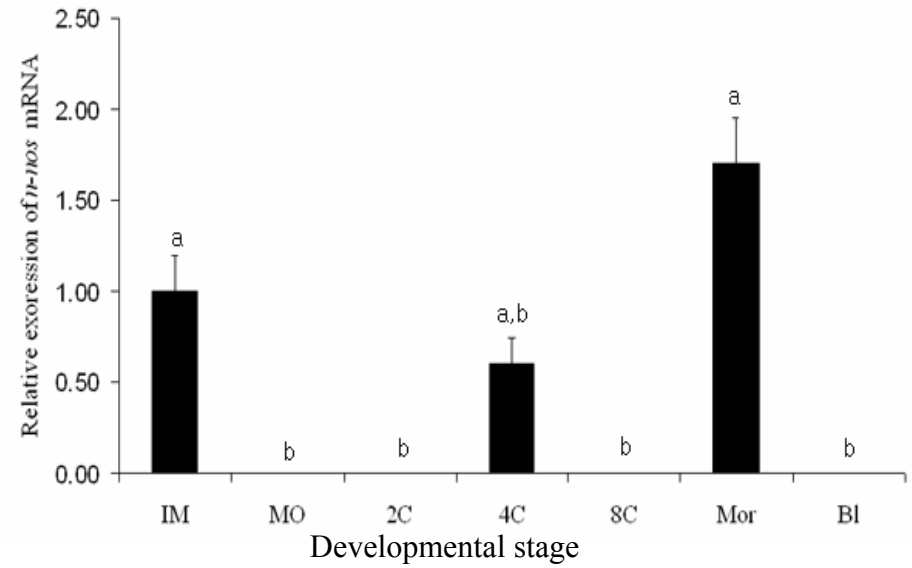

Figure 13: The relative distribution of *nNOS* isoform mRNA expression within oocytes and early embryonic developmental stages. immature (IM) and mature (MO) oocytes then embryos at two-cell (2C), four-cell (4C), eight-cell (8C), morula (Mor) and blastocyst (Bl) stages. Values with different letters differ significantly ($p \leq 0.05$)

The neuronal NOS gene transcript well expressed in immature oocytes was absent in the following stages except at 4-cell and morulae stage. Neuronal NOS from maternal origine degraded progressively till the embryonic becomes active at morulae stage.

4.4 Expression and localization of NOS protein during preimplantation development

As NOS gene expression was detected in unfertilized oocytes and preimplantation embryos, immunocytochemistry was used to determine whether NOS proteins are expressed in oocytes and embryos. Expression of eNOS and iNOS was detected in oocytes and embryos during the preimplantation period as expected (figure 14A). The pattern of immunostaining, however, seemed to exhibit slight differences depending on the NOS isoform investigated although both are stable throughout this period.

After omitting each primary antibody, fluorescence was not observed (figure 14A-k and B-k) indicating that the staining was specific for each of the NOS proteins.

In germinal vesicle-stage oocytes (c), iNOS immunoreactivity was localized in the germinal vesicle and cytoplasm. In matured oocytes (d) the iNOS staining accumulated mostly around the condensed chromosomes as indicated in red color.

Inducible NOS protein was distributed in fine discret granule uniformly in the cytoplasm and around nuclei of oocytes and embryos. The intensity of staining was however weaker in the perinuclear areas than the cytoplasm. Embryos at the eight-cell (h) and morulae (i) stages exhibited distinct positive zone surrounding the cell boundary. Staining was distributed at the periphery of the cytoplasm in zygote (e) and throughout the preimplantation period whereas the ICM in blastocyst (j) showed weaker labeling for iNOS than the trophectoderm cells.

Endothelial NOS protein was diffuse throughout the cytoplasm of oocytes to blastocyst stages with a weak staining in the nuclei as described by Nishikimi et al. (2001). In cumulus oocyte complex (a, b) the unstained zona pellucida delimitates the ooplasm from granulosa cells layers whith less eNOS protein than the oocyte which exhibited positive granular reaction uniformly distributed throughout the cytoplasm. In germinal vesicle-stage oocytes (c), eNOS immunoreactivity was uniformly localized in the cytoplasm and germinal vesicle. In matured oocytes (d), the eNOS immunofluorescence mostly accumulated around the condensed chromosomes. In embryos eNOS was predominantly in the peripherical areas of the cytoplasm and the staining was most intensive towards the junctions between blastomeres (2C, 4C, 8C and morulae). The staining was apparently more concentrated in the ICM at blastocyst stage than in the trophectoderm cells (j).

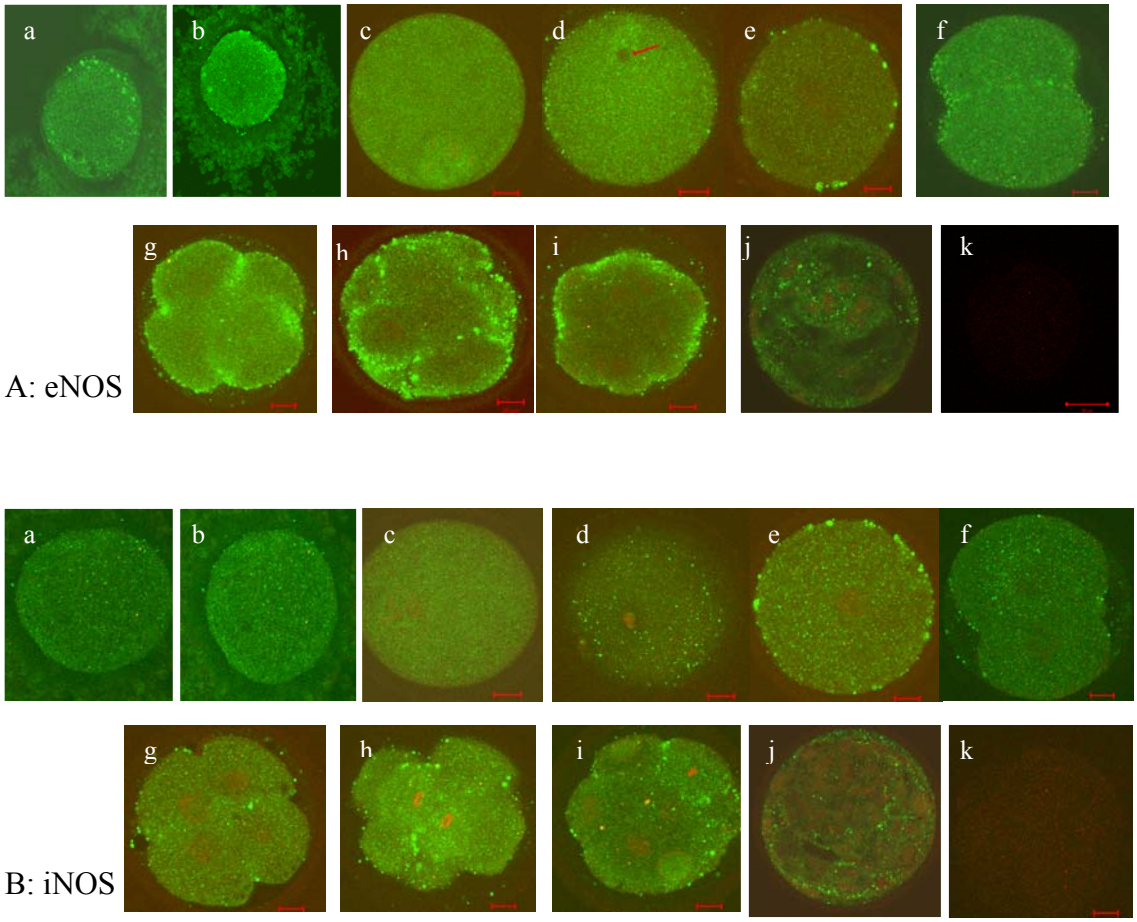

Figure 14: Subcellular localization of eNOS (A) and iNOS (B) proteins in bovine *in vitro* derived oocytes and in preimplantation embryos. Immature (a) and mature (b) cumulus oocytes complex, immature (c) and mature (d) oocytes then embryos at the zygote (e), two-cell (f), four-cell (g), eight-cell (h), morula (i) and blastocyst (j) stages were stained by indirect immunofluorescence using anti-NOS antibody. Controls (k) were stained without anti-NOS antibodies. In red are nucleus stained by propidium iodide. Scale bars represent 20 µm.

These predominant granules may represent eNOS in some organelles such as Golgi apparatus, mitochondria, endoplasmic reticulum or plasma membrane since this isoform is known to be membrane-bound.

4.5 Western blot analysis for endothelial NOS

To confirm that NOS was present in the bovine ovary, follicles, granulosa cells and oocytes, we performed immunoblots on ovarian, follicular, granulosa and oocytes homogenates.

Western blot analysis revealed the presence of immunoreactive proteins corresponding to eNOS (135-kDa) in extracts of bovine ovary and follicles. The amount of eNOS protein was similar (independent of the size of the follicle or the tissue-extract specificity) in these tissues (Fig. 15) in conformity with the transcript pattern observed at RT-PCR. These data support the immunostaining results and show that eNOS protein is present in bovine follicle and ovary and by extrapolation eventually in granulosa cells, oocytes and embryos (to be studied).

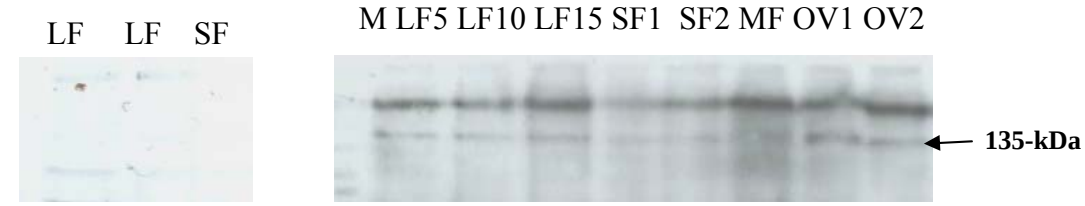

Figure 15: Western blot analysis for eNOS in follicles and ovaries. We observed that eNOS was present in large follicle (LF), middle follicle(MF), small follicle (SF), ovary (Ov) with an apparent molecular mass of 135 kDa and was not detectable when primary eNOS antibody was omitted (lane LF, LF, SF in negative control). The amount of eNOS was constant in all tissues

5 Discussion

In light of studies focused on NO production and emphasizing its crucial importance during the maturation, fertilization and embryogenesis (Manser et al. 2004, Tranguch et al. 2003, Nakamura et al. 2002, Nishikimi et al. 2001, Chen et al. 2001, Kuo et al. 2000, Jablonka-Shariff and Olson 1998, Lim and Hansel 1998) we investigated the participation of the bovine oocytes and preimplantation embryos in NO synthesis and development-associated functions. The purpose of this study was to determine whether nitric oxide synthases genes were synthesized by bovine oocytes and preimplantation embryos in a regulated manner during development.

In the present work, the role, the identification plus expression profile and the localization of NOS (endothelial and inducible isoforms) protein product in oocytes and preimplantation embryos of bovine were investigated *in vitro* system by application of NOS inhibitor (L-NAME), PCR, and immunofluorescence staining using antibodies against eNOS and iNOS respectively. Our results clearly demonstrate that significant level of NO is produced during in vitro development of bovine oocytes and preimplantation embryos.

5.1 Inhibitory effects of L-NAME on development of embryos *in vitro*

These data indicate that bovine embryos cultured in medium containing NO inhibitor, from the one-cell to the blastocyst stage, were developmentally affected becoming nonviable at high doses (10 mM L-NAME) as compared to the control group (0 mM) or their counterparts incubated only in maturation medium. Previous reports in mice and rats have shown that embryos cultured in medium containing NOS inhibitors were developmentally delayed (Biswas et al. 1998, Gouge et al. 1998, Nishikimi et al. 2001, Tranguch et al. 2003, Manser et al. 2004). We believe that the difference of inhibition of oocytes and zygote is due to the presence of maternal factors regulating development or mitosis. The time that activation of embryonic NOS transcription occurs may explain that the treated oocytes did not respond in the same manner as the one-cell embryos. Developing in normal medium newly synthesized NO in the absence of L-arginine competitor is possible but may be made impaired in presence of excess L-NAME. It is

likely that a physiological level of NO is required for oocyte survival and subsequent development (Nishikim et al. 2001). Addition of low concentrations of NOS inhibitor could reduce the excessively generated NO during development, but NO concentrations below the physiologically required amount, due to addition of high concentrations of NOS inhibitor, may impair embryo survival. In our study the optimum concentration was 1 mM. We have demonstrated indirectly that NO is produced in preimplantation embryos and that its production is required for normal embryonic development. The results from exogenous effect of pharmacological substances are conflicting. The development of mouse embryos beyond the two-cell stage in vitro was completely inhibited by L-NAME whereas L-NIO and AG had no effect (Nishikimi et al. 2001). L-NAME is generally known to have a higher potency in inhibiting eNOS than do L-NIO and AG, both relatively selective inhibitors for iNOS (Boer et al. 2000). This difference of effect of NOS inhibitors could be explained by the inactivation of both eNOS and iNOS or even all three isoforms activities by L-NAME. Probably when iNOS only is blocked by L-NIO or AG, other NOS isoforms may supply its action so that embryo development is no more or less affected. Studies on single-NOS knockout mice revealed that, reproductive abnormalities exist such as lower ovulatory efficiency, compromised erectile function, shorter and less variable estrous cycles, and reduced ovulation, atypical oocytes morphology (Jablonka-Shariff et al. (1999b), Tempfer et al. (2000), Sengoku et al. (2001)). However, no abnormalities in preimplantation embryo development in single knockout mice were seen but double knockout mice loss during development has been reported by Tranguch and Huet-Hudson in 2003, although the stage at which embryonic loss occurs is not known.

Oxygen free radical species, including NO, generated intracellularly, are required for the progression of embryos through the two-cell stage and probably also for the transition between maternal and embryonic gene expression in mouse embryos (Nishikimi et al. 2001). Nitric oxide is a labile gas molecule with half-life time of about some seconds (< 6 sec)(Thomas et al. 2001). Direct measurement of NO in the context of its involvement in cellular signalling is virtually impossible. We rely on the second level evidence (NOS or cGMP) to draw conclusions. In many tissues, NO can mediate its effects through the cGMP pathway, this may be at least in part, embryos (in mouse) as proposed by Chen et al. (2001) and Tranguch et al. (2003) in mouse embryos.

Embryo development may be continuously affected by newly synthesized NO during *in vitro* culture (Lim and Hansel 1998) and continuous removal of NO may be beneficial to optimize prehatching development as shown here by L-NAME addition. However embryos themselves do not appear to produce toxic amounts of NO, as indicated by the high cleavage and blastocyst rates in control than the treatment in this study. De novo production of the NO metabolites in medium containing L-arginine could not be detected after *in vitro* culture of bovine embryo for periods up to 174 h by Lim and Hansel (1998) and murine by Nishikimi et al. (2001). It looks like in unstimulated or normal medium embryos do not produce nitrite at detectable amounts.

We did not assess whether all oocytes were recovered mature by evaluating germinal vesicle breakdown and presence of polar bodies. Furthermore, we cannot prove that all recovered oocytes were fertilized. Thus, we cannot exclude that oocytes that did not develop to two-cell stage or blastocyst stage were not fertilized or immature. This has to be kept in mind when interpreting the results of our study.

An alternative approach to better understand NO stage-specific role could be the analysis of mRNA patterns of different isoforms.

5.2 Expression profile of NOS genes

In bovine, not all stages of preimplantation embryos or oocytes showed quantifiable mRNA expression for all three NOS genes, contrasting with our qualitative identification data or previously reported results in mouse by Tranguch and colleagues in 2003. Endothelial and inducible NOS were significantly expressed in oocytes, 2-cell and 4-cell embryos. Embryonic gene activation of iNOS was observed at blastocyst. Only two embryonic stages (4-cell and morulae) and immature oocyte showed measurable nNOS transcripts. This study shows, for the first time, the profile of mRNA for all NOS isoforms in preimplantation embryos and in particular in bovine, providing further evidence for the importance of NO production in mammalian embryo development. Analysis of stage-specific patterns reveals potentially complex interactions between these isoforms since they are stage-specific isoform regulated. These data attested that not only NO is required for preimplantation embryo

development but also multiple NOS isoforms are present to ensure its production at each stage except may be at 8-cell.

Previously in our laboratory using microarray technique, eNOS was revealed significantly ($p \leq 0.05$) downregulated at blastocyst stage compared to oocyte stage (Mamo 2004). At the same time iNOS was differently regulated at these two stages but not at significant level. It is known that developmental arrest at the time when embryonic gene activation occurs is caused by disturbance of transcriptional activity or redox regulation in embryos (Telford et al. 1990). In this case the susceptibilty of blastocyst to NO reduction may correspond to the initiation of embryonic genome activation of nNOS and iNOS. Before this stage embryos relayed on the maternal accumulated mRNA and proteins. Our NOS profiles seemed to confirm the NADPH-diaphorase staining experiments, which shows two peaks of NOS activity in mouse embryos during the preimplantation period (Nishikimi et al. 2001). According to these authors the first peak occurred immediately after fertilization (in zygote) then maintained consistant in two-cell and the second one at morulae-blastocyst stage. In sea urchin eggs, Kuo et al. (2000) found an increase in the activity of NOS after fertilization, and reported that NOS and NO-related bioactivities were necessary and sufficient for successful egg activation at fertilization. It could be inferred that the role of NOS and the related metabolism in egg activation is conserved through evolution from sea urchins to mammals. The second NADPH-diaphorase peak is paralleling our nNOS and iNOS mRNA content whereas the first peak corresponds to eNOS and iNOS activities at (zygote and) two-cell stage (high transcript). In our study mRNA transcript in zygote is missing. Moreover, L-NAME decreased NADPH-diaphorase activity at two-cell stage; this evidences once more the probable correlation between the NOS enzyme and the mRNA status. Taken together, the reduced developmental competence or nonviability of embryos could result from their incapability to activate the NO production at some critical time. Detectable nitrite generation by bovine macrophages is preceded by the induction of iNOS mRNA in liver (Adler et al. 1995). The complexity of NOS catalysis, reflected in the diversity of its cofactors and cosubstrates, certainly leads to important cell-specific differences in NOS regulatory pathways (Michel and Feron 1997). Our result shows that NOS mRNAs decreased in mature oocyte compare to immature oocyte partially in accordance with Nakamura and colleagues. In fact,

Jablonka-Shariff and Olson, 2000 demonstrated that the eNOS derived NO is a key modulator of oocyte meiotic maturation by using L-NAME with whild type in vitro and eNOS knock-out oocytes. In contrast Nakamura et al. 2002 proposed that the iNOS-NO-(cGMP) axis may play an important role in oocyte meiotic maturation. According to Nakamura et al. 2002 the lowering of NO is necessary to stimulate GVBD by decreasing cGMP.

The iNOS transcript seems to be constantly expressed in embryos than the supposed constitutives ones (nNOS and eNOS). Real-time PCR has proven itself to be valuable tool for analysing gene expression at a single embryo level (El-Halawany 2003). The variability observed between NOS isoforms (from 7-10 oocytes/embryos) in the expression levels can not be attributed to the technique (real-time PCR alone) since high reproducibility between identical replicates was prerequisite to validate the results. Therefore it could be assumed that the main factor contributing to the observed variability are the differences among individuals in each isoform. Moreover the potential variation that exists in mRNA expression among genetically heterogenous eggs and embryos has been reported by De Sousa et al. (1998c) in bovine.

More evidence of NOS presence in oocytes and embryos may come from cytochemical and immunological studies.

5.3 Expression and localization of eNOS and iNOS during preimplantation development

In this study, oocytes and preimplantation embryos showed immunostaining for endothelial and inducible isoforms of NOS proteins in conformity with the qualitative identification. Immunocytochemistry studies showed that eNOS and iNOS proteins were present in rodents oocytes and embryos as reported in mouse by Gouge et al. (1998) and Nishikimi et al. (2001) in contrast to Gagioti et al. (2000) who did not evidence these isoforms in mouse delayed blastocyst. The heterogeneous localization of eNOS is known to vary depending on the species or degree of confluency of cells (Govers et al. 2002). In this study eNOS was predominantly in the peripherical areas of the cytoplasm and the staining was most intensive towards the junctions between blastomeres (2C, 4C, 8C and morulae). 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 et al. 1998). The mechanisms regulating the intracellular distribution of eNOS between the Golgi and plasma membrane pools and the physiological role underlying their distinct regulation remain unknown. In germinal vesicle-stage oocytes, NOS immunoreactivity was localized diffusely in the cytoplasm and nucleus but was accumulated around the condensed chromosomes in mature oocytes. NO is thought to react with thiol residues of effector proteins in a diffusion-limited reaction, however the greatest amount of NO is in the perinuclear region of cells in localized S-nitrosylation reactions (Gow et al. 2002). Moreover NO generated in the Golgi or perinuclear area would be more highly concentrated in membranes of the Golgi complex or around the nucleus and more apt to produce autocrine actions than NO synthesized in the plasma membrane, which may readily diffuse to extracellular targets (Fulton et al. 2004). Our results show variations in NOS protein in stage dependent way suggesting NOS-derived NO regulation in relation probably with its roles in oocyte meiotic maturation (Jablonka-Shariff et al. 2000, Nakamura et al. 2002), trophoblast outgrowth and implantation (Gagioti et al. 2000, Navaro et al. 1997, Sengoku et al. 2001). Unfortunately, at present, any correlation between the amount and localization of the NOS activities and the two NOS isoforms cannot be explained. Inducible NOS is known to increase substantially after fertilization in ovulated mouse oocytes and embryos (Nishikimi et al. 2001). Nitrate and nitrite, metabolites of NO, were detected in the conditioned medium of embryos supplemented with L-arginine by Gouge et al. (1998) and Athanassakis et al. (2000) only in presence of toxic stimuli to the embryos, such as IFNγ, TNFα and LPS in contrast to Lim and Hansel (1998**)** in bovine; Nishikimi et al. (2001) in murine who could not reveal the presence of these metabolites in medium of embryos cultured with or without L-arginine using the Griess assay. Therefore, it can be considered that an optimum amount of NO is required for physiological regulation of preimplantation development, but lower or higher concentrations of NO generated in embryos may play a potential role in early embryonic death. The lack of parallelism between iNOS, eNOS proteins and their mRNA may be due to the dissociation between transcription and translation as reported by Jablonka-Shariff and Olson (1997). In fact they observed that after ovulation, iNOS mRNA decreases, and iNOS protein increases in mouse ovary.

Another confirmation of NOS protein expression in the bovine ovary, follicles, granulosa cells and oocytes, was carried out by immunoblots analysis. Western blot analysis revealed the presence of immunoreactive protein corresponding to eNOS (135-kDa) in ovary and follicles extracts. There was no difference in eNOS protein in these tissues in conformity with the transcript pattern observed at RT-PCR. These data support the qualitative PCR results and show that eNOS protein is present in bovine follicle and ovary and by extrapolation eventually in granulosa cells, oocytes and embryos (to be studied). Unfortunately iNOS could not be detected in the same samples. Previous reports showed that both eNOS and iNOS localized in mouse oocyte (Nishikimi et al. 2001; Mitchell et al. 2004). In swine eNOS was detected in the oocyte but not in proliferated cumulus cells (Hattori et al. 2001) contrasting with Grasselli et al. (2001) who found the secretion of eNOS in freshly selected granulosa cells but not iNOS even after culture with cytokine hTNF-α, a well known iNOS inductor. These different results might be due to different surroundings (Hanafy et al. 2001) or different animals, and alternatively NO function (which may differ at the oocyte or embryo development). The NOS deficiency on ovulatory capacity and early embryonic development in mouse is supposed to be modulated by genetic background (Hefler et al. 2002). They suggested a role for strain-specific modifier genes. The genetic background and species specificity should not be neglated since eNOS seems to be more accessible in porcine than in rodent. Inducible NOS is mostly evocated in rat and mouse at oocyte maturation. Both NOS isoforms may be involved in bovine according to our result.

5.4 Nitric oxide role

Nitric oxide has dual functions in the variety of cell types (Bu et al. 2003): inhibiting or stimulating depending on the type of cell, duration or amount of exposure. But the same NOS isoform may play entirely distinct biological roles when expressed in different tissues, and pathways outlined in one tissue must not necessarily pertain when the same isoform is expressed in a different cell (Michel and Feron 1997). The presence of NOS protein in cultured oocytes and embryos provided the possibility of studying NO actions on embryo development *in vitro.* Several lines of evidence support multiple roles for NO within oocytes and embryos. Until now, no clear role for NO in preimplantation

development has emerged, largely due to different culture media and animals being used in different studies. However NO has been shown to negatively influence embryo development in vitro at high doses from exogenous pharmacological substances like SNP, DETA which are NO donors (Barroso et al. 1998, Jablonka-Shariff and Olson 2000 and Sengoku et al. 2001). Other direct appreciation of NO deprivation is achieved by administration of substances derived from L-arginine such as L-NAME, L-NNA, L-NMMA or AG (Purcell et al. 1999, Chen et al. 2001, Tranguch et al. 2003, Manser et al. 2004). Manipulation of L-arginine competitors is a standard method of affecting NO production, used because direct measurements of NO are technically difficult due to its very short half-life (3-5 sec in biological tissue as reported by Thomas et al. 2001).

As NOS genes role is not yet clear, using microarray eNOS was clustered according to its expression pattern with genes involved in cell growth and differentiation whereas iNOS was similar to signal transduction activities genes (Mamo 2004). Together with i/n-NOS mRNA at morulae and blastocyst, we can hypothezise that NO is involved in embryo compaction, cavitation and cell differentiation. NO role during the activation of mammalian oocytes is progressively better understood. NOS inhibitors (L-NAME or L-NNA at 1 mM) suppressed the formation of first polar body of the oocytes in cumulus enclosed oocytes in mouse but no effect on germinal vesicle break down was observed (Bu et al. 2003). This optimal dose exhibited no effect on oocyte meiotic maturation of denuded oocytes. Our results showed no significant effect of L-NAME on cleavage rate suggesting that oocyte meiosis may be not affected.

Different pathways exist (figure 16) between NO-inhibited spontaneous meiotic maturation and NO-stimulated meiotic maturation of mouse oocytes (Bu et al. 2004). It was suggested that oocytes may release NO from eNOS as a signal for somatic cells to steadily suppress the development of cumulus cells (Hattori et al. 2001). Recently, Huo et al. (2005) demonstrated that iNOS-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte. At the same time, exogenous NO from S-nitroso-N-acetylpenicillamine (SNAP) was able to induce parthenogenetic activation of pig oocytes matured *in vitro* (Petr et al. 2005). Moreover these authors concluded that NO-dependent activating stimulus seems inadequate because it did not induce the exocytosis of cortical granules and also, the cleavage of parthenogenetic embryos was very low and embryos did not develop beyond the stage

of eight blastomeres. At culture when L-NAME was implemented, we observed a slight stimulation of the blastocyst rate at 0.1 and 1 mM doses compare to control whereas at the same time 10 mM inhibits bovine embryo development. If intracellular NOS is regulated similarly in cultured oocytes and embryos, then embryos with NO deprivation could maintain development to control embryos by utilizing intracellular NOS until exhaustion. Embryos cultured with L-NAME appeared healthy until 8-cell (data not shown), which probably indicates the time taken to reach critically low levels of NO. We do not have evidence on the mechanism by which this effect is occurring, but this observation warrants further examination. Previous reports found that NO/cAMP/PKA and NO/cGMP/PKG might be functional in mouse meiotic maturation (Bu et al. 2003, Bu et al. 2004).

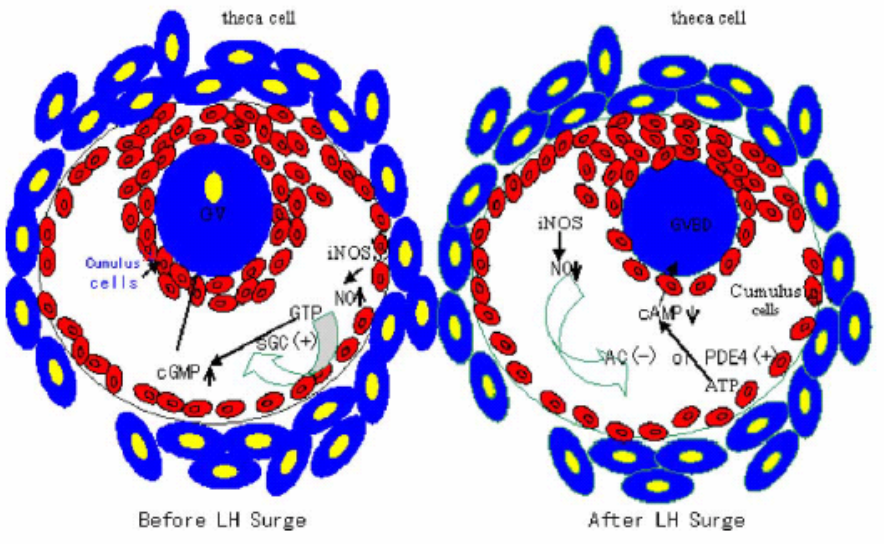

Figure 16: The physiological significance of NO in the process of meiotic maturation. Before the coming LH surge, the high concentration of NO produced by iNOS induces the increase of intrafollicular cGMP and the maintaining of meiotic arrest. The incoming of LH surge suppresses iNOS expression. The lower concentration of NO induces the decrease of intrafollicular cAMP and the meiotic resumption (Bu et al. 2004).

Till now cGMP mechanism is evocated mostly but it seems that other pathways are involved to execute the varieties of roles by NO in oocytes and embryos. Another possibility is that NO may limit oxygen consumption at the blastocyst stage at the level of mitochondrial cytochrome c oxidase (Manser et al. 2004) or act through nitrosylation

(Gow et al. 2002). Taken together it is clear that NO contributes to oocyte meiotic maturation and early embryo development but is not alone or dominant. Its association with PLC-zeta at GVBD or/and fertilization should be considered in calcium oscillations sustainance.
More investigations are needed to better establish the exact role played by each isoform at the critical step of embryogenesis beginning from maturation and fertilization.

In conclusion our results demonstrated that addition of NOS inhibitor adversely affected bovine embryo development similar to Biswas et al. 1998 *in vivo* or Lim and Hansel (1998) *in vitro* and confirm that NOS isoforms are present in oocytes and embryos as previously reported in murine (Tranguch et al. 2003, Kim et al. 2004). Preimplantation embryo may be highly sensitive to alteration of NO levels, and even subtle changes of NO levels could interfere with their development. Differences in required amount of NO and the sensitivity to cytotoxicity of NO in each developmental stages may also suggest that NO/NOS system is tightly regulated in developmental stage and isoform specific manner as seen in the profile. In definitive, these results demonstrate the importance and relevance of NO, in normal embryo development *in vitro* and the compensatory effect of the different isoform. Experiments in this series should be continued using specific inhibitors of NOS, to assess their effects upon embryos growth *in vitro*. Nitric oxide and NOS quantity (protein) are necessary to better understand the regulation of these genes.

5.5 Future prospects

Nitric oxide influences on the maturation of cumulus cell-enclosed bovine oocytes has to be clearly established in term of NO (metabolites), mRNA and proteins levels in immature or mature stages. The temporal characteristics of the NO rise may give us clues to its physiological role. These parameters should also be detected in cumulus cells to be able to distinguish NO from these cells from oocytes themselves. NO and apoptosis need to be clarified at embryonic development by looking closely to genes involved in cell apoptosis simultaneously with NOS genes. Apoptosis could be examined using a terminal deoxynucleotide-transferase-mediated dUTP-biotin nick end

labeling (TUNEL) method to determine whether embryos that arrested were undergoing DNA fragmentation. NOS protein details by using the electronic microscopy to specify the organells hosting each isoform may be an important approach of prospection. Profiling of NOS in *in vivo* oocytes and embryos should be undertaken to compare the transcript of these genes with their counterparts from *in vitro* since it is proven that culture conditions affect mRNA expression.

6 Summary

The present study was carried out, (i) to investigate the role of NO in bovine oocytes and preimplantation embryos development, (ii) to identify and profile the temporal expression pattern of NOS genes in preimplantation developmental stages and (iii) to detect and localize the protein products of iNOS and eNOS during this period.

The investigation of NO role in bovine oocytes and preimplantation embryos development was carried out by application of L-NAME, inhibitor of NO production, in maturation and culture media. To determine if inhibition of embryo development by L-NAME can be achieved at oocytes level, COC were cultured for 24h in either maturation medium alone (control) or with L-NAME at 1 and 10 mM. The *in vitro* development of bovine embryos was monitored daily to day 2 then at d7 and d9. All embryos developed normally through the two-cell stage to blastocyst stage. No significant effect of the inhibitor was obtained when oocytes were matured in presence of L-NAME as indicated by a blastocyst rate of 28 %, 19 % and 22 % at 0, 1 and 10 mM respectively. However, high level of arrested blastocyst rates (81 % and 78 % at 1 and 10 mM respectevely) are indicative of an impairement of normal development. To determine whether NO affects preimplantation embryo development, fertilized oocytes were cultured in either CR1 medium alone (control) or with L-NAME at 0.1, 1 and 10 mM. L-NAME inhibited normal embryo development past the eight-cell stage at 10 mM, significantly ($p \leq 0.05$) compare to non treated group. Very low percentage of embryos developed to blastocyst at 10 mM (1 %) compared to the control group (15 %). The inhibitory effect of L-NAME was reduced when its concentration was decreased to 0.1 or 1 mM (more embryos developed to the blastocyst stage) with blastocyst rate ranging from 15 %, 22 % to 28 % at a level of 0.1, 1 and 10 mM L-NAME respectively. Similarly, whether embryos that remained unaffected after L-NAME applications in maturation were susceptible or not to NO reduction, COC were matured in presence of L-NAME, fertilized without then cultured with NO-inhibitor. L-NAME inhibited normal embryo development past the eight-cell stage at 10 mM like in culture. In general the application of the inhibitor had no effect on the cleavage rates either at maturation, culture or both at any concentration compared to the control group. A great percentage of oocytes incubated with L-NAME cleaved regardless of the dose applied:

71 %; 63 % and 63 % cleavage rates respectively at 0, 1 and 10 mM were observed in maturation medium. During culture the following cleavage rates, 67 %, 71 %, 68 % and 66 % have been obtained at 0, 0.1, 1 and 10 mM L-NAME, respectively.

These data demonstrated that L-NAME inhibited the embryo development to blastocyst stages in a concentration-dependent manner at certain conditions or stage.

Before quantifying NOS genes we determine which isoforms are expressed in oocyte and embryos at different stages of preimplantation development, by using RT-PCR with GAPDH as reference gene. mRNAs have been isolated from ten pooled embryos at immature and mature oocytes, 2-cell, 4-cell, 8-cell, or seven morulae and blastocyst, then reverse transcribed to cDNAs with random primer. Specific primers have been designed from bovine or human NOS sequences using primer express software (version 2.0, ABI) to amplify different length fragments. These primers have been tested for their specificity to produce single specific band in each sample and used for isolation of the fragments which were subsequently cloned into plasmid vector. Following dideoxy chain termination sequencing, we obtained sequence information on NOS clones using BLAST program. Amplification of a 137-bp fragment of the eNOS and 167-bp fragment of the iNOS gene were evident in all pooled oocyte and embryo samples and also in the positive control. The expression pattern of these genes changed through development. To confirm these changes, real-time PCR technology has been used to examine the expression level of all three eNOS, iNOS and nNOS genes. The specific mRNA transcripts have been examined throughout the different stages of bovine preimplantation development. The quantitative reliability of our reaction conditions was established by measuring C_T values generated from spectrophotometrically quantified amounts of plasmid serial dilutions (10 up to 10^8 copy numbers) which carry isolated fragment. The resulting values were used to generate a standard curve to correlate the template copy numbers versus C_T values. To correct for both mRNA quality and quantity, cDNA amount values were adjusted to the reference gene histone 2a. Since the investigated genes were all expressed at immature oocyte, this stage has been selected as a calibrator, or 1 x and each of the normalized target value was divided by it to generate the relative expression levels. The iNOS and eNOS genes were well expressed in oocytes, 2-cell and 4-cell embryos but were far less prevalent in other developmental stages, except iNOS at blastocyst stage. The pattern of NOS expression was different in

relation to oocytes steady state which shows low level of expression of both NOS in mature oocyte compare to immature oocyte probably in relation with their role at maturation. The significantly ($p \leq 0.05$) increased transcript level at two-cell embryo stage compare to mature oocyte may be due to the contribution of NOS from spermatozoa (Herrero et al. 2003). The absence of quantifiable eNOS/iNOS at 8-cell and morulae in contradiction to the qualitative identification is surprising. This could be attributed to the determining amount of these genes in the whole embryo. A significantly expression of iNOS was observed at blastocyst compare to earlier stages (eight cell, morulae). Neuronal NOS transcript well expressed in immature oocytes was absent in the further embryonic stages except at 4-cell and morulae stages.

As NOS expression was detected in unfertilized oocytes and preimplantation embryos, immunocytochemistry was used to determine whether NOS proteins are present in oocytes and embryos. IVP bovine immature and mature oocytes, zygote, 2-cell, 4-cell, 8-cell, morulae and blastocyst stages embryos were washed three times in PBS, fixed in 4 % paraformaldehyde in cytoskeleton buffer overnight at 4 °C. They were permeabilized by 1h incubation in 0.5 % Triton-X100 then blocked in 3 % BSA in PBS for 1h followed by washing using PBS solution. The specimens were incubated for 1h at room temperature with anti-eNOS monoclonal antibody (1:200) and anti-iNOS polyclonal antibody (1:80). After three consecutive washes with PBS, oocytes and embryos were further incubated for 1h with secondary anti-rabbit IgG FITC conjugated antibody (1:500) in dark. In order to visualize the nucleus of the cells, they were finally incubated with 0.5µg/ml propidium iodide. Negative controls were processed in the same manner but the primary antibodies were omitted. After an ultimate washing with PBS, they were mounted on glass slides in Vectashield mounting medium, protected with coverslip, sealed with nail polish and examined under laser scanning microscope. Expression of eNOS and iNOS was detected in oocytes and embryos as expected, however the pattern of immunostaining, seemed to exhibit slight differences depending on the NOS isoform. In germinal vesicle-stage oocytes, iNOS immunoreactivity was mainly localized in the germinal vesicle and nucleus but after GVBD, the immunoreactivity accumulated around the condensed chromosomes. Inducible NOS protein was distributed in fine discret granule uniformly in the cytoplasm and around nuclei (weaker) of oocytes and embryos. Embryos at the eight-cell and morulae stages

exhibited distinct positive zone surrounding the cell boundary. Staining was distributed mostly at the periphery of the cytoplasm in zygote and throuought the preimplantation period but the ICM showed weak labeling for iNOS than the trophectoderm cells.

Endothelial NOS protein was diffuse throughout the cytoplasm of oocytes to blastocyst stages with a weak staining in the nuclei. In germinal vesicle-stage oocytes, eNOS immunoreactivity was mainly localized in the germinal vesicle but after GVBD, the staining mostly accumulated around the condensed chromosomes. In embryos eNOS was predominantly in the peripherical areas of the cytoplasm and the staining was most intensive towards the junctions between blastomeres. These predominant granules may represent eNOS in some organelles such as Golgi apparatus, mitochondria, endoplasmic reticulum or plasma membrane since this isoform is known membrane-bound. The staining was apparently more concentrated in the ICM at blastocyst stage than in the trophectoderm cells.

In conclusion, the current study demonstrated that addition of NOS inhibitor adversely affected bovine oocyte maturation or/and embryo development and supports the previous finding of the NO role in oocyte and embryogenesis. Moreover, a clear difference in the gene expression levels between isoform of in IVP embryos has been observed. The exact roles of these genes during meiotic maturation or preimplantation development are poorly understood, therefore, analysis of their expression levels could be a key step to understand their role during this period. Even real-time PCR has proven itself to be valuable tool for analysing gene expression at a single cell scale, not all the genes can be validate by methods such as quantitative real time PCR. Therefore, combination of more methods is the most suitable way in this kind of work. In definitive, these results demonstrate the importance and relevance of NO, in normal embryo development *in vitro* and the compensatory effect of the different isoform. Nitric oxide amount and NOS quantity in term of protein are necessary to better understand the regulation of these genes.

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