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).

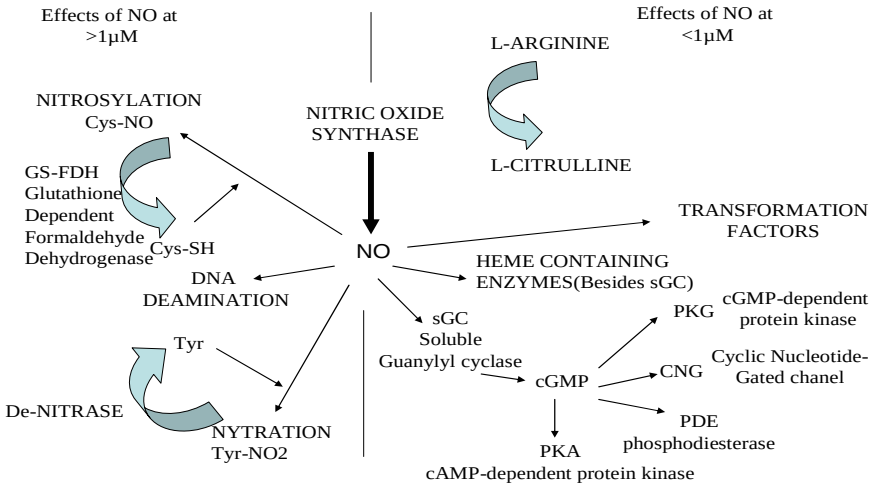

Figure 1: Summary of nitric oxide transduction (Hanafy et al. 2001)

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).

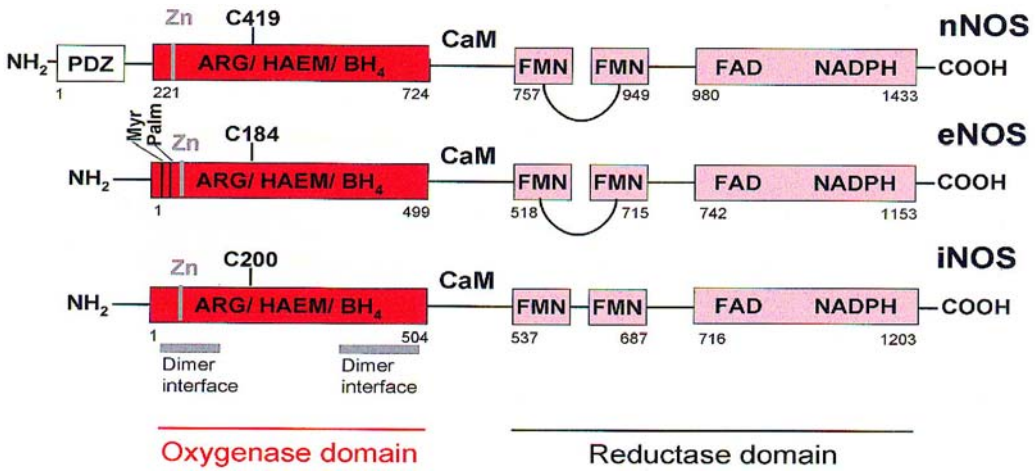

Figure 2: Domain structure of human nNOS, eNOS and iNOS (Alderton et al. 2001)

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