# Gene expression and apoptosis in bovine embryos during *in vitro* culture and *in vivo* development

Knijn, Hiemke Maria
Gene expression and apoptosis in bovine embryos during *in vitro* culture and *in vivo* development
2004

Dissertation Utrecht University, Faculty of Veterinary Medicine
-with a summary in Dutch
ISBN 90-393-3590-7

Realisation of printing by Atalanta, Houten

Cover illustration: Lisanne van der Voort

# Gene expression and apoptosis in bovine embryos during *in vitro* culture and *in vivo* development

## Gen expressie en apoptose in runder embryo's tijdens *in vitro* kweek en *in vivo* ontwikkeling

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universtiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 5 februari 2004 des middags te 2.30 uur

door Hiemke Maria Knijn, geboren op 25 augustus 1966 te Amersfoort

Promotoren: Prof. Dr. G.C. van der Weijden
Department of Farm Animal Health, section Reproduction, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Prof. Dr. H. Niemann
Department of Biotechnology, Institute for Animal Science (FAL), Neustadt, Germany

Co-promotoren: Dr. S.J. Dieleman
Department of Farm Animal Health, section Reproduction, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Dr. P.J.M. Hendriksen
Department of Urology, Erasmus Medical Center, Rotterdam, The Netherlands

Dr. P.L.A.M. Vos
Department of Farm Animal Health, section Reproduction, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

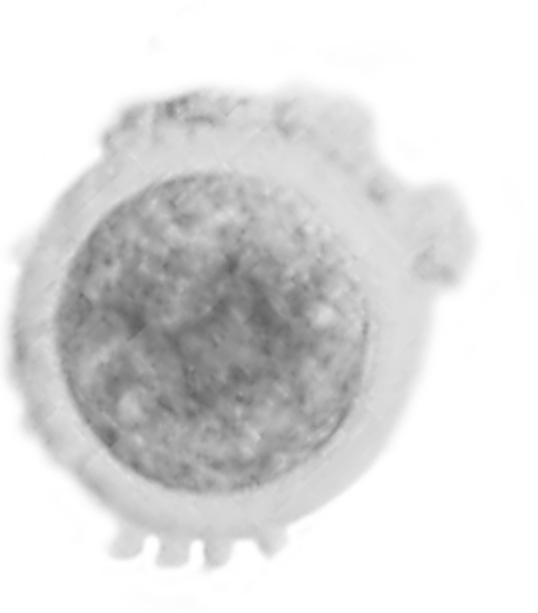
The investigations described in this thesis were financially supported by the Graduate School for Animal Health and the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Printing of this thesis was financially supported by the Graduate School for Animal Health and the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht University, Intervet Nederland BV, Holland Genetics, Bodinco and Dierenartsen coöperatie AUV.

# Contents

# Chapter 1

## General Introduction



## Introduction

In cattle the first attempts for *in vitro* fertilization were already performed in the late sixties of the past century (Sreenan, 1970) but it was not successful until 1982 when the first calves were born after transfer of completely *in vitro* produced embryos (Brackett *et al.*, 1982). Since then many attempts have been made to improve the multi-step process of *in vitro* production of embryos by refining the procedures of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes and *in vitro* culture of embryos (IVC). In 2001, worldwide 109.205 bovine embryos were produced *in vitro* (Thibier, 2002). Although the current *in vitro* production techniques have been improved a lot, it still cannot mimic the *in vivo* situation completely. Not only the efficiency of the *in vitro* procedure to produce morulae and blastocysts is lower compared to the *in vivo* situation (Merton *et al.*, 2003), also the quality of the produced morulae and blastocysts is impaired. The pregnancy rate after transfer of *in vitro* produced embryos is, in general, markedly reduced and a small but significant percentage of calves have distinct developmental abnormalities, known as “Large Offspring Syndrome” (LOS) (Hasler *et al.*, 1995; Galli and Lazzari, 1996; Kruip and den Daas, 1997; van Wagtendonk-de Leeuw *et al.*, 2000; Farin *et al.*, 2001).

In this chapter, the different steps during embryo development are introduced and the effects of *in vitro* production on the quality of the developing embryo, with special focus on gene expression and apoptosis, are discussed.

## Embryo Development

Development from the stage of immature oocyte to the formation of a blastocyst is a very complex process. During development *in vivo* several major steps can be distinguished: prematuration, maturation and fertilization of the oocyte and development of the zygote into a viable embryo.

During the growth phase of the oocyte, within a follicle with a diameter up to 2 mm, the oocyte builds up a store of mRNAs and proteins (Fair *et al.*, 1997) to support later development. When the follicle reaches a diameter of 2- to 3 mm, the intensive mRNA synthesis by the oocyte ceases although transcription is not completely inactivated (Fair *et al.*, 1995; 1996). Accumulation of mRNAs and proteins is crucial for later development since bovine oocytes that have not completed their growth phase fail to develop to the blastocyst stage in *in vitro* embryo production systems (Pavlok *et al.*, 1992; 1993; Lonergan *et al.*, 1994). During each estrus cycle, in general, 2-3 waves of follicular growth occur. The occurrence of follicular waves is predominantly regulated by surges of FSH in the peripheral blood (Adams *et al.*, 1992). A cohort of follicles grows from 3- to 8-mm and when they reach 8-9 mm there is a divergence whereby one follicle increases

in size, becoming larger than the other follicles and is referred to as the dominant follicle (Savio *et al.*, 1988). During the luteal phase of the cycle the dominant follicle of the first wave undergoes atresia but during the last wave, the growth of the dominant follicle is accompanied by an increased LH pulse frequency which presumably induces the start of prematuration of the oocyte that is completed shortly before the LH surge (Wiltbank *et al.*, 2002)(Figure 1).

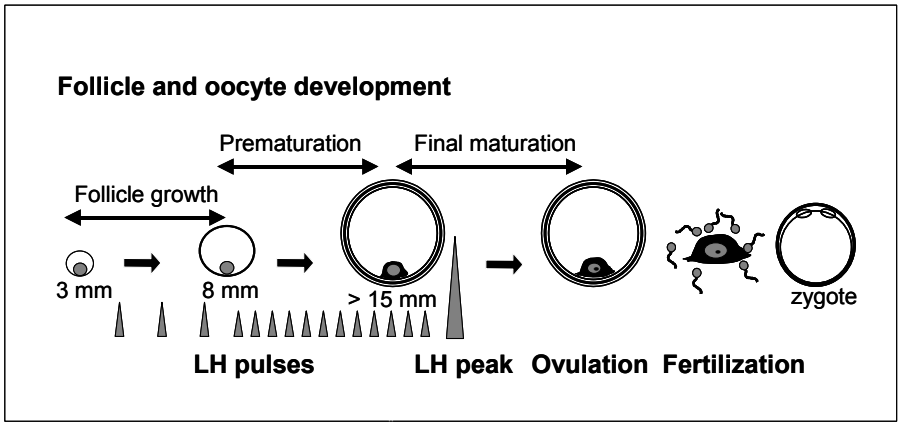



**Figure 1.** Phases of follicle growth, prematuration of the oocyte, development of preovulatory follicle and oocyte, and fertilization (adapted from Dieleman *et al.*, 2002).

During prematuration, ultrastructural changes become apparent in the nucleus and cytoplasm of the oocyte, such as undulation of the nuclear membrane, vacuolization of the nucleolus, more superficial localization of the clusters of cortical granules, and beginning retraction of corona cell extensions (Hyttel *et al.*, 1997). When the LH surge occurs, the oocyte within the dominant follicle will undergo final maturation and will respond to LH with morphological and molecular changes including germinal vesicle breakdown and resumption of meiosis. During the first meiotic division, one set of chromosomes is extruded as the first polar body. The oocyte then progresses through the second meiotic division and arrests at the metaphase stage (MII). From this time ovulation takes place and the oocyte enters the oviduct where it needs to be fertilized before further development occurs. After fusion of the bovine oocyte and spermatozoon, the nuclear envelope of the penetrated spermatozoon desintegrates. The tightly packed DNA of the sperm head decondenses and looses its characteristic appearance. At the same time, the oocyte expels its second polar body and completes its meiotic maturation. The oocyte and sperm haploid sets of DNA form two pronuclei, a maternal and a paternal, which remain distinct until their

membranes break down and the maternal and paternal chromosomes mix for the first time, during the first division of the zygote (Betteridge and Fléchon, 1988).

The first cell divisions are driven by mRNAs derived from the oocyte, maternal mRNA. The major genome activation, that occurs in cattle at the 8- to 16-cell stage (Camous *et al.*, 1986; Kopecny *et al.*, 1989; Telford *et al.*, 1990; Memili and First, 1998; 2000), is known as the maternal embryonic transition period (MET) (Figure 2). A minor genome activation has been demonstrated by studies using long-term exposure of 2- to 4-cell embryos to $^{3}$H-uridine followed by autoradiography and revealed that transcriptional activity can be detected earlier in development (Plante *et al.*, 1994; Hyttel *et al.*, 1996; Viuff *et al.*, 1996; for review Kanka, 2003). However, development can still proceed to the 8-cell stage in the presence of a transcriptional inhibitor like $\alpha$-amanitin (Liu and Foote, 1997).

After the MET, cell divisions depend on embryo derived gene transcripts. Compaction of the morula is the next important morphogenetic event in embryo development and depends on expression of molecules mediating cell to cell adhesion (Boni *et al.*, 1999). In bovine this starts from the 32-cell stage onwards (Betteridge and Fléchon, 1988; Van Soom *et al.*, 1997) . Compaction is followed by cavitation that is mediated by fluid transfer across the outer blastomeres, eventually forming a fluid-filled cavity. Simultaneously with cavitation, the first 2 embryonic cell lines, the outer epithelial trophectoderm and the undifferentiated inner cell mass (ICM) are formed (Watson, 1992).

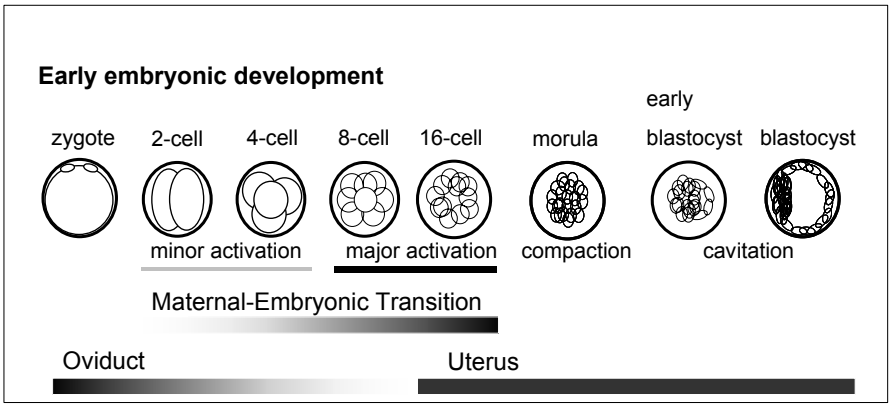



**Figure 2.** Scheme of stages of early embryonic development occurring in the oviduct and uterus. Maternal transcripts are essential to sustain the first few cell cycles of the early embryo until major activation of the embryonic genome during the 4$^{th}$ cell cycle. (adapted from Dieleman *et al.*, 2002).

**Differences between *in vitro* production and *in vivo* development of embryos**

Basically there are two major differences between *in vitro* production and *in vivo* development of embryos. First, the selection of gametes and second, the environmental differences during maturation and fertilization of the oocyte and early embryonic development.

*The selection of gametes*

Female gamete

*In vivo* a very strong selection of oocytes takes place; in the cow, only one or two follicles per cycle ovulate. Even in assisted reproduction techniques in which superovulation procedures are applied, a certain selection of oocytes occurs; only the follicles that have responded to superstimulation will ovulate. In most *in vitro* production systems that are used, no stringent selection of oocytes is applied because all oocytes from follicles between 3- to 8 mm are used. Furthermore, the oocytes collected from 3- to 8 mm follicles either miss the prematuration period (that occurs *in vivo* when the follicle grows from 8 mm to the maximum size)(Figure 1) or they are atretic, a process of degeneration of the follicle during which the follicle will finally be eliminated.

Male gamete

*In vivo* an extensive selection procedure of male gametes occurs during the journey through the female tract and only few reach the ampulla at the time of ovulation (Gualtieri and Talevi, 2003). During *in vitro* fertilization, this selection is much less extensive and although the protocols advanced a lot during the last decades (Bavister, 2002) they still bypass several steps of the natural sperm selection process (Gualtieri and Talevi, 2003).

*The environment of maturation, fertilization and early embryonic development;*

The second big difference is the environment in which maturation and fertilization of the oocyte and early embryonic development occurs. *In vivo*, the oocyte matures within the follicle, is fertilized in the oviduct in which also the embryo develops up to the 8- to 16-cell stage (Newcomb *et al.*, 1976; Hackett *et al.*, 1993), then the embryo is transported to the uterus in which further development occurs (Figure 2). The embryo itself and the oviduct/uterus produce many factors, such as growth factors and specific proteins, energy substrates, ions and vitamins that can affect the development of the embryo (Leese, 1988; Ellington, 1991; Gandolfi, 1994). Despite many efforts to improve culture media it still is not possible to completely mimic the environment of the follicle for maturation of the oocyte and the oviduct and uterus for fertilization and early embryonic development.

The consequences of these two major differences are twofold;

1) The morula/blastocyst yield is much lower after *in vitro* production compared to *in vivo* development. *In vivo*, after insemination of normally cyclic cows, approximately 85% of the ovulated oocytes will develop into a blastocyst (Asdell, 1964; Sreenan *et al.*, 2001). In contrast, in most ovum pick-up/*in vitro* production procedures performed in commercial practice, the developmental competence of the oocyte reaches only 10-30% (Merton *et al.*, 2003).
2) The final aim of assisted reproduction techniques is the production of healthy offspring. However, *in vitro* produced embryos do result in lower pregnancy rates, higher rates of abortion and a higher frequency of calves born with impaired health, known as “Large Offspring Syndrome” (Hasler *et al.*, 1995; Galli and Lazzari, 1996; Kruip and den Daas, 1997; van Wagtendonk-de Leeuw *et al.*, 2000; Farin *et al.*, 2001). Commonly reported abnormalities associated with LOS include increased calf birth weight, perinatal mortality, hydrops of the allantois and abortions. Van Wagtendonk-de Leeuw et al. (2000) reported that calves born after transfer of *in vitro*-produced embryos cultured in co-culture medium supplemented with fetal calf serum were on average 6% heavier at birth and the perinatal mortality was 2.2% higher compared to calves born after artificial insemination. Improvement of culture systems using Synthetic Oviductal Fluid (SOF) without supplementation of fetal calf serum diminished the number of aberrations but did not solve the problem completely. Calves born after transfer of embryos produced in SOF had a significantly lower birth weight compared to calves born after transfer of embryos produced in co-culture medium but the ease of calving was significantly more difficult than after artificial insemination.

Recent studies indicated that the quality of the oocyte is the main factor affecting, *in vitro*, the proportion of immature oocytes that develop to the blastocyst stage, but it still is less clear what the critical part is of the embryo production process, influencing the quality of the morulae and blastocysts. Clearly, any modification of the culture environment during any step of development from the oocyte until the blastocyst stage could have major effects on the quality of the developing embryo.

**Embryo quality**

Development of an oocyte into a blastocyst is a parameter for developmental competence of the oocyte but implies nothing with regards to the quality of the developed blastocyst. The ultimate test of the quality of a blastocyst is its ability to establish pregnancy and produce a healthy calf. For economical and logistic reasons this test is impractical and therefore alternative methods to assess blastocyst quality are used. The quality of bovine *in vitro*-produced embryos is impaired in comparison to that of the "golden standard", their *in vivo*-derived counterparts, according to several quality parameters such as morphology (Van Soom and de Kruif, 1992), cell number and inner cell mass number (Van Soom *et al.*, 1997), kinetics of development (Holm *et al.*, 2002), tolerance to cryopreservation (Enright *et al.*, 2000; Rizos *et al.*, 2002b), chromosomal abnormalities (Hyttel *et al.*, 2001; Viuff *et al.*, 2001), metabolism (Thompson, 1997; Khurana and Niemann, 2000), gene expression (Wrenzycki *et al.*, 1996; Eckert and Niemann, 1998; Lequarre *et al.*, 2001; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a; Lonergan *et al.*, 2003b) and apoptosis (for review Lonergan *et al.*, 2003a). Except for the quality parameters morphology and kinetics of development, all parameters are analyzed using invasive techniques and therefore no direct relations between these parameters and the ability of the morula/blastocyst to establish implantation and the emergence of LOS can be determined. Nevertheless, it has been hypothesized that higher implantation rates are related to embryos with high cell number and low degree of chromosomal abnormalities (Viuff *et al.*, 2001) as are fast development and good morphology (Hasler *et al.*, 1995). The morphology parameter also appears to be an indicator for LOS, i.e. increased birth weight. Merton et al. (1998) showed that calves born after transfer of *in vitro*-produced embryos of grade 2 according to criteria of Robertson and Nelson (1998) were 1.2 kg heavier then grade 1 embryos. Altered gene expression patterns in the embryo, especially IGF2R (Young *et al.*, 2001) and glucose transporter genes (Lazzari *et al.*, 2002) are also associated with the emergence of LOS.

The research in the present thesis, focuses in particular on two parameters of embryo quality, gene expression and apoptosis. Therefore, these are discussed more extensively in the following paragraphs.

*Gene expression*

In mice, approximately 15700 genes are expressed during preimplantation development (Stanton *et al.*, 2003) and, in cattle, this number will presumably be similar. A proper expression in a well-orchestrated manner of all developmentally important genes is essential for appropriate development of an embryo. Data for bovine embryos indicate that currently used *in vitro* conditions cannot fully mimic

the *in vivo* situation with regard to mRNA expression (Wrenzycki *et al.*, 1996; Eckert and Niemann, 1998; Lequarre *et al.*, 2001; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a; Lonergan *et al.*, 2003b). However, when a suboptimal environment of the embryo causes deviations in the expression of developmentally important genes, the embryos can demonstrate an enormous resiliency and can adapt to their environment (Niemann and Wrenzycki, 2000). It has been hypothesized that in the extreme, when the capacity for compensation is overloaded, development is arrested or that suboptimal conditions may result in abnormal early embryonic development probably resulting in for example “LOS”.

Deviant expression of mRNA transcripts between *in vitro*-cultured and *in vivo*-developed embryos can originate from two routes:

1. Change of the stability of mRNA
   Degradation of maternally derived mRNA occurs during the first cell divisions and at the time of the major genome activation most transcripts are vanished. Two transcript degradation pathways function in the early Drosophila embryo. The first ‘maternal’ pathway is driven by maternally encoded factors that are recruited by *cis*-acting RNA degradation elements independently of whether the transcript is translationally active or translationally repressed (Tadros *et al.*, 2003). The second ‘zygotic’ pathway becomes active 2 h after fertilization. The joint action of both pathways is necessary for elimination of maternally derived transcripts prior to MET (Bashirullah *et al.*, 1999). It could be hypothesized that similar pathways exist in bovine embryos and that the selection of oocytes during *in vitro* production from relatively small follicles could affect the degradation of mRNA by the maternal pathway. Furthermore, suboptimal culture conditions could affect the ’zygotic’ pathway and perturb the degradation of mRNA.
   Another cause of deviations in the stability of mRNA is suggested by the detection of significantly shortened poly(A) tails after *in vitro* maturation of the oocyte since the extent of the poly(A) tail at the 3’ end of the transcripts has emerged as an important regulatory element for determining their stability (Brevini-Gandolfi *et al.*, 1999).
   In conclusion, it can be hypothesized that suboptimal culture conditions during prematuration and maturation of the oocyte can perturb degradation of maternally derived mRNA whereas suboptimal conditions during *in vitro* culture, especially during the maternal embryonic transition, can disturb degradation of embryonically derived mRNA.

2. Change in the level of transcription of mRNA
   As mentioned earlier, in bovine, transcription of mRNA from the embryonic genome starts with a minor activation at the 2- to 4-cell stage

followed by a major genome activation at the 8- to 16-cell stage. Several causes for deviations in the level of transcription of the embryonic genome during *in vitro* production of embryos are suggested:

a. Suboptimal culture conditions such as nutritional imbalance and oxidative stress can cause up- or down-regulation of expression of developmentally important genes. It can be hypothesized that sub-optimal culture conditions have their effect especially at the time that the embryonic genome is activated.

b. Alterations of epigenetic modifications of embryonic expression patterns are supposed to be induced by *in vitro* production techniques. Epigenetics include gene-regulating activity that is not directly related to the DNA sequence itself but is primarily based on the methylation of the DNA sequence that, in mammals, is essential for the regulation of transcription during development and differentiation. Methylation has also long been known to be involved in imprinting, the process by which certain alleles are expressed or silenced depending on the parental sex from which they inherited (Sapienza, 1990). Aberrant expression patterns of imprinted genes have been found in mice and humans (Moore and Reik, 1996) and were related to specific phenotypes, e.g. Beckwith-Wiedemann syn-drome, a congenital overgrowth disorder in human (Reik *et al.*, 1995). This suggests that “LOS” could be caused by a change of the expression pattern of developmentally important genes in the early embryo, especially genes that are subject to imprinting (Eggenschwiler *et al.*, 1997; Niemann and Wrenzycki, 2000; Young and Fairburn, 2000).

c. Perturbation of inactivation of the X-chromosome can cause aberrant expression of X-Linked gene transcripts in *in vitro*-produced embryos (Wrenzycki *et al.*, 2002). In female embryos, one of the two X chromo-somes is inactivated during early embryogenesis to make X-linked gene dosages equivalent to the male embryos that possess a single X-chromosome (Lyon, 1961). Expression of the X inactive-specific transcript (Xist) is thought to be essential for the initiation of X chro-mosome inactivation during female embryo development. In the mouse, male and female embryos initially express Xist RNA. However, this expression is followed by a preferential reduction of Xist RNA in male embryos, indicating that dosage compensation for the X chromosome requires downregulation of Xist RNA in male embryos, in conjunction with the production of stable Xist transcripts in female embryos (Latham *et al.*, 2000). When this subtle regulation is

disturbed, a difference in the timing of locus inactivation on the X chromosome may occur, that can cause aberrant expression of X-Linked gene transcripts.

Many studies revealed differences in expression of developmentally important genes between *in vitro*- and *in vivo*-derived embryos (Wrenzycki *et al.*, 1996; Eckert and Niemann, 1998; Lequarre *et al.*, 2001; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a; Lonergan *et al.*, 2003b) and even minor differences in the *in vitro* culture systems can induce differential expression of gene transcripts (Wrenzycki *et al.*, 2001; Rizos *et al.*, 2002a).

*Apoptosis*

The term apoptosis was introduced by Kerr et al. (1972) to describe morphologically distinct features of programmed cell death including; cell shrinkage with cytoplasmic and organelle condensation, chromatin condensation, endogenous DNA-degradation and nuclear fragmentation, blebbing of the plasma membrane and cell fragmentation. This process differs from necrosis in the mechanism of induction. Necrosis is induced by lethal events of chemical, biological or physical origin while by contrast, apoptosis requires the co-ordination of gene-directed energy-dependent biological processes (Cotter *et al.*, 1990). This process of apoptosis, can be viewed as a cascade-like sequence of events (Figure 3).

The apoptosis cascade can be divided into three sets of sequential stages: *Initiation stages* include the induction of the cascade, e.g., by ligand-receptor interactions leading to first proteolytic events. *Execution stages* start with the activation of the execution caspases. Their activation is referred to as the ‘point of no return’ since, once activated these proteases degrade a variety of proteins resulting in irreversible damage of the cell. *Apoptotic death* is the result of a very complex cascade of events that finally leads to the collapse of the nucleus and the cell itself. Even in this final stage the cell does not release intracellular components, thus avoiding inflammatory reactions.

Two pathways, a receptor dependent and a receptor independent, can induce apoptosis. The receptor dependent pathway is activated by specific ligand-receptor interactions that lead to the formation of a signaling complex and subsequently activates a family of so-called initiator caspases. These are responsible for the first proteolytic events e.g. cleavage of cytoskeletal and related proteins including actin and fodrin (a membrane-associated cytoskeletal protein). These early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface. The flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is another early apoptotic event and is achieved by the activity of enzymes such as translocase.

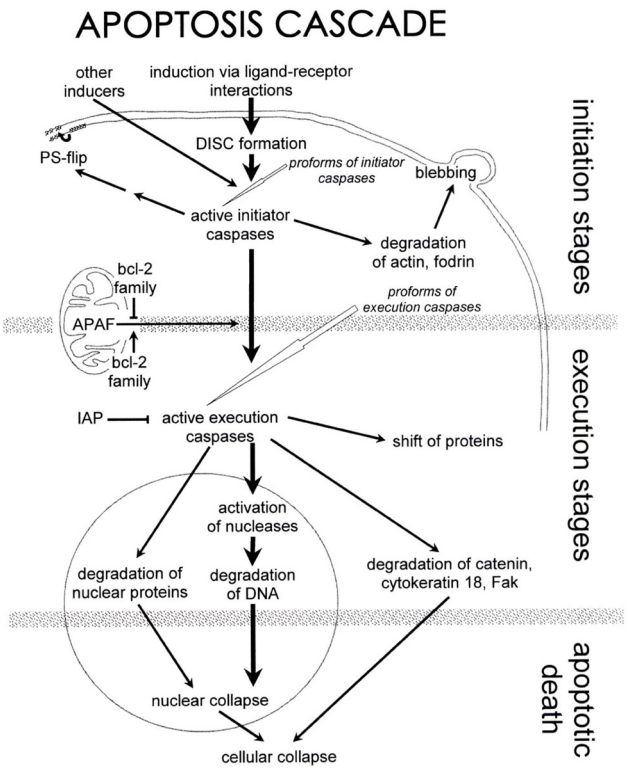



**Figure 3.** The apoptosis cascade in cells (adapted from Huppertz *et al.*, 1999). DISC = death-inducing signaling complex; PS-flip = phosphatidylserine flip; APAF = apoptotic protease activating factors; IAP = inhibitors of apoptosis proteins; fak = focal adhesion kinase; bcl-2 = b-cell lymphoma-2

Cells use this externalization of phosphatidylserine as a signal for events such as attraction of macrophages or induction of the coagulation cascade. Furthermore, the initiator caspases cleave and hereby activate a second subpopulation of caspases known as execution caspases. The second pathway is receptor independent and can be activated by cellular stress that directly induces pro-apoptotic bcl-2 family members (bad, bak, bax, bcl-xs, bid, bik, hrk, mtd) to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c that activates apoptotic protease activating factor (APAF) (Zimmermann *et al.*, 2001). The anti-apoptotic bcl-2 family members (A1, bcl-2, bcl-w, bcl-xl, bfl-1, brag-1, mcl-1, NR13) work to prevent cytochrome c release from the mitochondria. Once activated, the execution caspases either directly or

by means of other proteases, cleave a broad array of proteins critical for cell survival. Execution caspases activate DNA fragmentation factor and other endonucleases resulting in specific fragmentation of DNA (Liu *et al.*, 1997). Eventually, the apoptotic cell is phagocytized. Many other genes are associated with the apoptotic cascade under which the heat shock proteins. Especially Heat shock protein 70 serves as a potent anti-apoptotic protein by reducing or blocking caspase activation and suppresses mitochondrial damage (Buzzard *et al.*, 1998).

**Apoptosis in the early embryo**

Apoptosis is a widespread feature in early embryos of many mammals. In unarrested human embryos no evidence of apoptotic cell death with detectable DNA degradation was observed prior to compaction (Hardy, 1999), but signs of apoptosis have been observed at the 8-cell stage in unarrested *in vitro*-produced bovine embryos (Byrne *et al.*, 1999; Matwee *et al.*, 2001). However, in embryos arrested in development, displaying no mitotic activity within 24 h, characteristics of apoptosis can be observed at earlier stages (Jurisicova *et al.*, 1996); whether these characteristics are a cause or a result of embryonic arrest is still unclear (Antczak and Van Blerkom, 1999).

More than 80% of mouse blastocysts produced *in vivo* contain at least one apoptotic cell (Hardy, 1997) and the incidence is higher (10-20%) in the inner cell mass than among trophectoderm cells (<3%). The same distribution has been reported in other species, such as rat (Pampfer, 2000) and bovine (Matwee *et al.*, 2000), whereas a more equal distribution of 6-7% dead cells in both the inner cell mass and the trophectoderm is observed in *in vitro*-cultured human blastocysts (Hardy *et al.*, 1989). Apoptosis in preimplantation embryos has been hypothesized to serve a variety of physiological purposes such as;

1. Elimination of ICM cells which still have the potential to form trophectoderm to reduce the risk of inappropriate ectopic trophectoderm formation during germ layer differentiation (Handyside and Hunter, 1986; Pierce *et al.*, 1989).
2. Removal of cells with chromosomal and nuclear anomalies, such as a- or binucleation (Hardy, 1999) or polyploid cells (Viuff *et al.*, 1999; 2001).
3. Removal of cells/embryos that fail to activate the embryonic genome, suggested by co-incidental detection of DNA fragmentation at the time of embryonic genome activation (Jurisicova *et al.*, 1998; Byrne *et al.*, 1999; Matwee *et al.*, 2000; Betts and King, 2001).
4. In mouse and other species there seems to be an optimal plateau of number of inner cell mass cells to support further development.

Possibly, apoptosis serves to regulate cell number when mitotic activity is high (Tam, 1988; de la Fuente and King, 1997). This could be a possible explanation why the level of apoptosis is higher in the inner cell mass compared to the trophectoderm.

Nevertheless, a high incidence of apoptotic cells is assumed to be related to reduced embryo quality. Studies reported that human and bovine blastocysts with a high and variable incidence of DNA fragmentation have a reduced cell number (Hardy *et al.*, 1989; Byrne *et al.*, 1999). In mouse, a significant relationship between the total number of cells of a blastocyst with viability after transfer is observed (Lane and Gardner, 1997). Also, in human, the incidence of apoptosis appears to be correlated with embryo quality; the proportion of dead cells ranged from <10% in day 6 blastocysts of good morphology to 27% in those of poor morphology (Hardy et al., 1989). It is assumed that cell death beyond a certain threshold is detrimental for further embryo development (Jurisicova *et al.*, 1998; Hardy, 1999) and prevents non-viable offspring (Byrne *et al.*, 1999).

Hypothetically, several routes can trigger increased levels of apoptosis in *in vitro*-produced embryos:

1. Suboptimal maturation, fertilization and culture conditions can induce increase of chromosomal (Viuff *et al.*, 2001) and nuclear abnormalities which can trigger apoptosis. Furthermore, any cellular stress during *in vitro* culture such as nutritional imbalance, for example glucose uptake deficiency, and oxidative stress can lead to apoptosis (Leunda-Casi *et al.*, 2002).
2. Absence of maternally and/or embryo derived growth factors that can function as cell survival factors (Brison and Schultz, 1997; Lighten *et al.*, 1998; Spanos *et al.*, 2000).
3. Improper activation of the maternal embryonic genome by individual cells during *in vitro* culture can trigger the onset of apoptosis.

**Aim and design**

The main purpose of the research presented in the thesis was a) to improve our understanding of the effects of *in vitro* production vs. *in vivo* development of embryos on the quality of bovine blastocysts and b) to pinpoint during which part of development, from immature oocyte to blastocyst, the quality is affected.

The research focused on quality parameters such as, the level of expression of genes related to development, the total cell number and inner cell mass number, the blastocyst formation rate and the incidence of apoptosis. Two approaches were chosen to determine during which part of the developmental

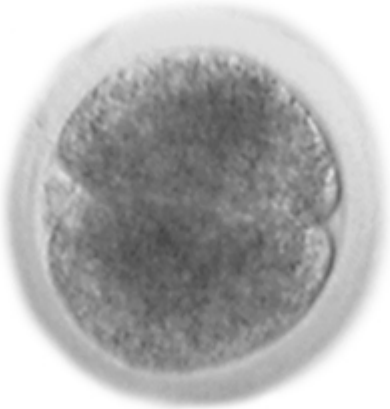
axis the quality of the embryos is affected. First, embryos were examined at different time points during early embryonic development *in vitro* vs. *in vivo*. Secondly, blastocysts were investigated after different routes of *in vitro* production vs. *in vivo* development, with special focus on 1) the effect of final maturation of the oocyte and 2) the effect of passing the period of MET, on quality of the blastocyst.

**Outline of the thesis**

**Chapter 1** presents the general introduction comprising the background and relationship of the processes involved in embryo development. In **Chapter 2**, the procedure is evaluated by which *in vivo* oocytes and embryos were collected for the research presented in the subsequent chapters of this thesis. The procedure encompasses superovulation with a fixed LH surge, which facilitates collection of oocytes and embryos at specific times of development. The relationships between changes of hormone concentrations, induced preovulatory follicles and yield of embryos in sufficient quantities for further research are investigated. In **Chapter 3**, the effect of different maturation regimens of the oocyte, *in vitro* vs. *in vivo*, on the level of expression of developmentally important gene transcripts in bovine blastocysts is studied. **Chapter 4** describes the consequences of culture completely *in vitro* vs. development entirely *in vivo* on the onset and level of morphological aspects of apoptosis in embryos from the 2-cell stage up to the blastocyst stage. **Chapter 5** reports on the effects of *in vivo* development until shortly before or after MET and subsequent culture *in vitro* until the time of blastocyst formation on cell number and inner cell mass number, blastocyst formation and on apoptosis in morulae and blastocysts. **Chapter 6** describes the consequences of various routes of *in vitro* and *in vivo* culture, with special focus on the period of MET, on the level of expression of gene transcripts involved in glucose metabolism and apoptosis in blastocysts. Finally, **Chapter 7** summarizes and discusses the findings with regards to the quality parameters studied in this thesis, in relation to implications for *in vitro* production of embryos and future research.

# Chapter 2

## Effects of superovulation with oFSH and norgestomet/GnRH-controlled release of the LH surge on hormone concentrations, and yield of oocytes and embryos at specific developmental stages



**Abstract**

The objective of this study was to evaluate a new superovulation procedure with oFSH after temporary suppression of the endogenous LH surge by norgestomet and followed by administration of GnRH, to collect bovine oocytes and embryos at specific developmental stages. Since 1999, our research group applies this superovulation procedure with postponement of the endogenous LH surge. To verify whether this procedure produces a sufficient number of oocytes and embryos at specific time points of development the procedure was validated regarding to hormonal characteristics, superovulatory response and both oocyte and embryo yield and quality at different times of *in vivo* development. The results demonstrate that the procedure used to postpone the occurrence of the preovulatory LH surge was effective in the majority of animals and therefore the oocytes and embryos were collected at the intended stage of development. The superovulatory response and both oocyte, embryo yield and quality were similar to the average yield in Europe reported by AETE. In conclusion, this super-ovulation procedure provides a valid tool to collect oocytes and embryos at specific stages of development.

**Authors:** Hiemke M. Knijn, Peter L.A.M. Vos, Wilma Fokker, Bert C. van der Weijden, Steph J. Dieleman
*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*

Submitted for publication

## Introduction

To study deviations in maturation of oocytes and in early embryonic development during *in vitro* culture, comparisons with *in vivo* derived oocytes and embryos at specific time points of development are a prerequisite. Although the single oocyte or embryo from untreated normally cyclic cows would be the “golden standard”, in general, superovulation methods are used to obtain larger numbers. However, superovulation procedures of cows result in large differences in superovulatory response (Monniaux *et al.*, 1983; Guilbault *et al.*, 1991; Kafi and McGowan, 1997), ovulation rate and embryo yield between individual cows and different superovulation protocols (Greve *et al.*, 1995; Kanitz *et al.*, 2002; Mapletoft *et al.*, 2002). Furthermore, the morula/blastocyst yield and quality is depending on many factors such as age of donor, genetic background, breed, drug used and many other factors (Hasler *et al.*, 1983; Lerner *et al.*, 1986; Barros and Nogueira, 2001). However, the most complicationg aspect of using superovulation treatment for collecting oocytes and embryos at specific time points during development, is the wide variation in the time of occurrence of the LH surge after prostaglandin (PG) administration. During superovulation the period between PG administration and occurrence of the LH surge varied between 26 and 54 h according to Callesen et al. (1988), and between 33 and 49 h according to Dieleman and Bevers (1987). In contrast, the period between luteolysis and the LH surge is on average 61 h during a normal estrous cycle (Dieleman *et al.*, 1986). Consequently, variations in time of start of multiple ovulations between individual cows and different superovulation procedures have been reported (Bevers and Dieleman, 1987; Callesen *et al.*, 1988; Wubishet *et al.*, 1991; Laurincik *et al.*, 1993). A rapid radioimmunoassay for LH analysis has been developed to determine the time of occurrence of the LH surge in individual cows which allows prediction of the onset of multiple ovulations (Dieleman and Bevers, 1987). Although this procedure facilitates collection of oocytes and embryos at specific developmental stages, it does not overcome the wide variation in timing of the start of ovulations. Application, therefore, of this procedure to experiments with larger numbers of cows appeared to be less feasible in particular when oocytes and embryos are collected for immediate elaborate experimentation.

To solve these problems, superovulation procedures have to be used in which the period between PG administration and the LH surge is fixed for example by inhibiting the endogenous LH surge and induction of an LH surge at a predetermined time resulting in accurate timing of the start of ovulations.

Three different methods to induce an LH surge at a fixed time during superovulation have been reported. First, suppression of the endogenous LH surge was carried out using a GnRH antagonist followed by induction of ovulation with an exogenous source (hCG) (Rieger *et al.*, 1989). Second, release of endogenous LH was inhibited by continuous GnRH agonist treatment causing the

anterior pituitary gland to become desensitized to endogenous and exogenous GnRH, and administration of LH at a fixed time produced an LH surge (D'Occhio *et al.*, 1997). Third, inhibition of the endogenous LH surge by using a progestagen block has been reported by our group (Vos *et al.*, 1994; van de Leemput *et al.*, 2001). The block due to a progesterone device or norgestomet implant, suppresses the release of endogenous LH from the pituitary gland. To time the release of the preovulatory LH surge, GnRH was administered at a fixed time, initiating final maturation and multiple ovulations. In superovulation procedures with an LH surge at a fixed time, the PG-LH surge interval in general is prolonged and approximates the duration of the corresponding period in normally cyclic cows. Such procedures are supposed to diminish individual cow variability by reducing inter-follicular heterogeneity and to give more follicles enough time to acquire all features necessary to respond to the LH signal (Vos *et al.*, 1994). With respect to the pulsatile secretion of LH and FSH, however, the mentioned methods to suppress the release of the LH surge have different effects. The pulsatile secretion of LH which is supposed to be required for accurate prematuration of the oocyte (Greve *et al.*, 1995), is already reduced during superovulation treatment (Bevers *et al.*, 1989; Ben Jebara *et al.*, 1994; Price *et al.*, 1999) but disappears completely during superovulation combined with GnRH agonist treatment (D'Occhio *et al.*, 1989; Gong *et al.*, 1995; 1996). In contrast, norgestomet treatment did not influence the pattern of LH secretion when implanted during the luteal phase of a normal cycle, and during the follicular phase the LH pulse frequency increased and the amplitude decreased (Taylor *et al.*, 1993). Furthermore, when animals received a 6 mg norgestomet implant and luteolysis was induced by PG administration, the profiles of LH secretion were similar to those detected during the follicular phase of a normal estrous cycle (Sanchez *et al.*, 1995).

Although follicular development is influenced by chronic treatment with GnRH agonist (Gong *et al.*, 1996), d'Occhio et al. (2000) reported apparently normal follicular growth and ovulation when combined with FSH superstimulation. In animals treated with a progesterone block the follicular function with regards to estradiol secretion and potential to ovulate remained unharmed (Vos *et al.*, 1994).

Conflicting results were reported on the embryo yield after superovulation treatments with prolongation of the preovulatory period. Rieger et al. (1990) showed that when the LH surge was delayed to 72 h after PG a higher proportion of transferable embryos were derived compared to a control group were no prolongation was performed. D' Occhio et al. (1997) used a GnRH agonist to induce ovulation with exogenous LH and obtained a similar number of transferable embryos when the LH surge was delayed for 12 h compared to the control group, and the number of transferable embryos decreased significantly after delaying ovulation for 24 h. Earlier studies using eCG for superovulation with a progesterone block showed that this results in a higher superovulatory

response and more ovulations (Vos *et al.*, 1994) but not more embryos were collected, possibly due to an impaired oviductal environment (van de Leemput *et al.*, 2001).

Since 1999 our research group applies a superovulation procedure comprising ovine FSH, with a prolonged preovulatory period using a norgestomet implant and a fixed LH surge induced by GnRH, to collect oocytes and embryos at specific stages of development for further research. The objective of this study is to verify if this procedure produces a sufficient number of both oocytes and embryos of good quality and if it is reliable for collection of oocytes and embryos at specific time points of development. This procedure was validated regarding to hormonal characteristics, superovulatory response and both oocyte and embryo yield and quality at different times of *in vivo* development.

## Materials and methods

*Experimental design*

This study comprises data collected during various experiments that have been performed between September 1999 and November 2002. The superovulation procedure was performed 238 times (number of animals = 221). Briefly, the method uses oFSH with temporary suppression of the endogenous release of the LH surge by norgestomet and induction of the LH surge by GnRH (Figure 1). Most animals were used only once, 14 animals two times and three animals three times, each time with at least a two months interval. To validate this superovulation procedure, characteristics of hormonal patterns (LH, estradiol-17ß and progesterone concentrations), superovulatory response and both oocyte and embryo yields and quality, at different times after the LH surge, were analyzed. Since the aim was different for the various experiments, not all parameters were observed in all animals.

*Animals and treatment*

Clinically healthy, non-lactating Holstein-Friesian cattle (n = 221) were selected from the experimental herd of the Veterinary Faculty of the Utrecht University based on cyclicity as established by measuring progesterone levels in peripheral blood samples taken three times a week during at least four weeks before the experiments started. The animals were fed silage and concentrate and supplied water ad libitum. The experiments were carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

The animals were synchronized using an ear implant for 9 days (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) and the single administration of 3 mg norgestomet and 5 mg estradiol-valerate im. The implant was removed after 9 days, and prostaglandin (PG, 15 mg Prosolvin

im; Intervet International BV) was administered 2 days before to ensure complete regression of the corpus luteum. On Day 8 of the synchronized cycle (estrus = Day 0) all follicles larger than 5 mm were ablated by transvaginal ultrasound guided puncturing to prevent the presence of a dominant follicle at the start of superstimulation and to induce a new follicular wave. On Day 9, that is between 0 and 12 h before the start of FSH administration, again an ear implant (3 mg norgestomet, Crestar) was inserted for 5 days but without the additional administration of norgestomet and estradiol-valerate. From Day 10 onwards, oFSH (Ovagen, ICP, Auckland, New Zealand) was administered im, twice daily with decreasing doses during 4 days. Specific doses were given to heifers or cows: the first day 2.0 or 3.5 mL, the second day 1.5 or 2.5 mL, the third day 1.0 or 1.5 mL and the fourth day 0.5 or 1.0 mL (in total 10 or 17 mL equivalent to 176 or 299 IU NIH-FSH-S1), respectively. Prostaglandin (22.5 mg PG, im) was administered concomitant with the fifth dose of FSH, and the ear implants were removed 2 days after PG, and then GnRH (1.0 mg gonadoreline im, Fertagyl or 0.02 mg busereline-acetaat im, Receptal, Intervet International BV) was administered to induce an LH surge.

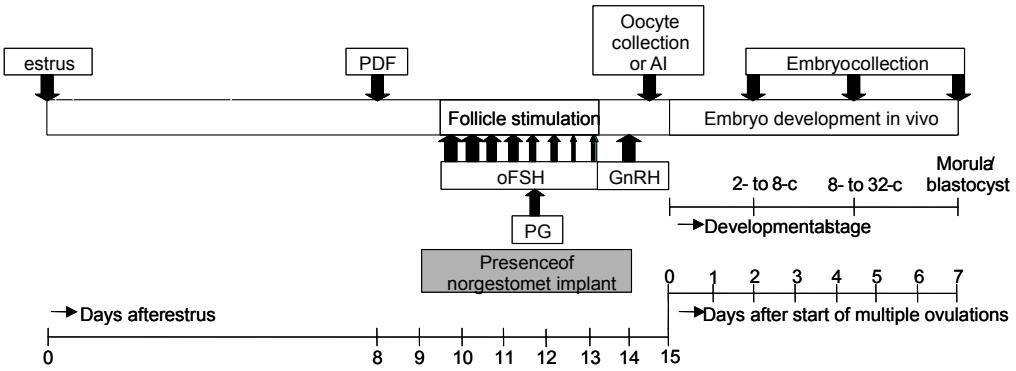



**Figure 1.** Schedule of treatment for superovulation with a fixed LH surge to obtain oocytes and embryos at specific times of *in vivo* development. PDF = Puncture dominant follicle; PG = administration of prostaglandin; oFSH = administration of 8 consecutive, decreasing doses of ovine follicle stimulating hormone; GnRH = administration of gonadotropin releasing hormone; AI = artificial insemination

*Blood samples*

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 4 hours 24 h before GnRH administration and thereafter every hour for 6 h. After immediate centrifugation at 4°C, plasma was stored at -25°C for hormone analysis.

*Collection of oocytes*

In 71 animals oocytes were collected from all follicles sized > 8 mm. Ovariectomy was performed in 21 animals by laparatomy through flank incision under local infiltration anesthesia. Ovaries were collected in 0.9% NaCl at 37°C and immediately transported to the laboratory and oocytes were obtained by aspiration of the follicles (OVX-group). Ovum pick-up was performed in 50 animals in which oocytes were collected from follicles using an ultrasound scanner (Pie Medical 100 Vet, Maastricht, The Netherlands with a 7.5 MHz sector probe and disposable 18 gauge needles) just before the LH surge or 24 h after the GnRH induced LH surge (OPU-group).

All oocytes were retrieved using an embryo recovery filter (Embryo Concentrator, Immuno Systems Inc., Spring Valley, WI) and were assessed on morphological criteria by stereomicroscopy. Oocytes were classified as good when they possessed at least 3 layers of compact cumulus cells at collection just before the LH surge or an expanded cumulus-cell mass at collection 24 h after the LH surge.

*Collection of embryos*

For embryo collection at specific time points, 167 animals were artificially inseminated 10 to 12 hours after the induced LH surge with semen from bulls of proven fertility. In 99 animals, at different times after the expected LH surge, embryos were collected ex vivo after slaughter at 72 h for the 8-cell group (n=37), 126 h for the 32-cell group (n=26) and 190 h for the blastocyst group (n=36), respectively. After slaughter, genital tracts were placed in saline (37°C) in a thermocontainer and transported immediately to the laboratory. The time period between slaughter and flushing ranged from 45 min to 2 h. Oviducts and uterine horns were flushed with PBS (PBS-ET; Bio Whitaker Europe, Verviers, Belgium) of 37°C using a blunt needle, from the infundibulum towards the uterine horn under gentle massage. The PBS was collected into an embryo recovery filter and the embryos were collected by rinsing the filter with saline supplemented with 0.005 % (w/v) BSA (A 6003; Sigma, St Louis, MO). The developmental stage of the embryonic structures was assessed by stereomicroscopy. At 72 h after the LH surge, the 1-cell and degenerated embryos, and at 126 h in addition the embryos at the 2- to 7- cell stage were characterized as non-viable. Moreover, at 190 h after the LH surge the embryonic structures that had not attained the morula or blastocyst stage were considered to be non-viable.

*Superovulation Response*

To evaluate the response to superovulation in animals in which oocytes were collected, the number of preovulatory follicles (i.e., follicles larger than 8 mm) present at the time of OVX or OPU was counted and in animals in which embryos

were collected, the number of corpora lutea on the ovaries were counted at the time of collection of embryos.

*Radioimmunoassays for progesterone, estradiol-17ß and LH*
Concentrations of progesterone and estradiol-17ß in plasma were estimated by a solid-phase $^{125}$I RIA method (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer as validated by Dieleman and Bevers (1987). The sensitivities were 0.05 µg/L and 2 ng/L, and the interassay coefficients of variation were 11% and 8.9%, respectively.

Concentrations of LH in plasma were estimated by a validated homologous RIA method as described earlier (Dieleman *et al.*, 1983a). The sensitivity was 0.4 µg/ L NIH-LH-B4. The intra- and interassay coefficients of variation were < 9%.

*Statistical analysis*
All values are expressed as mean ± SD unless indicated otherwise. Pearson correlation coefficients were calculated between the number of preovulatory follicles, corpora lutea and viable embryos in the blastocyst group and estradiol-17ß concentration and between the recovery rate of embryonic structures and the number of corpora lutea. Differences of $P < 0.05$ were considered significant.

## Results

*Characteristics of hormonal patterns during and after superovulation*

Progesterone
In all animals in which progesterone was analyzed (n=212) values decreased to <1.0 µg/L within 24 h after PG administration, indicating that luteal regression had occurred.

LH
The interval between PG administration and maximum of the LH surge was 54.2 ± 3.6 h (n=238). The interval between GnRH administration and maximum of the LH surge was 2.2 ± 0.5 h (n=238) and the amplitude of the LH surge was 26.5 ± 9.6 µg/L (n=238) (Figure 2A).

In 185 animals, LH concentrations were analyzed every four hours at least 20 h before GnRH administration. In the majority of the treatments (91.8%) LH concentrations were at a basal level of on average 0.9 ± 0.1 µg/L to reach a maximum concentration 2 hours after GnRH administration and return to basal levels, 2.1 ± 0.1 µg/L, 6 hours after GnRH. In 15 animals a deviated LH surge

was observed. Three different types of deviations (Figure 2B) could be distinguished;

Type 1; an increase of LH just before or just after GnRH administration (n=7)

Type 2; an increasing LH concentration starting 10h before GnRH administration (n=6)

Type 3; a LH peak before the GnRH administration occurred (n=2)

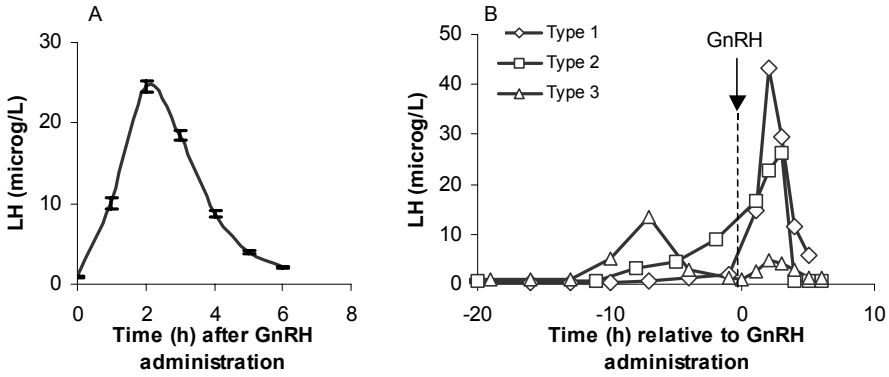



**Figure 2.**

A. Concentrations (mean ± sem, n=238) of LH (µg/L) in peripheral blood of animals treated for superovulation with oFSH with a norgestomet/GnRH postponed LH surge relative to the time of GnRH administration.

B. Examples of different types of deviated LH profiles in peripheral blood of animals superovulated with oFSH with a norgestomet/GnRH postponed LH surge relative to the time of GnRH administration.

Estradiol-17ß

The estradiol-17ß concentration at the onset of the superovulation treatment with oFSH was 3.7 ± 1.6 ng/L (n = 195) and increased to 35.2 ± 30.7 ng/L (n=217) at the time of the maximum LH concentration. At 22 to 24 after the maximum of the LH surge, the estradiol-17ß concentration was decreased to 7.3 ± 2.6 ng/L (n = 70).

*Superovulation Response*

The superovulatory response measured as number of follicles calculated at the time of puncturing of the follicles after OVX or during OPU was 21.4 ± 13.7 (n = 21) and 23.9 ± 12.1 (n = 50), respectively. A significant correlation between maximum of the estradiol-17ß concentration and the number of preovulatory follicles (r=0.60, $P < 0.05$, n = 70) was observed (Figure 3A).

In the animals that were slaughtered before embryo collection by uterine flushing, the superovulatory response was calculated according to number of

corpora lutea at the time of flush, 17.4 ± 11.7 (n = 167). A significant correlation between maximum of the estradiol-17ß concentration and the number of corpora lutea (r =0.75, P < 0.01, n=147) was observed (Figure 3B).

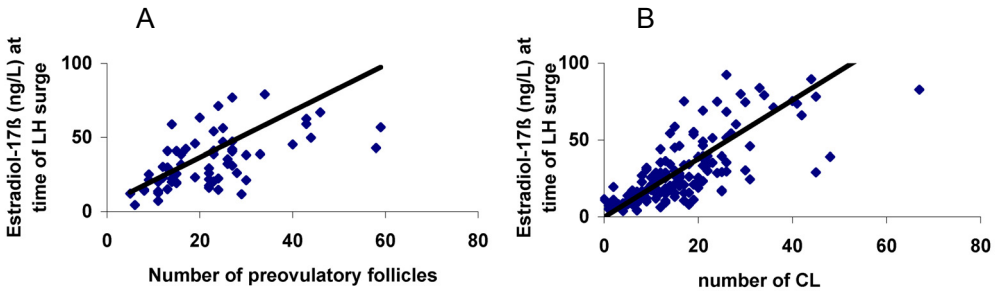



**Figure 3.**

A. Correlation between the estradiol-17ß concentration (ng/L) at the time of the LH surge and number of preovulatory follicles in animals treated for superovulation using oFSH with a norgestomet/GnRH postponed LH surge.

B. Correlation between the estradiol-17ß concentration (ng/L) at the time of the LH surge and number of corpora lutea in animals treated for superovulation using oFSH with a norgestomet/GnRH postponed LH surge.

*Oocyte and embryo, yield and quality*

In the OVX-group and OPU-group 19.5 ± 12.9 (n = 21) and 14.8 ± 6.9 (n = 50) oocytes per cow were collected, respectively. The recovery rate calculated as the number of oocytes collected relative to the number of preovulatory follicles was higher in the OVX-group (87%) than in the OPU-group (62%). The quality of the oocytes in the OVX-group and OPU-group were similar, 84% and 85% of the collected oocytes were characterized as good oocytes, respectively.

No significant differences were observed in the recovery rate of embryos after flushing of the genital tract, calculated as number of collected embryonic structures relative to the number of CL, between the different times of collection, 76%, 65% and 70% in the 8-cell-, 32-cell- and blastocyst groups respectively. The recovery rate of embryonic structures was negatively correlated with the number of CL (r=-0.17, P<0.05, n= 167 animals) (Figure 4).

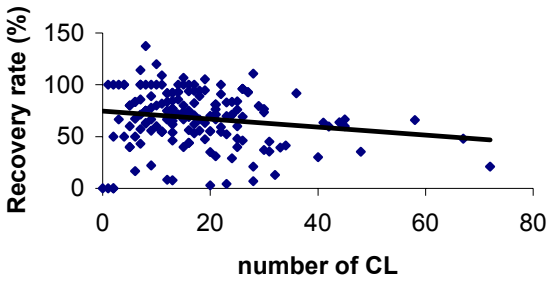



**Figure 4.** Correlation between recovery rate of embryonic structures calculated as number of collected embryonic structures relative to number of CL in animals superovulated with oFSH with a norgestomet/GnRH postponed LH surge.

The number of collected oocytes in the OPU and OVX groups combined was 16.2 ± 9.3 oocytes (n = 71) and the number of embryonic structures collected after flushing was 10.9 ± 7.4 (n = 37), 11.9 + 9.3 (n = 26) and 10.9 + 5.6 (n = 36) for the, 8-cell-, 32-cell- and blastocyst groups, respectively. In the combined OPU and OVX group, 84% of the collected oocytes were characterized as good oocytes and 78%, 51% and 65% of the collected embryonic structures were characterized as viable in the 8-cell, 32-cell- and blastocyst groups, respectively (Figure 5).

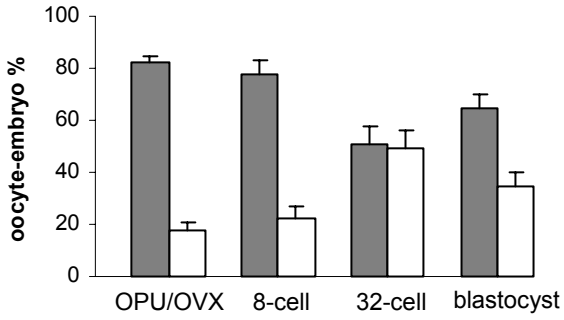



**Figure 5.** Percentage (± sem) of oocytes in combined OPU- and OVX group and number of embryos in the 8-cell-, 32-cell- and blastocyst groups of good/viable (dark grey bars) and bad/non-viable (open bars) quality, collected from animals treated for superovulation using oFSH with a norgestomet/GnRH postponed LH surge.

No correlation was observed between maximum of the estradiol-17ß concentration and the number of viable embryos collected in the blastocyst group (r = 0.24, n = 36).

*Individual variation in cow response*

Over 80% of the animals showed a normal superovulatory response, i.e. between 5 to 30 follicles sized > 8 mm or 5 to 30 corpora lutea per cow. The reponse on the superovulation treatment was poor in a small proportion of the animals (<4%), that is a response less than 5 follicles sized > 8 mm or less than 5 corpora lutea (Figure. 6).

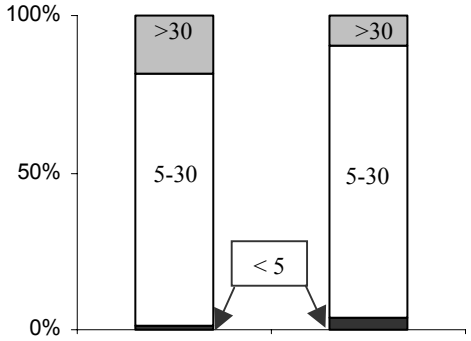



**Figure 6.** Distribution of animals treated for superovulation using oFSH with a norgestomet/GnRH postponed LH surge with low (<5), normal (5 to 30) and high (>30) number of follicles sized > 8 mm or corpora lutea.

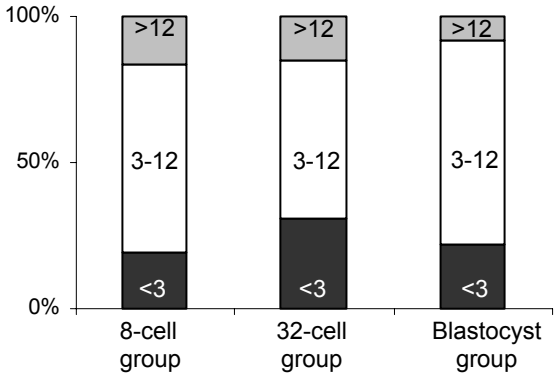



**Figure 7.** Distribution of animals with low (< 3), normal (3 to 12), and high (> 12) number of viable embryos in the 8-cell-, 32-cell- and blastocyst group, collected after treatment for superovulation using oFSH with a norgestomet/GnRH postponed LH surge.

At the time of embryo collection, 65%, 54% and 69% of the animals, in the 8-cell-, 32-cell- and blastocyst group, respectively, had a normal viable embryo yield i.e. between 3 to 12 embryos. In 18.9%, 30.8% and 22.2% in the 8-cell-, 32-

cell and blastocyst group, respectively, less than 3 viable embryos were collected and even in 11%, 23% and 8%, no viable embryos were collected (Figure 7).

## Discussion

The procedure used to postpone the occurrence of the preovulatory LH surge was effective in the majority of animals. In 91.8% of the animals treated for superovulation using oFSH with a norgestomet postponed, GnRH induced LH surge, a single LH surge was observed with a maximum at 2.2 ± 0.5 h after GnRH administration, suggesting that the observed LH surge was exclusively due to pituitary response to GnRH. Only in two cases the release of the LH surge was not prevented during norgestomet treatment and a clear LH surge occurred before GnRH administration. This probably would have caused, in less than 1% of the animals, start of multiple ovulations to occur at an earlier time than expected, and consequently oocytes or embryos would have been collected at a different stage of development than intended. The average maximum level of LH is similar to previous observations from our group (Bevers and Dieleman, 1987; van de Leemput *et al.*, 2001) using a eCG/anti-eCG superovulation protocol with norgestomet postponement of the LH surge and induction of the LH surge with GnRH, but the maximum was higher compared to a spontaneous LH surge (Bevers and Dieleman, 1987; van de Leemput *et al.*, 2001) during superovulation. The effect of the superovulation protocol evaluated in the present study was not examined with regards to the pulsatile secretion of LH and needs to be elucidated.

The follicular function appeared to be unaffected by the norgestomet implant, since the estradiol-17ß concentration being low at the time of start of the superovulation protocol reached a maximum level concurrent with the maximum of the LH surge. The preovulatory follicles apparently acquired the necessary characteristics to respond to the induced LH surge, as indicated by the decline in estradiol-17ß concentration after the maximum of the preovulatory LH surge. The observed maximum estradiol-17ß concentration of 35.2 ± 30.7 ng/L is in agreement with studies using a superovulation protocol with a spontaneous LH surge where values ranged from 30-80 ng/L (Lafri *et al.*, 2002). However, in a previous study of our group (van de Leemput *et al.*, 2001) using eCG in stead of FSH, the mean maximum estradiol-17ß concentration was 110 ng/L in animals with a spontaneous LH surge and 145 ng/L in animals with a norgestomet postponed, GnRH induced LH surge. Our results confirm the hypothesis that during superovulation with FSH preparations, which are low in luteinizing hormone bioactivity, the concentration of estradiol-17β in plasma is lower compared to superovulation with eCG (Ben Jebara *et al.*, 1994; Takagi *et al.*, 2001).

In this study, the superovulatory response, measured by counting the number of follicles sized > 8 mm or of corpora lutea at day 2 to 7 after ovulation, is in accordance with earlier observations for superovulation with eCG with a norgestomet postponed, GnRH induced LH surge (van de Leemput *et al.*, 2001) and for superovulation with FSH with a spontaneous LH surge (Gradela *et al.*, 1996). In the animals that were used for the collection of embryos the superovulatory response was lower compared to the superovulatory response in the OVX- and OPU groups, indicating that not all follicles would have ovulated in the latter group. It could be speculated that the oocytes characterized as bad were collected from follicles that would not have ovulated. In accordance with earlier studies, the maximum estradiol-17ß concentration was positively correlated with the superovulatory response i.e. the number of preovulatory follicles (Bevers and Dieleman, 1987), and with the number of corpora lutea but not with the number of viable embryos collected at 190 h po, indicating that estradiol-17ß is correlated to the superovulatory response but is not coherent with viable embryo yield.

The oocyte yield and quality of the collected oocytes was similar to earlier studies using eCG with a norgestomet postponed, GnRH induced LH surge (Dieleman and Bevers, 1987; van de Leemput *et al.*, 2001). No negative effects of eCG superovulation with a norgestomet postponed, GnRH induced LH surge on the potential of the oocytes to develop into a blastocyst had been observed (van de Leemput *et al.*, 2001).

The recovery rate of embryonic structures was correlated with the number of corpora lutea showing that overstimulation of follicular activity has negative effects on embryo recovery. Similar yields of embryonic structures were collected at the three different times of embryonic development. The ratio between viable and non-viable was higher when embryos were collected at an early embryonic stage, 8-cell stage compared to later stages, 32-cell and blastocysts. This is not surprising because embryos that are not able to activate the embryonic genome will not develop further then the 8- to 16-cell stage. Earlier studies reported a decrease in embryo yield after superovulation of cows with eCG together with norgestomet to postpone the LH surge, possibly caused by an impaired oviductal environment (van de Leemput *et al.*, 2001). In the present study no negative effects were observed. The morulae/blastocyst yield at Day 7 of *in vivo* development, 190 h after the LH surge, was similar to the average yield in Europe reported by the AETE, 5.6 transferable embryos per cow (AETE, 2002). No transfer of embryos to donor cows has been performed in this study so therefore the capacity of the embryos, collected after superovulation treatment as described in this study, to establish pregnancies is not known. Some indications that the quality of the embryos collected from cows superovulated using FSH with postponement of the LH surge by progesterone or a GnRH-agonist, with induction of LH, was not declined, were reported by Gouveia Nogueira et al

(2002). In their study the pregnancy rate after transfer of these embryos was similar compared to embryos obtained from cows treated for superovulation with a spontaneous LH surge.

A large variation in individual cow response was observed in this study. Although more than 96% of the animals responded on the superovulation treatment with more than 5 preovulatory follicles or corpora lutea in 11%, 23% and 8% of the animals in the 8-cell-, 32-cell- and blastocyst group respectively, no viable embryos were collected.

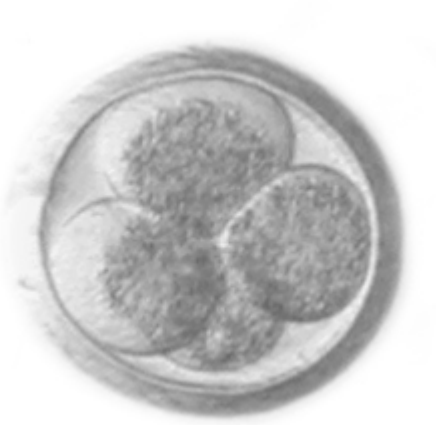
In conclusion, our superovulation protocol using oFSH with a norgestomet postponed, GnRH induced LH surge provides a valid tool to collect oocytes and embryos at specific stages of development. However this protocol does not substantially improve the viable embryo yield at Day 7 and is no solution for the differences in embryo yield between individual cows.

**Acknowledgments**

The authors thank Thea Blankenstein and Christine Oei for performing all radio immuno assay hormone analysis and Elly Zeinstra and Henk Heuveling for excellent technical assistance. We thank the animal handlers for their expert managing of the animals.

# Chapter 3

## Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*



**Abstract**

Bovine embryos produced *in vitro* differ substantially from embryos produced *in vivo* in the mRNA expression patterns of genes important for development. Several factors in the *in vitro* production systems have profound effects on embryonic mRNA expression patterns. The effects of the type of maturation on the expression pattern of genes important for development in blastocysts produced *in vitro* have not yet been investigated. The aim of the present study was to investigate the effects of various maturational protocols on the relative abundance of a panel of six marker genes, indicative of compaction and cavitation, metabolism, stress susceptibility and RNA processing, in bovine blastocysts produced *in vitro*. Four groups of blastocysts were analysed by a sensitive semi-quantitative RT-PCR assay. Blastocysts were produced *in vitro* from oocytes of different origin from: 1) 3-8 mm follicles; 2) preovulatory follicles before the LH surge; and 3) preovulatory follicles 24 h after the LH surge. The first two groups were matured *in vitro*, whereas the third group had undergone maturation *in vivo*. A fourth group comprised blastocysts developed entirely *in vivo*. Expression of glucose transporter-1 was significantly ($p<0.05$) higher, and expression of desmocollin-2 and plakophilin tended to be higher ($p<0.1$) for *in vivo* (group 4) compared with *in vitro* blastocysts (group 1), whereas no differences were found for heat shock protein 70.1, E-cadherin and poly(A) polymerase. Expression of the six transcripts did not differ among blastocysts produced *in vitro* from oocytes of groups 1, 2 and 3. Results indicate that alterations in the relative abundance of these transcripts in blastocysts produced *in vitro* cannot primarily be attributed to the origin of the oocyte, but are likely to have been induced by post-maturation or fertilization culture conditions.

**Authors:** Hiemke M. Knijn[1], Christine Wrenzycki[2], Peter J.M. Hendriksen[1], Peter L.A.M. Vos[1], Doris Herrmann[2], Bert C. van der Weijden[1], Heiner Niemann[2] and Steph J. Dieleman[1]

[1]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*

[2]*Department of Biotechnology, Institute for Animal Science (FAL), Neustadt, Germany*

Reproduction (2002) 124, 365-375

## Introduction

In cyclic cows, embryos develop after fertilization of oocytes that originate from an ovulatory follicle. These follicles have undergone the processes of selection, growth and dominance until estrus, when the LH surge initiates the final maturation approximately 24 h before ovulation (Dieleman et al., 1983b; Fortune, 1994). In contrast, for production (IVP) of viable embryos *in vitro,* oocytes are used that differ from oocytes originating from ovulatory follicles *in vivo* in that they are: 1) derived from immature, subordinate follicles of 2-8 mm in diameter, and 2) matured *in vitro*, outside the follicle. Oocytes from small follicles (<2-3 mm) are less likely to develop *in vitro* into blastocysts than oocytes from larger (>3 mm) follicles (Pavlok et al., 1992; Blondin et al., 1997; Van de Leemput et al., 1999). A comparative analysis of the ultrastructure of oocytes derived from dominant, preovulatory follicles with that of oocytes from subordinate, small follicles revealed first signs of maturation (referred to as 'capacitation' or 'prematuration') in the dominant follicle before the LH surge (Assey et al., 1994; Hyttel et al., 1997). Bovine oocytes contain large but changing populations of mRNAs and proteins. The oocyte supplies mRNAs that sustain embryonic development up to the stage of maternal-embryonic transition, and a few mRNAs persist throughout development until the blastocyst stage (Memili and First, 1999). In particular, the oocyte contains transcription factors involved in the minor and major activation of the embryonic genome at the 2- to 4-cell stage and 8-cell stage, respectively (Viuff et al., 1996). So, within the mammalian oocyte, genes have to be properly imprinted; furthermore, oocyte-derived factors are important for changes in methylation and for recognition of imprinted genes that do not need to be demethylated (Young and Fairburn, 2000). The mRNA contents of the oocyte are related to the developmental competence of the zygote after fertilization (Lequarre et al., 1997). Furthermore, the length of the poly-A tail of RNA in bovine oocytes was to some extent correlated with the quality of the resulting blastocyst (Brevini-Gandolfi et al., 1999). The amounts of mRNA encoding for Na/K-ATPase, Cu-Zn superoxide dismutase (SOD), basic fibroblast growth factor (bFGF), cyclin A and B in bovine oocytes are affected by the maturation medium (Watson et al., 2000). The intrinsic quality of the oocyte is a key factor in determining blastocyst yields (Sirard and Blondin, 1996; Rizos et al., 2002). These findings indicate that prematuration and maturation of the oocyte affect patterns of gene expression at the blastocyst stage.

Various differences have been described between embryos derived *in vitro* and *in vivo* (Holm and Callesen, 1998; Niemann and Wrenzycki, 2000) including morphology (Van Soom and de Kruif, 1992; Van Soom et al., 1997a), number and allocation of cells (Van Soom et al., 1996, Viuff et al., 2001), frequency of apoptosis and mixoploidy (Gjørret et al., 2001; Viuff et al., 1999; 2001), tolerance

to cryopreservation (Niemann, 1995; Enright et al., 2000), embryonic metabolism (Khurana and Niemann, 2000) and expression profile of specific mRNAs (Wrenzycki et al., 1996; 2001a; Eckert and Niemann, 1998; Lequarre et al., 2001). The relative abundance of several gene transcripts thought to be critically involved in preimplantation development is affected by the choice of basic culture medium and the type of protein supplement (Wrenzycki et al., 1999, 2001a). The effects of different maturational protocols on the relative abundance of genes important in development in blastocysts derived *in vitro* have not yet been investigated.

The aim of the present study was to determine the effects of prematuration-maturation (*in vitro* versus *in vivo*) of the oocyte on the relative abundance of a panel of six 'marker' genes important in development: glucosetransporter-1 (Glut-1), desmocollin-2 (Dc-2), E-cadherin (E-cad), plakophilin (plako), heat shock protein 70.1 (Hsp 70.1) and poly (A) polymerase (poly A). These genes were selected because they are indicative of various mechanisms in preimplantation bovine development *in vitro*. Plako and Dc-2 are of embryonic origin, whereas the other genes are of maternal and embryonic origin. All six transcripts are sensitive markers for compaction and cavitation, metabolism, RNA-processing and stress susceptibility, and thereby indicate the quality of blastocysts (Wrenzycki et al., 1999; 2001a). The effects of different oocyte origins on blastocyst gene expression were investigated by collecting and analysing four groups of blastocysts from: a) oocytes lacking the preovulatory development (*In vitro* group); b) oocytes prematured *in vivo* and matured *in vitro* (Pre LH group); c) oocytes prematured and matured *in vivo* (Post LH group); and d) blastocysts developed entirely *in vivo* (*In vivo* group). Oocytes derived from these different sources were subjected to *in vitro* fertilization and cultured to blastocysts *in vitro* under identical conditions.

## Materials and methods

*Experimental Design*

Four groups of blastocysts that differed with regard to either the origin of the oocyte or conditions of embryo development were collected. In the first group (*In vitro* group), oocytes from ovaries obtained from an abattoir were matured, fertilized and cultured *in vitro* up to the blastocyst stage. In the second and third groups, two groups of cows were synchronized and treated with FSH to stimulate a large population of follicles to undergo the normal events of prematuration. The oocytes were collected either before the LH surge (Pre LH group) or 24 h after the induced LH surge (Post LH group). The oocytes from the Pre LH group were matured *in vitro*, and were then fertilized and cultured up to the blastocyst stage

simultaneously with the oocytes from the Post LH group (Figure 1). In the fourth (*In vivo* group) group, blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. Oocytes and blastocysts were collected from Holstein Friesian cows. Oocytes were recovered from cows at a commercial abattoir for the *In vitro* group, whereas donor animals from the experimental herd of the Veterinary Faculty of Utrecht University were used for the other three groups.

*Animals*

For the Pre LH (n = 8) and Post LH groups (n = 9), 17 clinically healthy, non-lactating Holstein Friesian cows were selected on the basis of progesterone concentrations in peripheral blood samples measured three times a week for at least four weeks before the experiment. The animals were fed 20 kg corn silage, 4 kg concentrate per day, and grass silage and water were supplied *ad libitum*. The cows were presynchronised using an ear implant for 9 days (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) together with 3 mg norgestomet and 5 mg estradiol-valerate im (Intervet International BV). Two days before the implant was removed, prostaglandin (15 mg Prosolvin im; Intervet International BV) was administered.

On Day 8 of the synchronized cycle (estrus = Day 0), all follicles larger than 5 mm were removed by transvaginal ultrasound-guided aspiration to avoid the inhibitory effects of follicular dominance on the developmental capacity of oocytes from subordinate follicles (Bungartz and Niemann, 1994). On Day 9 of the estrus cycle, the cows received another ear implant (3 mg norgestomet, Crestar) for 5 days, but not further combined with norgestomet and estradiol-valerate. From Day 10 of the estrus cycle onwards, the cows received o-FSH i.m. (Ovagen ICP, Auckland, New Zealand) twice a day in decreasing doses during 4 days (3.5 mL, 2.5 mL, 1.5 mL and 1.0 mL; in total 17 mL equivalent to 299 IU NIH-FSH-S1). Prostaglandin (22.5 mg i.m.) was administered together with the fifth dose of FSH, and 55 h later the ear implants were removed (Figure 1).

The day after the last FSH dose, transrectal ultrasound technology was used to collect oocytes from cows with more than eight follicles larger than 8 mm. For the Pre LH group, cows (n = 8) were ovariectomized 2 h after removal of the implant. In the Post LH group, cows (n = 9) received GnRH (1.0 mg Fertagyl in 10 mL saline im; Intervet International BV) at the time of removal of the implant and were ovariectomized 26 h after receiving GnRH. Ovariectomy was performed by laparatomy through a flank incision under local anaesthetic, using lidocaine cum adrenaline (Alfasan, Woerden, The Netherlands). Ovaries were collected in 0.9% (w/v) NaCl at 37°C and were immediately transported to the laboratory. Heparinized blood samples were collected from the jugular vein every day during the experimental cycle and every hour after removal of the second implant for 6 h

or until ovariectomy. After immediate centrifugation at 1800 **g** for 10 min at 4°C, plasma was stored at -25°C.

The seven cows in the *In vivo* group were treated with 3000 IU eCG (Intergonan; Intervet,Tönisvorst, Germany) between Day 9 and Day 13 of the estrus cycle, and with prostaglandin (Estrumate; Shering-Plough, Munich, Germany) 48 h later. At estrus the donors were inseminated twice at an interval of 12 h with semen of a bull with proven fertility. At Day 7 after insemination, blastocysts were recovered by non-surgical flushing of the uterine horns with 300 mL PBS (Sigma, St Louis, MO, U.S.A.) supplemented with 1% newborn calf serum (NBCS, No. 295957; Boehringer, Mannheim, Germany) using established procedures. Only blastocysts of morphological grades I and II (Robertson and Nelson, 1998) were stored in a minimum volume of PBS with 0.1% (w/v) polyvinyl alcohol (PVA; Sigma) and stored at -80°C.

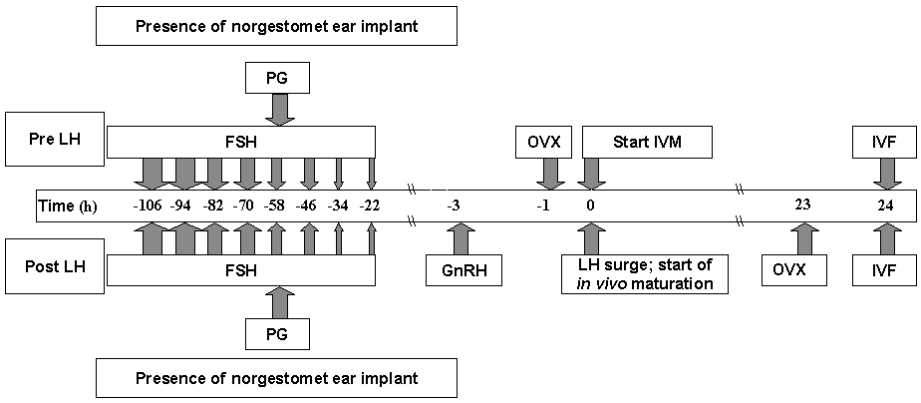



**Figure 1.** Schedule of treatments for superovulation with a fixed LH surge to collect prematured oocytes from FSH-treated cows for *in vitro* maturation (Pre LH) or *in vivo* maturation (Post LH), and subsequent simultaneous *in vitro* fertilization (IVF).
FSH = administration of eight consecutive, decreasing doses of FSH; Time = the time relative to start of *in vitro* maturation (Start of IVM); PG = administration of prostaglandin; GnRH = administration of gonadotropin releasing hormone; LH surge; start *in vivo* maturation = the time at which the maximum of the GnRH-induced LH surge occurs, starting *in vivo* maturation; OVX= ovariectomy.

*Radioimmunoassays for progesterone and LH*
Concentrations of progesterone in plasma were estimated by a solid-phase $^{125}$I-radioimmunoassay (Coat-A-Count TKPG; Diagnostic Products Corporation, Los

Angeles, CA) according to the instructions of the manufacturer as validated by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol/ L, the intra- and interassay coefficients of variation were 8 and <11%, respectively.

Concentrations of LH in plasma were estimated by a validated homologous radioimmunoassay (Dieleman et al., 1983a). The sensitivity was 0.4 µg/ L NIH-LH-B4. The intra- and interassay coefficients of variation were < 9%.

*Collection of oocytes and in vitro maturation*

*In vitro* group. Bovine ovaries were collected in a thermos flask at a local abattoir from Holstein Friesian cows of unknown reproductive status and were then transported to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 3-8 mm follicles, but only those with a multi-layered compact cumulus investment were used for the experiments. An average of ten usable COCs was collected per ovary. Selected COCs were rinsed once with Hepes-buffered M199 supplemented with 10% fetal calf serum (FCS; Gibco BRL, Paisley, UK) and once with maturation medium. Groups of 35 oocytes were randomly allocated to wells of 4-well culture plates (Nunc A/S, Roskilde, Denmark) containing 500 µL maturation medium (M199; Gibco BRL, Paisley, UK) supplemented with 10% FCS, 0.01 IU/mL porcine FSH (Sigma), and 0.01 IU/mL equine LH (Sigma) per well and then cultured for 22 h (39°C, 5% $CO_2$ in humidified air).

Pre LH group. Immediately after ovariectomy, COCs were aspirated from putative preovulatory follicles > 8 mm and were included in the experiment on the basis of a minimum of three layers of compact cumulus investment. COCs of one cow were then processed in one well for *in vitro* maturation similar to the COCs of the *In vitro* group.

Post LH group. Immediately after ovariectomy at 24 h after the LH surge, COCs matured *in vivo* were aspirated from preovulatory follicles > 8 mm and were selected based on an expanded cumulus-cell mass. COCs with a compact cumulus investment and degenerated oocytes were discarded. Selected COCs from each cow were kept together in maturation medium in a single well until fertilization.

The interval between ovariectomy and transfer of COCs to maturation (Pre LH group) or fertilization (Post LH group) medium was 30-40 minutes. The duration of maturation was 24 h for the Pre LH group (*in vitro*) and 24 h *in vivo* (ovariectomy 24 h after the LH surge). Ovulation of fully matured oocytes occurs 24 h after the LH surge (Dieleman et al., 1983b). Maturation was assessed by determining expansion of the cumulus cells. Cytological studies have revealed that in our system > 80% of the oocytes reach the metaphase II stage.

*In vitro fertilization and embryo culture (IVF and IVC)*
Procedures for *in vitro* fertilization (IVF) were performed as described by Izadyar et al. (1996). In brief, matured oocytes of the *in vitro*, Pre LH and Post LH groups were fertilized in wells of 4-well culture plates. All oocytes were fertilized at the same time with the same batch of semen from one bull with proven fertility. Before fertilization, oocytes in each well were rinsed twice with 2 mL Hepes-buffered M199 medium.

Frozen-thawed semen used for IVF was centrifuged over a Percoll gradient for 30 min at 700 **g** at 25°C. The sperm sample was collected by removing the gradient except for the last 150 µL containing the sperm pellet. COCs were transferred to 0.43 mL fertilization medium (Fert-TALP) (Parrish et al., 1988) but without glucose and with 10 µL/mL penicillin-streptomycin (Gibco BRL) instead of gentamycin. Sperm suspension (20 µL; final concentration $0.5x10^6$ cells/mL), 20 µL heparin (final concentration 10 µL/mL; Sigma), and 20 µL PHE (consisting of 20 µM D-penicillamine, 10 µM hypotaurine, 1 µM adrenaline; Sigma) were added to the fertilization medium. After 18-20 h of incubation at 39°C, with 5% $CO_2$ in humidified air, cumulus cells were completely removed from presumptive zygotes by vortexing for 3 min; a maximum of 10 zygotes was placed in 20 µL droplets of synthetic oviductal fluid (SOF) medium under oil (Squibbs oil, Princeton, USA) (Van Wagtendonk-de Leeuw et al., 2000). Zygotes of the Pre LH and Post LH group from each animal were cultured in individual droplets. *In vitro* culture (IVC) was performed at 39°C in a humidified atmosphere containing 5% $CO_2$, 7% $O_2$ and 88% $N_2$. On Day 4 of culture all cleavage stages were transferred to fresh culture droplets, and the proportion of each cleavage stage was assessed. On Day 7 after insemination, blastocysts and expanded blastocysts were collected whereas morulae and early blastocysts were cultured for one additional day. On Day 8, additional blastocysts and expanded blastocysts were collected. Day 1 of culture was defined as beginning immediately after completion of IVF. Embryos were washed four times in PBS with 0.1% (w/v) PVA (PBS-ET, Bio Whittaker Europe, Verviers) and transferred to 0.5 ml cups in a minimum volume (≤ 5 µl) of PBS with 0.1% (w/v) PVA and frozen at -80°C. Analysis of cultured embryos was confined to those in morphological grades I and II (Robertson and Nelson, 1998).

*Selection of the embryos for RT-PCR*
For each gene, embryos from as many different cows as possible were analysed. Furthermore, similar numbers of embryos collected at days 7 and 8 were assigned to each mRNA analysis. Depending on the type of gene, four to five transcripts were analysed per embryo and at least five embryos per gene were determined. The relative abundance of mRNAs from the six different genes

important in the development of a preimplantation embryo was determined in single bovine blastocysts by semi-quantitative RT-PCR.

*Isolation of RNA*

Poly $(A)^+$ RNA from a single embryo was isolated using a Dynabeads mRNA DIRECT kit (Dynal A.S., Oslo, Norway). RNA was isolated following the manufacturer's instructions with minor alterations (Wrenzycki et al., 1999). In brief, frozen embryos were thawed by adding 30 µL lysis buffer (100 mM Tris-HCL pH 7.5; 500 mM LiCl, 10 mM EDTA pH 8, 1% (w/v) LiDS (SDS); and 5 mM dithiothreitol). As an internal standard 1.0 pg rabbit globin RNA (Life Technologies BV, Eggenstein, Germany) was added to each embryo. The samples were mixed for 10 s, centrifuged for 15 s at 12 000 **g**, and left for 10 min at room temperature. Dynabeads (5 µL; Dynal) were added and mixed for 5 min at room temperature. The samples were put in the magnetic separator to remove the lysis buffer and leave the Dynabeads. The Dynabeads were washed four times, once with 40 µL washing buffer A (10 mM Tris-HCL pH 7.5; 0.15 M LiCl; 1 mM EDTA; and 0.1% (w/v) LiDS) and three times with 40 µL washing buffer B (10 mM Tris-HCl pH 7.5; 0.15 M LiCl; and 1 mM EDTA). After removal of washing buffer B, 11 µL sterile $H_2O$ was added and incubated at 65°C for 2 min to elute mRNA from the Dynabeads. The cups were put into the magnetic separator again; the supernatant was removed and immediately used for reverse transcription.

*Reverse transcription*

Poly$(A)^+$ RNA isolated from a single embryo was reverse transcribed into cDNA in a total volume of 20 µl. The reaction mixture consisted of 1x RT buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; Perkin Elmer Biosystems, Vaterstatten, Germany), 5 mM $MgCl_2$, 1 mM of each dNTP (Amersham, Brunswick, Germany), 2.5 µM random hexamers (Perkin Elmer), 20 IU RNase inhibitor (Perkin Elmer) and 50 IU MuLV reverse transcriptase (Perkin Elmer). The samples were centrifuged for 15 s at 12000 **g** and were overlaid with mineral oil to prevent evaporation. One sample was prepared with 1.0 pg rabbit globin RNA and 10 µL $H_2O$. As negative controls, tubes without an RNA sample and tubes with an RNA sample, but without reverse transcriptase were analysed. The reverse transcription reaction was carried out in a thermocycler (Biometra, Triothermoblock; Biometra, Göttingen, Germany) for 10 min at 25°C, 60 min at 42°C, 5 min at 99°C and was kept on ice once the reaction was completed.

*PCR*

Immediately after reverse transcription the samples were subjected to PCR amplification. PCR was performed with a volume of the reverse transcriptase

reaction corresponding to 0.1 (desmocollin-2 and glucose transporter-1), 0.2 (Heat shock protein, E-cadherin and poly(A)polymerase) and 0.4 (plakophilin) embryo equivalents in a final volume of 50 µL, containing 1x PCR buffer (20 mM Tris-HCl; 50 mM KCl, pH 8.4; Life Technologies), 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1 µM of each sequence-specific primer (0.5 µM for globin primers). A 'hot start' PCR was performed to obtain specific amplification. During the 'hot start' 1 IU Taq DNA polymerase (Life Technologies) was added at 72°C. The sequences and positions of the primers used, the annealing temperature, the fragment sizes of the expected products, and the sequence references are shown (Table 1). The products of each primer pair were sequenced to confirm the identity of the RT-PCR fragments.

Running a linear cycle series established the optimal cycle number at which the transcript was amplified exponentially for the PCR reaction. The PCR programme started at 97°C for 2 min, decreased to 72°C for 2 min (hot start) and was followed by a number of cycles, which depended on the gene (Table 1), of 15 s at 95°C for denaturation, 15 s at the annealing temperature of the specific gene (Table 1) and 15 s at 72°C for primer extension. After finishing these cycles a final extension of 5 min at 72°C was performed, followed by cooling to 4°C. A PTC-200 thermocycler (MJ Research, Watertown, MA) was used.

*Detection and semi-quantification of RT-PCR products*

RT-PCR product (25 µL) with 5 µL of 10x loading buffer (0.25% (w/v) xylenecyanol and 25 mM EDTA in 50% (w/v) glycerin) was loaded on to a 2% (w/v) agarose gel in TBE buffer (90 mM Tris; 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 µg/mL ethidium bromide and subjected to electrophoresis. The concentration of ethidium bromide in the running buffer and the gel was the same. After electrophoresis at 100 V for 5 min and at 80 V for 40 min, the fragments were visualised on a 312 nm UV-transilluminator. A CCD camera (Quantix, Photometrics, Munich, Germany) and IP Lab spectrum (IP Lab Gel, Signal Analytics Corporation, Vienna, VA) were used to digitize the image of the gel. Densitometric scanning using a computer-assisted image analysis system (IP Lab Gel; Signal) was used to quantify the signal intensity of each band. The relative abundance of the mRNA of the different genes was estimated by dividing the intensity of the band of the gene of interest by the intensity of the globin band from the same sample.

The recovery rate of the RNA was estimated for all of the embryos. It was calculated as the ratio between the intensity of the globin bands with and without RNA extraction. On average, 42% of the poly(A)$^+$ RNA of the blastocyst was extracted, which is similar to percentages obtained by Wrenzycki et al. (1999).

**Table 1**. Primers used for PCR

| Genes | Primer sequence and positions | Cycle no. | Annealing temp (°C) | Fragment size (bp) | Sequence references (EMBL accession no.) |
|---|---|---|---|---|---|
| Globin | 5'primer (241-260) = GCAGCCACGGTGGCGAGTAT<br>3'primer (555-657) = GTGGGACAGGAGCTTGAAAT | 27 | 60 | 257 | Cheng et al. (1986) (X04751) |
| Desmocollin-2 (Dc-2) | 5'primer (2085-2109) = CTCCTGGCGATGACAAAGTGTATTC<br>3'primer (2503-2527) = GCCGATCCTCTTCCTTCGTAGTTAT | 31 | 57 | 443/397 (insertion of 46 bp, 2396-2441) | Koch et al. (1992) (M81190) |
| Plakophilin (Plako) | 5'primer (1337-1361) = CCCGTGGACCCCGAGGTCTTCTTCA<br>3'primer (1580-1604) = CGGTGTAGGCGTTGCGGGCGTTGTA | 35 | 64 | 268 | Heid et al. (1994) (Z37975) |
| Glucose transporter-1 (Glut-1) | 5'primer (1609-1638) = AGGAGCTGTTCCACCCCCTGGGAGCTGACT<br>3'primer (1906-1935) = TGTGGGTGAAGGAGACTCTGGCTGATAAAA | 32 | 59 | 327 | Boado and Pardridge (1991) (M60448) |
| Poly(A)polymerase (Poly A) | 5'primer (886-915) = GTTTCCTCGGTGGTGTTTCCTGGGCTATGC<br>3'primer (1108-1137) = TGGAGTTCTGTTGTGGGTATGCTGGTGTAA | 35 | 57 | 252 | Raabe et al. (1991) (X63436) |
| Heat shock protein 70.1 (HSP) | 5'primer (1861-1890) = AAGGTGCTGGACAAGTGCCAGGAGGTGATT<br>3'primer (2319-2348) = ACTTGGAAGTAAACAGAAACGGGTGAAAAA | 36 | 59 | 488 | Gutierrez and Guerrioro (1995) (U09861) |
| E-cadherin (E-cad) | 5'primer (1486-1515) = CTCAAGCTCGCGGATAACCAGAACAAAGAC<br>3'primer (1785-1814) = AGGCCCCTGTGCAGCTGGCTAAATCAAAG | 33 | 55 | 332 | Ringwald et al. (1987) (X06339) |

*Statistical analysis*
Rates of blastocyst formation were analysed with logistic regression and $P \leq 0.05$ was considered significant. For the relative abundance of the gene transcripts, an analysis of variance was performed using the nlme library of S-PLUS 2000 with random cow effects (Pinheiro and Bates, 2000). Data are presented as mean ± SEM.

## Results

*Response to superovulation treatment (Pre LH and Post LH groups)*
No LH surge was observed in peripheral blood of cows in the Pre LH group (Figure 2). The cows that were treated with GnRH (Post LH group) showed a clear LH surge at 2 h after the administration of GnRH; in cows from which embryos were collected for RT-PCR, maximum LH concentration was 24.5 ± 2.5 µg/L (n=7; Figure 2).

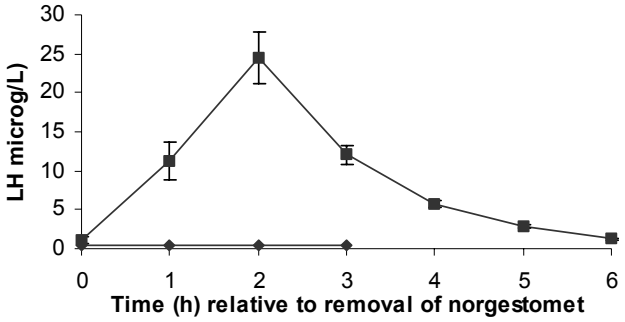



**Figure 2.** Concentration (mean ± SEM) of LH in peripheral blood of FSH-PG-norgestomet – treated cows without GnRH (Pre LH, n=8;ɔ) and with GnRH administered (Post LH, n=7;■); GnRH was administered at time of removal of the implant.

Signs that an LH surge had occurred before the implant was removed were observed in four cows: cumulus cells were moderately expanded in two cows from the Pre LH group and two cows of the Post LH group had already ovulated. Oocytes from these animals were excluded from the experiment.

The superovulation response in the Pre LH- and the Post LH group was 30.3 ± 10.5 (mean ± SD, n=6) and 23.1 ± 10.0 (mean ± SD, n=7) follicles > 8 mm on average per cow, respectively.

*Oocyte recovery and in vitro culture*
In the Pre LH group, 166 COCs were collected from 182 follicles of preovulatory size from six cows (recovery rate 91.2%). COCs that did not have an intact multi-

layered compact cumulus investment or that already showed cumulus expansion were excluded (n=26) from analysis. In the Post LH group, 136 oocytes were collected from 162 follicles of preovulatory size from seven cows (recovery rate 84.0%). Denuded oocytes and COCs not showing cumulus expansion (n=20) were excluded. In the *In vitro* group, 333 oocytes were collected and used for IVM, IVF and IVC.

Cleavage rates on day 4 of culture were similar in the three IVC groups (average 75%; Table 2). On day 7 after insemination, most (60 to 80%) of the blastocysts had already formed. Rates of blastocyst formation at day 7 were significantly ($p<0.05$) different in the Pre LH, Post LH and *in vitro* groups. The overall rate of blastocyst formation was significantly ($p<0.05$) higher in the Pre LH group (50%; Table 2) than in the Post LH (31.0%) and *in vitro* groups (26.4%). Most of the blastocysts in the three IVC groups were already expanded: 64.3%, 52.7% and 54.5% for Pre LH, Post LH and *in vitro* group embryos, respectively.

**Table 2.** Rates of blastocyst formation after *in vitro* culture (IVC) and IVF of *in vivo*-or *in vitro*-matured oocytes obtained from preovulatory follicles from FSH-stimulated cows compared with rates of *in vitro*-matured oocytes from 3-8 mm follicles.

| | Number of oocytes After IVF Day 1 [1]) | Number of cleaved embryos day 5 (%) | Number of blastocysts | | |
|---|---|---|---|---|---|
| | | | day 7 [2]) (%) | Day 8 (%) | Total |
| Preovulatory follicles | | | | | |
| Pre LH after *in vitro* maturation | 140 | 123 (87.9) | 51 (36.4)[a] | 19 (13.6)[a] | 70 (50.0)[a] |
| Post LH after *in vivo* maturation | 116 | 85 (73.3) | 29 (25.0)[b] | 7 (6.0)[a] | 36 (31.0)[b] |
| 3-8 mm follicles | | | | | |
| after IVM | 333 | 233 (70.0) | 55 (16.5)[c] | 33 (9.9)[a] | 88 (26.4)[b] |

[1] Oocytes were collected from preovulatory follicles before (Pre LH) or 24 h after (Post LH) an induced LH surge. Day 1 is start of IVC. Percentages of cleavage and blastocyst formation are calculated relative to the number of (non)-fertilized oocytes at day 1 of IVC.

[2] At day 7 of IVC, blastocysts were collected and stored for RT-PCR, and morulae and early blastocysts were cultured for an additional day to be collected as blastocysts at day 8.

[a-c] Values with different superscripts within one column are significantly different ($P<0.05$).

*Relative abundance of genes in single blastocysts derived from different oocyte origins*

For RT-PCR products (Figure 3), the bands represent gene transcripts derived from 0.1 – 0.4 parts of a bovine blastocyst. As negative controls, RNA or reverse transcriptase was omitted during the reverse transcriptase reaction. No amplified products were found at any time in the negative controls during these experiments.

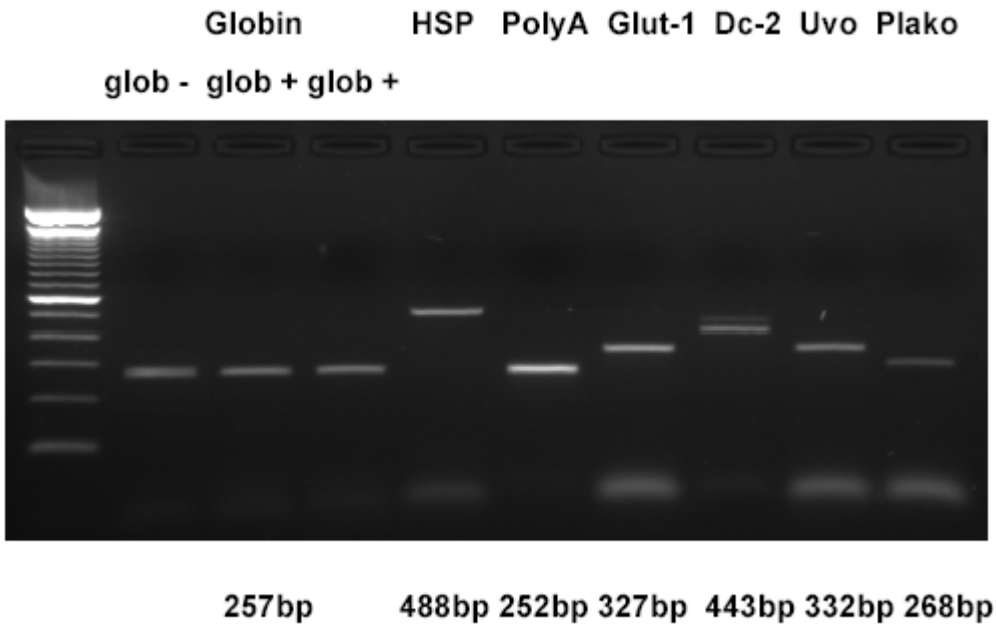



**Figure 3.** Expression of six genes important to development as detected by RT-PCR in a 0.1 to 0.4 proportion of a single bovine blastocyst; digitalized image after electrophoresis on an agarose gel containing ethidium bromide. PCR products are indicated at the top for heat shock protein 70.1 (HSP), poly(A)polymerase (Poly A), glucose transporter-1 (Glut-1), desmocollin-2 (Dc-2), E-cadherin (E-cad) and plakophilin (Plako), and for the RNA extraction control globin (- ext = without extraction; + ext = with extraction). Expected fragment sizes are indicated at the bottom; bpl = 100 bp ladder as marker for fragment size.

The relative abundance of Glut-1 was significantly ($p<0.05$) higher in the *in vivo* group compared with the Pre LH-, Post LH- and *in vitro* groups (Figure 4). The relative abundance of Dc-2 and plako tended to be higher in the fourth group (*in vivo*) compared with the other three groups, although this difference was not

significant. No significant differences were detected in any of the gene transcripts between the three *in vitro* groups (*in vitro*-, Pre LH- and Post LH groups; Figure 4).

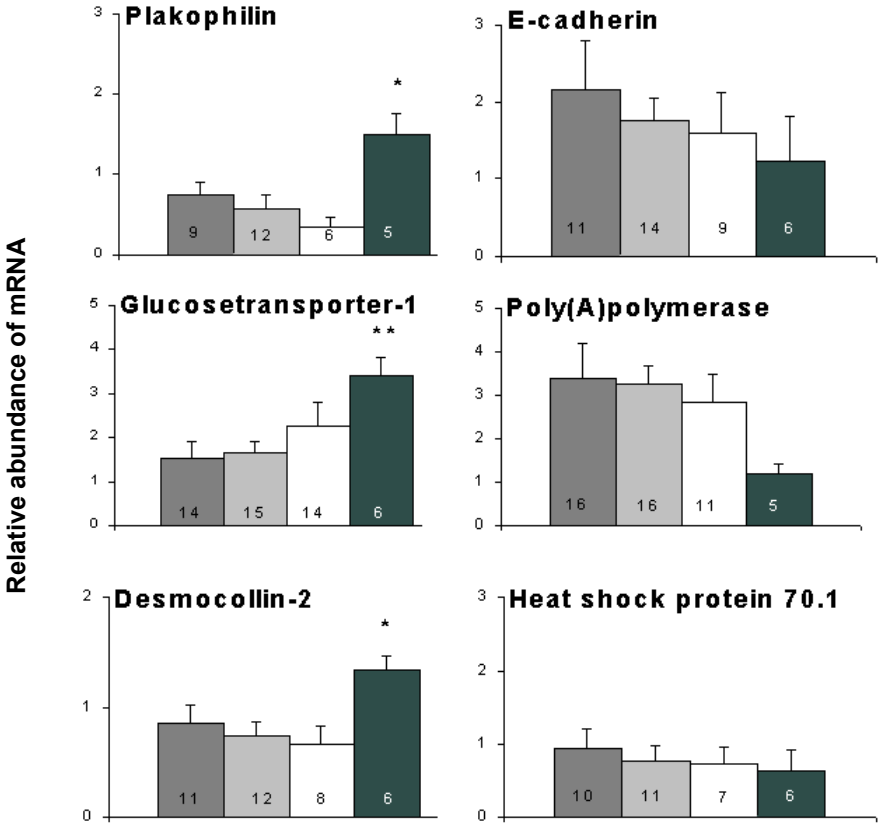



**Figure 4.** Relative abundance of transcripts of six genes important to development as detected by semi-quantitative RT-PCR in single bovine blastocysts originating from oocytes subjected to different modes of maturation. Embryos were derived from: 1) immature oocytes from 3-8 mm follicles after IVM, IVF and IVC (*In vitro* group; dark grey bars); 2) prematured oocytes from preovulatory-sized, FSH-stimulated follicles after IVM, IVF and IVC; (Pre LH group; light grey bars); 3) prematured oocytes from preovulatory-sized, FSH-stimulated follicles after *in vivo* maturation, IVF and IVC (Post LH group; open bars); and 4) similar oocytes as to those in 3) but also with fertilization and development entirely *in vivo* (*In vivo group;* black bars). Bars represent the mean ± SEM relative abundance of a number of blastocysts as indicated in the bars; each value was the mean of at least five different cows. Asteriks indicate difference from all groups: * P<0.1; **P<0.05.

## Discussion

In the present study the effects of different maturation regimens of bovine oocytes on the relative abundance of transcripts from six genes important in development in blastocysts were investigated. *In vitro* conditions have profound effects on the patterns of expression of a range of gene transcripts in bovine and murine embryos (Ho et al., 1994; 1995; Niemann and Wrenzycki, 2000). The present data show for the first time that prematuration-maturation does not necessarily affect the relative abundances of the genes and indicate that *in vitro* maturation may not be the critical factor contributing to variation in mRNA content between *in vitro*- and *in vivo*-derived embryos. The differences between *in vivo*- and *in vitro*-derived blastocysts were probably not due to variation in the rates of development. In parallel experiments, blastocysts derived *in vivo* and *in vitro* as well as those derived from pre LH and post LH groups had similar numbers of cells.

The semi-quantitative RT-PCR assay used in the present study provides sensitive and highly reproducible results from both pooled and single bovine embryos. It has constantly been updated and increased in sensitivity (Wrenzycki et al., 1999; 2001a; 2001b; 2002). The validity of the assay has been demonstrated previously and revealed efficient amplification of both the globin standard and the RNA of choice (Wrenzycki et al., 2000). Its accuracy compares favourably with current real-time RT-PCR techniques (Stenman et al., 1999). Similar to endpoint RT-PCR, real-time RT-PCR uses standards against which the amounts of mRNA of the genes of choice are compared (Freeman et al., 1999; Steuerwald et al., 2000; Bustin, 2000). The densitometric analysis of ethidium bromide stained agarose gels described in the present study is a well established and sensitive enough approach to detect even subtle differences in amounts of mRNA in different biological materials (Grover et al., 2001; Ringhoffer et al., 2001). The degree of variation with this approach is rather small (Grover et al., 2001), but can be further decreased by an optimized real-time RT-PCR protocol (Bustin, 2000). We have calculated the number of replicates necessary to obtain statistically significant differences to accommodate any inherent variability. This approach has recently proven valid for single bovine cloned and IVP embryos (Wrenzycki et al., 2001b; 2002). The differences in patterns of expression described previously were related to the increased incidence of the Large Offspring Syndrome (LOS) in offspring derived from IVP or cloned bovine embryos (Niemann and Wrenzycki, 2000; Niemann et al., 2002). Even subtle changes in patterns of expression of specific genes were shown to have marked biological effects such as predisposition to tumourigenesis (Yan et al., 2002) and may be causally involved in LOS.

The mRNAs measured in blastocysts in the present study were probably transcribed during embryonic development and did not represent mRNAs that

remained from the maternal mRNA pool. Plako, Dc-2 and E-cad are predominantly expressed from the morula stage onwards (Wrenzycki et al., 1999; 2001a). The mRNAs of Glut-1 and poly(A) were found throughout early development up to the blastocyst stage, which is indicative of both maternal and embryonic origin, with a burst after maternal-embryonic transition of genomic activity (Wrenzycki et al., 1999). The different amounts of mRNA observed in blastocysts derived from oocytes matured *in vitro* or *in vivo* in the present study are supported by findings from histological and biochemical studies. Embryos developed *in vitro* frequently form the blastocoel earlier than *in vivo,* without proper compaction, and have fewer cells than do *in vivo*-grown embryos (Van Soom et al., 1997b). Dc-2 and plako molecules are involved in intercellular communication structures and compaction (Collins et al., 1995). The lower expression of Dc-2 and plako observed in blastocysts of the *in vitro* group might explain the impaired compaction in embryos produced *in vitro*. Differences in metabolism between bovine embryos produced *in vitro* and those developed *in vivo* have been detected, especially with regard to lactate production and glucose metabolism (Khurana and Niemann, 2000). The decreased amount of Glut-1 in *in vitro* embryos found in the present study probably reflects differences in energy metabolism between blastocysts developed *in vitro* and *in vivo*.

A novel aspect of the present study was related to expression of the panel of genes in blastocysts derived from prematured oocytes (Pre LH group) and oocytes matured *in vivo* (Post LH group). The results of the present study indicate that blastocysts developed from Pre LH and Post LH oocytes are not different with respect to patterns of gene expression from those derived from oocytes that lacked a period of prematuration and *in vivo* maturation. Nevertheless, the mode of oocyte prematuration and maturation may affect the developmental competence after cleavage into morulae and blastocysts (Hyttel et al., 1997).

The rate of blastocyst development in the Pre LH group was higher than in the *In vit*ro group, which is in accordance with earlier observations and confirms the stimulatory effects of prematuration on oocyte developmental competence. The rate of blastocyst development in the Post LH group differed from recent findings in which exceptionally high rates of blastocyst development were found (Van de Leemput et al., 1999; Hendriksen et al., 2000). When two cows with low rates of blastocyst development were excluded from analysis, the rate of blastocyst development of the Post LH group increased to approximately 45% and the relative abundance of the genes did not change significantly. The high proportion of well-expanded blastocysts indicates the inherent high capacity of the blastocysts for development.

The results of the present study indicate that *in vitro* maturation - as the first step in IVP of embryos - has improved significantly (Bavister, 1995; Keskintepe and Brackett, 1996). Blastocysts derived from oocytes that were matured differently, but cultured identically, did not show significant differences with

respect to the panel of gene transcripts investigated in the present study. The differences may therefore be related to the *in vitro* conditions imposed on fertilized oocytes. This contention is supported by previous findings for sheep and cattle, in which zygotes were subjected to different culture conditions and developed abnormal phenotypes (Young et al., 1998; Sinclair et al., 2000). Nevertheless, it could be useful to analyse gene expression in oocytes immediately after completing different maturation protocols. However, this would require a different panel of gene transcripts to be informative, including genes involved on cell cycle regulation, meiotic competence and cumulus expansion. Little is known about transcriptional activity in bovine oocytes. Duration of oocyte maturation and quality of COCs affected transcription of cyclooxygenase-2 and various prostaglandin E receptors in *in vitro* culture (Calder et al., 2001). Furthermore, it would be interesting to determine rates of transcription in the embryos in which development was arrested at an early stage and thus did not reach the blastocyst stage.

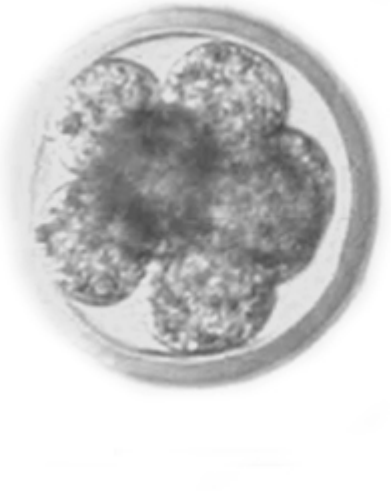
In conclusion, the difference in the relative abundance of the six gene transcripts found between blastocysts produced *in vitro* and *in vivo* can probably be attributed to embryo culture conditions after maturation and fertilization. Prematuration and maturation are thought to be critically involved in the ability of the oocyte to support early development (Sirard and Blondin, 1996; Hyttel et al., 1997, Rizos et al., 2002). Whether blastocysts derived from oocytes matured *in vivo* or *in vitro* differ with regard to quality has yet to be determined. It should be noted that this study was limited to a panel of six gene transcripts and it is possible other gene transcripts would behave differently. cDNA array technology enables simultaneous determination of a potentially unlimited number of gene transcripts and it has already been applied to the diagnosis of various forms of human cancer, human autoimmune diseases and the characterization of murine embryonic stem cells (Alizadeh et al., 2000; Kelly and Rizzino, 2000; Rogge et al., 2000). A prototype of a suitable cDNA array for single bovine embryos has recently become available (Brambrink et al., 2002) and its broader application will also improve determination of patterns of gene expression patterns in embryos derived from IVP or cloning.

## Acknowledgments

The authors thank Thea Blankenstein, Elly Zeinstra and Sandra Wilkening for technical assistance and animal handlers and surgery assistants for management of the animals. The authors also gratefully acknowledge Jan van de Broek and Hans Vernooy for advice on statistical analysis.

# Chapter 4

## Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*



**Abstract**

The postimplantation developmental potential of embryos can be affected by various forms of cell death, such as apoptosis, at preimplantation stages. However, correct assessment of apoptosis is needed for adequate inference of the developmental significance of this process. This study is the first to investigate the independent chronological occurrence of apoptotic changes in nuclear morphology and DNA degradation (detected by the TUNEL reaction) and incidences of nuclei displaying these features at various preimplantation stages of bovine embryos produced both *in vivo* and *in vitro*. Different elements of apoptosis were observed at various developmental stages and appeared to be differentially affected by *in vitro* production. Nuclear condensation was observed from the 6-cell stage *in vitro* and the 8-cell stage *in vivo*, whereas the TUNEL reaction was first observed at the 6-cell stage *in vitro* and the 21-cell stage *in vivo*. Morphological signs of other forms of cell death were also observed in normally developing embryos produced both *in vivo* and *in vitro*. The onset of apoptosis seems to be developmentally regulated in a stage-specific manner, but discrete features of the apoptotic process may be differentially regulated and independently modulated by the mode of embryo production. Significant differences in indices of various apoptotic features were not evident between *in vivo*-, and *in vitro*-produced embryos at the morula stage, but such differences could be observed at the blastocyst stage, where *in vitro* production was associated with a higher degree of apoptosis in the inner cell mass.

**Authors:** Jakob O. Gjørret[1], Hiemke M. Knijn[2], Steph J. Dieleman[2], Birthe Avery[3], Lars-Inge Larsson[1], Poul Maddox-Hyttel[1].

[1]*Department of Anatomy and Physiology, Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark.*

[2]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan, 7 3584 CL Utrecht, The Netherlands.*

[3]*Department of Clinical Studies, Reproduction, Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark.*

Biology of Reproduction (2003) 69, 1193-1200

## Introduction

Functional mechanisms for disposal of cells that are in excess, in the way, abnormal or potentially dangerous, are crucial during development and for tissue homeostasis of multicellular organisms. The phenomenon of cell death is firmly established in preimplantation development of mammalian embryos (for reviews, see Hardy, 1997; Betts and King, 2001), and roles and regulation of these processes are currently under investigation. In classic terms, necrosis and apoptosis are two types of cell death that can be differentiated by their morphological appearance (Wyllie *et al.*, 1980; Majno and Joris, 1995). However, several intermediate forms exist (Leist and Jaattela, 2001) and several types of cell death may occur during normal embryonic development. Necrosis, an accidental form of cell death resulting from a direct injury, commonly affects cells in clusters and inflicts damage on their neighbours by triggering an inflammatory response, whereas apoptosis, a highly conserved process, is a far more regulated and suicidal form of cell death that occurs in single cells. Apoptosis has received an increasing amount of attention because of its potential role in early embryonic loss and in cellular responses to stress and suboptimal developmental conditions (Devreker and Hardy, 1997; Moley *et al.*, 1998; Wuu *et al.*, 1999; Matwee *et al.*, 2000; 2001; Paula-Lopes and Hansen, 2002). However, an accurate assessment of the different forms of cell death is needed to adequately infer their biological significance during preimplantation development.

Morphological characteristics of apoptotic cell death, such as chromatin condensation and marginalization and nuclear fragmentation by karyorhexis (Majno and Joris, 1995), are visible in unarrested morula and blastocyst stage embryos produced both *in vivo* and *in vitro*. Such nuclear changes are observed in 70-80% of all *in vitro*-produced blastocysts from mice (Handyside and Hunter, 1986) and humans (Hardy *et al.*, 1989), and in practically all blastocysts from cattle (Byrne *et al.*, 1999), and the presence of these changes has been taken as evidence of apoptotic activity. Ultrastructural studies of blastocysts have revealed extensive chromatin and cytoplasmic condensation, nuclear and cell fragmentation with intact organelles, and phagocytosis (El-Shershaby and Hinchliffe, 1974; Enders *et al.*, 1982).

The TUNEL reaction (Gavrieli *et al.*, 1992) enables *in situ* detection of apoptotic cells by labeling of extensive oligonucleosomal DNA fragmentation generated by endogenous DNAse activity during the apoptotic process. Initial application of this reaction assay to preimplantation embryos (Jurisicova *et al.*, 1996) has opened a new line of research, and it has been used for observation of apoptotic cell death in cleavage, morula and blastocyst stages from many species (Brison and Schultz, 1997; Pampfer *et al.*, 1997; Long *et al.*, 1998; Moley *et al.*, 1998; Yang *et al.*, 1998; Byrne *et al.*, 1999; Hardy, 1999; Wuu *et al.*, 1999; Matwee *et al.*, 2000; Hinck *et al.*, 2001; Kölle *et al.*, 2002; Makarevich and

Markkula, 2002; Neuber *et al.*, 2002; Paula-Lopes and Hansen, 2002). Unfortunately, the TUNEL reaction fails to determine how DNA degradation is generated, because nuclei of cells undergoing necrosis are also labeled (Grasl-Kraupp *et al.*, 1995) and inadequate tissue handling may induce sufficient DNA damage to generate labeling of apparently normal nuclei (Negoescu *et al.*, 1996). Apoptosis may not always be associated with extensive DNA degradation (Cohen *et al.*, 1992; Chautan *et al.*, 1999), and when it occurs, DNA degradation appears to be a relatively late event in the apoptotic process (Collins *et al.*, 1997). The morphological appearance of the TUNEL reaction in necrotic cells is, however, somewhat different because the mode of nuclear disintegration is by karyolysis rather than by karyorhexis as seen in apoptosis (Majno and Joris, 1995; Darzynkiewicz *et al.*, 2001). Therefore, morphological evaluation must be performed when the apoptotic mode of cell death is to be quantified, but more than one feature of apoptosis must be observed for correct identification of the process (Darzynkiewicz *et al.*, 2001). Variation in the assessment of apoptotic incidence in preimplantation embryos may arise because of discrepancy in definitions. Some researchers regard TUNEL-positive nuclei as apoptotic without implying morphological evaluation, others regard both TUNEL-positive nuclei and nuclei with apoptotic morphology but without TUNEL reaction as apoptotic, and others rely only on morphological changes for quantification of apoptosis. Therefore, results are not always directly comparable among studies.

The chronology of onset of apoptotic cell death in preimplantation embryos has been debated, because various characteristics of this complex process, such as cell and nuclear fragmentation, DNA degradation, and phagocytosis, emerge at different developmental stages. There is little evidence of apoptotic cell death with detectable DNA degradation prior to compaction in unarrested human and mouse embryos with normal morphological appearance that have been produced either *in vivo* or *in vitro* (Handyside and Hunter, 1986; Brison and Schultz, 1997; Hardy, 1999), but signs of apoptosis have been observed at the 8-cell stage in similar *in vitro*-produced bovine embryos (Byrne *et al.*, 1999; Matwee *et al.*, 2000). However, in embryos arrested in development, displaying no mitotic activity within 24 h, characteristics of apoptosis can be observed at earlier stages (Jurisicova *et al.*, 1996) whether these characteristics are a cause or a result of embryonic arrest is still unclear (Antczak and Van Blerkom, 1999). Because different markers of apoptotic cell death are needed concurrently for exact identification (Darzynkiewicz *et al.*, 2001), a thorough knowledge of their occurrence and regulation is crucial for adequate evaluation of apoptosis in preimplantation embryos. To our knowledge, this is the first report of the independent chronological appearance of both apoptotic nuclear morphology and DNA degradation during bovine pre-implantation development *in vivo* and of direct comparisons to similarly staged embryos developed *in vitro*.

In this study we investigate the chronological occurrence of two key apoptotic markers: 1) changes in nuclear morphology, such as nuclear and chromatin condensation and nuclear fragmentation, typical for apoptosis, and 2) DNA degradation detectable by the TUNEL reaction. We assessed these markers at various preimplantation stages of bovine embryos with expected developmental kinetics that were produced either *in vivo* (*in vivo* embryos) or *in vitro* (*in vitro* embryos) We wanted to determine a) whether the markers could be found at different developmental stages and whether *in vitro* production (IVP) alters the chronological appearance and ratios of embryos displaying these markers, thereby providing information about the underlying cell death machinery, and b) whether any differences in the incidence and distribution of apoptotic cell death in bovine morulae and blastocysts produced either *in vivo* or *in vitro*, could be observed using a scoring system encompassing both markers simultaneously. These results can be used as a reference for future mechanistic studies and should stimulate the use of similar combinations of markers in other experiments.

## Materials and methods

*In vivo embryo production*

*In vivo*-produced embryos were collected from normal cyclic Holstein-Friesian heifers (n=24), which were synchronized and superovulated with postponement and monitoring of the LH surge as described previously (van de Leemput *et al.*, 2001) with some modification. Dominant follicle ablation was performed on Day 8 (synchronized estrus = Day 0), and subsequent superovulation (Day 10) was instigated with sheep FSH (Ovagen; ICP, Auckland, New Zealand), which was given i.m. twice daily for 4 days in a decreasing regimen (total dose of 299 IU NIH-FSH-S1). A 3 mg progesterone ear implant (Crestar; Intervet International BV, Boxmeer, The Netherlands) inserted concurrently with the first administration of FSH and was removed 96 h after insertion, and 21 mg of a GnRH analogue (Buserelin-acetate; Receptal, Intervet) was given i.m. 12 h after the last FSH injection. All animals were inseminated 12-14 h after the GnRH administration with frozen-thawed semen from a known fertile bull according to standard procedures. The LH surge was monitored as previously described (Viuff *et al.*, 2001) using a validated RIA method (Dieleman *et al.*, 1983b). The LH surge was observed ~2 h after GnRH administration, and ovulation was expected 24 h after the surge.

All animals were killed, and embryos were collected as previously described (Viuff *et al.*, 2001). Embryonic developmental stage and general morphologic appearance were assessed by stereo microscopy. Embryos at different developmental stages were collected at the following time points post ovulation (p.o.): 2-cell stage at 38 h p.o., 3- to 8-cell stage at 48 h p.o.; 9- to 16-cell stage at

81 h p.o., morula stage at 131 h p.o., and blastocysts stage at 168 h p.o. Collected embryos were washed briefly in PBS with BSA, fixed for 1 h at room temperature in 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) in PBS, transferred to 1% PFA, and stored at 4°C until further analysis.

*In vitro embryo production*

*In vitro*-produced embryos were harvested from 4 experimental replicates following standard procedures previously described (Avery *et al.*, 1998; Avery and Greve, 2000), with all incubations were performed in a Heraeus incubator at 38.8°C in humidified air with 5% $CO_2$. Oocytes were derived from cattle abattoir ovaries, and *in vitro*-matured oocytes were *in vitro* fertilized (IVF) according to standard procedures using frozen-thawed semen from a known fertile bull. Quality of semen used for embryo production both *in vivo* and *in vitro* was regarded as equal with respect to support of subsequent embryo development. Following IVF, 20-25 inseminated oocytes were added to culture drops and cocultured with bovine oviduct epithelial cells until harvesting.

Embryonic developmental stage and general morphologic appearance were assessed by stereo microscopy, and embryos were collected at the following time points postinsemination (p.i.): 2-cell stage at 32 h p.i.; 3- to 8-cell stage at 40 h p.i.; 9- to 16-cell stage at 100 h p.i.; morula stage at 117 h p.i.; and blastocysts stage at 160 h p.i. Collected embryos were washed and fixed as above, and specimens were stored for no longer than 2 weeks before further processing.

*TUNEL and confocal microscopy*

Nuclei with DNA degradation were detected using a cell death detection technique based on the TUNEL principle (Graviele et al., 1992) with Fluorescein-conjugated dUTP as described previously (Byrne et al., 1999; Brison and Schultz, 1997) with minor modifications. Fixed embryos were subjected to TUNEL reaction (In Situ cell death detection kit; Roche, Hvidovre, Denmark). Extensive DNA fragmentation was induced in positive controls by incubation in 50 U/ml DNase (RQ1; Promega; Bie & Berntsen, Rødovre, Denmark) prior to the TUNEL reaction, and negative controls were generated by omitting terminal transferase from the reaction. Labeled embryos were all incubated in 0.1 mg/ml of RNase A (Sigma, St. Louis, MO), and DNA was counterstained with 10 µg/ml propidium iodide (PI; Sigma). Embryos at the 2-cell to morula stages were mounted on glass slides in 10-15 µl Flouroguard anti-fade (BioRad, Hercules, CA) under coverslip compression, but to conserve spherical morphology blastocysts were taken through an increasing gradient of Vecta-Shield anti-fade (Vector-Labs., Burlingame, CA) and mounted in pure Vecta-Shield with 0.05 µg/ml PI within a plastic ring, placed between the glass slide and coverslip, to prevent blastocyst compression. Slides were stored at 4ºC for up to 7 days before fluorescence microscope evaluation.

All specimens were examined on a DM-RB fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) with 16/40x PL Fluotar/0.75 oil objectives and appropriate filters for red (PI) and green (Fluorescein) fluorescence detection. Scoring of nuclei in 2-cell to morula stage embryos was performed according to the criteria described below. Selected 2-cell to morula stage embryos and all blastocysts were subsequently subjected to confocal laser-scanning microscopy on a Leica TCS4D microscope (Leica Laser Technik, GmbH, Heidelberg, Germany) using an argon/krypton laser at 488 and 568 nm and two-channel scanning for detection of fluorescein isothiocyanate and PI respectively. Complete Z series of 20-25 optical sections at 3- to 4-µm intervals were acquired from each embryo using Leica Scanware software. With this sectioning interval, all nuclei appeared on at least two consecutive images, thereby assuring that all nuclei of an embryo were registered. Image stacks were reconstructed with a Silicon Graphics octane computer (SGI, Mountain View, CA) equipped with an Imaris image analysis software package (Bitplane AG, Zurich, Switzerland), and reconstructed confocal images were used for scoring of nuclei in the trophoblast (Tb) and inner cell mass (ICM) separately in each blastocyst. Allocation of nuclei to each embryonic compartment was based on position in the reconstructed images, and nuclei of Tb cells covering the ICM i.e. the polar Tb were for practical reasons included in the ICM, and the remaining mural trophoblast constituted the Tb compartment.

*Scoring of Nuclei*

The total number of nuclei was counted during scoring, and nuclear morphology was assessed on the basis of PI staining and was scored as being normal, condensed (i.e. pyknotic), or fragmented. Normal nuclei displayed loose reticulated chromatin content and sharp delineations, whereas condensed nuclei exhibited stronger PI staining of compacted chromatin in a decreased volume when compared with normal nuclei within the same embryo (Figure 1A). Condensed nuclei displayed sharp delineations, were often spherical in shape, and contained either a homogenous chromatin content or chromatin aggregated in marginalized clumps along the nuclear envelope, which sometimes gave the nucleus a lobulated appearance. Fragmented nuclei had two or more condensed chromatin fragments, also with sharp delineations (Figure 1B). A cluster of nuclear fragments confined in an area comparable to or smaller than the volume of a normal nucleus was regarded as originating from a single nucleus. Conversely, when two fragments were separated by a distance of at least the diameter of an average nucleus, they were regarded as originating from different nuclei. Some nuclei displayed a different mode of disintegration; they had increased PI staining intensity but lacked a reduction in volume and had an unclear or fluffy delineation (and were often TUNEL-positive; Figure 1C), and they were sometimes fragmenting into numerous minute elements in an expanded

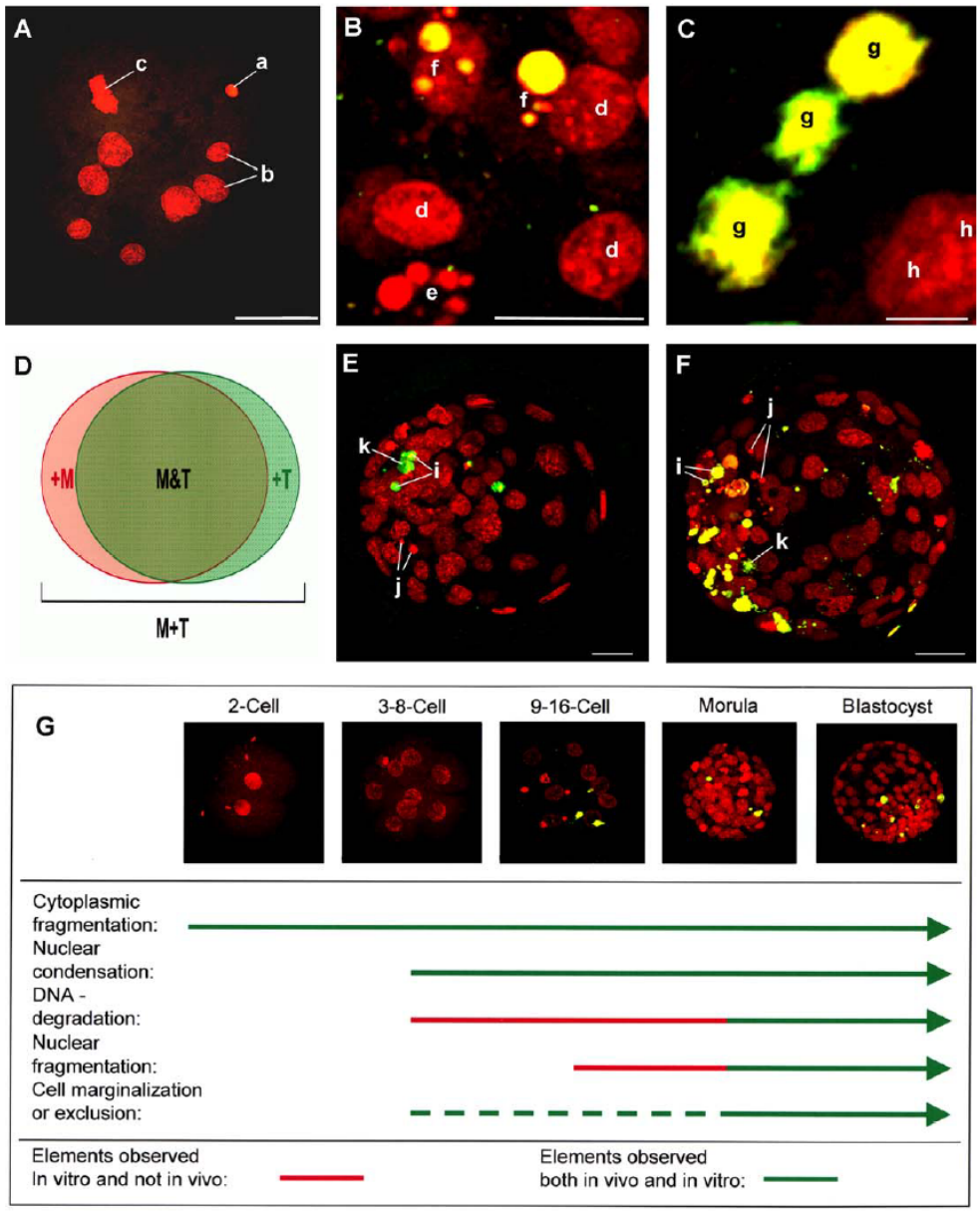



**Figure 1.** (A) Confocal laser scanning image of a 10-cell *in vivo*-produced bovine embryo. The chromatin content is stained by PI (red), fragmented DNA is labeled by the TUNEL reaction (green) and colocalization with PI is indicated as yellow. A highly condensed (pyknotic) but not fragmented nucleus (a) can be distinguished from normal appearing nuclei (b) with reticulated

chromatin content and the condensed chromosomes organized in a metaphase plate (c). The condensed nucleus is observed in a slightly marginalized blastomere at the periphery of the cell aggregation made up by the other embryonic blastomeres; however, it is not yet displaying the TUNEL reaction. (B) Confocal laser scanning images of embryonic nuclei. Note the normal appearing nuclei (d) and nuclei displaying typical morphological features of apoptosis (nuclear condensation and fragmentation by karyorhexis) without (e) or with (f) the TUNEL reaction. Nuclei and fragments thereof are all displaying sharp delineations. (C) Confocal laser scanning images of embryonic nuclei. Note the nuclei displaying the TUNEL reaction and a lack of volume reduction but with an unclear or fluffy delineation (g) when compared with normal nuclei (h: two partially overlaying nuclei). (D) Schematic diagram presenting the classification of nuclei according to two markers of apoptosis. Nuclei only displaying the morphological characteristic of apoptosis belong to the area termed +M (red), nuclei only displaying the biochemical characteristic of apoptosis (the TUNEL reaction) belong to the area termed +T (green), nuclei displaying both features (and fulfilling the criteria for apoptosis in this study) belong to the area termed M&T (overlap), and all nuclei displaying either one or both characteristics are defined as M+T (red and green). E and F) Confocal laser scanning images of *in vivo*-produced (E) and *in vitro*-produced (F) blastocysts. Note the presences of nuclei displaying apoptotic morphology with (i) or without (j) the TUNEL reaction and nuclei with the TUNEL reaction but no apparent nuclear condensation (k). Nuclei displaying apoptotic features are predominantly present in the ICM. (G) Schematic presentation of chronological occurrence of cytoplasmic fragmentation, changes in nuclear morphology, DNA degradation, and cell marginalization at various stages of bovine preimplantation development *in vivo* and *in vitro*. Cytoplasmic fragmentation, nuclear condensation, and cell marginalization (broken line) and extrusion (solid line) occur concurrently in embryos from the two production systems, whereas nuclear fragmentation and DNA degradation can be observed at earlier stages *in vitro* than *in vivo*. Bars = 20 μm.

volume. These nuclei were not classified as apoptotic because they could represent necrotic or other types of cell death, but they were included for calculation of the total numbers of nuclei.

DNA degradation was assessed by observation of a distinct TUNEL reaction of chromatin, and the nuclear origin of labeled material was verified by colocalization with PI staining. According to the criteria described above, nuclei were classified as: a) normal, b) displaying morphological characteristics of apoptosis (nuclear condensation with or without fragmentation; +M), c) displaying the biochemical characteristic of apoptosis (TUNEL-positive; +T), d) displaying both morphological and biochemical characteristics of apoptosis (M&T), or e) displaying one or both of the characteristics of apoptosis (M+T) (Figure 1D). In this study, nuclei were only regarded as apoptotic if in addition to being TUNEL-positive they also displayed apoptotic morphology, i.e., they were allocated to the M&T subset of nuclei. Indices (percentages) based on total number of nuclei were calculated for each subset respectively in morulae and separately for the ICM and Tb compartment and in total in blastocysts. To validate the scoring procedure, nuclei of ten randomly selected blastocysts were reassessed three times and variation between repeated scorings was less than 8% for all parameters.

*Statistical analysis*

Ratios of *in vivo*-produced and *in vitro*-produced embryos at different stages displaying at least one nucleus with apoptotic morphology and TUNEL reaction were compared by Chi-square tests, whereas indices of +M, +T, M&T, and M+T were compared using either unpaired Student t-tests with Welch correction after Kolmogoro-Smirnov test for normality or with a Mann-Whitney U-tests, when the data did not follow a normal (Gaussian) distribution. Correlation analysis between cell numbers and incidence of apoptotic nuclei in blastocysts was performed by a Pearson test assuming normal distribution of data. All tests were performed with a GraphPad InStat version 3.05 statistic software package (Graph Pad Software Inc., San Diego, CA).

## Results

*Chronological changes in apoptotic morphology and DNA degradation.*

A total of 213 preimplantation *in vivo* embryos and 201 preimplantation *in vitro* embryos were analyzed; the distribution of embryos at various developmental stages is presented in Table 1. Embryos subjected to preincubation in DNase (positive controls) displayed the TUNEL reaction in all nuclei, whereas when terminal transferase was omitted (negative controls) no labelling of any nuclei was observed (data not shown). Apoptotic morphology was not observed in any

2-cell embryos (Table 1), but it was first observed as nuclear condensation in a 6-cell *in vitro* embryo and an 8-cell *in vivo* embryo. Thus, apoptotic morphology was virtually not observable prior to the 4$^{th}$ cell cycle. A condensed nucleus of an 8-cell *in vitro* embryo displayed some degree of nuclear lobulation, but a classic apoptotic pattern of nuclear fragmentation was not observed until the 9-cell stage in *in vitro* embryos and the 21-cell stage in *in vivo* embryos. Thus, nuclear condensation could be observed at earlier developmental stages than nuclear fragmentation and appeared almost simultaneously *in vitro* and *in vivo*, whereas nuclear fragmentation occurred earlier *in vitro* than *in vivo*.

The earliest observation of the TUNEL reaction was in a condensed but not fragmented nucleus of a 6-cell *in vitro* embryo, which represented the earliest observation of apoptosis in the present study. The first observation of the TUNEL reaction *in vivo* was made in a condensed but not fragmented nucleus of a 21-cell embryo, whereas the first observations of nuclei displaying both nuclear condensation and fragmentation in combination with the TUNEL reaction were made in a 19-cell *in vitro* embryo and a 28-cell *in vivo* embryo. TUNEL reactions in nuclei without apoptotic morphology were first observed in an 18-cell *in vitro* embryo and a 60-cell *in vivo* embryo. However, apoptotic morphology and TUNEL reaction were observed in only a few embryos prior to the 16-cell stage, and when comparing the ratios of *in vivo* and *in vitro* embryos displaying at least one nucleus with either apoptotic morphology or apoptotic morphology and the TUNEL reaction (Table 1), no significant differences were observed at any stage (P>0.1).

**Table 1.** Numbers of embryos analyzed and occurrence and distribution of apoptotic changes in nuclear morphology and TUNEL reaction at different preimplantation stages of bovine embryos produced *in vivo* and *in vitro*. No differences in percentages were observed at any stage between *in vivo*- and *in vitro*-produced embryos (P>0.1, Chi-square test).

| | *In vivo* | | | *In vitro* | | |
|---|---|---|---|---|---|---|
| Developmental stage | n[A] | Morphology[B] | Morphology & TUNEL[C] | n[A] | Morphology[B] | Morphology & TUNEL[C] |
| 2 Cell | 33 | 0% (0/33) | 0% (0/33) | 31 | 0% (0/31) | 0% (0/31) |
| 3-8 Cell | 44 | 2% (1/44) | 0% (0/44) | 57 | 4% (2/57) | 2% (1/57) |
| 9-16 Cell | 38 | 11% (4/38) | 0% (0/38) | 36 | 25% (9/36) | 3% (1/36) |
| 17-Morula | 38 | 63% (24/38) | 47%(18/38) | 46 | 57% (26/46) | 43% (20/46) |
| Blastocyst | 35 | 100%(35/35) | 97%(34/35) | 31 | 100%(31/31) | 100%(31/31) |

[A] Number of embryos analyzed at each developmental stage.

[B] Percentage of embryos containing at least one nucleus displaying morphological characteristics of apoptosis.

[C] Percentage of embryos containing at least one nucleus displaying both morphological and biochemical (TUNEL) characteristics of apoptosis (apoptotic nuclei).

When nuclei with apoptotic morphology (with or without the TUNEL reaction) were observed in precompaction embryos, it predominantly occurred in marginalized blastomeres (Figure 1A). When such marginalized blastomeres were observed in the perivitelline space of postcompaction embryos, they were considered evidence of cell extrusion. These blastomeres were relatively large and bulky and often contained highly condensed nuclei, with or without fragmentation and the TUNEL reaction. However, some of these extruded blastomeres contained nuclei displaying a morphology that was not classified as apoptosis. Marginalization of blastomeres was observed from the 9- to the 16-cell stage in both *in vitro* embryos and *in vivo* embryos. At all embryonic stages examined, cytoplasmic fragments of various sizes with diffuse PI staining but no observable chromatin content were seen in both *in vivo* and *in vitro* embryos. Various cell changes indicating apoptosis are presented in Figure 1G.

*Incidences of apoptotic changes in morulae and blastocysts.*

In morulae and blastocysts, 84.1% and 87.7% of the TUNEL-positive nuclei (+T) observed *in vivo* and *in vitro*, respectively, also displayed apoptotic morphology (M&T). Thus, according to our criteria 15.9% and 12.3% of the TUNEL-positive nuclei were not regarded as apoptotic. At these stages, only 66.8% and 57.1% of nuclei with apoptotic morphology (+M) also displayed the TUNEL reaction (M&T) *in vivo* and *in vitro*, respectively. Thus, 33.2% and 42.9% of the nuclei with apoptotic morphology *in vivo* and *in vitro*, respectively, were not regarded as apoptotic.

*In vivo* morulae contained more nuclei (77.3 ± 6.6) than their *in vitro* counterparts (43.3 ± 3.4; $P<0.0001$), but when comparing percentages of nuclei displaying apoptotic morphology (+M; 3.4 ± 0.7% *in vivo* vs. 6.1 ± 1.4% *in vitro*), TUNEL reaction (+T; 2.4 ± 0.6% *in vivo* vs. 3.1 ± 0.1% *in vitro*), apoptosis (M&T; 2.0 ± 0.5% vs. 2.9 ± 0.9% *in vitro*), and one or both of the features (M+T; 3.9 ± 0.7% *in vivo* vs. 6.5 ± 1.5% *in vitro*), no differences were observed ($P>0.05$ for all parameters). At the blastocyst stage, no differences in numbers of cells in the ICM, Tb or the total blastocyst were observed between *in vivo* and *in vitro* embryos, but the percentages of nuclei scored as +M, +T, M&T, and M+T were all higher in the ICM than in the Tb regardless of production system ($P<0.01$; Figure 1, E and F). However, several differences were observed between *in vivo* and *in vitro* blastocysts (Table 2). The percentages of +M, +T, M&T, and M+T nuclei were all significantly lower for *in vivo* total blastocysts than for *in vitro* total blastocysts (+M, $P<0.0005$; +T, $P<0.05$; M&T, $P<0.05$; M+T, $P<0.001$), and these differences were the result of significant differences in percentages for the ICM (+M, $P<0.001$; +T, $P<0.05$; M&T, $P<0.05$; M+T, $P<0.001$), whereas no significant differences were observed for the Tb ($P>0.05$ for all parameters).

**Table 2.** Mean (±SEM) number of cell in the trophoblast (Tb), the inner cell mass (ICM), and the total blastocyst and percentage of nuclei displaying morphological changes of apoptosis (+M), TUNEL reaction (+T), both markers concurrently (M&T), and one or both (M+T) markers for bovine blastocysts produced either *in vivo* (n = 35) or *in vitro* (n = 31).

| | *In vivo* | | | *In vitro* | | |
|---|---|---|---|---|---|---|
| Classification[a] | Tb | ICM | Total | Tb | ICM | Total |
| No.cells | 62.1 ± 6.0 | 106.5 ± 5.8 | 168.5± 10.6 | 70.3 ± 5.7 | 105.4 ± 7.1 | 175.7 ± 11.3 |
| +M (%) | 2.4 ± 0.5 | 6.1 ± 0.7[b] | 4.8 ± 0.6[b] | 3.6 ± 0.6 | 11.2 ± 1.0[c] | 8.2 ± 0.7[c] |
| +T (%) | 2.4 ± 0.6 | 4.8 ± 0.7[b] | 3.4 ± 0.6[b] | 3.1 ± 0.5 | 7.3 ± 0.6[c] | 5.6 ± 0.6[c] |
| M&T (%) | 1.8 ± 0.5 | 4.2 ± 0.5[b] | 3.3 ± 0.5[b] | 2.5 ± 0.4 | 6.5 ± 1.0[c] | 4.9 ± 0.6[c] |
| M+T (%) | 2.9 ± 0.5 | 6.8 ± 0.8[b] | 5.4 ± 0.6[b] | 4.2 ± 0.6 | 12.1 ± 1.2[c] | 8.9 ± 0.8[c] |

[a] Percentages based on number of nuclei displaying morphological characteristics of apoptosis or TUNEL reaction divided by the number of nuclei in each embryonic compartment or in the total blastocyst.

[b,c] Values with different superscripts are significant different from those in the same embryonic compartment of *in vivo*- versus *in vitro*-produced embryos.

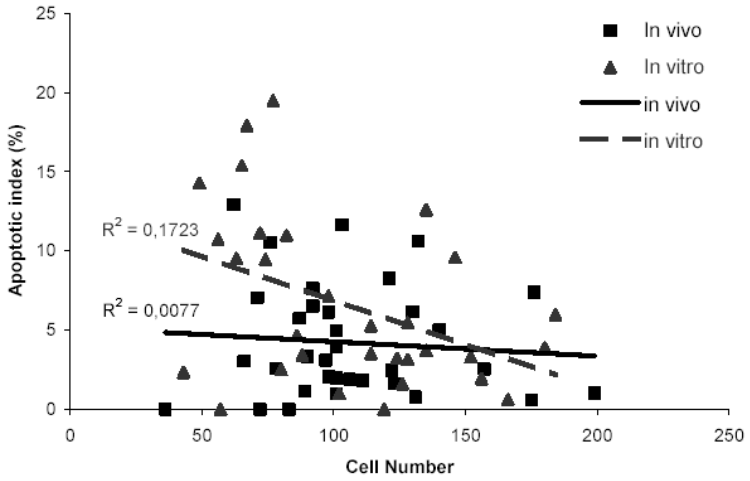



**Figure 2.** Relationship between cell number and apoptotic index in the ICM of bovine blastocysts produced either *in vivo* or *in vitro*. $R^{2a}$ and $R^{2b}$ are the correlation coefficients for observations made in the ICM *in vivo* and *in vitro*, respectively.

No significant correlation between total numbers of nuclei and the percentages of apoptotic nuclei were observed in blastocysts produced either *in vivo* ($R^2$=0.0186, P=0.4342) or *in vitro* ($R^2$=0.1109, P=0.0671)(data not shown). However, when comparing data for the ICM and Tb separately, a significant correlation between cell number and apoptotic incidence was observed in the ICM of *in vitro* embryos ($R^2$=0.1723, P=0.0202) but not *in vivo* embryos ($R^2$=0.0077, P=0.6170)(Figure 2). No such correlation was evident in the Tb of *in vivo* embryos ($R^2$=0.003239, P=0.7454) or *in vitro* embryos ($R^2$=0.01370, P=0.5307) (data not shown).

## Discussion

Occurrence and percentage of nuclei displaying morphological changes compatible with apoptotic cell death and DNA degradation detectable with the TUNEL reaction were assessed in this study. Only nuclei concurrently displaying features of both apoptotic morphology and the TUNEL reaction were regarded as apoptotic. This approach gives a relatively conservative estimate of apoptotic activity; however, there are several reasons for applying both morphological and biochemical markers simultaneously in assessing apoptosis. Morphological evaluation is crucial because different cell death pathways share biochemical features (Majno and Joris, 1995; Darzynkiewicz *et al.*, 2001; Leist and Jaattela, 2001). A substantial proportion of TUNEL-positive nuclei did not display concurrent apoptotic morphology, indicating that necrosis or possibly other modes of cell death may occur in apparently normal *in vivo* and *in vitro* embryos. However, TUNEL-positive nuclei lacking apoptotic morphology may also represent blastomeres suffering the fate of secondary necrosis (Wyllie, 1997; Darzynkiewicz *et al.*, 2001), which is a normal process in apoptotic cells that are not being adequately removed by phagocytosis. Biochemical markers of apoptosis are equally important to proper assessment. Nuclei displaying apoptotic morphology but no TUNEL reaction were observed at all embryonic stages after the 8-cell stage regardless of production system. Such a lack of TUNEL reaction may have different reasons. Extensive endogenous DNA degradation is a relatively late event in the apoptotic cascade (Collins *et al.*, 1997), and some nuclei with apoptotic morphology and no TUNEL reaction may not have reached this stage yet. Other possibilities could be misinterpretation of prophase nuclei, which may display increased staining intensity caused by higher chromatin content than that seen in interphase nuclei, or the irregular appearance of metaphase plates or telophase nuclei on confocal optical sections, making them hardly distinguishable from condensed and lobulated or fragmenting nuclei. These problems emphasize the need for adequate staining and confocal imaging procedures, even though great efforts were invested in these areas. Nuclei also

may become fragmented by mechanisms independent of the apoptotic program, such as uncontrolled chromosome segregation during mitosis, which may generate micronuclei-like structures by dislodgement of chromosomes. The complex expression of morphological and biochemical markers of apoptosis highlights the need for a combination of criteria when occurrence and function of apoptosis is assessed (Darzynkiewicz *et al.*, 2001). This is the first study to investigate the independent chronological appearance of apoptotic nuclear morphology and DNA degradation during preimplantation development both *in vivo* and *in vitro*, and the results support the argument for implementing such an approach when quantification of apoptosis in embryos is desired.

Expression of different morphological and molecular elements of the apoptotic program may be developmentally regulated in mammalian embryos. Cellular fragmentation has been reported in the earliest stages of development both here and in other studies (Hardy, 1997; 1999), but it is controversial whether this feature as such represents true apoptotic activity (Antczak and Van Blerkom, 1999). However, the fragmentation of cells into membrane-bound elements is a key feature of the apoptotic process (Wyllie *et al.*, 1980), and its occurrence in cleavage stages, without other signs of apoptosis, may represent a specific activation of a subsection of the apoptotic machinery responsible for this characteristic. In this study, extensive nuclear condensation became apparent at the 6-cell stage concurrently with the first observation of DNA degradation in *in vitro* embryos. This observation is consistent with those of previous studies of bovine *in vitro* embryos, where apoptosis was first observed at the 8-cell stage as indicated by either apoptotic morphology and the TUNEL reaction (Byrne *et al.*, 1999) or the TUNEL reaction alone (Matwee *et al.*, 2000; Paula-Lopes and Hansen, 2002). This is the first study to investigate the comparable chronological appearance of apoptotic markers in both *in vivo*-produced and *in vitro*-produced bovine embryos. IVP in this species seems to affect the developmental regulation of DNA degradation, because this apoptotic element was not detectable before the 21-cell stage *in vivo*. The occurrence of DNA degradation *in vivo* is more consistent with observations in other species, where apoptotic morphology or TUNEL reaction was not observed prior to blastocyst formation around the 32-cell stage in mouse *in vivo* and *in vitro* embryos (Handyside and Hunter, 1986; Brison and Schultz, 1997), or prior to compaction in normal developing human *in vitro* embryos (Hardy, 1999). Progress to the 8-cell stage coincides with the time of major genome activation in cattle, whereas this event occurs around the 2-cell stage in mouse and human embryos (Memili and First, 2000). The concurrence of genome activation with competence for DNA degradation in bovine *in vitro* embryos may be caused by deviant activation of apoptosis suppressing genes in some of these embryos (Jurisicova *et al.*, 1998a; Matwee *et al.*, 2001). The present results indicate that passing this point of development under presumably optimal conditions *in vivo* does not result in a similar activation of the apoptotic

program, suggesting a tentative relation between accelerated apoptotic activity and deviant genome activation in preimplantation bovine embryos. However, the onset of apoptosis is not observed before compaction in both mouse and human *in vitro* embryos, possibly because the relation between genome activation and onset of apoptosis is not as straightforward or because the IVP systems are more optimized in these species.

Fragmentation of condensed nuclei by karyorhexis is another key element of apoptosis that may be affected by IVP. This feature was not observed before the morula stage *in vivo*, but it seemed accelerated to the 9- to 16-cell stage *in vitro*. Thus, different features of apoptosis appeared at various developmental stages, they were differentially affected by IVP, and their appearance may be dependent on species-specific characteristics.

In cattle, the appearance of different apoptotic elements is not fixed, and IVP affects their occurrence. Various molecular components of the apoptotic cascade are present in early cleavage stages of mouse (Exley *et al.*, 1999), human (Warner *et al.*, 1998) and bovine (Kölle *et al.*, 2002; Paula-Lopes and Hansen, 2002) embryos. These findings are supported by results from chemical induction of the TUNEL reaction at stages where apoptosis is not occurring spontaneously in both mouse (Weil *et al.*, 1996) and bovine (Matwee *et al.*, 2000) *in vitro* embryos. Such results indicate that cleavage stage blastomeres constitutively process the machinery to run the apoptotic program if adequately provoked. However, whereas chemical induction may activate the full apoptotic machinery, stressors or sublethal insults may only partially activate the process in preimplantation embryos. A recent study of bovine *in vitro* embryos documented that heat stress can induce the TUNEL reaction at the late 8- to 16-cell stage but not at the 2- to 4-cell stage (Paula-Lopes and Hansen, 2002); however, an effect of heat stress on subsequent cell numbers was observed in 2- to 4-cell embryos, and nuclear fragmentation was found in a heat stressed 2-cell embryo, documenting that this feature of apoptosis was affected by the treatment. Thus, the difference in effect of IVP and stress on developmental regulation of nuclear fragmentation and DNA degradation may indicate governance of these discrete apoptotic features by separate mechanisms in early embryo stages. This hypothesis is supported by results in other cell systems, where changes in nuclear morphology and DNA degradation are probably differentially regulated (Collins *et al.*, 1997). Culture conditions that cause an increased incidence of the TUNEL reaction in preimplantation embryos (Pampfer *et al.*, 1997; Wu *et al.*, 2000) have no significant effect on the incidence of nuclear fragmentation. If the gradual occurrence of different apoptotic features during preimplantation development reflects a progressive release of repression of these elements in the constitutively present program, then the release of this repression could possibly be individually modulated for each feature by ambient conditions such as IVP procedures and various stressors. Thus, premature occurrence of different

apoptotic features may serve as indicators of stressors that may affect one but not necessarily all features of apoptosis in early embryonic stages.

The postimplantation developmental potential or embryo quality is likely to be affected by apoptotic incidence in preimplantation stages. Thus, the degree and patterns of cell fragmentation have an impact on implantation and development (Alikani *et al.*, 1999), and culture conditions that decrease embryonic cell number and increase the apoptotic incidence also decrease implantation rates, increase fetal resorption, and lower fetal birth weight upon embryo transfer (Wuu *et al.*, 1999). A negative correlation between embryonic cell number and incidence of the TUNEL reaction has been established in both mouse (Brison and Schultz, 1997; Devreker and Hardy, 1997) and bovine embryos (Byrne *et al.*, 1999), and the incidence of the TUNEL reaction is higher in mouse *in vitro* embryos than in similar *in vivo* embryos (Brison and Schultz, 1997). In the present study, almost every *in vivo* and *in vitro* blastocyst displayed at least one apoptotic nucleus. This finding is consistent with those of previous studies (Byrne *et al.*, 1999; Matwee *et al.*, 2000; Neuber *et al.*, 2002) and indicates the universal occurrence of this cellular process during normal bovine development. Although the appearance of apoptotic characteristics was developmentally accelerated by IVP, these features were only observed in insignificant numbers of precompaction embryos. Likewise, incidence of apoptosis at the morula stage was not significantly affected. These results indicate that even though IVP affects the chronological occurrence of apoptosis, a substantial impact on apoptotic incidence may not occur prior to compaction. However, a stage-specific decrease in apoptosis was previously reported in bovine *in vitro* morulae (Byrne *et al.*, 1999) and could explain the lack of difference in apoptotic activity at this specific stage. The higher number of cells of *in vivo* morulae could have been generated by erroneous inclusion of blastocysts that may have collapsed during the flushing and collection procedure, thereby biasing this experimental group.

The incidence of apoptotic nuclei and nuclei displaying apoptotic features was higher in bovine *in vitro* blastocysts than in their *in vivo* counterparts. This finding is similar to that for mouse embryos (Brison and Schultz, 1997) and supports a relation between incidence of cell death and developmental potential. Apoptotic incidence was higher in the ICM than in the Tb compartment regardless of production system, as has been observed in other studies of *in vitro* blastocysts from cattle (Matwee *et al.*, 2000; Makarevich and Markkula, 2002; Neuber *et al.*, 2002), mice (Devreker and Hardy, 1997) and rats (Pampfer, 2000). However, such a difference is not apparent in human embryos (Hardy *et al.*, 1989). Differences observed at the blastocysts stage were specifically based on differences of cell death activity in the ICM; no differences were observed in the Tb compartment between production systems. This finding may have substantial importance, because the pluripotent ICM forms the future embryo proper, and damaging effects only affecting this embryonic compartment may result in

blastocysts that appear normal at the stereo microscopical level but that carry subcellular deviations that could impact on developmental competence (Maddox-Hyttel *et al.*, 2003).

Apoptosis occurs during normal preimplantation development of bovine embryos produced *in vivo* and *in vitro*. By examining nuclear morphology and the incidence of the TUNEL reaction, apoptosis was identified from the 6-cell stage *in vitro* and the 21-cell stage *in vivo*, and a higher incidence of apoptotic cells was observed in blastocysts derived *in vitro* than in their *in vivo* counterparts. This difference at the blastocyst stage was specifically based on higher levels of apoptosis in the *in vitro* ICM.

**Acknowledgments**

The authors would like to thank Anne Dorthe Rasmussen for technical assistance with *in vitro* embryo production, Susanne Holm Kristiansen, for technical assistance with the TUNEL reaction, Michael Hansen for help with confocal equipment, Jytte Nielsen for technical assistance with image processing, Bo Martin Biby for advice on statistical procedures, and to technical personnel animal care and handling.

# Chapter 5

## Consequences of *in vivo* development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos

**Abstract**

Bovine embryos produced *in vitro* differ considerably in quality from embryos developed *in vivo*. The *in vitro* production system profoundly affects the competence to form blastocysts, the number of cells of the total embryo and of the inner cell mass (ICM), and the incidence of apoptosis. To our knowledge, the effects of different postfertilization regimens before and after completion of the 4$^{th}$ embryonic cell cycle on these aspects have not yet been investigated. In the present study, we assessed the blastulation rate by stereo microscopy and the cell number of the total embryo, of the ICM, and of cells with apoptotic changes by confocal laser-scanning microscopy after staining with propidium iodide and TUNEL. Two groups of embryos were developed in heifers, after superovulation, until 45 or 100 h postovulation (po) and, after collection on slaughter, were further cultured *in vitro* until Day 7 po. A third and fourth group comprised embryos that were produced entirely *in vitro* or *in vivo*. The results indicate that passage *in vivo* of the 4$^{th}$ cell cycle does not prevent acceleration of the formation of the blastocoele *in vitro* but may be the critical factor contributing to a higher cell number in the total blastocyst and its ICM. The lower quality of *in vitro*-produced embryos can be attributed to the ICM having less viable cells because of a lower number of cells and a higher incidence of apoptosis that appears to be determined before completion of the 4$^{th}$ cell cycle.

**Authors:** Hiemke M. Knijn[1], Jakob O. Gjørret[2], Peter L.A.M. Vos[1], Peter J.M. Hendriksen[1], Bert C. van der Weijden[1], Poul Maddox-Hyttel[2], Steph J. Dieleman[1]
[1]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*
[2]*Department of Anatomy and Physiology, Royal Veterinary and Agricultural University, DK-1850 Frederiksberg C, Denmark*

Biology of Reproduction (2003) 69, 1371-1378

## Introduction

Despite much effort to improve the technique of *in vitro* production of bovine embryos, differences remain between *in vitro* - and *in vivo*-derived embryos. These differences are twofold. First, in general, 30% of the oocytes derived from slaughterhouse ovaries or obtained by transvaginal ovum pick-up are competent to develop into a blastocyst (Merton *et al.*, 2003), whereas *in vivo*, approximately 90% of ovulated oocytes from cyclic cows are fertilized after insemination with very few embryos being lost up to Day 8 (Sreenan *et al.*, 2001). Second, the quality of the *in vitro*-derived embryo is diminished in comparison to that of embryos developed *in vivo*. After transfer of embryos produced *in vitro*, approximately one-third do not implant or do not attach, and the health of a small proportion of the newborn calves is impaired (van Wagtendonk-de Leeuw *et al.*, 2000).

A variety of morphological and biochemical parameters have been studied in relation to the developmental competence of the oocytes and to embryo quality during *in vitro* production. These studies showed that bovine embryos produced *in vitro* differ from their *in vivo* counterparts in many aspects, such as morphology, cell number of the total embryo and of the inner cell mass (ICM) (van Soom *et al.*, 1997), expression of specific genes (Wrenzycki *et al.*, 1996; Knijn *et al.*, 2002), and incidence of chromosomal abnormalities (Viuff *et al.*, 2001).

In the above-mentioned studies, *in vivo*-developed morulae and blastocysts were compared with embryos for which maturation, fertilization and culture had been performed *in vitro*. Recently, some data have been reported concerning the effects of the respective, separate steps of the *in vitro* procedure on embryo quality. *In vivo*-occurring prematuration and maturation of the oocyte are crucial for enhancing the competence of the oocyte to develop *in vitro* into a blastocyst (Hendriksen *et al.*, 2000, review), but they have less influence on the quality of the embryo. Different modes of maturation, *in vitro* or *in vivo*, of prematured oocytes did not influence the level of expression of six developmentally important genes in blastocysts (Knijn *et al.*, 2002), but they did have a moderate effect on the incidence of chromosomal aberrations in cells of the blastocyst (Dieleman *et al.*, 2002). The postfertilization period appears to be the most important step in determining the quality of the embryo. Postfertilization culture *in vitro* reduced the postthaw survival rate of blastocysts following cryopreservation (Rizos *et al.*, 2002b) and modified expression of different genes (Rizos *et al.*, 2002a). To unravel the effects of *in vitro* culture (IVC) versus *in vivo* development on the quality of the embryo, we investigated morulae and blastocysts after temporary development *in vivo* and subsequent culture *in vitro*. The blastulation rate is a criterion for evaluating the quality of *in vitro*-produced embryos (Bavister, 1995). In the cow, blastulation occurs at an earlier point of time after insemination in

embryos derived *in vitro* than in embryos developed entirely *in vivo* (van Soom *et al.*, 1997). In particular, the presence of serum during culture accelerates the formation of the blastocoele (Pinyopummintr and Bavister, 1994). As a measure for cleavage rates, the number of cells of the total embryo and of the ICM has often been used to evaluate embryo quality. Small differences between *in vitro*- and *in vivo*-derived embryos already occur during development up to the 8-cell stage (Viuff *et al.*, 2001), but thereafter, until the blastocyst stage, the cell number is significantly smaller after IVC (Thompson, 1997; Enright *et al.*, 2000; Viuff *et al.*, 2001). The cell number of the ICM is also decreased after IVC (van Soom *et al.*, 1997, Viuff *et al.*, 2001). The cell number of the total embryo and the ICM both appeared to be dependent on the culture medium used (van Soom *et al.*, 1997). Therefore, the environment during early embryonic development clearly plays a role determining the cell numbers of the embryo.

Although apoptosis can be considered to be a normal process in preimplantation embryos to eliminate deviating cells, a high incidence of apoptotic cells is correlated with abnormal morphology of the embryo (Hardy *et al.*, 1989). In *in vitro*-produced mouse blastocysts, the percentage of apoptotic cells is significantly higher than in their *in vivo*-developed counterparts (Brison and Schultz, 1997; Hardy, 1997). Also, data from the cow indicate that *in vitro*-produced blastocysts possess a higher level of apoptosis than embryos developed completely *in vivo* (Gjørret *et al.*, 2001). Furthermore, the presence or absence of factors in the culture medium can affect the incidence of apoptosis in mouse (Brison and Schultz, 1997; Hardy, 1997, Moley *et al.*, 1998) and bovine embryos (Byrne *et al.*, 1999). It has been suggested that “survival factors” produced by the embryo itself and by the maternal reproductive tract regulate the incidence of apoptosis (Brison and Schultz, 1997; Hardy, 1997). Although the environment during early embryonic development evidently influences the level of apoptosis in mammalian blastocysts, it is not known at which stage during IVC increased levels of apoptosis are induced.

In the present study, embryos that had been collected *in vivo* before or after the major genome activation were cultured. The major genome activation presumably starts during the fourth cell cycle (de Sousa *et al.*, 1998) that, *in vivo*, takes place between 45 to 100 h postovulation (po) (Vos *et al.*, 2003). Therefore, embryos from superovulated heifers were flushed from the genital tract 45 and 100 h po and, subsequently, cultured until Day 7 po. For comparison, Day 7 embryos that were derived *in vitro* from slaughterhouse oocytes or after development entirely *in vivo* were analyzed. The quality of the embryos was assessed by determining the blastulation rate, the cell number of the total embryo and of the ICM, and the level of apoptosis in the embryo.

## Materials and Methods

*Animal treatment*

To obtain embryos at precisely defined times after ovulation, heifers were treated for superovulation using a procedure with a controlled LH surge as described by Vos et al., (1994) with slight modifications. Clinically healthy, nonlactating Holstein-Friesian heifers were selected from the experimental herd of the Veterinary Faculty of Utrecht University on the basis of cyclicity, as established by measuring progesterone levels in peripheral blood samples taken three times a week for at least four weeks before the experiment started. The heifers were fed silage and concentrate and were supplied water ad libitum. The experiments were carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University in December 2000 (first session, n=32 heifers) and October 2001 (second session, n=27 heifers).

The heifers were presynchronized using an ear implant for 9 days (3 mg norgestomet; Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet and 5 mg estradiol-valerate, i.m.. Two days before the implant was removed, prostaglandin (PG)(15 mg, Prosolvin; Intervet International BV) was administered i.m. to ensure complete regression of the corpus luteum. On Day 8 of the synchronized cycle (estrus = Day 0), all follicles larger than 5 mm were ablated by transvaginal, ultrasound-guided puncturing to synchronize follicular development. At the time of puncturing, one of the animals was excluded from the experiment, because it showed cystic ovaries. On Day 9, the remaining heifers (n = 58) were administered another ear implant (Crestar) for 5 days, but without the additional administration of norgestomet and estradiol-valerate. From Day 10 onward, ovine FSH (Ovagen; ICP, Auckland, New Zealand) was administered i.m., twice daily, with decreasing doses during 4 days: on the first day, 2.0 mL; on the second day, 1.5 mL; on the third day, 1.0 mL; and on the fourth day, 0.5 mL (10 mL in total, equivalent to 176 IU NIH-FSH-S1). Prostaglandin (22.5 mg Prosolvin) was administered i.m. concomitant with the fifth dose of FSH, and 55 h later, the ear implants were removed and GnRH administered (1.0 mg Fertagyl in 10 mL saline i.m.; Intervet International BV) to induce an LH surge. Heparinized blood samples from the jugular vein were collected daily during the experimental cycle, every 3 h from 24 h after PG administration and every hour during the period 7-h period after removal of the second implant. After immediate centrifugation (1800 **g** for 10 min) at 4°C, plasma was stored at -25°C. All animals were inseminated 12-14 h after GnRH administration with two straws of semen from a known-fertile bull (one straw/uterine horn). In both sessions, the heifers were assigned at random to the three *in vivo* groups. The time point of 24 h after the LH surge was taken as the starting time of multiple ovulations. The time between expected ovulation and

slaughter to collect embryos was 45-48 h for the 45 h *in vivo* group, 100-103 h for the 100 h *in vivo* group and 165-168 h for the *in vivo* group (Figure 1).

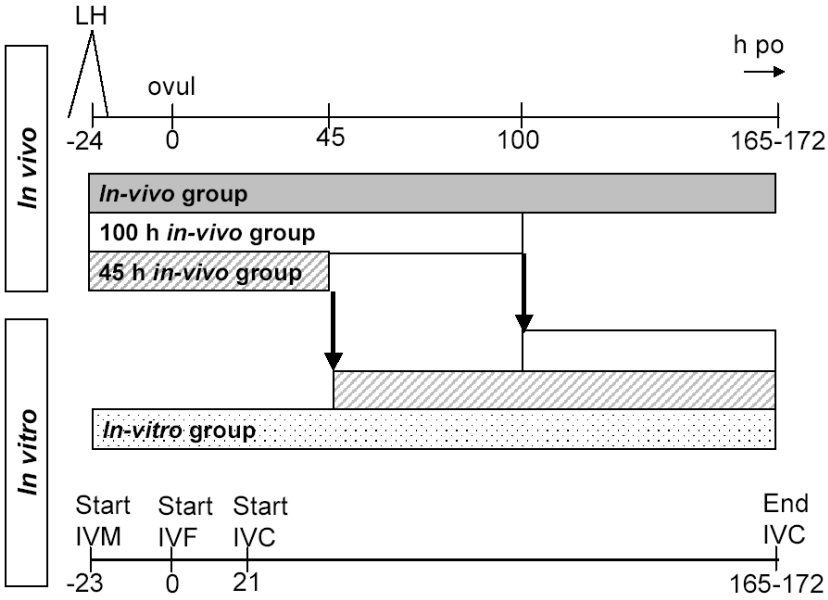



**Figure 1.** Schedule of temporary development *in vivo* (upper panel) and subsequent culture *in vitro* (lower panel) to collect embryos at Day 7 po from FSH-stimulated heifers. The arrow indicates the time of transition from development *in vivo* to culture *in vitro*. For comparison, the schedule for embryos produced completely *in vitro* is depicted (lower panel). IVM, *In vitro* maturation; IVF, *in vitro* fertilization

*Embryo collection for the in vivo groups*

After slaughter, genital tracts were placed in saline (37°C) and transported immediately to the laboratory in a thermocontainer. Per genital tract, the number of corpora lutea on the ovaries was counted. The time period between slaughter and flushing ranged from 45 min to 2 h. Oviducts and uterine horns were flushed with PBS (PBS-ET; Bio Whitaker Europe, Verviers, Belgium) of 37°C using a blunt needle and from the infundibulum towards the uterine horn under gentle massage. For the 45 h *in vivo* group, only the oviducts with the top of the uterine horn were flushed. The PBS was collected into an embryo-recovery filter (Embryo Concentrator; Immuno Systems Inc., Spring Valley, WI), and the embryos were collected by rinsing the filter with saline supplemented with 0.005 % (w/v) BSA (A 6003; Sigma, St Louis, MO).

*Embryo culture, in vivo groups*

The embryos were transferred to the culture medium within 2 h after slaughter. The developmental stage of the viable and nonviable embryos (i.e., of all

embryonic structures) was assessed by stereo microscopy. The 1-cell and degenerated embryos, as well as, at 100 h po, the embryos at the 2- to 7-cell stage were characterized as nonviable and excluded from further investigation. Before culture, embryos were washed in culture medium consisting of synthetic oviduct fluid medium (SOF) with BSA as described by van Wagtendonk-de Leeuw et al. (van Wagtendonk-de Leeuw *et al.*, 2000). The embryos were grouped per heifer and at most, 10 ex vivo-collected embryos were placed in 20 µL droplets of SOF under oil (Mineral oil for IVF; Reproline medical GmbH, Rheinback, Germany) (van Wagtendonk-de Leeuw *et al.*, 2000). *In vitro* culture (IVC) was performed at 39°C in humidified air of 5% $CO_2$, 7% $O_2$ and 88% $N_2$. Morulae and blastocysts were collected after 120 h of culture for the 45 h *in vivo* group and 72 h for the 100 h *in vivo* group (Figure 1).

*Embryo Culture, in vitro group*

A control group of embryos was collected that was produced completely *in vitro*. Cumulus-oocyte complexes were aspirated from 3- to 8-mm follicles of ovaries that had been collected at a local abattoir, but only those with a multilayered, compact cumulus investment were used for the experiments. The cumulus-oocyte complexes were rinsed once with Hepes-buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL) and once with maturation medium M199 supplemented with 10% FCS, 0.01 IU/mL recombinant human FSH (Organon, Oss, The Netherlands), 11.36 µg/mL of cysteamine (M-6500, Sigma) and 1% (v/v) penicillin-streptomycin (Gibco BRL). Groups of 35 oocytes were allocated at random to each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µL of maturation medium with the additions described above. After maturation for 22 h (39º C, 5% $CO_2$ in humidified air), all oocytes were fertilized.

Procedures for *in vitro* fertilization (IVF) were performed as described by Parrish et al., (1988) with minor modifications (Izadyar *et al.*, 1996) using semen from the same bull as used in the *in vivo* experiments for artificial insemination. After 21 h of incubation (39°C, 5% $CO_2$ in humidified air), the presumptive zygotes were freed from cumulus cells by vortexing for 3 min, and a maximum of 10 zygotes was placed in 20 µL droplets of SOF and cultured as described above. All cleavage stages were transferred to fresh culture droplets at Day 5 after the start of fertilization. Morulae and blastocysts were collected 165 h after the start of fertilization.

*Determination of embryonic stage and fixation*

The developmental stage (morula, early blastocyst, blastocyst, expanded blastocyst and hatched blastocyst) of all embryonic structures at Day 7 was assessed and scored by stereo microscopy. The embryonic structures that had not attained the morula or blastocyst stage at 165-172 h po (*in vivo* groups) or

after fertilization (*in vitro* group) were excluded from further analysis. These embryos were considered to be nonviable.

Half of all collected morulae and blastocysts were used for TUNEL; the other half was stored for another study. For TUNEL, embryos were washed three times with PBS containing 1 mg/mL polyvinylacohol (P-8136, Sigma) and then fixed in 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 1-2 h at room temperature. After fixation, the embryos were stored in 1% paraformaldehyde in PBS and were processed for TUNEL within 2 weeks.

*TUNEL Assay*

Nuclei with degraded DNA were detected by using a cell death-detection technique based on TUNEL principle (Gavrieli *et al.*, 1992) using fluorescein-conjugated dUTP as described previously (Brison and Schultz, 1997), (Byrne *et al.*, 1999) with minor modifications. The embryos were washed three times in PBS with polyvinylpyrrolidone (PBS/PVP; 1 mg/mL; Sigma), permeabilized for 1 h in PBS with 0.5% (v/v) Triton X-100 (Sigma), and washed twice in PBS/PVP. As positive controls, embryos were incubated in 50 U DNAse/mL PBS (RQ1; Promega, Bie & Bernsten, Rødovre, Denmark) for 30 min at 37°C and then washed two times in PBS/PVP. Embryos were incubated in 10 µL of terminal deoxynucleotidyl transferase and 90 µL fluorescein-conjugated dUTP (TUNEL, In situ Cell Death Detection kit; Roche, Hvidovre, Denmark) for 60 min at 37°C in the dark. For negative controls, embryos were not incubated with the terminal transferase enzyme. The embryos were washed twice in the Triton X-100 in PBS and once in PBS/PVP. Embryos were washed one time in Tris-buffer (40 mM Tris, 10 mM NaCl, and 6 mM $MgCl_2$; pH 8.0) and then incubated with 0.1 mg/mL RNAse A (Sigma) in Tris-buffer for 1 h at 37°C in the dark. The nuclear material was counterstained with 10 µg/mL of propidium iodide (PI; Sigma) in the Tris-buffer for 45 min at 37°C in the dark. Embryos were then transferred through a gradient of Vecta-Shield (Vector laboratories, Burlinghame, CA) at 50%, 75%, and 100% (v/v) in PBS in the dark, with each step lasting for 30 min; in the 100% Vecta-Shield, 0.05 µg PI per mL was added. Then, the embryos were mounted on a slide with ring enforcement in 4 µl of the 100% Vecta-Shield solution and covered with a coverslip. Slides were stored at 4°C for up to 7 days before confocal laser-scanning was performed.

*Microscopy and Image Processing*

The embryos were subjected to confocal laser-scanning microscopy on a Leica TCS4D microscope (Leica Laser Technik GmbH, Heidelberg, Germany) fitted with 25/40x PL Fluotar/0.75 oil objectives. An argon/krypton laser was used for excitation at 488 and 568 nm, and two-channel scanning was performed with double-dichroic DD488/568 beam splitter and a band-pass BP530 barrier and long pass OG590 barrier filter for detection of TUNEL reaction and PI, respective-

ly. A complete Z series of 20-25 optical sections at 3- to 4-µm intervals was acquired from each embryo using Leica Scanware software (Leica Laser Technik), and image stacks were reconstructed on a Silicon Graphics computer equipped with an Imaris image-analysis software (Bitplane, Zurich, Switzerland) package.

*Quantitative analysis of TUNEL labeling and apoptosis*

Digitally recombined, composite images were analyzed using the Imaris software. All 20-25 optical sections were divided using a standard grid over each layer to count all nuclei as a measure of the cell number of the total embryo. Nuclei were scored for TUNEL labeling, signs of fragmentation and condensation. Cells were judged to be apoptotic when the nucleus displayed both the biochemical feature (TUNEL labeling) and the morphological feature (fragmentation and/or condensation) as described by Gjørret et al. (2003b). The apoptotic index of the embryos was calculated as the percentage of apoptotic cells relative to the total number of cells. Allocation of nuclei to ICM and Trophectoderm (TE) was based on their position in the reconstructed images (Figure 2F). The nuclei belonging to the polar TE were counted together with the ICM nuclei, leading to an overestimation of the ICM. Differential staining of the ICM to provide a more accurate number of nuclei for the ICM (van Soom *et al.*, 1996) was not performed, because this technique cannot be combined with TUNEL labeling.

Validation of the counting of the cells and TUNEL positive (T+) cells was done on a random selection of 10% (20/194) of all embryos. After renewed allocation of the nuclei to the ICM and TE, counting was performed a second time. The proportional difference between the first and second countings was 0.9% for the cell number per total embryo and 1.5% for the level of T+ nuclei. For the number of nuclei in the ICM and TE, the differences were both 1.6% and, for the level of T+ in the ICM and TE the differences were 2.3% and 4.3%, respectively.

*Radioimmunoassays for progesterone and LH*

Concentrations of progesterone in plasma were estimated by a solid-phase $^{125}$I RIA method (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions as validated previously (Dieleman and Bevers, 1987). The sensitivity was 0.15 nmol/ L, and the intra- and interassay coefficients of variation were 8 and <11%, respectively.

Concentrations of LH in plasma were estimated by a validated, homologous RIA method as described previously (Dieleman *et al.*, 1983a). The sensitivity was 0.4 µg/L NIH-LH-B4. The intra- and interassay coefficients of variation were <9%.

*Statistical analysis*
Correlation analysis between cell number and incidence of apoptosis was performed by linear regression test. All tests were performed using SPSS 8.0 statistical software (SPSS Inc., Chigaco, IL).

## Results

*LH*
No differences were observed between the two sessions with regard to the LH surge. On average, it occurred 2.5 h after the administration of GnRH, with a maximum level (mean ± SEM) of 22.4 ± 1.2 µg/L (n=56). In two heifers an increase of the LH concentration was observed some hours before the GnRH administration. The LH data of these two animals were not included.

*Embryo collection in vivo*
In the majority of the heifers, a variable proportion of the collected embryonic structures was characterized as nonviable. The embryos of the few animals (n=7) with an exceptionally high proportion of nonviable embryonic structures (>85%) were not used for the experiment.

The superovulatory responses with regard to the number of corpora lutea and the recovery rate of the embryonic structures relative to the number of corpora lutea were similar between the two sessions. The recovery rate was not different for the 45 h *in vivo*, 100 h *in vivo* and *in vivo* groups being 75% (n=18 heifers), 66% (n=13), and 70% (n=20), respectively. The developmental stages of the embryos flushed at 45 h, 100 h, and Day 7 po are shown in Tables 1 and 2.

**Table 1**. Number and characteristics of embryonic structures collected at 45 and 100 h po from FSH-stimulated heifers.

| Group (n *) | Tot. | Nonviab. | Viable embryos (n [%]) ** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (n) | (n [%]) ** | Developmental stage (c = cell) | | | | | | |
| | | | 2-4 c | 5-7 c | 8 c | >8 c | 8-15 c | 16-32 c | morula |
| 45 h *in vivo* | 167 | 9 | 9 | 48 | 98 | 3 | | | |
| (18) | | (5) | (5) | (29) | (59) | (2) | | | |
| 100 h *in vivo* | 163 | 44 | | | | | 32 | 62 | 25 |
| (13) | | (27) | | | | | (20) | (38) | (15) |

* number of heifers.

** percentages nonviable embryos (Nonviab)are calculated relative to the total number of embryonic structures collected; viable embryos were cultured until Day 7 po.

*Embryo culture results and developmental rate*

A significantly higher (P<0.05) proportion of the embryos selected for culture developed *in vitro* to the morula or blastocyst stage when culture was started after 100 h of development *in vivo* (90%) than after 45 h (68%) (Table 2). In both the 45- and 100 h *in vivo* groups, the proportion of embryos developing further *in vitro* was much higher than the proportion of oocytes developed completely *in vitro* to the morula and blastocyst stage (29% for the *in vitro* group). The proportion of embryos at the blastocyst stage was high in the 45- and 100 h *in vivo* groups (91% and 100%, respectively), similar to that in the *in vitro* group (91%), and the proportion of embryos at the morula stage was low. On the contrary, after development entirely *in vivo*, the proportions of embryos at the morula and blastocyst stage were more alike (43% and 57%, respectively)(Table 2).

**Table 2.** Rates of morula and blastocyst formation at Day 7 po in relation to time of development *in vivo* and culture *in vitro*.

| Group(n *) | Non-viable embryos (n) | Number of viable embryos | | | | | |
|---|---|---|---|---|---|---|---|
| | | Total (h) | Developmental stage (n [%]) ** | | | | |
| | | | morula | early blastoc. | blastocyst | expanded blastocyst | hatched blastocyst |
| *In vitro* (279) | 138 *** | 81 | 7 (9)$^{a}$ | 7 (9) | 16 (20)$^{a}$ | 50 (61)$^{a}$ | 1 (1)$^{a}$ |
| 45 h *in vivo* (158) | 51 | 107 | 10 (9)$^{a}$ | 16 (15)$^{a}$ | 19 (18)$^{a}$ | 51 (47)$^{a}$ | 11 (10)$^{b}$ |
| 100 h *in vivo* (119) | 12 | 107 | 0 (0)$^{b}$ | 7 (6)$^{b}$ | 21 (20)$^{a}$ | 62 (58)$^{a}$ | 17 (16)$^{bp}$ |
| *In vivo* (170) | 42 | 128 | 55 (43)$^{c}$ | 17 (13) | 17 (13)$^{a}$ | 33 (26)$^{b}$ | 6 (5)$^{q}$ |

$^{abc}$ or $^{pq}$ Values with different superscripts within a column are significantly different (P<0.05, $chi^2$ test with Yates correction); all other comparisons are not significantly different.

* total number of oocytes (*in vitro* group) and embryos (45- and 100 h *in vivo* group) put into culture, or collected at Day 7 po (*in vivo* group).

** percentages of developmental stages are calculated relative to the total number of viable embryos.

*** non-cleaved structures (n=60) had been removed at Day 5 of culture.

*Cell count*

In general, the cell number of the total embryo was significantly lower for embryos produced completely *in vitro* compared to that of embryos developed entirely *in*

*vivo* (Table 3). The cell numbers of embryos in the 45 h *in vivo* group were not significantly different from those in the *in vitro* and the *in vivo* group at corresponding stages. However, in the 100 h *in vivo* group, the cell number of the embryos at the expanded blastocysts and hatched blastocyst stages was significantly higher than that in the *in vitro* group and similar to that the *in vivo* group. At the blastocyst stage, the cell number of the total embryo tended to increase concurrently with extension of the period of development *in vivo*.

**Table 3.** Cell number of total embryos at morula and blastocyst stages at Day 7 po in relation to time of development *in vivo* and culture *in vitro*.

| Group | Cell number * | | |
|---|---|---|---|
| | morula + early blastocyst | blastocyst | expanded + hatched blastocyst |
| *In vitro* | $113 \pm 10^{a}$ (6) | $114 \pm 14^{a}$ (5) | $160 \pm 8^{a}$ (23) |
| 45 h *in vivo* | $88 \pm 6^{ab}$ (9) | $127 \pm 12^{a}$ (9) | $175 \pm 8^{ab}$ (33) |
| 100 h *in vivo* | ND * | $143 \pm 14^{a}$ (11) | $201 \pm 8^{b}$ (37) |
| *In vivo* | $128 \pm 9^{b}$ (28) | $166 \pm 11^{a}$ (18) | $205 \pm 21^{b}$ (15) |

* Values are expressed as the mean ± SEM. Numbers in parentheses are the number of embryos.

[ab] Values with different superscripts within a column are significantly different ($P<0.05$, ANOVA and Bonferroni test).

* ND, Not determined. The few collected early blastocysts that were assigned to this study were lost.

In 15% of the embryos that were characterized as blastocyst, expanded blastocyst, or hatched blastocyst by stereo microscopy, the respective numbers of nuclei of the ICM and TE could not be assessed because of collapse of the blastocoele. As shown in Table 4, the average cell number of the ICM was significantly lower in embryos of the *in vitro* group over all stages with a blastocoele (blastocyst, expanded blastocyst and hatched blastocyst) than those of the *in vivo* group. Likewise, the cell number of the ICM in the 45 h *in vivo* group was not significantly different from that in the *in vitro* and *in vivo* group. As observed for the cell number of the total embryo, the embryos at the expanded blastocyst and hatched blastocyst stages in the 100 h *in vivo* group again showed a significantly higher number of cells, but now for the ICM, than in the *in vitro* group and a number similar to that in the *in vivo* group. For the TE, the number of cells was markedly lower than that in the corresponding ICM, showing only minor variation between groups at specific developmental stages.

**Table 4.** Cell number of ICM and TE cells of embryos at blastocyst stages at Day 7 po in relation to time of development *in vivo* and culture *in vitro*.

| Group | Cell number * | | | | | |
|---|---|---|---|---|---|---|
| | blastocyst | | expanded + hatched blastocyst | | all blastocysts | |
| | ICM | TE | ICM | TE | ICM | TE |
| *In vitro* | 89 ± 14[a] (2) | 45 ± 9[a] (2) | 101 ± 6[a] (22) | 62 ± 4[a] (22) | 100 ± 5[a] (24) | 61 ± 4[a] (24) |
| 45 h *in vivo* | 104 ± 12[a] (5) | 45 ± 4[a] (5) | 114 ± 6[ab] (31) | 63 ± 4[a] (31) | 112 ± 6[ab] (36) | 61 ± 3[a] (36) |
| 100 h *in vivo* | 119 ± 13[a] (7) | 57 ± 2[a] (7) | 129 ± 6[b] (36) | 72 ± 3[a] (36) | 128 ± 5[b] (43) | 68 ± 3[a] (43) |
| *In vivo* | 130 ± 13[a] (8) | 45 ± 4[a] (8) | 124 ± 13[ab] (14) | 78 ± 10[a] (14) | 127 ± 8[b] (22) | 64 ± 7[a] (22) |

* Values are expressed as the mean ± SEM. Numbers in parentheses are the number of blastocysts.

[ab] Values with different superscripts within a column are significantly different (P<0.05, ANOVA and Bonferroni test).

*Apoptosis*

From all nuclei with clear signs of TUNEL labeling, 98% were condensed, and 79% were condensed and fragmented (Figure 2). The apoptotic index of the embryos is shown in Table 5. In general, it tended to be higher in the *in vitro* group at all embryonic stages than in the corresponding stages in the 45 and 100 h *in vivo* groups and the *in vivo* group. However, the difference was significant only between the expanded blastocyst and hatched blastocyst stages of the in vitro group and the 45 h *in vivo* group. The average apoptotic index over all embryos, regardless of the developmental stage, was rather similar between the 45- and 100 h *in vivo* groups and the *in vivo* group. The apoptotic index in blastocysts (blastocysts, expanded blastocysts, and hatched blastocysts) of all four groups was significantly higher in the ICM than in the TE (Table 6 and Figure 2E and 2F). In the 45 h *in vivo* group and the *in vivo* group, the apoptotic index in the ICM was significantly lower than that in the in vitro group.

The distribution of the embryos according to low (<5%), average (5-10%), and high (>10%) levels of apoptosis in the total embryo was not different between the four groups (results not shown).

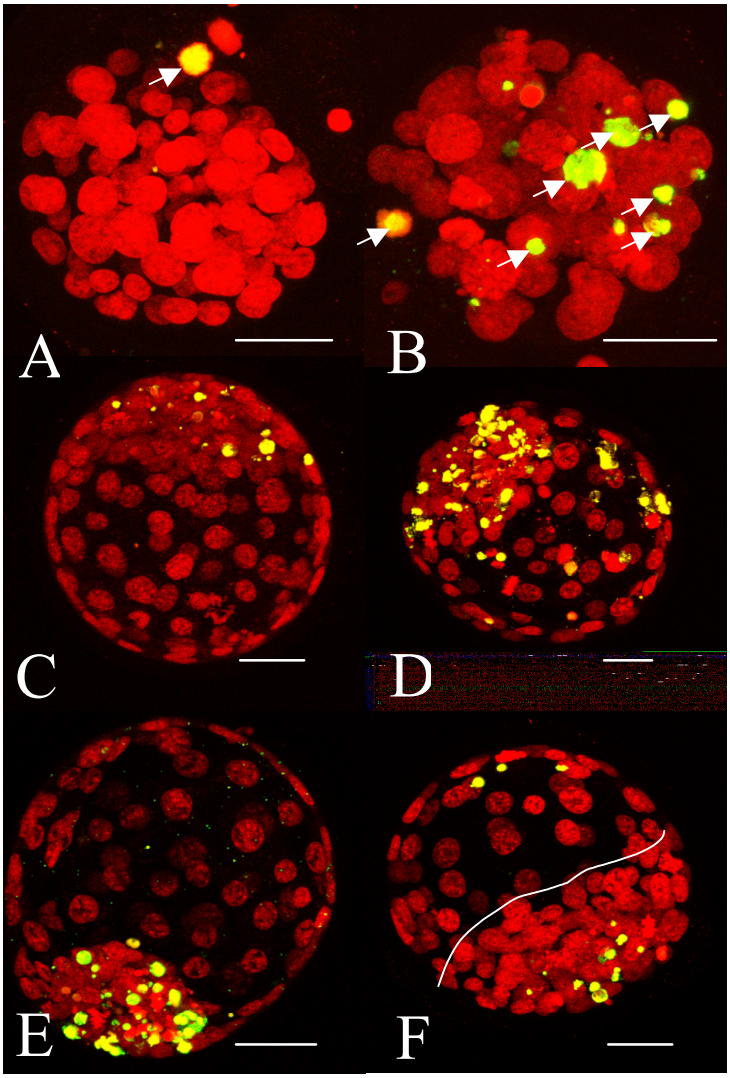



**Figure 2.** Digitally recombined, confocal laser-scanning images illustrating apoptosis in embryos at Day 7 po representative for embryos obtained from FSH-stimulated heifers after temporary or entire development *in vivo* or after production *in vitro* from oocytes of 3- to 8-mm follicles. Chromatin content is stained red with PI. Fragmented DNA is labeled green by TUNEL reaction and colocalization is observed as yellow. A) Morula with one condensed apoptotic nucleus. B) Morula with several apoptotic nuclei. C) Blastocyst with few apoptotic nuclei. D) Blastocyst with many apoptotic, condensed and fragmented nuclei. E) Blastocyst with apoptotic nuclei only in the ICM. F) Blastocyst with line drawn on a visual basis between ICM and TE for separate counting of the nuclei. Bar = 30 µm.

**Table 5.** Apoptosis in embryos at morula and blastocyst stages at Day 7 po in relation to time of development *in vivo* and culture *in vitro*.

| Group | Apoptotic index[1] per developmental stage | | | |
|---|---|---|---|---|
| | morula + early blastocyst | blastocyst | Expanded + hatched blastocyst | all embryos |
| *In vitro* | 9.3 ± 2.5[a] (6) | 11.7 ± 5.1[a] (5) | 8.9 ± 1.3[a] (23) | 9.4 ± 1.2[a] (34) |
| 45 h *in vivo* | 8.6 ± 4.2[a] (9) | 9.3 ± 2.9[a] (9) | 5.0 ± 0.6[b] (33) | 6.4 ± 1.0[a] (51) |
| 100 h *in vivo* | ND * | 7.4 ± 1.0[a] (11) | 7.9 ± 0.8[ab] (37) | 7.6 ± 0.7[a] (48) |
| *In vivo* | 7.9 ± 0.2[a] (28) | 7.4 ± 0.8[a] (18) | 5.0 ± 0.8[ab] (15) | 7.1 ± 0.6[a] (61) |

[1]Apoptotic index is expressed as the mean ± SEM and is calculated as the percentage of cells displaying both TUNEL labeling and fragmentation and/or condensation of the nuclei relative to the total number of cells. Numbers in parentheses are the number of embryos.

[ab] Values with different superscripts within a column are significantly different (P<0.05, ANOVA and Bonferroni test).

* ND, Not determined. Embryos at morula and early blastocyst stage were not available in this group.

**Table 6.** Apoptosis in ICM and TE cells of blastocysts at Day 7 po in relation to time of development *in vivo* and culture *in vitro*.

| Group | Apoptotic index[1] | |
|---|---|---|
| | ICM | TE |
| *In vitro* (24) | 12.4 ± 1.6[aA] | 6.3 ± 1.7[aB] |
| 45 h *in vivo* (36) | 6.3 ± 0.7[bA] | 4.1 ± 0.8[aB] |
| 100 h *in vivo* (43) | 9.1 ± 0.9[abA] | 5.5 ± 0.7[aB] |
| *In vivo* (26) | 7.1 ± 0.9[bA] | 2.9 ± 0.5[aB] |

[1]Apoptotic index is expressed as the mean ± SEM and is calculated as the percentage of cells in the ICM or TE displaying both TUNEL labeling and fragmentation and/or condensation of the nuclei relative to the total number of cells of the ICM or TE .

[ab] Values with different superscripts within a column are significantly different (P<0.05, ANOVA and Bonferroni test).

[AB] Values with different superscripts within a row are significantly different (P<0.05, Student *t*-test).

Although the correlation between cell number of the total embryo and apoptotic index was significant ($R^2$=0.026, P<0.05) (Figure 3), the decrease of the apoptotic index with increasing cell number was only marginal.

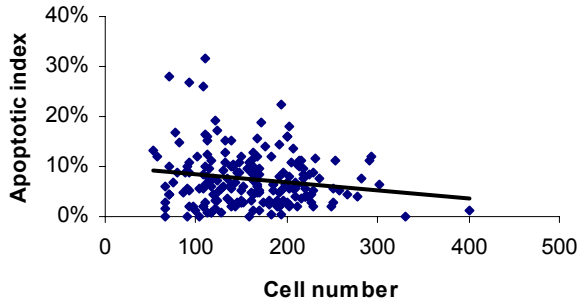



**Figure 3.** Correlation between cell number of the total embryo and apoptotic index of all embryos (n=194) at Day 7 po from the four groups (in vitro, 45- and 100 h *in vivo*, and *in vivo* groups).

## Discussion

In the present study the effects of different postfertilization regimens before and after completion of the 4$^{th}$ cell cycle of bovine preimplantation embryos on developmental competence and on the cell number and apoptotic index in blastocysts were investigated. Production entirely *in vitro* from IVM to complete IVC profoundly influences early embryonic development. Culture conditions affect the blastocyst formation rate (Thompson, 1997; van Soom *et al.*, 1997; Viuff *et al.*, 2001, Ahern and Gardner, 1998) and reduce embryo quality both by decreasing the number of cells of the total embryo and of the cells allocated to the ICM and by increasing the incidence of apoptosis in murine (Brison and Schultz, 1997; Hardy, 1997) and bovine blastocysts (Gjørret, 2003b). The present data show, to our knowledge for the first time, that passage *in vivo* of the 4$^{th}$ cell cycle does not prevent acceleration of the formation of the blastocoele *in vitro* and may be the critical factor contributing to a higher cell number of the total blastocyst and its ICM. Another novel aspect is related to the finding that development *in vivo* until occurrence of the 4$^{th}$ cell cycle appears to be essential in determining the incidence of apoptosis, particularly in the ICM of expanded blastocysts.

The rate of development into morula and/or blastocyst stages in the in vitro group was much lower than in the groups with temporary or total *in vivo* development, which is in accordance with earlier observations that prematuration

and maturation are decisive factors for oocyte developmental competence (Hendriksen *et al.*, 2000; Dieleman *et al.*, 2002). The rate of blastocyst development relative to the number of viable embryos in the *in vivo* group was lower than in the other three groups, concurrent with a higher rate of morula development. The present results indicate that *in vivo* initiation of blastulation occurs after 100 h po and may be mediated by factors from the uterine environment. The majority of the embryos used for further IVC were collected before (45 h *in vivo* group, 92% at the 5- to 8-cell stages) or after (100 h *in vivo* group, 79% at the 8- to 32-cell stages) completion of the 4$^{th}$ cell cycle. The rate of development into morula and/or blastocyst stages relative to the embryos selected for culture was higher in the 100 h *in vivo* group than in the 45 h *in vivo* group, which seems to indicate that passage of the 4$^{th}$ cell cycle may stimulate embryo development. However, this difference appears to result from selection of embryos for IVC. In the 100 h *in vivo* group, a considerably higher proportion of nonviable embryos (27%), which was similar to that in the *in vivo* group, had been discarded for IVC compared to the proportion in the 45 h *in vivo* group (5%). When the proportion of embryos developed to the morula and/or blastocyst stages is calculated over the total number of embryonic structures, the values are almost identical (45 h *in vivo* group, 107/167=64%; 100 h *in vivo* group, 107/163=66%). This is in agreement with earlier studies, in which the developmental competence of *in vitro*- or *in vivo*-derived zygotes did not change with further culture *in vitro* or *in vivo* in the ewe oviduct (Rizos *et al.*, 2002b, Enright *et al.*, 2000). The present data suggest that part of *in vivo*-derived, 5- to 8-cell stage embryos are not competent to develop beyond the 4$^{th}$ cell cycle, regardless of in vitro or *in vivo* conditions. The differences between the embryos of the four in vitro and *in vivo* groups were probably not caused by a low number of embryos per volume during culture. In an earlier study, the rate of blastocyst development decreased significantly below 72% when less than four 8-cell stage embryos were cultured in 50 µL SOF droplets under oil, and the number of ICM cells decreased (Ahern and Gardner, 1998). In the present study, a higher ratio of, on average, nine embryos per 20 µL was employed.

The number of cells of the total embryo, as a measure of the growth rate, at 7 days po was higher in the 100 h *in vivo* and the *in vivo* group than in the in vitro group. In the 45 h *in vivo* group, the number was intermediate. This pattern was particularly evident in the expanded blastocysts constituting the major proportion of the embryos at the respective blastocyst stages. It is interesting to note that the growth rate of embryos in the in vitro group was much slower than of that of *in vivo* embryos but not as much as observed earlier using Menezo B2-coculture (Viuff *et al.*, 2001 and 1999) in stead of SOF (as used in the present study). Effects of different culture media on the growth rate have been shown by van Soom et al. (van Soom *et al.*, 1996). The number of ICM cells of embryos in the four groups followed a pattern parallel to that observed for the total number of

embryo cells, which is in accordance with the higher number of ICM cells reported for *in vivo* developed embryos versus embryos after IVC (van Soom *et al.*, 1997). It was suggested that contact with the maternal tract might be of importance to switch on certain genes that encode for developmentally important processes, such as tight junction formation in the case of inner cell allocation. The fact that the total number of cells and of the ICM in the 100 h *in vivo* group was similar to that in the *in vivo* group suggests that the growth rate is defined at 100 h po, when the $4^{th}$ cell cycle is completed. It is speculated that *in vivo*, the embryonic cells are more stimulated to cleave because of interaction with the oviduct. However, a higher cell death, specifically at earlier stages of development, may have been the cause of the lower cell numbers in the in vitro group. Cell death appears to be correlated with cell number in mouse and human blastocysts (Hardy, 1997), and in the present study, embryos with low cell numbers showed a higher apoptotic index than embryos with high cell numbers.

The incidence of apoptosis in the in vitro group was higher than in the 45 h *in vivo* group and the *in vivo* group, particularly in expanded blastocysts and their ICM. In the 100 h *in vivo* group, the apoptotic index was intermediate, which may have been caused by the change from *in vivo* to the in vitro environment. Stress has been reported to increase apoptosis. Recently, it was demonstrated that heat stress can induce TUNEL labeling at the late 8- to 16-cell stages, but not at earlier stages (Paula-Lopes and Hansen, 2002). In the 45 h *in vivo* group, stress caused by change of environment possibly did not affect the incidence of apoptosis at the blastocyst stage, because *in vivo*, the apoptotic machinery is not apparent before the 21-cell stage as no DNA degradation was observed before that stage (Gjørret et al., 2003b). Although in the bovine apoptosis is not evident before the 21-cell stage, various molecular components may already be present at early cleavage stages, as reported in the mouse (Exley *et al.*, 1999), human (Warner *et al.*, 1998), and bovine embryos (Kölle *et al.*, 2002; Paula-Lopes and Hansen, 2002). Furthermore, in the 45 h *in vivo* group, the apoptotic cells that were eventually induced probably had already been phagocytosed during further culture until collection at Day 7 po. Phagocytosis has been observed in human and bovine embryos (Hardy, 1999; Gjørret *et al.*, 2003a). The entire time span for apoptosis, from the beginning of cell rounding and blebbing to the final lysis of the cell, takes 12-24 h (Wyllie *et al.*, 1980; Collins *et al.*, 1997), which implies that the apoptotic nuclei we observed form a “snapshot”. Overall, the present data show small differences in the apoptotic index of the total embryo between groups, which indicates that SOF provides an environment in which apoptosis in bovine embryos occurs in a manner fairly similar to that *in vivo*. Kölle et al. (2002) observed an even lower level of apoptosis in embryos cultured in SOF compared to that in our in vitro group. In other studies, in bovine embryos using Menezo B2-coculture (Gjørret *et al.*, 2001) and murine embryos (Jurisicova *et al.*, 1998a), the

difference between completely *in vitro*- and entirely *in vivo*-derived embryos was more pronounced.

The variable overestimation of the number of cells of the ICM did influence the ICM:TE ratio but not the relation of the apoptotic indexes between the four groups. In all four groups, the apoptotic index of the ICM was significantly higher than that of the TE, which is in agreement with studies in bovine (Watson *et al.*, 2000; Gjørret *et al.*, 2001, Neuber *et al.*, 2002) and murine (Hardy and Handyside, 1996; Hardy, 1997; Kamjoo *et al.*, 2002) embryos. It is assumed that apoptosis in the ICM may regulate this cell population, because the number of ICM cell reaches a plateau in later-stage blastocysts without decrease in mitotic division (Handyside and Hunter, 1986). At the blastocyst stage, apoptosis likely acts to eliminate cells that are damaged, are in excess, are no longer required, are developmentally incompetent, or have acquired TE potential. This cellular 'quality control' within the ICM is critical. This lineage forms the fetus and contains the germline, and furthermore, an aberrant TE:ICM ratio has been suggested to be related with the large offspring syndrome of *in vitro*-produced embryos (Thompson *et al.*, 1995).

In the cow, passage of the 4$^{th}$ cell cycle coincides *in vivo* with the period of major genome activation. In other species, this period appears to induce competence for apoptosis (Matwee *et al.*, 2000) and to activate expression of genes that serve to suppress cell death in developing embryos (Sible *et al.*, 1997; Stack and Newport, 1997; Jurisicova *et al.*, 1998a). The present study shows that passing the period of major genome activation *in vitro* or *in vivo* does not affect the level of apoptosis in bovine Day 7 embryos.

In conclusion, development *in vivo* during the first 100 h po up to the 5$^{th}$ cell cycle is decisive for the cell number of the total embryo and the ICM in the expanded blastocyst at 7 days po. However, timing of blastulation appears to be affected by uterine factors after 100 h po. The level of apoptosis appears to be determined *in vitro* in SOF at an earlier stage of development, before 45 h po.

**Acknowledgments**

The authors thank Holland Genetics (Arnhem, The Netherlands) for supplying SOF culture medium. The Department of Molecular Cell Biology, Institute of Biomembranes, Utrecht University is appreciated for the use of their computer facilities and Scientific Volume Imaging BV (Hilversum, The Netherlands) for supplying Imaris software. We are grateful to Elly Zeinstra, Christine Oei, Thea Blankenstein, Henk Heuveling and Jan Joop Harkema for excellent technical assistance and to animal handlers for management of the animals.

# Chapter 6

## *In vitro* and *in vivo* culture effects on mRNA expression of genes involved in metabolism and apoptosis in bovine embryos

**Abstract**

Bovine blastocysts produced *in vitro* differ substantially from their *in vivo*-derived counterparts with regard to glucose metabolism, level of apoptosis and mRNA expression patterns. An aberrant maternal embryonic transition of transcriptional control due to deficient *in vitro* culture conditions could be involved in these differences. The aims of the present study were to identify the critical period of culture during which the differences in expression of gene transcripts involved in glucose metabolism are induced and to determine gene transcripts involved in apoptosis that are differentially expressed in *in vitro*- and *in vivo*-produced blastocysts. Relative abundances of glucose transporters Glut-1, Glut-3, Glut-4 and Glut-8 and transcripts involved in the apoptotic cascade BAX, BCL-XL, XIAP and HSP-70.1 were analyzed by a semi-quantitative RT-PCR assay in single blastocysts produced *in vitro* or *in vivo* for specific time intervals, i.e. prior to or after maternal embryonic transition. Culture environment *in vitro* vs. *in vivo* affected expression of glucose transporter transcripts, Glut-3, Glut-4 and Glut-8. However, the critical period during culture responsible for these changes, prior to or after maternal embryonic transition, could not be determined. Furthermore, no effect of culture regimen on the mRNA expression of BAX, BCL-XL and HSP 70.1 could be observed. These data show that expression of XIAP transcripts in expanded blastocysts is affected by *in vitro* culture. Our findings do not support the hypothesis that maternal embryonic transition is critical in inducing the aberrations in gene expression patterns studied here.

**Authors:** Hiemke M. Knijn[1], Christine Wrenzycki[2], Peter J.M. Hendriksen[1], Peter L.A.M. Vos[1], Elly C. Zeinstra[1], Bert .C. van der Weijden[1], Heiner Niemann[2] and Steph J. Dieleman[1]

[1]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands*

[2]*Department of Biotechnology, Institute for Animal Science (FAL), Neustadt, Germany*

Submitted for publication

## Introduction

*In vitro*-produced bovine embryos differ from their *in vivo*-derived counterparts in numerous parameters, including morphology (Van Soom *et al.*, 1997), metabolism (Khurana and Niemann, 2000), tolerance of low temperatures (Leibo and Loskutoff, 1993), mRNA expression (Niemann and Wrenzycki, 2000) and *in vivo* development (van Wagtendonk-de Leeuw *et al.*, 2000). Various studies reported deviations from the normal mRNA pattern in *in vitro*-produced bovine blastocysts versus their *in vivo* counterparts (Wrenzycki *et al.*, 1996; Lequarre *et al.*, 2001; Knijn *et al.*, 2002; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a). However, it is unknown when precisely during *in vitro* culture these molecular deviations are induced. A recent study revealed that the maturation step during *in vitro* production probably is not the critical step and it is rather during the postfertilization period that these differences in quality between embryos cultured *in vitro* or *in vivo* are induced (Knijn *et al.*, 2002; Rizos *et al.*, 2002b).

The earliest stages of development are dependent on maternally-derived messages stored in the oocyte. As development progresses, maternal RNAs and proteins are depleted and embryo-derived mRNAs gain control of development. This shift from dependence on oocyte-derived to embryo-produced messages is referred to as maternal-to-embryonic transition (MET). The onset of MET has been investigated in embryos of a variety of species using $\alpha$-amanitin, a specific RNA polymerase II inhibitor (for review see Telford *et al.*, 1990). In bovine preimplantation development, a minor embryonic genome activation has been described at the 2-cell stage (Hyttel *et al.*, 1996; Viuff *et al.*, 1996; Memili *et al.*, 1998). Addition of $\alpha$-amanitin to the culture medium during the earliest stages of development blocks transcription, and in the absence of embryonic transcription the bovine embryos can only survive until the 8- to 16-cell stage (Telford *et al.*, 1990; Memili and First, 1998). An aberrant MET due to deficient *in vitro* culture conditions could be involved in molecular deviations frequently observed in *in vitro*-produced (IVP) blastocysts.

Concomitant with major embryonic genome activation, the metabolism switches from utilization of lactate and pyruvate to glucose as the main energy source (Rieger *et al.*, 1992; Khurana and Niemann, 2000). As the blastocyst forms, glucose consumption increases, requiring specific regulation of glucose transport by the early embryo. Glucose transport across the plasma membranes in mammalian cells is mediated by an active sodium-dependent transport and a passive energy-independent transport via facilitative glucose transporters (Glut). Glut-3 is thought to play a crucial role in the uptake of maternal glucose in the bovine (Augustin *et al.*, 2001) as well as in the mouse (Pantaleon *et al.*, 1997). In the mouse, Glut-8 is critical for blastocyst survival (Pinto *et al.*, 2002). Deviating mRNA expression patterns of Glut-1, Glut-3 and Glut-4 transcripts were found in *in vitro*-produced bovine embryos (Knijn *et al.*, 2002; Lazzari *et al.*, 2002).

A decreased glucose transport was associated with enhanced apoptosis most markedly at the blastocyst stage (Moley *et al.*, 1998; Chi *et al.*, 2000). The level of apoptosis in bovine blastocysts is affected by the culture environment and increased by suboptimal conditions (Gjørret *et al.*, 2003; Knijn *et al.*, 2003). The molecular basis of enhanced apoptosis in *in vitro*-produced blastocysts has not yet been studied in bovine embryos. Regulation of apoptosis depends upon a cascade comprising pro-apoptotic and anti-apoptotic proteins. The ratio between these proteins determines whether a cell survives or undergoes apoptosis (Oltvai *et al.*, 1993; Wyllie, 1995). Messenger-RNA of BCL-XL, an anti-apoptotic protein, is present in mouse embryos at the 2-cell, 8-cell and blastocyst stages (Exley *et al.*, 1999). Messenger-RNA transcripts for the pro-apoptotic protein BAX are found throughout preimplantation development in the mouse (Exley *et al.*, 1999). Expression of BAX mRNA in bovine blastocysts was affected by the culture method (Rizos *et al.*, 2002a). The extent of apoptosis also seems to be correlated with activity of the X–chromosome-linked Inhibitor of Apoptosis (XIAP) and Heat Shock Protein (HSP 70.1). This relationship has not yet been investigated for bovine embryos.

The goal of the present study was twofold: 1) to identify the critical period of culture during which the differences in expression of gene transcripts involved in glucose metabolism between *in vitro*- and *in vivo*-cultured bovine blastocysts are induced, and 2) to determine gene transcripts involved in apoptosis that are differentially expressed by the embryonic genome in *in vitro*- and *in vivo*-cultured bovine blastocysts.

## Materials and Methods

*Experimental design*

The relative abundances of transcripts of the apoptosis-related genes BAX, BCL-XL, XIAP and HSP 70.1 transcripts were analyzed in early blastocysts, blastocysts and expanded blastocysts produced *in vitro* (In vitro group (1)) or *in vivo* either after slaughter (*In vivo* slaughter group (4)) or by non-surgical uterine flushing of the donor animals (*In vivo* flush group (5)). The relative abundance of the glucose transporter transcripts, Glut-1, Glut-3, Glut-4 and Glut-8 were analyzed in expanded blastocysts produced *in vitro* (1) or *in vivo* (5) collected after uterine non-surgical flushing. To identify the critical period of embryo culture in which differences in gene expression are induced, two categories of expanded blastocysts were analyzed, one *in vivo* developed until 45 h (45 h *In vivo* group (2)) and the other until after MET (100 h *In vivo* group (3)) followed by culture *in vitro*. A schematic drawing of the experimental design is shown in Figure 1.

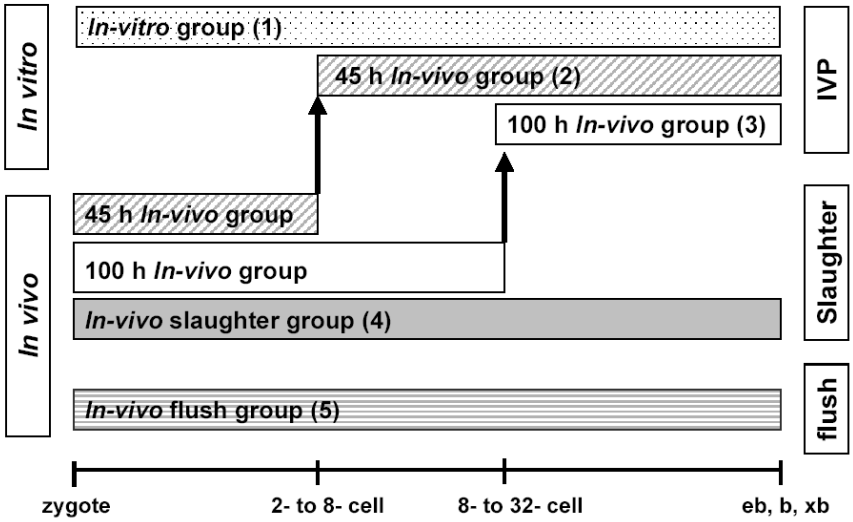



**Figure 1.** Schedule for production of bovine blastocysts for analysis of mRNA expression to identify MET-induced aberrations. Group number is indicated in bars.

To investigate if the specific gene transcripts are of maternal origin, the relative abundance of all gene transcripts were analyzed in 8- to 16-cell embryos cultured with or without α-amanitin.

*Animal treatment*

Experiments were carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Selected heifers were superovulated using a method with a fixed LH surge as described previously (Knijn *et al.*, 2003). Briefly, animals (n=59) were pre-synchronized, and on Day 8 of the synchronized cycle (oestrus = Day 0) follicles larger than 5 mm were ablated by transvaginal ultrasound guided aspiration to synchronize follicle development. On Day 9, the heifers received an ear implant (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) for 5 days, without additional administration of norgestomet and oestradiol-valerate. From Day 10 onwards, oFSH (Ovagen; ICP, Auckland, New Zealand) was administered i.m., twice daily with decreasing doses during 4 days (in total 10 mL equivalent to 176 IU NIH-FSH-S1). Prostaglandin (22.5 mg Prosolvin; Intervet International BV) was administered i.m. concomitant with the fifth dose of FSH. Fifty-five h later ear implants were removed and GnRH was administered (1.0 mg Fertagyl in 10 mL saline i.m.; Intervet International BV) to induce the LH surge. Heparinized blood samples were collected from the jugular vein daily during the experimental phase, every 3 h from 24 h after PG admini-

stration and every hour during 7 h after removal of the implant. All animals were inseminated 12 to 14 h after GnRH administration with two straws of semen from a bull with known fertility (one straw per uterine horn). The heifers were randomly assigned to three experimental groups. LH concentrations were analyzed by radioimmunoassay (Dieleman *et al.*, 1983). The animals showed an LH surge on average 2.2 h after GnRH administration. The time point of 24 h after the LH surge was taken as the onset of ovulation. The time interval between expected ovulation and slaughter to collect embryos was 45 to 48 h for the 45 h *In vivo* group (2), 100 to 103 h for the 100 h *In vivo* group (3) and 165 to 168 h for the *In vivo* slaughter group (4). In addition, two other groups of *in vivo*-developed embryos were collected. The first group was collected after superovulation described above and by non-surgical flushing of the uterine horns with 500 mL ET-PBS at Day 7 post ovulation (po). The second group was collected from donor cows superovulated with a single injection of 3000IU eCG (Intergonan; Intervet, Tönisvorst, Germany) between Days 9 and 13 of the oestrus cycle followed 48 h later by administration of cloprostenol (Estrumate; Essex, Munich, Germany). When oestrus was detected 48 h later, the donors were inseminated twice at an interval of 12 h with semen of a bull with proven fertility. At Day 7 after insemination, blastocysts were recovered by non-surgical flushing of the uterine horns with 300 mL PBS (Sigma, St. Louis, MO) supplemented with 1% newborn calf serum (NBCS, No. 295957; Boehringer, Mannheim, Germany) using established procedures. Embryos collected after non-surgical uterine flushing are referred to as *In vivo* flush group (5).

*Embryo collection after slaughter, 45 h In vivo- (2), 100 h In vivo- (3) and In vivo slaughter group (4)*
After slaughter, genital tracts were placed in saline (37°C) in a thermocontainer and transported to the laboratory within 45 min to 2 h. The number of corpora lutea was counted. Oviducts and uterine horns were flushed with PBS (PBS-ET; Bio Whittaker Europe, Verviers, Belgium) at 37°C using a blunt needle, from the infundibulum towards the uterine horn under gentle massage. For the 45 h *In vivo* group (2), only the oviducts and the top of the uterine horn were flushed. The fluid was collected into an embryo recovery filter (Embryo Concentrator; Immuno Systems Inc., Spring Valley, WI) and the embryos were isolated by rinsing the filter with saline supplemented with 0.005 % (w/v) BSA (A 6003; Sigma).

*Embryo culture, 45 h In vivo- (2) and 100 h In vivo group (3)*
Embryos were transferred to culture medium within 2 h after slaughter. The developmental stage of the embryonic structures was assessed by stereomicroscopy at 100x magnification. The 1-cell and degenerated embryos, and at 100 h po also embryos at the 2- to 7-cell stage, were characterized as non-viable and excluded from culture. Prior to culture, embryos were washed in

medium consisting of synthetic oviduct fluid medium (SOF) with BSA as described earlier (van Wagtendonk-de Leeuw *et al.*, 2000; Knijn *et al.*, 2003). A maximum of 10 collected embryos per heifer were placed in 20 µL droplets of SOF under oil (Mineral oil for IVF, Reproline Medical GmbH, Rheinback, Germany) at 39°C in humidified air of 5% $CO_2$, 7% $O_2$ and 88% $N_2$. Early blastocysts, blastocysts and expanded blastocysts were collected after 120 h of *in vitro* culture for the 45 h *In vivo* group (2) and 72 h for the 100 h *In vivo* group (3).

*Embryo culture, In vitro group (1)*
Cumulus oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles of ovaries that had been collected at a local abattoir. Those oocytes with a multi-layered compact cumulus investment were used for the experiments. COCs were rinsed once with Hepes buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL) and once with maturation medium M199 supplemented with 10% FCS, 0.01IU/mL recombinant hFSH (Organon, Oss, The Netherlands), 11.36 µg/ml cysteamine (M-6500, Sigma) and 1% (v/v) penicillin-streptomycin (Gibco BRL). Groups of 35 oocytes were randomly allocated to each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µL maturation medium with the above supplements. After maturation for 23 h at 39º C, 5% $CO_2$ in humidified air, all oocytes were fertilized *in vitro*.

Procedures for *in vitro* fertilization (IVF) were performed as described earlier (Parrish *et al.*, 1988) with minor modifications (Izadyar *et al.*, 1996), using semen from the same bull as used for the 45 h *In vivo*- (2) and 100 h *In vivo* (3) groups, *In vivo* slaughter group (4) and *In vivo* flush group (5). After 21 h of co-incubation (39°C, 5% $CO_2$ in humidified air), presumptive zygotes were liberated from cumulus cells by vortexing for 3 min and a maximum of 10 zygotes were placed in 20 µL droplets of SOF and cultured as described above. All cleavage stages were transferred to fresh culture droplets at Day 5 after the start of fertilization. Early blastocysts, blastocysts and expanded blastocysts were collected 165 h after the start of fertilization.

*Total collection of blastocysts*
The number of blastocysts collected at Day 7 po were 73 in the In vitro group (1) (7 early blastocysts (eb), 16 blastocysts (b) and 50 expanded blastocysts (xb)); 86 in the 45 h *In vivo* group (2) (16 eb, 19 b and 51 xb); 90 in the 100 h *In vivo* group (3) (7 eb, 21 b and 62 xb); and 67 in the *In vivo* slaughter group (4) (17 eb, 17 b and 33 xb).

*Inhibition of transcription from the embryonic genome*
For inhibition of embryonic transcription, presumed zygotes were produced *in vitro* as described above in two replicates. After fertilization, presumed zygotes were randomly assigned to three groups of 50 each. The first group served as a control without α-amanitin. In the second group 10 mM α-amanitin was added to the culture medium from the start of culture onwards, 24 h after fertilization (d0 group). In the third group, 48 h after the start of fertilization the culture medium was replaced by medium supplemented with 10 mM α-amanitin (d1 group). At 96-100 h after the start of fertilization the embryos were scored and 8- to 16-cell embryos were collected. To ascertain whether the α-amanitin concentration terminated development at major genome activation, two groups of 50 zygotes were cultured, one supplemented with 10 mM α-amanitin at the start of culture and one group without α-amanitin, until Day 7 post-fertilization, and scored for embryonic development.

*Storage of embryos*
All collected embryos were washed four times in PBS with 0.1% (w/v) PVA and transferred to 0.5 ml Eppendorf vials in a minimum volume (≤ 5 µl) of PBS with 0.1% (w/v) PVA and frozen at -80°C. Analysis of cultured embryos was confined to those in morphological grades I and II (Robertson and Nelson, 1998).

*Determination of relative mRNA abundance in individual embryos by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)*
Poly $(A)^+$ RNA from a single embryo was isolated as previously described (Wrenzycki *et al.*, 1999). Prior to RNA isolation, 1 pg of rabbit globin RNA (Gibco BRL, Gaithersburg, MD) was added as an internal standard. Isolated Poly$(A)^+$ RNA was used immediately for reverse transcription which was carried out in a total volume of 20 µl. The reaction mixture consisted of 1x RT buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, Perkin Elmer Biosystems, Vaterstatten, Germany), 5 mM $MgCl_2$, 1 mM of each dNTP (Amersham, Brunswick, Germany), 2.5 µM random hexamers (Perkin Elmer), 20 IU RNase inhibitor (Perkin Elmer) and 50 IU MuLV reverse transcriptase (Perkin Elmer). As negative controls, tubes without RNA samples were analyzed. The RT reaction was carried out in a PTC-200 thermo-cycler (MJ Research, Watertown, MA) for 10 min at 25°C, 60 min at 42°C, 5 min at 99°C and after finishing immediately put on ice. Immediately after reverse transcription the samples were subjected to polymerase chain reaction (PCR). PCR was performed with embryo equivalents as described in Table 1 and 50 fg of globin RNA (corresponding to 0.05 embryo equivalents) in a final volume of 50 µL, containing 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4, Life Technologies BV, Eggenstein, Germany), 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1 µM of each sequence-specific primer (0.5 µM for globin primers). A ”hot start” PCR was performed to obtain specific amplification. During the “hot start”

1 IU Taq DNA polymerase (Life Technologies BV) was added at 72°C. The sequences and positions of the primers used, the annealing temperature, the fragment sizes of the expected products, and the sequence references are summarized in Table 1.

Running a linear cycle series established the optimal cycle number at which the transcript was amplified exponentially for the PCR reaction. The PCR program started at 97°C for 2 min, followed by 72°C for 2 min (hot start) and was then followed by a number of cycles depending on the gene (Table 1) of 15 sec at 95°C for denaturation, 15 sec at the annealing temperature of the specific gene (Table 1) and 15 sec at 72°C for primer extension. After these cycles were finished a post dwell of 5 min at 72°C was done followed by cooling to 4°C. A PTC-200 thermocycler was used.

For detection and semi-quantification of RT-PCR products, electrophoresis was performed with 25 µL RT-PCR product that was loaded with 5 µL of 10x loading buffer (0.25% (w/v) xylenecyanol and 25 mM EDTA in 50% (w/v) glycerin). A 2% (w/v) agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) with 0.2 µg/mL ethidium bromide was used. The concentration of ethidium bromide in the running buffer was the same as in the gel. After running at 100 V for 5 min and at 80 V for 40 min, the fragments were visualised on a 312 nm UV-transilluminator. A CCD camera (Quantix, Photometrics, Munich, Germany) and IP Lab spectrum (IP Lab Gel, Signal Analytics Corporation, Vienna, VA) were used to digitise the image of the gel. Densitometric scanning using a computer-assisted image analysis system (IP Lab Gel) quantified the signal intensity of each band. The relative abundance of the mRNA of the different genes was estimated by dividing the intensity of the band of the gene of interest by the intensity of the globin band from the same sample (Wrenzycki *et al.*, 1999).

Four transcripts were analyzed per blastocyst with 10 to 16 replicates for the glucose transporter genes, 5 to 15 replicates for the apoptosis-related genes, with the exception of BCL-XL in eb of the In vitro group (1) and *In vivo* slaughter group (4), b in the *In vivo* slaughter group (4) and xb in the *In vivo* flush group (5), for which 2 to 4 replicates were analyzed due to the low number of available embryos. The 8- to 16-cell embryos from the $\alpha$-amanitin experiment were pooled in groups of 3 from which 3 to 5 gene transcripts were analyzed in 4 to 8 replicates per gene.

Relative abundance was calculated on a per cell basis per embryo because the average number of cells per embryo is significantly different between *in vitro*- and *in vivo*-derived embryos and among different stages. The results of previous studies were used as a reference to estimate cell numbers in this study (Lazzari *et al.*, 2002; Knijn *et al.*, 2003)(Table 2).

**Table 2.** Average cell number of total embryos blastocyst stages at Day 7 po in relation to time of development *in vivo* and culture *in vitro*

| Group | Cell number[1] | | |
|---|---|---|---|
| | early blastocyst | blastocyst | expanded blastocyst |
| In vitro* | 106 ± 10 (3) | 114 ± 14 (5) | 160 ± 8 (23) |
| 45 h *In vivo** | n.a.*** | n.a. | 176 ± 8 (30) |
| 100 h *In vivo** | n.a. | n.a. | 189 ± 8 (30) |
| *In vivo** | 118 ± 9 (8) | 166 ± 11 (18) | 181 ± 14 (13) |
| *In vivo* flush** | 98 ± 6 (15) | 121 ± 9 (15) | 158 ± 46 (20) |

[1] Values are expressed as the mean + SEM. Numbers in parentheses are the number of embryos

* Cell numbers were used from Knijn et al. (2003).

** Cell numbers of Lazarri et al. (2002) were used with results obtained from embryos of donor cows treated for superovulation with eCG.

*** n.a., Not applicable. These stadia were not used for analyses.

*Sex determination*

For the embryos that were analyzed for the relative abundance of XIAP, a sex determination was performed. DNA was collected from the supernatant of the RNA extraction and prepared using the Microcon YM-100 (42412; Millipore, Eschborn, Germany) columns according to the manufacturer's instructions. Embryonic sex was determined by PCR analysis using bovine-specific and Y-chromosome-specific primers (Table 1) as recently described (Wrenzycki *et al.*, 2002).

*Statistical analysis*

Relative abundances were analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kologorov-Smirnov test with Lilliefor correction) and testing for equal variance (Levene Median test), ANOVA followed by multiple paired comparisons using the Tukey test were employed. Relative abundances for XIAP transcripts between male and female embryos were compared using Student *t*-test. Sex ratio was compared with the expected ratio of 50% using chi-square analysis. Data on the relative abundance are presented as mean ± SEM. Differences of $P \leq 0.05$ were considered significant.

**Table 1.** Primers and conditions used for PCR of bovine embryos

| Genes | Primer sequence and positions | Annealing temp (°C) | No. cycles and embryo equivalent for blastocyst/8-16 c | Fragment size (bp) | Sequence references (EMBL accession no.) |
|---|---|---|---|---|---|
| Globin | 5' (241-260): GCAGCCACGGTGGCGAGTAT | 60 | 27/27 | 257 | Cheng *et al.*, 1986 (X04751) |
| | 3' (555-657): GTGGGACAGGAGCTTGAAAT | | 0.05/0.05 | | |
| Glucose transporter-1 | 5' (1609-1638): GGAGCTGTTCCACCCCCTGGGAGCTGACT | 59 | 33/35 | 327 | Boado and Pardridge, 1991 |
| (Glut-1) | 3' (1906-1935): GTGGGTGAAGGAGACTCTGGCTGATAAAA | | 0.1/0.9 | | (M60448) |
| Glucose transporter-3 | 5' (1129-1152): CCTTGGAGGGATGGCTTTTTGTTC | 58 | 34/36 | 259 | Bennett *et al.*, 1995) (l39214 |
| (Glut-3) | 3' (1364-1387): CGTGGCTGAGGGGAAGAGCAGTCC | | 0.1/0.5 | | |
| Glucose transporter-4 | 5' (1663-1692): GGAGCAGGAAGTGAAACCCAGCACAGAACT | 59 | 39/41 | 277 | Abe *et al.*, 1997) (D63150 |
| (Glut-4) | 3' (1919-1939): GCTAACCACAACACAAATAATCCAAGAGGT | | 0.5/0.5 | | |
| Glucose transporter-8 | 5' (184-204): CCTCGCTTCCTGCTGTCTCA | 58 | 36/38 | 244 | Augustin et al., unpublished data |
| (Glut-8) | 3' (408-428): CCTCTGGTAGAAACTCCTCC | | 0.2/0.5 | | (AF321324) |
| BAX | 5' (72-96): CCTTTTGCTTCAGGGTTTCATCCAG | 63 | 35/37 | 232 | Reyes and Cockerell, 1998 |
| | 3' (281-304): CTCACCGCCGACTTTACAAAAGAC | | 0.1/0.5 | | (U92569) |
| BCL-X long | 5' (47-72): ATGGAGCCACTGGCCACAGCAGAAG | 60 | 34/36 | 307 | Amills and Bouzat, unpublished |
| | 3' (329-354): TCCATAACCACTCAGCCTAGCGTTG | | 0.2/0.5 | | data (AF245489) |
| XIAP | 5' (83-107): AATATGCAAAGTGAATCCTCTGTC | 52 | 35/37 | 229 | Gutierrez-Adan et al., |
| | 3' (288-314): CTCCTCCCGATTTACTAACCTTCG | | 0.2/0.5 | | unpublished data (AF458770) |
| Heat shock protein 70.1 | 5' (1861-1890): AAGGTGCTGGACAAGTGCCAGGAGGTGATT | 59 | 36/38 | 488 | Gutierrez and Guerriero, 1995 |
| (HSP 70.1) | 3' (2319-2348): ACTTGGAAGTAAACAGAAACGGGTGAAAAA | | 0.2/0.9 | | (U09861) |
| Y-Chromosome specific | 5' CCTCCCCTTGTTCAAACGCCCGGAATCATT | 60 | 34 | 210 | PCT WO 86/07095 |
| | 3' TGCTTGACTGCAGGGACCGAGAGGTTTGGG | | | | |
| Bovine specific | 5' AGGTCGCGAGATTGGTCGCTAGGTCATGCA | 60 | 34 | 300 | PCT WO 86/07095 |
| | 3' AAGACCTCGAGAGACCCTCTTCAACACGT | | | | |

## Results

*Effects of in vivo development on relative abundance of genes involved in apoptosis and glucose metabolism*

Apoptosis

Representative gel photographs of the semi-quantitative RT-PCR analysis in early blastocysts, blastocysts and expanded blastocysts of BAX, BCL-XL, and HSP 70.1 transcripts developed *in vitro* or *in vivo* (Figure 2A), for XIAP in male and female (Figure 2B) and PCR analysis of the sex of the embryo (Figure 2C) are shown in Figure 2.

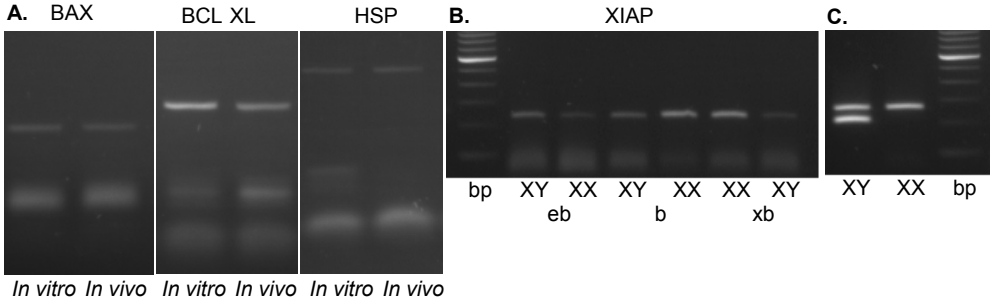



**Figure 2.** Photographs of representative agarose gels from semi-quantitative RT-PCR analysis of **A.** transcripts of BAX, BCL-XL and HSP 70.1 **B.** transcripts of XIAP in male and female early blastocysts, blastocysts and expanded blastocysts and base pair ladder (bp) **C.** PCR analysis of the sex of the embryos.

No effects of *in vitro* vs. *in vivo* culture on the relative abundance of BAX and BCL-XL transcripts in eb, b and xb were observed (Figure 3). mRNA expression of HSP 70.1 transcripts was significantly higher in eb, b and xb in the *In vivo* slaughter group (4), collected after slaughter of the animals, than in the In vitro group (1) and *In vivo* flush group (5).

To ensure the validity of this finding and to exclude a possible effect of different superovulation procedures, we analyzed the relative abundance of HSP 70.1 in expanded blastocysts that were collected from animals after exactly the same superovulation treatment as used for the animals of the *In vivo* slaughter group (4), with the sole difference that embryos were collected by non-surgical uterine flushing. Embryos from cows treated with different superovulation protocols did not differ in HSP 70.1 mRNA levels when collected by non-surgical uterine flushing (data not shown).

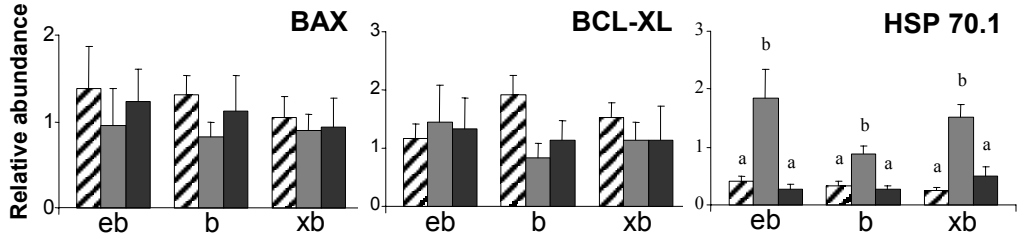



**Figure 3.** Expression pattern of BAX-, BCL-XL- and HSP 70.1 transcripts in the *In vitro* group (1) (striped bars), *In vivo* slaughter group (4) (dark grey bars) and *In vivo* flush group (5) (black bars) in single early blastocysts, blastocysts and expanded blastocysts. Bars with different superscripts differ significantly.

In early blastocysts the expression patterns of XIAP transcripts were similar in males and females in the In vitro group (1) as well as in the *In vivo* flush group (5). In blastocysts, mRNA expression of XIAP was significantly higher in females in the In vitro group (1) as well as in the *In vivo* flush group (5). However, expression of XIAP transcripts in expanded blastocysts was different between *in vitro* vs. *in vivo* culture: the relative abundance of XIAP transcripts was similar in male and female *in vitro*-produced expanded blastocysts, but in the *in vivo*-developed embryos the expression of XIAP was significantly higher in female expanded blastocysts than in their male counterparts (Figure 4). Sex ratio in the eb, b and xb of the In vitro group (1) and the *In vivo* flush group (5) was similar (data not shown), which is in accordance with earlier studies using the same culture conditions (van Wagtendonk-de Leeuw *et al.*, 2000).

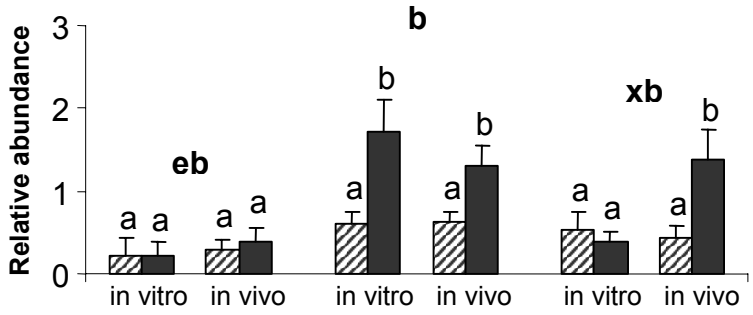



**Figure 4.** Expression pattern of XIAP transcript in single male (striped bars) and female (black bars) early blastocysts, blastocysts and expanded blastocysts produced *in vitro* and *in vivo*. Bars with different superscripts differ significantly.

Glucose metabolism

A representative gel photograph of the semi-quantitative RT-PCR analysis of glucose transporter 1, 3, 4 and 8 transcripts in expanded blastocysts is shown in Figure 5.

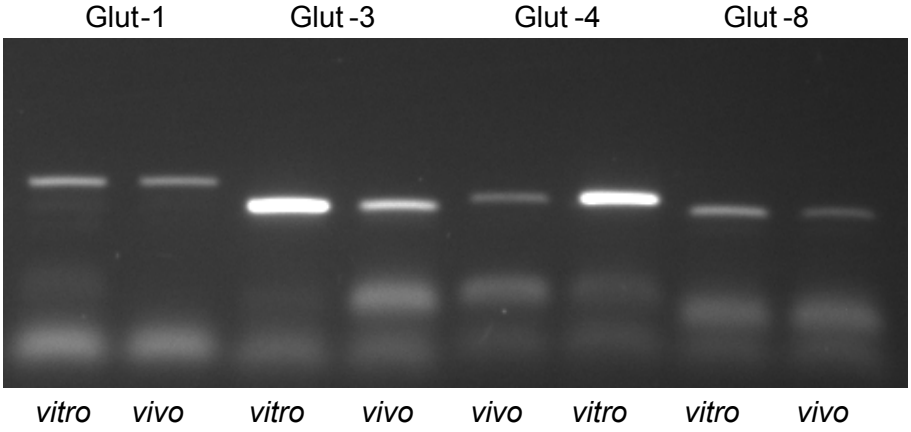



**Figure 5.** Photograph of representative agarose gel from semi-quantitative RT-PCR analysis of transcripts of glucose transporters 1, 3, 4 and 8 in completely *in vitro*- (1) and *in vivo*- (5) developed expanded blastocysts.

*In vitro* culture affected mRNA abundance of Glut-3, Glut-4 and Glut-8 in expanded blastocysts (Figure 6). A significant difference for Glut-3, Glut-4 and Glut-8 was observed between *in vitro*-produced and *in vivo*-developed expanded blastocysts. The relative abundance of Glut-1, Glut-4 and Glut-8 transcripts in expanded blastocysts developed *in vivo* until 45 h (2) or 100 h (3) po and further cultured *in vitro* was not different from that of expanded blastocysts produced *in vitro* (1) or *in vivo* (5). Expression of Glut-3 transcripts was significantly higher in the 100 h *In vivo* group (3) compared to the 45 h *In vivo* group (2) and *In vivo* flush group (5).

*Inhibition of transcription from the embryonic genome*

At 96-100 h after the start of fertilization a total of 68, 70 and 54 8- to 16-cell embryos were collected for the control, d0 and d1 groups, respectively. No significant difference in the percentage of embryos developed until the 8- to 16-cell stage was observed between the control, d0 and d1 groups (68%, 70% and 54%, respectively). In the absence of α -amanitin, 28% of zygotes developed to blastocysts. In the presence of α -amanitin no embryo reached the morula /blastocyst stage.

Treatment with α-amanitin at the start of culture as well as 24 h thereafter inhibited transcription from BCL-XL, XIAP, HSP 70.1, Glut-1, Glut-3 and Glut-8.

However, mRNA level of BAX was not affected by treatment with α-amanitin (data not shown). No transcripts for Glut-4 were detectable in 8- to 16-cell embryos.

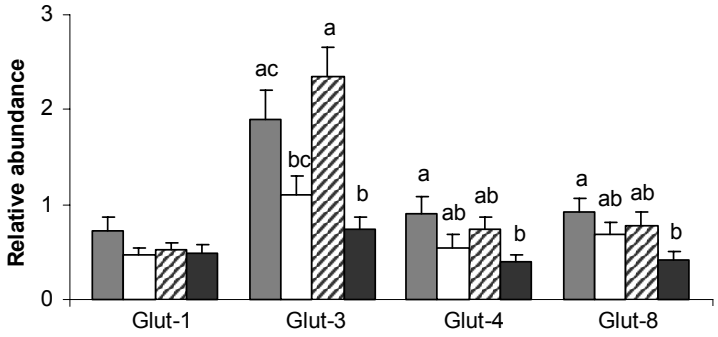



**Figure 6.** Expression pattern of glucose transporters transcripts in single expanded blastocysts after culture *in vitro* (1) (grey bars), embryos obtained at 45 h (2) (open bars) and 100 h (3) po (striped bars) from heifers stimulated for superovulation and cultured *in vitro* until the expanded blastocyst stage, and expanded blastocysts, from heifers stimulated for superovulation, developed entirely *in vivo* (5) (black bars) and collected by non-surgical uterine flushing. Bars with different superscripts differ significantly**.**

## Discussion

The present study investigated the effect of specific periods of *in vitro* and *in vivo* culture on mRNA expression of genes involved in apoptosis and glucose metabolism. Expression of gene transcripts involved in apoptosis, BAX, BCL-XL and HSP 70.1 was not affected by *in vitro* vs. *in vivo* culture. However, the relative abundance of HSP 70.1 transcripts was altered due to the collection protocol. In *in vitro*-produced expanded blastocysts the X-chromosome seems to be inactivated prematurely, suggesting that a low mRNA expression of XIAP could possibly be associated with higher levels of apoptosis. Although *in vitro* culture affected expression of glucose transporter genes, the critical period during which culture induced differential expression could not yet be determined.

The difference previously found in the level of apoptosis between *in vitro*- vs. *in vivo*-developed embryos suggests a difference in expression of genes involved in the apoptotic cascade (Gjørret *et al.*, 2003b; Knijn *et al.*, 2003). However, no effect of *in vitro* vs. *in vivo* culture on expression of apoptosis-related genes BAX and BCL-XL was observed in this study. A higher BAX expression in blastocysts produced in SOF medium compared to blastocysts developed *in vivo* has been reported (Rizos *et al.*, 2002a). This could be due to supplementation of the

culture medium with fetal calf serum. It is known that serum affects the mRNA expression of several genes and thereby affects the quality of embryos (Wrenzycki *et al.*, 1999; Rizos *et al.*, 2003). Messenger-RNA of HSP 70.1 transcripts was increased in all blastocyst stages collected ex-vivo at Day 7. This is in contrast to previous studies in which no differences between blastocysts derived *in vitro* in serum-free medium or *in vivo* were detected (Wrenzycki *et al.*, 2001; Lazzari *et al.*, 2002). By adding a group in which the embryos underwent the same treatment as the *In vivo* slaughter group (4), but the embryos were collected by non-surgical uterine flushing, we conclude that the differences in HSP 70.1 mRNA expression are caused by the collection protocol. This finding confirms that HSP 70.1 is a sensitive indicator of embryonic stress (Wrenzycki *et al.*, 1999) and indicates that even minor manipulations of embryos can lead to alterations in relative abundances.

Expression of XIAP transcripts was higher in female blastocysts irrespective of the production method. Recently it was shown that XIAP mRNA expression was increased in female bovine blastocysts produced *in vitro* compared to their male counterparts (Jimenez *et al.*, 2003). Here, we show that no dosage compensation is observed in *in vivo*-produced female expanded blastocysts. Speculatively, the premature inactivation of the X-chromosome in *in vitro*-produced expanded blastocysts is related to the increased level of apoptosis in *in vitro*-produced expanded blastocysts (Gjørret *et al.*, 2003b). For other X-linked gene transcripts a delay or lack of dosage compensation by *in vitro* culture has been reported (Wrenzycki *et al.*, 2002).

This study has shown that in bovine expanded blastocysts, expression of gene transcripts involved in glucose metabolism is affected by *in vitro* culture. For the first time, it was demonstrated that Glut-8 is actively transcribed by *in vivo*-developed expanded blastocysts and is up-regulated, similar to Glut-3 and Glut-4 transcripts by *in vitro* culture. The post-fertilization period appears critical in determining the quality of the embryo (Knijn *et al.*, 2002), and we hypothesized that the MET *in vitro* vs. vivo is crucial for regulating gene transcripts. However, expression of Gluts in embryos derived from the 45 h *In vivo* group (2) and the 100 h *In vivo* group (3) were not significantly different from the In vitro group (1) and from the *In vivo* flush group (5), with the exception of Glut-3. This finding suggests that MET *in vitro* or *in vivo* is not likely to be involved in aberrant mRNA expression patterns induced by suboptimum culture conditions. The high expression of Glut-3 transcript in the 100 h group could be due to the shift from *in vivo* to in vitro environment as Glut-3 is supposed to be a sensitive marker for embryo environment (Lazzari *et al.*, 2002). In contrast, expression of Glut-1 was not affected by the production method, which is in contrast to results of our earlier study (Knijn *et al.*, 2002) and could be due to the use of embryos of different developmental stages. In the mouse, expression of Glut-8 appears simultaneously with expression of the IGF-1 receptor and coincides with

movement of the embryo from the oviduct to the uterus where 10-fold higher concentrations of IGF-1 and insulin are found (Pinto *et al.*, 2002). A reduced Glut-8 expression, induced by Glut-8 antisense, was associated with increased apoptosis in murine blastocysts and poor pregnancy outcome (Pinto *et al.*, 2002) suggesting that the overall decrease in glucose transport is responsible for apoptosis in early embryos. Possibly, regulation of Glut-8 may be an additional anti-apoptotic mechanism in the blastocyst to maximize glucose utilization at this critical turning point in development, when the embryo switches from using pyruvate as its main energy substrate to using glucose (Wales, 1986; Carayannopoulos *et al.*, 2000).

The $\alpha$-amanitin treatment effectively blocked embryonic development. Furthermore, it blocked expression of Glut-1, Glut-3, Glut-8, BCL-XL, XIAP and HSP-70.1 transcripts from the embryonic genome and all maternally- derived transcripts were degraded at the 8- to 16-cell stage. These gene transcripts are of embryonic origin and transcribed after MET. The exception was that expression of BAX mRNA was not decreased in 8- to 16-cell stage embryos after treatment with $\alpha$-amanitin. It is possible that maternally-derived BAX transcripts are stable and not yet degraded in the 8- to 16-cell stage embryo. A possible mechanism to stabilize maternal transcripts was detected in Drosophila where expression of HSP 70.1 seems to be regulated at the level of transcript elongation rather than binding of RNA polymerase II to the promoter (Gilmour and Lis, 1986), and this might hold true for BAX as well.

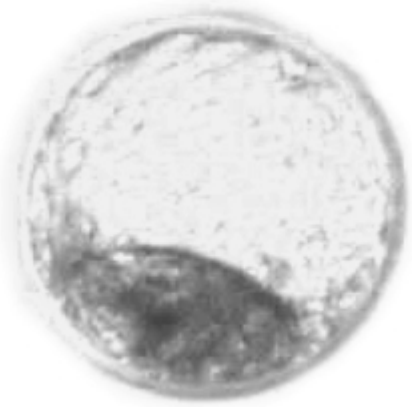
In conclusion, results of the present study did not show effect of culture regimen on the mRNA expression of BAX, BCL-XL and HSP 70.1. Expression of HSP 70.1 transcripts was up-regulated by the collection protocol. Expression of XIAP transcripts in expanded blastocysts seems to be affected by *in vitro* culture, suggesting that this could be one of the reasons for increased apoptosis. Culture environment *in vitro* vs. *in vivo* affected expression of glucose transporter transcripts, Glut-3, Glut-4 and Glut-8; however, the critical period during culture responsible for this, prior to or after MET, could not yet be determined.

**Acknowledgments**

We are grateful to Christine Oei, Thea Blankenstein, Henk Heuveling, Klaus-Gerd Hadeler and Doris Herrmann for excellent technical assistance, and to animal handlers for management of the animals. The critical reading of the manuscript by Ms Jane Collins, Boston is highly appreciated. The authors thank Holland Genetics (Arnhem, The Netherlands) for supplying SOF culture medium. Netherlands Organization for Scientific Research supported this study.

# Chapter 7

## Summarizing Discussion



## Introduction

During the last decade, the use of assisted reproduction techniques has been increased and refined a lot, especially in bovine. Nevertheless, bovine embryos produced *in vitro* have a lower developmental capacity following transfer than their *in vivo* counterparts. Large field trials under commercial conditions have shown that the pregnancy rate after transfer of *in vitro*-produced embryos is, in general, significantly reduced (Hasler *et al.*, 1995; Galli and Lazzari, 1996; van Wagtendonk-de Leeuw *et al.*, 2000). Furthermore, in the offspring born after transfer of *in vitro*-produced embryos, a higher percentage of calves is born with the "Large Offspring Syndrome" (LOS), compared to that in the offspring of the *in vivo*-developed counterparts (Kruip and den Daas, 1997; Young *et al.*, 1998; Taverne *et al.*, 2002), although the outcome varies depending on the culture system used (Thompson *et al.*, 1995; van Wagtendonk-de Leeuw *et al.*, 2000). To ensure healthy offspring we need 1) to improve the conditions during *in vitro* production to mimic the natural conditions as closely as possible, and 2) to select the best quality embryos for transfer. The best parameter for quality of blastocysts would be the ability of the embryo to establish pregnancies after transfer, and healthy offspring. However, this is a very impractical parameter and therefore other methods to assess blastocyst quality are applied such as, morphology (van Soom and de Kruif, 1992), total cell number and inner cell mass number (van Soom *et al.*, 1997), kinetics of development (Holm *et al.*, 2002), tolerance to cryopreservation (Enright *et al.*, 2000; Rizos *et al.*, 2002b), chromosomal abnormalities (Hyttel *et al.*, 2001; Viuff *et al.*, 2001), metabolism (Thompson, 1997; Khurana and Niemann, 2000), gene expression (Wrenzycki *et al.*, 1996; Eckert and Niemann, 1998; Lequarre *et al.*, 2001; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a; Lonergan *et al.*, 2003b) and apoptosis (Byrne *et al.*, 1999). The work presented in this thesis has focused on a) identifying differences between *in vitro*-cultured and *in vivo*-developed bovine embryos, with a special focus on gene expression and apoptosis, and b) determining the critical period during *in vitro* production responsible for these differences. This chapter summarizes the major findings and considers their implications for further research.

## The culture system, a complicating factor

In general, because of a huge diversity that exists between *in vitro* culture systems used, it is complicated to compare the different reports about differences between *in vitro*-cultured and *in vivo*-developed embryos. In particular, the supplementation of fetal calf serum to the culture medium had distinct effects on the embryo with regards to developmental rate, cell number, gene expression,

cryosensitivity and level of apoptosis (Wrenzycki *et al.*, 1999; Holm *et al.*, 2002; Rizos *et al.*, 2003). The emergence of symptoms related to LOS was significantly reduced by using SOF medium without the addition of fetal calf serum compared to buffalo rat liver co-culture medium with the addition of 10% fetal calf serum (van Wagtendonk-de Leeuw *et al.*, 2000). Therefore, in most of the research presented in this thesis, *in vitro* culture was performed in SOF medium without the addition of fetal calf serum.

**Timing of the developmental stages**

To study differences between *in vitro*-cultured and *in vivo*-developed embryos, it is important to compare embryos of the same stage after corresponding times of development. During *in vitro* production, the time when oocytes are transferred to fertilization medium is known. Nevertheless, there is a range from 1 to 6 h after transfer of the oocyte to the fertilization medium before penetration of the oocyte by sperm occurs (Xu and Greve, 1988; Kochhar *et al.*, 2003). For *in vivo*-developed embryos, it is necessary to know the exact time of ovulation to estimate the start of fertilization and embryonic development. During super-ovulation procedures, large differences between individual cows with regards to the onset of ovulations are reported (Dieleman and Bevers, 1987; Callesen *et al.*, 1988). The superovulation protocol with temporary suppression of the endogenous LH surge by norgestomet, followed by administration of GnRH to induce the LH surge at a fixed time, used in all experiments reported in this thesis, was designed to diminish the variation in onset of ovulation to obtain an accurate timing of the developmental stage of the embryo (Vos *et al.*, 1994; van de Leemput *et al.*, 2001). In **Chapter 2**, this superovulation protocol was evaluated by analyzing hormonal characteristics, superovulation response and both oocyte and blastocyst, yield and quality. This study showed that an LH surge occurred earlier than expected in less then 1% of the animals, and consequently in these cases oocytes and embryos could have been collected at a different stage of development than intended. Both yield and morphological quality of the oocytes and embryos were similar to those used in common superovulation protocols. Therefore, the superovulation protocol used, with temporary suppression of the endogenous LH surge, provides a valid tool to collect sufficient numbers of good quality oocytes and embryos of accurately defined timing of developmental stages.

**Emergence of differences in quality during early embryonic development**

The emergence of differences in quality observed at the blastocyst stage may find their origin at preceding stages of development. Therefore, the stage of development at which these differences first become apparent can likely provide information about the underlying mechanisms responsible for these differences. In **Chapter 4**, it is shown that different elements of apoptosis could be observed at various developmental stages in *in vitro*-cultured and *in vivo*-developed embryos. Nuclear condensation was first observed at the 6-cell stage *in vitro* and the 8-cell stage *in vivo* whereas DNA degradation, detected by the TUNEL reaction, was first observed at the 6-cell stage *in vitro* and the 21-cell stage *in vivo*. In this study, the embryos were cultured in Menezo-B2 medium supplemented with 10% fetal calf serum. Corresponding data are not available for embryos cultured in SOF medium. As mentioned earlier, the composition of the culture medium can affect the quality of the developing embryo. The apoptotic index in blastocysts cultured in Menezo-B2 supplemented with 10% fetal calf serum was lower than in blastocysts cultured in SOF medium with or without 10% fetal calf serum (Gjørret, 2003). This was supported by our findings in **Chapters 4 and 5,** were we reported a higher apoptotic index in embryos cultured in SOF without fetal calf serum (**Chapter 5**) compared to embryos cultured in Menezo-B medium (**Chapter 4**). Whether the first emergence of different elements of apoptosis is also affected by the composition of the culture medium needs to be elucidated. With regards to chromosomal abnormalities, Viuff et al. (1999; 2001) reported a higher incidence in embryos cultured in Menezo-B2 medium compared to embryos developed entirely *in vivo*. Already from day 2 of development onwards, at the 4-cell stage up to the blastocyst stage, more chromosomal abnormalities were observed after *in vitro* culture. Further, it was shown that from day 3 onwards, *in vivo*-developed embryos contained significantly more cells than their *in vitro* produced counterparts. These findings were supported by our results in **Chapter 5,** where we reported a significantly lower total cell count in *in vitro*-produced morulae and blastocysts, using SOF medium without the addition of fetal calf serum, compared to their *in vivo* developed counterparts. It is clear that the occurrence of apoptosis, the incidence of chromosomal abnormalities, and the total number of cells in blastocysts, originate from earlier stages of development. These three features are probably related. As mentioned before, a possible role for apoptosis in preimplantation embryos is supposed to be the elimination of abnormal cells. Therefore, it can be assumed that lower cell numbers will be found when the percentage of abnormal cells, i.e. with chromosomal aberrations, increases. However, it is also possible that the higher cell number in *in vivo*-developed embryos is not caused by a lower incidence of apoptosis but is due to a shorter duration of cell cycles. Holm et al. (2002) showed that the duration of cell cycles varied with the route of production. It

would be very interesting to develop techniques that provide more information on the mechanisms that result in apoptosis of cells in preimplantation embryos by combining staining for apoptosis with techniques identifying chromosomal abnormalities.

Gene expression patterns were also altered by *in vitro* production at an early stage of development. Expression of several developmentally important gene transcripts was analyzed in embryos of different developmental stages from zygotes to blastocysts, after maturation and fertilization *in vitro* and culture *in vitro* or *in vivo* in ewe oviduct (Lonergan *et al.*, 2003b). Depending on the transcript, differences between *in vitro*-cultured and *in vivo*-developed embryos were already evident as little as 10 h after initiation of culture and were still evident at the blastocyst stage (Lonergan *et al.*, 2003b). In **Chapter 6,** a similar rapid response in the expression of a specific gene was observed. Within a few hours after slaughter of the animals and collection of the embryos from the genital tract, an upregulation of the expression of HSP 70.1 was observed in all blastocyst stages.

Also morphologically, completely *in vitro*-cultured embryos differ from *in vivo*-developed embryos from the first cell cycle onwards. They appear darker due to higher lipid contents (Thompson, 1997), blastomeres are more swollen and the perivitelline space is smaller at all precompaction stages, and at the morula stage, compaction is less evident (van Soom and de Kruif, 1992).

In conclusion, already at the earliest stages of development *in vitro*-cultured embryos differ from their *in vivo*-developed counterparts in many different aspects. The intriguing questions are:
1) Is the embryo capable to cope with the induced differences and will it develop into a “normal” blastocyst and later into a healthy calf?
2) Do these deviations have a deleterious effect and stop further development of the embryo? 3) Does development continue, despite these deviations, and cause aberrations in the developing calf?

**A critical period during development from oocyte to blastocyst?**

Differences in embryo quality between *in vitro*-cultured and *in vivo*-developed blastocysts may originate from all steps of *in vitro* production, i.e. oocyte maturation and fertilization and embryo culture. In this thesis, the effect of oocyte maturation and embryo culture on the quality of the blastocyst was studied to identify critical timepoints responsible for the differences between completely *in vitro*-cultured and entirely *in vivo*-developed blastocysts.

*Oocyte maturation*

Many studies have been performed investigating the effect of the origin of the oocyte, *in vitro*- versus *in vivo*-matured, on its ability to develop to the blastocyst stage. Some studies reported a crucial effect of *in vivo* maturation on the competence of the oocyte to develop to the blastocyst stage (Greve *et al.*, 1987; Leibfried-Rutledge *et al.*, 1987; van de Leemput *et al.*, 1999). However, in these studies, the developmental competence between oocytes derived from slaughterhouse ovaries, from follicles of 2- to 8-mm diameter, were compared with oocytes collected *in vivo* after the LH surge. The oocytes collected from the 3- to 8-mm follicles lacked the period of preovulatory development in comparison with the oocytes collected *in vivo* after the LH surge, and therefore, they probably missed not only the final maturation *in vivo* but also the prematuration events occurring before the LH surge (Hendriksen *et al.*, 2000). When the developmental competence of oocytes collected *in vivo* just before the LH surge were compared with oocytes collected 24 h after the LH surge conflicting results were reported: Rizos et al. (2002b), reported a higher developmental competence with oocytes matured *in vivo* and our research group did not find a difference in developmental competence of the oocyte to develop to the blastocyst stage (Dieleman *et al.*, 2002). Possibly, minor differences in the composition of the maturation media, for example the supplementation of different FSH preparations, are crucial in determining the competence of the *in vitro*-matured oocyte. Reports on the effect of the mode of final maturation on the quality of the blastocyst are limited. Watson et al. (2000) showed that the maturation conditions *in vitro* can affect the cell number of the blastocyst. This raises the question whether differences in quality, found between *in vitro*-cultured and *in vivo*-developed blastocysts, could originate from the maturation regimen. In **Chapter 3,** it was shown that the expression of six developmentally important genes in bovine blastocysts was not affected by the maturation regimen. The six genes studied are involved in various mechanisms important in preimplantation development such as cavitation and compaction (desmocollin-II, E-cadherin and plakophilin), metabolism (glucose transporter-1), initiation of translation of mRNA (Poly(A)) and stress (heat shock protein 70.1). No significant difference was observed in the expression of these genes between blastocysts developed from oocytes collected just before the LH surge or 24 h after the LH surge. The expression levels were similar as in blastocysts developed from oocytes collected from slaughterhouse ovaries, whereas the expression of three genes was higher in blastocysts developed entirely *in vivo* compared to the three groups of blastocysts developed from oocytes derived from slaughterhouse ovaries, oocytes collected from cows before the LH surge and 24 h after the LH surge. Nevertheless, the mode of oocyte maturation can have an effect on the expression of other gene transcripts. Our finding was in accordance with a recent study of Rizos et al., (2002b) in which they reported that final maturation of the oocyte either *in vitro* or *in vivo* had no

effect on the cryosensitivity of bovine blastocysts. This suggests that maturation is not the determining step causing the differences in quality between *in vitro*-cultured and *in vivo*-developed blastocysts, based on the quality parameters gene expression and cryosensitivity. On the other hand, final maturation *in vivo* appeared to reduce the incidence of chromosomal abnormalities in conjunction with an increase of the cell number in blastocysts, in comparison to blastocysts derived after *in vitro* maturation, *in vitro* fertilization and *in vitro* culture (Dieleman *et al.*, 2002).

*Early embryonic development*

To determine which part of *in vitro* culture was responsible for the differences found between completely *in vitro*-cultured and entirely *in vivo*-developed embryos, special focus was put on the period of the maternal embryonic transition (MET), that occurs in bovine during the 4$^{th}$ cell cycle, by collecting embryos developed *in vivo* until either before the 4$^{th}$ cell cycle or after the 4$^{th}$ cell cycle and thereafter cultured further *in vitro* until Day 7.

In **Chapter 5**, it is reported that the proportion of oocytes developing to the morula/blastocyst stage was not altered when embryos were grown *in vivo* until or just after the MET in comparison to embryos that develop entirely *in vivo* to this stage. This indicates that *in vitro* culture does not influence the developmental competence of the oocyte to form a blastocyst. This was in accordance with recent studies showing that the post-fertilization period did not affect the proportion of oocytes developing to the blastocyst stage (Enright *et al.*, 2000; Rizos *et al.*, 2002a; 2002b; 2003). However, the post-fertilization period seems to have an effect on the quality of the embryo in terms of cryotolerance (Rizos *et al.*, 2002b; 2003) and abundance of several gene transcripts (Rizos *et al.*, 2002a; 2003).

**Chapters 5 and 6** describe the effect of the *in vitro* culture step on a range of quality parameters such as, total cell number and inner cell mass number, blastulation rate, level of apoptosis and expression of developmentally important genes. In **Chapter 5**, it is shown that the total cell number and inner cell mass number were significantly higher in entirely *in vivo*-developed morulae and blastocysts compared to their completely *in vitro*-cultured counterparts. The level of apoptosis was significantly higher in completely *in vitro*-cultured expanded blastocysts compared to entirely *in vivo*-developed expanded blastocysts. However, no significant difference was observed at the morula, early blastocyst and blastocyst stages. It seems that the period before the MET is decisive for the difference in the level of apoptosis in expanded blastocysts. For the difference in total number of cells and number of inner cell mass cells the period up to the 5$^{th}$ cell cycle, after the MET, seems to be the decisive period. Development of embryos *in vivo*, until before or after MET and subsequent culture *in vitro*, apparently did not affect the time of initiation of blastulation. However, when

embryos develop entirely *in vivo* to the blastocyst stage, blastulation occurred at a later time. This “delayed blastulation” *in vivo*, seems to originate from the period after the MET and may be mediated by factors originating from the uterine environment since, in bovine, development up to the 8- to 16-cell stage embryo occurs in the oviduct and further development takes place in the uterus (Leese, 1988; Ellington, 1991; Gandolfi, 1994). In **Chapter 6**, striking differences in expression of gene transcripts involved in glucose transport between completely *in vitro*- and entirely *in vivo*-derived blastocysts were reported. The critical period responsible for this, before or after the MET, could not be determined since probably the expression of the glucose transporter genes studied is deviated gradually during the entire culture period. This finding is in accordance with a recent study indicating that the quality, i.e. cryosensitivity, of blastocysts cultured partly in the ewe oviduct (*in vivo*) and partly in SOF medium (*in vitro*) was gradually impaired when the embryos spend increasing time in SOF culture medium (Lonergan *et al.*, 2003c).

As mentioned earlier, in **Chapters 4 and 5**, it is reported that the level of apoptosis in completely *in vitro*-cultured expanded blastocysts is higher than in their *in vivo*-developed counterparts. To unravel the molecular basis for this difference, the expression of four gene transcripts involved in the apoptotic cascade was studied in **Chapter 6**. No differences in expression were observed between *in vitro*-cultured and *in vivo*-developed expanded blastocysts. Clearly, as discussed in **Chapter 1**, many proteins are involved in the apoptotic cascade and the expression of other genes involved in apoptosis may be deviated by *in vitro* culture.

In conclusion, the culture step seems to be the most important period in determining several features related to blastocyst quality. It appears, however, that no unambiguous answers can be presented to the question from which specific period during *in vitro* culture, before or after MET, the quality differences that are reported between *in vitro*-cultured and *in vivo*-developed blastocysts originate.

## Implications

There are still severe deficiencies regarding to our understanding of the significance of the observed differences between *in vitro*-cultured and *in vivo*-developed embryos with respect to later stages of embryonic development and further development into healthy offspring. For example, it is unclear whether upregulation of glucose transporter genes in *in vitro*-cultured embryos or increased level of apoptosis as reported in **Chapters 5 and 6** is favorable for the embryo, showing that it is able to adapt to a suboptimal environment? Or is it a sign of an embryo “in trouble”? Furthermore, deviant mRNA levels of genes do

not necessarily imply deviations in their functionality with respect to translation to proteins. To collect more information about the functionality of mRNA, immuno-histochemical studies have to be conducted to analyze the presence of the proteins.

The SOF culture medium without the addition of fetal calf serum seems to create an environment for preimplantation embryos that resembles the *in vivo* situation better than co-culture or culture-systems with the addition of fetal calf serum. As mentioned earlier, the introduction of SOF culture medium without the addition of fetal calf serum diminished the number of aberrations related to LOS (van Wagtendonk-de Leeuw *et al.*, 2000). Moreover, the data on apoptosis presented in this thesis show only small differences in the apoptotic index of embryos cultured in SOF medium compared to embryos development entirely *in vivo* (**Chapter 5**). Nevertheless, the differences in expression of gene transcripts, especially the glucose transporter gene transcripts, between embryos cultured in SOF and *in vivo*-developed embryos (**Chapter 6)** indicate that the culture conditions still need to be improved.

**Future considerations**

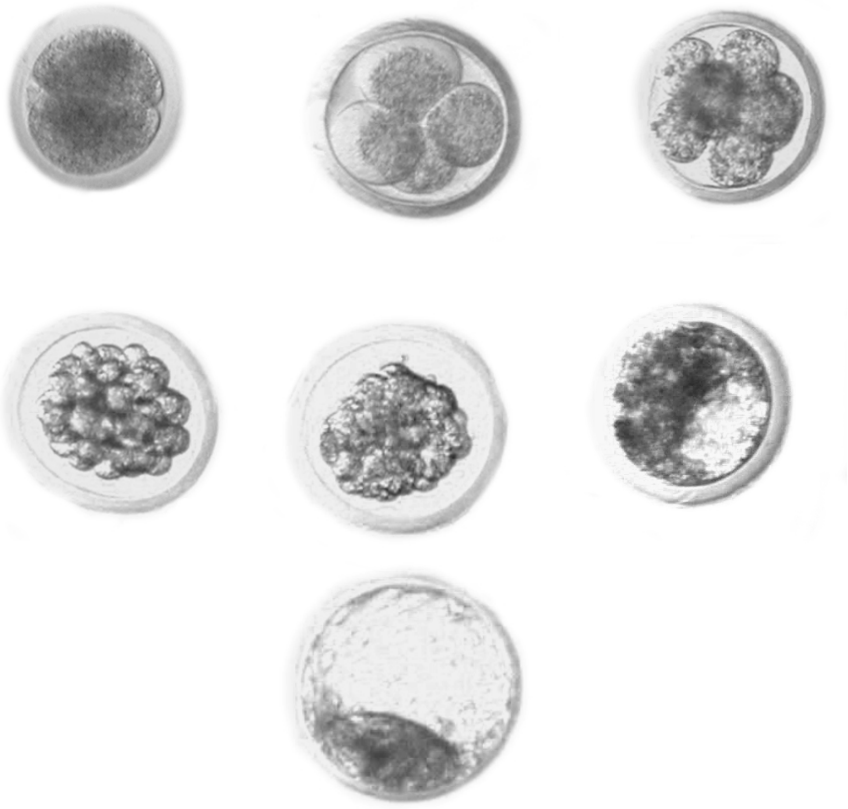
Overlooking all fundamental differences between *in vitro* culture and *in vivo* development it is remarkable that *in vitro* production of embryos functions at the level as it does nowadays. It is clear that there is an enormous flexibility of preimplantation bovine embryos to adapt to the environment. Nevertheless, *in vitro* embryo production techniques should mimic the natural conditions as closely as possible. To improve *in vitro* embryo techniques it is necessary to know all deviations induced by *in vitro* culture and which part of the embryo production technique is responsible for these aberrations.

Knowledge about the consequences of *in vitro* fertilization on the quality of the developing embryo is very limited up till now. The number of accessory sperm cells found in the zona pellucida of *in vivo* zygotes varies from only 5-75 (Hawk, 1988). In contrast, *in vitro*-matured oocytes are, in general, still enclosed by cumulus cells at fertilization, and the sperm-oocyte ratio is very high *in vitro*, as the customary concentration of sperm cells in the fertilization medium is 1-2 x $10^6$ sperm per ml. Until now it has been very difficult to study the fertilization step separately because of technical problems. A recently developed method of transfer or flush of zygotes and embryos directly in or out the bovine oviduct under endoscopic guidance (Besenfelder *et al.*, 2001) opens new possibilities to study the several steps of *in vitro* production separately, especially fertilization.

A very promising tool to collect an enormous amount of information about differences in expression of genes in oocytes and all stages of the preimplantation embryo is the microarray technique. Preliminary results

comparing immature and *in vitro* matured bovine oocytes as reported by Dalbiès-Tran and Mermillod (2003) using a human cDNA array were promising, but results from bovine arrays applied to oocytes and embryos at specific developmental stages are expected in the near future and will provide more detailed information. The challenge will be to discover genes that are the key players in determining the ability of oocytes to develop into healthy offspring and to identify marker genes that can serve as optimal quality parameters for bovine embryos.

# References



## References

**Adams GP, Matteri RL, Kastelic JP, Ko JC and Ginther OJ** (1992) Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers *J Reprod Fertil* **94** 177-188

**AETE** (2002) Proceedings of the 18th scientific meeting of the Association Européenne de Transfert Embryonnaire, The Netherlands

**Ahern T and Gardner D** (1998) Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass *Theriogenology* **49** 194 abstract

**Alikani M, Cohen J, Tomkin G, Garrisi GJ, Mack C and Scott RT** (1999) Human embryo fragmentation in vitro and its implications for pregnancy and implantation *Fertil Steril* **71** 836-842

**Antczak M and Van Blerkom J** (1999) Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains *Hum Reprod* **14** 429-447

**Asdell SA** (1964) Patterns of mammalian reproduction. Constable & Co Ltd, London

**Avery B and Greve T** (2000) Effects of ethanol and dimethylsulphoxide on nuclear and cytoplasmic maturation of bovine cumulus-oocyte complexes *Mol Reprod Dev* **55** 438-445

**Avery B, Hay-Schmidt A, Hyttel P and Greve T** (1998) Embryo development, oocyte morphology, and kinetics of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to in vitro maturation *Mol Reprod Dev* **50** 334-344

**Barros CM and Nogueira MF** (2001) Embryo transfer in Bos indicus cattle *Theriogenology* **56** 1483-1496

**Bashirullah A, Halsell SR, Cooperstock RL, Kloc M, Karaiskakis A, Fisher WW, Fu W, Hamilton JK, Etkin LD and Lipshitz HD** (1999) Joint action of two RNA degradation pathways controls the timing of maternal transcript elimination at the midblastula transition in Drosophila melanogaster *Embo J* **18** 2610-2620

**Bavister BD** (1995) Culture of preimplantation embryos: facts and artefacts. *Human Reproduction Update* **1** 91-148

**Bavister BD** (2002) Early history of in vitro fertilization *Reproduction* **124** 181-196

**Ben Jebara MK, Carrière PD and Price CA** (1994) Decreased pulsatile LH secretion in heifers superovulated with eCG or FSH *Theriogenology* **42** 685-694

**Besenfelder U, Havlicek V, Mosslacher G and Brem G** (2001) Collection of tubal stage bovine embryos by means of endoscopy. A technique report *Theriogenology* **55** 837-845

**Betteridge KJ and Fléchon J-E** (1988) The anatomy and physiology of pre-attachment bovine embryos *Theriogenology* **9** 155-187

**Betts DH and King WA** (2001) Genetic regulation of embryo death and senescence *Theriogenology* **55** 171-191

**Bevers MM and Dieleman SJ** (1987) Superovulation of cows with PMSG: variation in plasma concentrations of progesterone, oestradiol, LH, cortisol, prolactin and PMSG and in number of preovulatory follicles *Anim Reprod Sci* **15** 37-52

**Bevers MM, Dieleman SJ, van Tol HT, Blankenstein DM and van den Broek J** (1989) Changes in pulsatile secretion patterns of LH, FSH, progesterone, androstenedione and oestradiol in cows after superovulation with PMSG *J Reprod Fertil* **87** 745-754

**Boni R, Tosti E, Roviello S and Dale B** (1999) Intercellular communication in in vivo- and in vitro-produced bovine embryos *Biol Reprod* **61** 1050-1055

**Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF and Dressel MA** (1982) Normal development following in vitro fertilization in the cow *Biol Reprod* **27** 147-158

**Brevini-Gandolfi TA, Favetta LA, Mauri L, Luciano AM, Cillo F and Gandolfi F** (1999) Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence *Mol Reprod Dev* **52** 427-433

**Brison DR and Schultz RM** (1997) Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha *Biol Reprod* **56** 1088-1096

**Buzzard KA, Giaccia AJ, Killender M and Anderson RL** (1998) Heat shock protein 72 modulates pathways of stress-induced apoptosis *J Biol Chem* **273** 17147-17153

**Byrne AT, Southgate J, Brison DR and Leese HJ** (1999) Analysis of apoptosis in the preimplantation bovine embryo using TUNEL *J Reprod Fertil* **117** 97-105

**Callesen H, Greve T and Hyttel P** (1988) Preovulatory evaluation of the superovulatory response in donor cattle *Theriogenology* **30** 477-488

**Camous S, Kopecny V and Flechon JE** (1986) Autoradiographic detection of the earliest stage of [3H]-uridine incorporation into the cow embryo *Biol Cell* **58** 195-200

**Chautan M, Chazal G, Cecconi F, Gruss P and Golstein P** (1999) Interdigital cell death can occur through a necrotic and caspase-independent pathway *Curr Biol* **9** 967-970

**Cohen GM, Sun XM, Snowden RT, Dinsdale D and Skilleter DN** (1992) Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation *Biochem J* **286** 331-334

**Collins JA, Schandi CA, Young KK, Vesely J and Willingham MC** (1997) Major DNA fragmentation is a late event in apoptosis *J Histochem Cytochem* **45** 923-934

**Cotter TG, Lennon SV, Glynn JG and Martin SJ** (1990) Cell death via apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells *Anticancer Res* **10** 1153-1159

**Dalbies-Tran R and Mermillod P** (2003) Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation *Biol Reprod* **68** 252-261

**Darzynkiewicz Z, Bedner E and Traganos F** (2001) Difficulties and pitfalls in analysis of apoptosis *Methods Cell Biol* **63** 527-546

**de la Fuente R and King WA** (1997) Use of a chemically defined system for the direct comparison of inner cell mass and trophectoderm distribution in murine, porcine and bovine embryos *Zygote* **5** 309-320

**de Sousa PA, Watson AJ, Schultz GA and Bilodeau-Goeseels S** (1998) Oogenetic and zygotic gene expression directing early bovine embryogenesis: a review *Mol Reprod Dev* **51** 112-121

**Devreker F and Hardy K** (1997) Effects of glutamine and taurine on preimplantation development and cleavage of mouse embryos in vitro *Biol Reprod* **57** 921-928

**Dieleman SJ and Bevers MM** (1987) Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows *J Reprod Fertil* **81** 533-542

**Dieleman SJ, Bevers MM, Poortman J and van Tol HTM** (1983a) Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood *J Reprod Fertil* **69** 641-649

**Dieleman SJ, Bevers MM, van Tol HTM and Willemse AH** (1986) Peripheral plasma concentrations of oestradiol, progesterone, cortisol, LH and prolactin during the oestrous cycle in cow, with emphasis on the peri-oestrous period *Anim Reprod Sci* **10** 275-292

**Dieleman SJ, Hendriksen PJM, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM and Vos PLAM** (2002) Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos *Theriogenology* **57** 5-20

**Dieleman SJ, Kruip TA, Fontijne P, de Jong WH and van der Weyden GC** (1983b) Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone *J Endocrinol* **97** 31-42

**D'Occhio MJ, Fordyce G, Whyte TR, Aspden WJ and Trigg TE** (2000) Reproductive responses of cattle to GnRH agonists *Anim Reprod Sci* **60-61** 433-442

**D'Occhio MJ, Gifford DR, Earl CR, Weatherly T and von Rechenberg W** (1989) Pituitary and ovarian responses of post-partum acyclic beef cows to continuous long-term GnRH and GnRH agonist treatment *J Reprod Fertil* **85** 495-502

**D'Occhio MJ, Sudha G, Jillella D, Whyte T, Maclellan LJ, Walsh J, Trigg TE and Miller D** (1997) Use of a GnRH agonist to prevent the endogenous LH surge and injection of exogenous LH to induce ovulation in heifers superstimulated with FSH: a new model for superovulation *Theriogenology* **47** 601-613

**Eckert J and Niemann H** (1998) mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-beta in bovine embryos derived in vitro or in vivo *Mol Hum Reprod* **4** 957-965

**Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM and Efstratiadis A** (1997) Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes *Genes Dev* **11** 3128-3142

**Ellington JE** (1991) The bovine oviduct and its role in reproduction: a review of the literature *Cornell Vet* **81** 313-328

**El-Shershaby AM and Hinchliffe JR** (1974) Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate *J Embryol Exp Morphol* **31** 643-654

**Enders AC, Hendrickx AG and Binkerd PE** (1982) Abnormal development of blastocysts and blastomeres in the rhesus monkey *Biol Reprod* **26** 353-366

**Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X and Boland MP** (2000) Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality *Theriogenology* **54** 659-673

**Exley GE, Tang C, McElhinny AS and Warner CM** (1999) Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos *Biol Reprod* **61** 231-239

**Fair T, Hulshof SC, Hyttel P, Greve T and Boland M** (1997) Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles *Mol Reprod Dev* **46** 208-215

**Fair T, Hyttel P and Greve T** (1995) Bovine oocyte diameter in relation to maturational competence and transcriptional activity *Mol Reprod Dev* **42** 437-442

**Fair T, Hyttel P, Greve T and Boland M** (1996) Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle *Mol Reprod Dev* **43** 503-512

**Farin PW, Crosier AE and Farin CE** (2001) Influence of in vitro systems on embryo survival and fetal development in cattle *Theriogenology* **55** 151-170

**Galli C and Lazzari G** (1996) Practical aspects of IVM/IVF in cattle *Anim Reprod Sci* **42** 371-379

**Gandolfi F** (1994) Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst *Theriogenology* **41** 95-100

**Gavrieli Y, Sherman Y and Ben-Sasson SA** (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation *J Cell Biol* **119** 493-501

**Gjørret J, Avery B and P M-H** (2003a) Active-Caspase, TUNEL and ultrastructure of apoptotic cell death in bovine blastocysts *Theriogenology* **59** 322 abstract

**Gjørret JO** (2003) Apoptosis in bovine embryos *Thesis, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark*

**Gjørret JO, Avery B, Larsson L-I, Schellander K and Hyttel P** (2001) Apoptosis in bovine blastocysts produced in vivo and in vitro *Theriogenology* **55** 321 abstract

**Gjørret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI and Maddox-Hyttel P** (2003b) Chronology of apoptosis in bovine embryos produced in vivo and in vitro *Biol Reprod* **69** 1193-1200

**Gong JG, Bramley TA, Gutierrez CG, Peters AR and Webb R** (1995) Effects of chronic treatment with a gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovarian function in heifers *J Reprod Fertil* **105** 263-270

**Gong JG, Campbell BK, Bramley TA, Gutierrez CG, Peters AR and Webb R** (1996) Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicle development in heifers continuously infused with a gonadotropin-releasing hormone agonist *Biol Reprod* **55** 68-74

**Gouveia Nogueira MF, Barros BJP, Teixeira AB, Trinca LA, D'Occhio MJ and Barros CM** (2002) Embryo recovery and pregnancy rates after the delay of ovulation and fixed time insemination in superstimulated beef cows *Theriogenology* **57** 1625-1634

**Gradela A, Esper CR and Rosa e Silva AAM** (1996) Plasma concentrations of progesterone, 17beta-estradiol and androstenedione and superovulatory response of Nelore cows (Bos indicus) treated with FSH *Theriogenology* **45** 843-850

**Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W and Schulte-Hermann R** (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note *Hepatology* **21** 1465-1468

**Greve T, Callesen H, Hyttel P, Hoier R and Assey R** (1995) The effects of exogenous gonadotropins on oocyte and embryo quality in cattle *Theriogenology* **43** 41-50

**Greve T, Xu KP, Callesen H and Hyttel P** (1987) In vivo development of in vitro fertilized bovine oocytes matured in vivo versus in vitro *J In Vitro Fert Embryo Transf* **4** 281-285

**Gualtieri R and Talevi R** (2003) Selection of highly fertilization-competent bovine spermatozoa through adhesion to the Fallopian tube epithelium in vitro *Reproduction* **125** 251-258

**Guilbault LA, Grasso F, Lussier JG, Rouillier P and Matton P** (1991) Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle *J Reprod Fertil* **91** 81-89

**Hackett AJ, Durnford R, Mapletoft RJ and Marcus GJ** (1993) Location and status of embryos in the genital tract of superovulated cows 4 to 6 days after insemination *Theriogenology* **40** 1147-1153

**Handyside AH and Hunter S** (1986) Cell division and death in the mouse blastocyst before implantation *Roux's Archives of Developmental biology* **195** 519-526

**Hardy K** (1997) Cell death in the mammalian blastocyst *Mol Hum Reprod* **3** 919-925

**Hardy K** (1999) Apoptosis in the human embryo *Rev Reprod* **4** 125-134.

**Hardy K and Handyside AH** (1996) Metabolism and cell allocation during parthenogenetic preimplantation mouse development *Mol Reprod Dev* **43** 313-322

**Hardy K, Handyside AH and Winston RML** (1989) The human blastocyst: cell number, death and allocation during late preimplantation development in vitro *Development* **107** 597-604

**Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE and Trimmer SA** (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results *Theriogenology* **43** 141-152

**Hasler JF, McGauley AD, Schermerhorn EC and Foote RH** (1983) Superovulatory responses of Holstein cows *Theriogenology* **19** 83-99

**Hawk HW** (1988) Gamete transport in the ovulated cow *Theriogenology* **29** 125-142

**Hendriksen PJM, Vos PLAM, Steenweg WN, Bevers MM and Dieleman SJ** (2000) Bovine follicular development and its effect on the in vitro competence of oocytes *Theriogenology* **53** 11-20

**Hinck L, Van Der Smissen P, Heusterpreute M, Donnay I, De Hertogh R and Pampfer S** (2001) Identification of caspase-3 and caspase-activated deoxyribonuclease in rat blastocysts and their implication in the induction of chromatin degradation (but not nuclear fragmentation) by high glucose *Biol Reprod* **64** 555-562

**Holm P, Booth PJ and Callesen H** (2002) Kinetics of early in vitro development of bovine in vivo- and in vitro- derived zygotes produced and/or cultured in chemically defined or serum- containing media *Reproduction* **123** 553-565

**Huppertz B, Frank HG and Kaufmann P** (1999) The apoptosis cascade--morphological and immunohistochemical methods for its visualization *Anat Embryol (Berl)* **200** 1-18

**Hyttel P, Fair T, Callesen H and Greve T** (1997) Oocyte growth, capacitation and final maturation in cattle *Theriogenology* **47** 23-32

**Hyttel P, Viuff D, Avery B, Laurincik J and Greve T** (1996) Transcription and cell cycle-dependent development of intranuclear bodies and granules in two-cell bovine embryos *J Reprod Fertil* **108** 263-270

**Hyttel P, Viuff D, Fair T, Laurincik J, Thomsen PD, Callesen H, Vos PLAM, Hendriksen PJM, Dieleman SJ, Schellander K, Besenfelder U and Greve T** (2001) Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos *Reproduction* **122** 21-30

**Izadyar F, Zeinstra E, Colenbrander B, Vanderstichele HM and Bevers MM** (1996) In vitro maturation of bovine oocytes in the presence of bovine activin A does not affect the number of embryos *Anim Reprod Sci* **45** 37-45

**Jurisicova A, Latham KE, Casper RF and Varmuza SL** (1998a) Expression and regulation of genes associated with cell death during murine preimplantation embryo development *Mol Reprod Dev* **51** 243-253

**Jurisicova A, Rogers I, Fasciani A, Casper RF and Varmuza S** (1998b) Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development *Mol Hum Reprod* **4** 139-145

**Jurisicova A, Varmuza S and Casper RF** (1996) Programmed cell death and human embryo fragmentation *Mol Hum Reprod* **2** 93-98

**Kafi M and McGowan MR** (1997) Factors associated with variation in the superovulatory response of cattle *Anim Reprod Sci* **48** 137-157

**Kamjoo M, Brison DR and Kimber SJ** (2002) Apoptosis in the preimplantation mouse embryo: effect of strain difference and in vitro culture *Mol Reprod Dev* **61** 67-77

**Kanitz W, Becker F, Schneider F, Kanitz E, Leiding C, Nohner HP and Pöhland R** (2002) Superovulation in cattle: practical aspects of gonadotropin treatment and insemination *Reprod Nutr Dev* **42** 587-599

**Kanka J** (2003) Gene expression and chromatin structure in the pre-implantation embryo *Theriogenology* **59** 3-19

**Kerr JF, Wyllie AH and Currie AR** (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics *Br J Cancer* **26** 239-257

**Khurana NK and Niemann H** (2000) Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo *Biol Reprod* **62** 847-856

**Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H and Dieleman SJ** (2002) Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo *Reproduction* **124** 365-375

**Knijn HM, Gjørret JO, Vos PLAM, Hendriksen PJM, van der Weijden GC, Maddox-Hyttel P and Dieleman SJ** (2003) Consequences of in vivo development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos *Biol* Reprod **69** 1371-1378

**Kochhar HS, Kochhar KP, Basrur PK and King WA** (2003) Influence of the duration of gamete interaction on cleavage, growth rate and sex distribution of in vitro produced bovine embryos *Anim Reprod Sci* **77** 33-49

**Kölle S, Stojkovic M, Boie G, Wolf E and Sinowatz F** (2002) Growth hormone inhibits apoptosis in in vitro produced bovine embryos *Mol Reprod Dev* **61** 180-186

**Kopecny V, Flechon JE, Camous S and Fulka J, Jr.** (1989) Nucleologenesis and the onset of transcription in the eight-cell bovine embryo: fine-structural autoradiographic study *Mol Reprod Dev* **1** 79-90

**Kruip TAM and den Daas JHG** (1997) In vitro produced and cloned embryos:effects on pregnancy, parturition and offspring *Theriogenology* **1997** 43-52

**Lafri M, Ponsart C, Nibart M, Durand M, Morel A, Jeanguyot N, Badinand F, De Mari K and Humblot P** (2002) Influence of CIDR treatment during superovulation on embryo production and hormonal patterns in cattle *Theriogenology* **58** 1141-1151

**Lane M and Gardner DK** (1997) Differential regulation of mouse embryo development and viability by amino acids *J Reprod Fertil* **109** 153-164

**Latham KE, Patel B, Bautista FD and Hawes SM** (2000) Effects of X chromosome number and parental origin on X-linked gene expression in preimplantation mouse embryos *Biol Reprod* **63** 64-73

**Laurincik J, Oberfranc M, Hyttel P, Grafenau P, Tomanek M and Pivko J** (1993) Characterization of the periovulatory period in superovulated heifers *Theriogenology* **39** 537-544

**Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H and Galli C** (2002) Cellular and molecular deviations in bovine in vitro-produced

embryos are related to the large offspring syndrome *Biol Reprod* **67** 767-775

**Leese HJ** (1988) The formation and function of oviduct fluid *J Reprod Fertil* **82** 843-856

**Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL and First NL** (1987) Development potential of bovine oocytes matured in vitro or in vivo *Biol Reprod* **36** 376-383

**Leist M and Jaattela M** (2001) Four deaths and a funeral: from caspases to alternative mechanisms *Nat Rev Mol Cell Biol* **2** 589-598

**Lequarre AS, Feugang JM, Malhomme O, Donnay I, Massip A, Dessy F and Van Langendonckt A** (2001) Expression of Cu/Zn and Mn superoxide dismutases during bovine embryo development: influence of in vitro culture *Mol Reprod Dev* **58** 45-53

**Lerner SP, Thayne WV, Baker RD, Henschen T, Meredith S, Inskeep EK, Dailey RA, Lewis PE and Butcher RL** (1986) Age, dose of FSH and other factors affecting superovulation in Holstein cows *J Anim Sci* **63** 176-183

**Leunda-Casi A, Genicot G, Donnay I, Pampfer S and De Hertogh R** (2002) Increased cell death in mouse blastocysts exposed to high D-glucose in vitro: implications of an oxidative stress and alterations in glucose metabolism *Diabetologia* **45** 571-579

**Lighten AD, Moore GE, Winston RM and Hardy K** (1998) Routine addition of human insulin-like growth factor-I ligand could benefit clinical in vitro fertilization culture *Hum Reprod* **13** 3144-3150

**Liu X, Zou H, Slaughter C and Wang X** (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis *Cell* **89** 175-184

**Liu Z and Foote RH** (1997) Effects of amino acids and alpha-amanitin on bovine embryo development in a simple protein-free medium *Mol Reprod Dev* **46** 278-285

**Lonergan P, Monaghan P, Rizos D, Boland MP and Gordon I** (1994) Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro *Mol Reprod Dev* **37** 48-53

**Lonergan P, Rizos D, Gutiérrez-Adán A, Fair T and Boland M** (2003a) Oocyte and Embryo Quality: Effect of Origin, Culture Conditions and Gene Expression Patterns *Reprod Domest Anim* **38** 259-267

**Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, De La Fuente J and Boland MP** (2003b) Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo *Biol Reprod* **69** 1424-1431

**Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P and Boland MP** (2003c) Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality *Reproduction* **126** 337-346

**Long CR, Dobrinsky JR, Garrett WM and Johnson LA** (1998) Dual labeling of the cytoskeleton and DNA strand breaks in porcine embryos produced in vivo and in vitro *Mol Reprod Dev* **51** 59-65

**Lyon MF** (1961) Gene action in the X-chromosome of the mouse (*Mus musculus L.*) *Nature* **190** 372-373

**Maddox-Hyttel P, Gjørret JO, Vatja G, Alexopopulo NI, Lewis I, Trounson AO, Viuff D, Laurincik J, Müller M, Tveden-Nyborg P and Thomsen PD** (2003) Morhological assessment of pre-implantation embryo quality in cattle *Reproduction Supplement* 103-116

**Majno G and Joris I** (1995) Apoptosis, oncosis, and necrosis. An overview of cell death *Am J Pathol* **146** 3-15

**Makarevich AV and Markkula M** (2002) Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during in vitro maturation and culture *Biol Reprod* **66** 386-392

**Mapletoft RJ, Steward KB and Adams GP** (2002) Recent advances in the superovulation in cattle *Reprod Nutr Dev* **42** 601-611

**Matwee C, Betts DH and King WA** (2000) Apoptosis in the early bovine embryo *Zygote* **8** 57-68

**Matwee C, Kamaruddin M, Betts DH, Basrur PK and King WA** (2001) The effects of antibodies to heat shock protein 70 in fertilization and embryo development *Mol Hum Reprod* **7** 829-837

**Memili E and First NL** (1998) Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development *Mol Reprod Dev* **51** 381-389

**Memili E and First NL** (2000) Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species *Zygote* **8** 87-96

**Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PLAM and Dieleman SJ** (2003) Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry *Theriogenology* **59** 651-674

**Merton JS, van Wagtendonk- de Leeuw AM and den Daas JHG** (1998) Factors affecting birthweight of IVP calves *Theriogenology* **49** 293 abstract

**Moley KH, Chi MM, Knudson CM, Korsmeyer SJ and Mueckler MM** (1998) Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways *Nat Med* **4** 1421-1424

**Monniaux D, Chupin D and Saumande J** (1983) Superovulatory responses of cattle *Theriogenology* **19** 55-81

**Moore T and Reik W** (1996) Genetic conflict in early development: parental imprinting in normal and abnormal growth *Rev Reprod* **1** 73-77

**Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C and Brambilla E** (1996) In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations *J Histochem Cytochem* **44** 959-968

**Neuber E, Luetjens CM, Chan AW and Schatten GP** (2002) Analysis of DNA fragmentation of in vitro cultured bovine blastocysts using TUNEL *Theriogenology* **57** 2193-2202

**Newcomb R, Rowson LEA and Trounson AO** (1976) The entry of superovulated eggs into the uterus. *In* "Egg transfer in cattle" (Rowson LEA, ed.), pp. 1-15. Commission of the European Communities, Luxembourg

**Niemann H and Wrenzycki C** (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development *Theriogenology* **53** 21-34

**Pampfer S** (2000) Apoptosis in rodent peri-implantation embryos: differential susceptibility of inner cell mass and trophectoderm cell lineages--a review *Placenta* **21 Suppl A** S3-10

**Pampfer S, Vanderheyden I, McCracken JE, Vesela J and De Hertogh R** (1997) Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-alpha in vitro *Development* **124** 4827-4836

**Parrish JJ, Susko-Parrish J, Winer MA and First NL** (1988) Capacitation of bovine sperm by heparin *Biol Reprod* **38** 1171-1180

**Paula-Lopes FF and Hansen PJ** (2002) Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon *Biol Reprod* **66** 1169-1177

**Pavlok A, Kopecny V, Lucas-Hahn A and Niemann H** (1993) Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by in vitro maturation and fertilization of oocytes from different growth categories of antral follicles *Mol Reprod Dev* **35** 233-243

**Pavlok A, Lucas-Hahn A and Niemann H** (1992) Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles *Mol Reprod Dev* **31** 63-67

**Pierce GB, Lewellyn AL and Parchment RE** (1989) Mechanism of programmed cell death in the blastocyst *Proc Natl Acad Sci U S A* **86** 3654-3658

**Pinyopummintr T and Bavister B** (1994) Development of bovine embryos in a cell-free culture medium: effects of type of serum, timing of its inclusion and heat inactivation *Theriogenology* **41** 1241-1249

**Plante L, Plante C, Shepherd DL and King WA** (1994) Cleavage and 3H-uridine incorporation in bovine embryos of high in vitro developmental potential *Mol Reprod Dev* **39** 375-383

**Price CA, Carrière PD, Gosselin N, Kohram H and Guilbault LA** (1999) Effects of superovulation on endogenous LH secretion in cattle, and consequences for embryo production *Theriogenology* **51** 37-46

**Reik W, Brown KW, Schneid H, Le Bouc Y, Bickmore W and Maher ER** (1995) Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by altered imprinting pattern in the IGF2-H19 domain *Hum Mol Genet* **4** 2379-2385

**Rieger D, Roberge S, Coy DH and Rawlings NC** (1989) Effects of an LHRH antagonist on gonadotrophin and oestradiol secretion, follicular development, oestrus and ovulation in Holstein heifers *J Reprod Fertil* **86** 157-164

**Rieger D, Walton JS, Johnson WH and Coy DH** (1990) The effect of treatment with an LHRH antagonist on the quality of day-7 embryos collected from superovulated holstein heifers *Theriogenology* **33** 307, abstract

**Rizos D, Gutiérrez-Adán A, Perez-Garnelo S, De La Fuente J, Boland MP and Lonergan P** (2003) Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression *Biol Reprod* **68** 236-243

**Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J and Gutiérrez-Adán A** (2002a) Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality *Biol Reprod* **66** 589-595

**Rizos D, Ward F, Duffy P, Boland MP and Lonergan P** (2002b) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality *Mol Reprod Dev* **61** 234-248

**Robertson I and Nelson R** (1998) Certification of the embryos. *Manual of the International Embryo Transfer Society,* **3rd Edn** 103-134 Eds DA Stringfellow and SM Seidel. International Embryo Transfer Society, Savoy, IL

**Sanchez T, Wehrman ME, Kojima FN, Cupp AS, Bergfeld EG, Peters KE, Mariscal V, Kittok RJ and Kinder JE** (1995) Dosage of the synthetic progestin, norgestomet, influences luteinizing hormone pulse frequency and endogenous secretion of 17 beta-estradiol in heifers *Biol Reprod* **52** 464-469

**Sapienza C** (1990) Parental imprinting of genes *Sci Am* **263** 52-60

**Savio JD, Keenan L, Boland MP and Roche JF** (1988) Pattern of growth of dominant follicles during the oestrous cycle of heifers *J Reprod Fertil* **83** 663-671

**Sible JC, Anderson JA, Lewellyn AL and Maller JL** (1997) Zygotic transcription is required to block a maternal program of apoptosis in Xenopus embryos *Dev Biol* **189** 335-346

**Spanos S, Becker DL, Winston RM and Hardy K** (2000) Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development *Biol Reprod* **63** 1413-1420

**Sreenan JM** (1970) In vitro maturation and attempted fertilization of cattle follicular oocytes *J Agricult Sci* **75** 393-396

**Sreenan JM, Diskin MG and Morris DG** (2001) Embryo survival rate in cattle:a major limitation to the achievement of high fertility *British Society of Animal Science* **1** 93-104

**Stack JH and Newport JW** (1997) Developmentally regulated activation of apoptosis early in Xenopus gastrulation results in cyclin A degradation during interphase of the cell cycle *Development* **124** 3185-3195

**Stanton JA, Macgregor AB and Green DP** (2003) Gene expression in the mouse preimplantation embryo *Reproduction* **125** 457-468

**Tadros W, Houston SA, Bashirullah A, Cooperstock RL, Semotok JL, Reed BH and Lipshitz HD** (2003) Regulation of maternal transcript destabilization during egg activation in Drosophila *Genetics* **164** 989-1001

**Takagi M, Kim IH, Izadyar F, Hyttel P, Bevers MM, Dieleman SJ, Hendriksen PJM and Vos PLAM** (2001) Impaired final follicular maturation in heifers after superovulation with recombinant human FSH *Reproduction* **121** 941-951

**Tam PPL** (1988) Postimplantation development of mitomycin C-treated mouse blastocysts *teratology* **37** 205-212

**Taverne MA, Breukelman SP, Perenyi Z, Dieleman SJ, Vos PLAM, Jonker HH, de Ruigh L, Van Wagtendonk-de Leeuw JM and Beckers JF** (2002) The monitoring of bovine pregnancies derived from transfer of in vitro produced embryos *Reprod Nutr Dev* **42** 613-624

**Taylor C, Rajamahendran R and Walton JS** (1993) Ovarian follicular dynamics and plasma luteinizing hormone concentrations in norgestomet-treated heifers *Anim Reprod Sci* **32** 173-184

**Telford NA, Watson AJ and Schultz GA** (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species *Mol Reprod Dev* **26** 90-100

**Thibier M** (2002) A contrasted year for the world activity of the animal embryo transfer industry *IETS newsletter* **20** 13-18

**Thompson JG** (1997) Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants *Reprod Fertil Dev* **9** 341-354

**Thompson JG, Gardner DK, Pugh PA, McMillan WH and Tervit HR** (1995) Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos *Biol Reprod* **53** 1385-1391

**van de Leemput EE, Vos PLAM, Hyttel P, van den Hurk R, Bevers MM, van der Weijden GC and Dieleman SJ** (2001) Effects of brief postponement of the preovulatory LH surge on ovulation rates and embryo formation in eCG/prostaglandin-treated heifers *Theriogenology* **55** 573-592

**van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, van der Weijden GC and Dieleman SJ** (1999) Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge *Theriogenology* **52** 335-349

**van Soom A, Boerjan M, Ysebaert MT and De Kruif A** (1996) Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media *Mol Reprod Dev* **45** 171-182

**van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M and de Kruif A** (1997) Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation *Biol Reprod* **57** 1041-1049

**van Soom A and de Kruif A** (1992) A comparative study of in vitro and in vivo derived bovine embryos. *In* "Proc 12th Int Cngr Anim Reproduc", pp. 1363-1365

**van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B and de Ruigh L** (2000) Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring *Theriogenology* **53** 575-597

**Viuff D, Avery B, Greve T, King WA and Hyttel P** (1996) Transcriptional activity in in vitro produced bovine two- and four-cell embryos *Mol Reprod Dev* **43** 171-179

**Viuff D, Hendriksen PJM, Vos PLAM, Dieleman SJ, Bibby BM, Greve T, Hyttel P and Thomsen PD** (2001) Chromosomal abnormalities and developmental kinetics in in vivo- developed cattle embryos at days 2 to 5 after ovulation *Biol Reprod* **65** 204-208

**Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H and Thomsen PD** (1999) A high proportion of bovine blastocysts produced in vitro are mixoploid *Biol Reprod* **60** 1273-1278

**Vos PLAM, Bevers MM, Willemse AH and Dieleman SJ** (1994) Effects of suppression by a progesterone-releasing intravaginal device and subsequent induction by GnRH of the preovulatory LH surge on follicular function in PMSG/PG-treated heifers *J Reprod Fertil* **101** 43-49

**Vos PLAM, Fokker HW, Knijn HM and Dieleman SJ** (2003) Embryonic development in vivo relative to the preovulatory LH surge after superovulation with controlled LH surge in heifers *Theriogenology* **59** 332 abstract

**Warner CM, Cao W, Exley GE, McElhinny AS, Alikani M, Cohen J, Scott RT and Brenner CA** (1998) Genetic regulation of egg and embryo survival *Hum Reprod* **13 Suppl 3** 178-190

**Watson AJ** (1992) The cell biology of blastocyst development *Mol Reprod Dev* **33** 492-504

**Watson AJ, De Sousa P, Caveney A, Barcroft LC, Natale D, Urquhart J and Westhusin ME** (2000) Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis *Biol Reprod* **62** 355-364

**Weil M, Jacobson MD, Coles HS, Davies TJ, Gardner RL, Raff KD and Raff MC** (1996) Constitutive expression of the machinery for programmed cell death *J Cell Biol* **133** 1053-1059

**Wiltbank MC, Gumen A and Sartori R** (2002) Physiological classification of anovulatory conditions in cattle *Theriogenology* **57** 21-52

**Wrenzycki C, Herrmann D, Carnwath JW and Niemann H** (1996) Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo *J Reprod Fertil* **108** 17-24

**Wrenzycki C, Herrmann D, Carnwath JW and Niemann H** (1999) Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA *Mol Reprod Dev* **53** 8-18

**Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Jr., Sirisathien S, Brackett B and Niemann H** (2001) Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos *Hum Reprod* **16** 893-901

**Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K and Niemann H** (2002) In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos *Biol Reprod* **66** 127-134

**Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X and Shi Y** (2000) Structural basis of IAP recognition by Smac/DIABLO *Nature* **408** 1008-1012

**Wubishet A, Kesler DJ, Graves CN, Spahr SL and Favero RJ** (1991) Preovulatory LH profiles of superovulated cows and progesterone concentrations at embryo recovery *Theriogenology* **35** 451-457

**Wuu YD, Pampfer S, Becquet P, Vanderheyden I, Lee KH and De Hertogh R** (1999) Tumor necrosis factor alpha decreases the viability of mouse blastocysts in vitro and in vivo *Biol Reprod* **60** 479-483

**Wyllie AH** (1997) Apoptosis: an overview *Br Med Bull* **53** 451-465

**Wyllie AH, Kerr JF and Currie AR** (1980) Cell death: the significance of apoptosis *Int Rev Cytol* **68** 251-306

**Xu KP and Greve T** (1988) A detailed analysis of early events during in vitro fertilization of bovine follicular oocytes *J Reprod Fertil* **82** 127-134

**Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW and Oh KS** (1998) Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos *Hum Reprod* **13** 998-1002

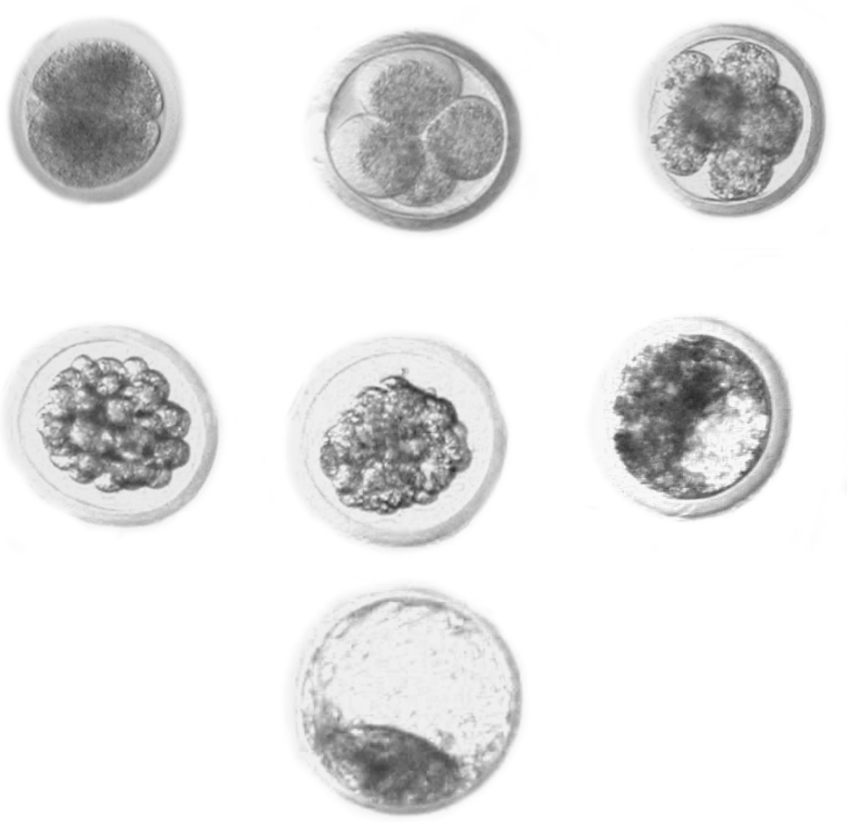
**Young LE and Fairburn HR** (2000) Improving the safety of embryo technologies: possible role of genomic imprinting *Theriogenology* **53** 627-648.

**Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I and Sinclair KD** (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture *Nat Genet* **27** 153-154

**Young LE, Sinclair KD and Wilmut I** (1998) Large offspring syndrome in cattle and sheep *Rev Reprod* **3** 155-163

**Zimmermann KC, Bonzon C and Green DR** (2001) The machinery of programmed cell death *Pharmacol Ther* **92** 57-70

# Samenvatting



## Samenvatting

De eerste pogingen om *in vitro* rundereicellen te fertiliseren werden al in de late zestiger jaren ondernomen maar pas in 1982 werd het eerste kalf geboren na transplantatie van een totaal *in vitro* geproduceerd embryo. Sindsdien is het *in vitro* productieproces veel verbeterd maar het is nog steeds onmogelijk om de *in vivo* situatie geheel na te bootsen. Niet alleen de efficiëntie van de *in vitro* procedure om morulae en blastocysten te produceren is lager dan in de *in vivo* situatie maar ook de kwaliteit van *in vitro* geproduceerde embryo's is minder dan die van *in vivo* ontwikkelde embryo's. Het drachtigheidspercentage na transfer van *in vitro* geproduceerde embryo's is in het algemeen aanmerkelijk lager vergeleken met transfer van *in vivo* ontwikkelde embryo's en een klein maar significant hoger percentage van de kalveren wordt geboren met afwijkingen die bekend staan als het "Large Offspring Syndrome".

De beste parameter om de kwaliteit van een embryo te bepalen is de mogelijkheid van het embryo om uit te groeien tot een gezond kalf. Dit is echter zeer onpraktisch en daarom worden er verschillende andere parameters gebruikt zoals morfologie, celaantal van het totale embryo en van de "inner cell mass", snelheid van ontwikkeling, metabolisme, chromosomale afwijkingen, expressie-niveau van genen en apoptose. In dit proefschrift worden verschillen beschreven tussen embryo's die *in vitro* gekweekt of *in vivo* ontwikkeld zijn met speciale aandacht voor het celaantal, expressie-niveau van genen en apoptose. Verder wordt onderzocht welke stappen tijdens het *in vitro* productieproces verantwoordelijk zijn voor de gevonden verschillen. De algemene strategie was om een deel van het ontwikkelingsproces *in vivo* te laten plaatsvinden met daarna een vervolg *in vitro*. De op deze wijze verkregen embryo's werden vergeleken met embryo's die volledig *in vitro* of volledig *in vivo* waren ontwikkeld. Speciale aandacht werd gegeven aan de maturatie van de eicel en de periode van activatie van het embryonale genoom dat in runderembryo's plaatsvindt tijdens de 4$^{de}$ celcyclus, in het 8- tot 16-cellige stadium.

Een overzicht van de verschillende stappen van embryo-ontwikkeling, mogelijke oorzaken van afwijkingen in de expressie van genen tijdens de vroege embryonale ontwikkeling en een omschrijving van apoptose en de mogelijke functie daarvan tijdens vroege embryonale ontwikkeling zijn beschreven in **Chapter 1**.

Om *in vitro* gekweekte- met *in vivo* ontwikkelde embryo's te kunnen vergelijken is het noodzakelijk dat de ontwikkelingsstadia van de embryo's nauwkeurig kunnen worden bepaald. Tijdens *in vitro* productie van embryo's is het ontwikkelingsstadium vrij nauwkeurig te bepalen maar door variatie in het ovulatietijdstip is

dit voor *in vivo* ontwikkelde embryo's veel onnauwkeuriger. In **Chapter 2** is een methode geëvalueerd waar met behulp van superovulatie met FSH en met norgestomet/GnRH, de LH piek met een nauwkeurigheid van een half uur geïnduceerd kan worden. Hierdoor wordt de variatie in het tijdstip van ovulatie kleiner en kan het ontwikkelingsstadium van het embryo nauwkeuriger worden bepaald. De LH piek was in meer dan 90% van de koeien op het verwachte tijdstip en de respons op de superovulatiebehandeling en de embryo-opbrengst waren voldoende. Geconcludeerd kan worden dat deze techniek een waardevolle methode is om embryo's van bepaalde stadia te verzamelen.

In **Chapter 3** is het effect beschreven van eicel-maturatie *in vitro* of *in vivo* op de mRNA expressie van zes genen in blastocysten. Deze zes genen waren geselecteerd omdat ze belangrijk zijn in verschillende processen tijdens de vroege embryonale ontwikkeling. Voor drie van deze genen was de mRNA expressie lager in geheel *in vitro*- vergeleken met compleet *in vivo* geproduceerde blastocysten. De mRNA expressie van deze genen in embryo's gekweekt uit eicellen die tot de maturatie of tot en met de maturatie *in vivo* ontwikkelde en daarna *in vitro* gefertiliseerd en gekweekt waren was hetzelfde als in de geheel *in vitro* geproduceerde embryo's. Geconcludeerd kan worden dat de maturatie van de eicel niet de belangrijkste factor was die verschillen in mRNA expressie van deze zes genen tussen compleet *in vitro* gekweekte embryo's en compleet *in vivo* ontwikkelde embryo's veroorzaakt.

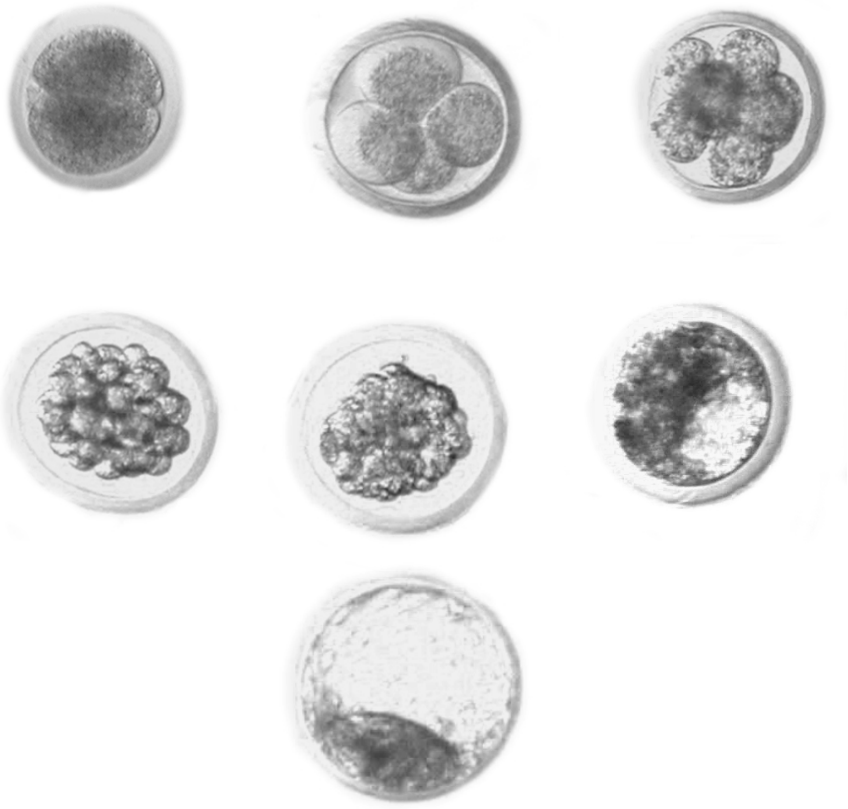
Volgens een aantal studies bevatten compleet *in vitro* ontwikkelde blastocysten meer apoptotische cellen dan hun *in vivo* tegenhangers. In **Chapter 4** is onderzocht op welk tijdstip van de ontwikkeling verschillende kenmerken van apoptose voor het eerst optreden in geheel *in vitro*- en compleet *in vivo* ontwikkelde embryo's. Eén kenmerk van apoptose, condensatie van de kern, trad eerder op tijdens *in vitro*- dan tijdens *in vivo* ontwikkeling, respectievelijk, 6-cellig versus 8-cellig. Een tweede kenmerk van apoptose, breuken in het DNA, werd in *in vitro* geproduceerde embryo's al waargenomen in het 6-cellige stadium terwijl in *in vivo* embryo's dit pas in het 21-cellige stadium optrad. **Chapter 5** beschrijft het effect van *in vitro* kweek tijdens de periode van activatie van het embryonale genoom op het celaantal van het embryo en het aantal cellen dat apoptose ondergaat in morulae en blastocysten. De resultaten toonden aan dat embryo's die vlak voor de 4$^{de}$ celcyclus van *in vivo* ontwikkeling over werden gezet naar *in vitro* kweek, in het blastocyst stadium, minder apoptotische cellen hadden dan geheel *in vitro* geproduceerde blastocysten en evenveel als compleet *in vivo* ontwikkelde blastocysten. Het totaal aantal cellen in blastocysten die geheel *in vitro* waren gekweekt was lager dan in blastocysten die compleet *in vivo* waren ontwikkeld. Het totaal aantal cellen was gelijk in geheel *in vitro* geproduceerde blastocysten en blastocysten die *in vivo* ontwikkelde tot voor de 4$^{de}$ celcyclus en

verder *in vitro* gekweekt werden, terwijl het celaantal in blastocysten die tot na de 4$^{de}$ celcyclus *in vivo* ontwikkelde en daarna *in vitro* gelijk was aan het celaantal van compleet *in vivo* ontwikkelde blastocysten.

In **Chapter 6** worden verschillen beschreven in de expressie van genen die betrokken zijn bij glucose-metabolisme en apoptose tussen compleet *in vitro* en compleet *in vivo* ontwikkelde blastocysten. Verder werd onderzocht of het passeren van de periode van activatie van het embryonale genoom *in vitro* een beslissende rol speelt. Er werden geen verschillen gevonden in de expressie van vier genen die betrokken zijn bij apoptose tussen geheel *in vitro*- en compleet *in vivo* ontwikkelde blastocysten. Drie genen die betrokken zijn bij glucose-transport kwamen hoger tot expressie in geheel *in vitro* geproduceerde embryo's. Het kon echter niet worden vastgesteld of de periode voor of na de activatie van het embryonale genoom hiervoor verantwoordelijk was.

Uit de resultaten van de experimenten beschreven in dit proefschrift lijkt de periode na de fertilisatie het belangrijkst voor het veroorzaken van verschillen in kwaliteit van een embryo, in dit proefschrift bepaald aan de hand van mRNA expressie van genen, totaal aantal cellen van het embryo en apoptose. Zowel de periode voor- als na de activatie van het embryonale genoom lijken belangrijk voor de verschillende aspecten van embryo-kwaliteit.

# Dankwoord



## Dankwoord

Aan het tot stand komen van dit proefschrift hebben vele, vele mensen bijgedragen en uiteraard wil ik iedereen daarvoor hartelijk bedanken!! Om te proberen aan alle mensen te denken en aan te geven op welke manier iedereen zijn/haar steentje bij heeft gedragen zal ik proberen te schetsen hoe de verschillende experimenten bedacht en uitgevoerd werden.

De eerste ideeën voor de experimenten werden met mijn enthousiaste co-promotorenteam, Steph Dieleman (vol met ideeën en nationale en internationale contacten om deze ook te verwezenlijken), Peter Hendriksen (gedreven en als kamergenoot was hij altijd bereid om alle opkomende vragen te bediscussiëren, ter plekke of tijdens een gezellig avondje) en Peter Vos (druk maar toch altijd de tijd-vindend om zijn kennis en kunde heel enthousiast bij te willen dragen) bedacht. Op de achtergrond stonden mijn promotoren Prof. Bert van der Weijden (die mij altijd de ruimte gaf om alle dingen te doen die nodig waren) en Prof. Heiner Niemann in Duitsland (who made my visits to Mariensee, Germany inspiring and fruitful) altijd klaar om mee te ”brainstormen”.

De praktische kanten werden besproken met Mart Bevers (die het “kweeklab” voor zijn rekening nam maar helaas de laatste experimenten niet meer heeft kunnen meemaken), Hans Lutz (die er, naast alle andere zaken, voor zorgde dat de koeien klaar stonden) en Frans van Kooi (die de logistiek in de kliniek verzorgde). Uiteindelijk stond iedereen paraat, de experimenten konden beginnen!!

De dierverzorgers (Freek, Irene, Nico M., Wim Brenk, Wim Broer, Sikko, Bert, Paul, Bart, Staas, Nico B., Gerrit, Paulco, Arie P., Arie W., Teus) behandelde de koeien zeer accuraat volgens de protocollen, dag en nacht werd er bloed getapt en behandeld. De dominante follikel punctie werd uitgevoerd met Henk Heuveling als onze steunpilaar. De bloedjes gingen naar het biochemisch laboratorium waar Thea Blankenstein en Christine Oei ervoor zorgden dat we alle hormoon-bepalingen op tijd tot onze beschikking hadden en daarnaast, tijdens en na de experimenten altijd zorgden voor kopjes thee en gezelligheid.

De koeien werden geopereerd met hulp van de operatieassistenten (Siebrand, Rik, Henk, Dick, Frans) of de chauffeurs zorgden (Willie, Piet, Jan, Rik) dat de koeien precies op tijd op het slachthuis arriveerde waar de baarmoeders verzameld werden door de operatie-assistenten of Jan Joop Harkema.

Ondertussen had Elly Zeinstra alles klaar staan op het embryo kweek-laboratorium om de eicellen en embryo’s die we verzamelden verder te verwerken. Om de “*in vitro* embryo’s” te kweken werkte er een heel internationaal “embryo-team” (Annadie, Anna Rita, Christine, Karianne, Jordi, Pachi, Omran, Nader, Patricia, Lenie, Erik, Theerawat) mee. Het medium om de embryo’s te kweken kon ik met één telefoontje regelen doordat Sybrand Merton en Lisette de Ruigh van Holland Genetics altijd direct bereid waren mee te werken.

Mijn collega's van de "leerstoel voortplanting" (Joyce, Dick, Maarten, Peter, Herman en Gerrit) leefde ondertussen mee en namen waar ze konden mijn diensten en onderwijsverplichtingen over. De andere runder-collegae (Annemarieke, Corein, Debora, Gerard, Ingrid, Jan van A., Jan L., Janneke, Jurjen, Karin, Kerstin, Leen, Mariska, Mark, Niek, Pia, Pim, Tine, Rogier, Ruurd, Steven, Wim) vroegen geïnteresseerd hoeveel embryo's ik had verzameld en zorgde voor de kopjes koffie en biertjes tussendoor met de nodige gezellige, "sterke" verhalen.

Eenmaal verzameld en gefixeerd moesten de embryo's natuurlijk geanalyseerd worden. De eerste RT-PCR experimenten heb ik samen met Lenie van Tol gedaan en met deze ervaring kon ik naar het Institute for Animal Science (FAL), Department of Biotechnology in Mariensee Duitsland om mijn embryo's te gaan analyseren. In Mariensee I got the opportunity to work in a very dedicated research team and received all the help I needed. The whole team made my several stays very successful, and my special thanks go to Christine Wrenzycki (for all her knowledge she wanted to share with me but also for her friendship) and to Doris Herrmann (for her patience and help in the laboratory). Voor ander analyse-werk van de embryo's moest er afgereisd worden naar Kopenhagen, Denemarken. In Copenhagen I received a warm welcome at the Department of Anatomy and Physiology of the Royal Veterinary Medicine and Agricultural University, especially by Prof. Poul Maddox-Hyttel and Jakob Gjørret who gave me all their help and all opportunities to use their facilities in a very friendly ambience.

Na al het laboratorium werk begon het analyse-werk van de verzamelde data. Hans Vernooy en Jan van de Broek hielpen met de statistiek. Meestal werd er eerst een abstract geschreven om de eerste data te kunnen presenteren op internationale congressen. Wim Bes zorgde er dan voor dat we altijd op tijd met de posters naar de congressen konden vertrekken.

"Last but not least" moesten de data natuurlijk nog worden opgeschreven in artikelen om te kunnen publiceren in internationale tijdschriften. Dit gebeurde onder de bezielende leiding van Steph met op de achtergrond de twee Peter's.

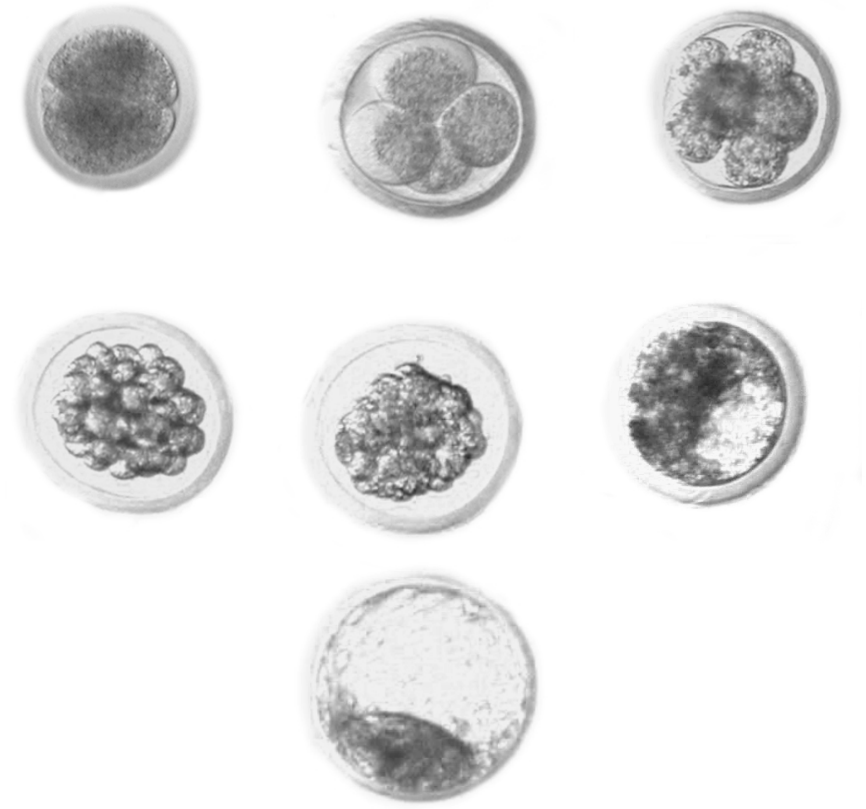
Iedereen héél héél hartelijk bedankt!!

Een aantal mensen die niet direct betrokken waren bij het onderzoek (Merel, Anna Rita, Irma, Els, Tine, Karin) waren getuigen van de werk/onderzoeks hoogtepunten en dips, luisterden en adviseerde waar ze konden en leverde hierdoor wel degelijk een bijdrage bij het tot stand komen van dit proefschrift.

Mijn twee paranimfen, Henk en Sandra, hebben mij, met name bij de laatste fase van het promoveren, met veel enthousiasme en precies het goede gevoel, bijgestaan en hebben geholpen om te proberen de promotiedag tot een feestdag te maken.

Mijn familie (Papa (hopelijk kan hij meekijken vanaf een wolkje), Mama, Jannemieke en Leon en kinderschare, Mirjam en Sandra) bedankt voor al jullie liefde.

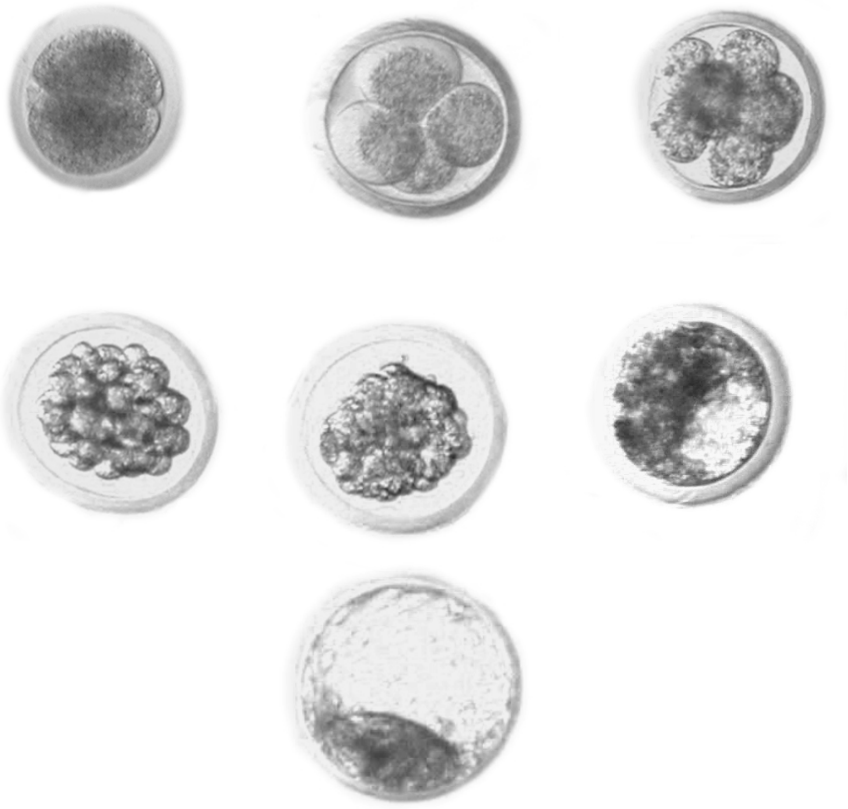
# Curriculum vitea



## Curriculum vitea

Hiemke Knijn werd op 25 augustus 1966 geboren te Amersfoort als dochter van Jan Knijn en Margot Knijn van den Hurk. In 1984 behaalde zij haar VWO diploma aan Het Rhedens Lyceum in Rozendaal. In datzelfde jaar begon zij de studie Medische Biologie aan de Universiteit Utrecht. Zij haalde haar doctoraal examen Medische Biologie in 1990, ondertussen was zij in 1988 tevens de studie Diergeneeskunde begonnen aan de Universiteit Utrecht. Tijdens deze studie periode deed zij onderzoekstages in Uppsala, Zweden en in Maputo, Mozambique. In 1996 studeerde zij af als dierenarts en werkte zij enkele jaren als landbouwhuisdieren practicus in verschillende dierartsenpraktijken. In 1998 kwam zij in dienst van de Faculteit Diergeneeskunde bij de toenmalige Vakgroep Bedrijfsdiergeneeskunde en Voortplanting en later bij de Hoofdafdeling Landbouwhuisdieren en begon in 1999 aan promotieonderzoek onder begeleiding van Bert van der Weijden, Heiner Niemann, Steph Dieleman, Peter Hendriksen en Peter Vos met dit proefschrift als resultaat.

# List of Publications



## List of publications

### *Refereed journals*

**Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H, and Dieleman SJ** (2002) Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived *in vitro* or *in vivo*. *Reproduction* **124**: 365-375

**Knijn HM, Gjørret JO, Vos PLAM, Hendriksen PJM, Van der Weijden BC, Maddox-Hyttel P, and Dieleman SJ** (2003) Consequences of *In vivo* development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. *Biol Reprod* **69**: 1371-1378

**Gjørret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI, and Maddox-Hyttel** (2003) Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*. *Biol Reprod* **69**: 1193-1200

**Dieleman SJ, Hendriksen PJM, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, and Vos PLAM** (2002) Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos *Theriogenology* **57**: 5-20

**Janszen BP, Knijn H, Van der Weyden GC, Bevers MM, Dieleman SJ, and Taverne MA** (1990) Flumethason-induced calving is preceded by a period of myometrial inhibition during luteolysis *Biol Reprod* **43**: 466-471

### *Conference poceedings*

**Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H, and Dieleman SJ** (2001) Effects of *in vitro* versus *in vivo* maturation on gene expression in single bovine blastocysts. *Theriogenology* **55:** 238 (runner-up in student competition)

**Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H, and Dieleman SJ** (2001) Comparison of expression of 6 developmentally important gene transcripts in day 7 and day 8 bovine blastocysts. *Proceedings of the 17$^{th}$ scientific meeting of the Association Europeenne de transfert embryonnaire, Lyon* (winner student competition)

**Knijn HM, Gjørret JO, Hendriksen PJM, Vos PLAM, Van der Weijden BC, and Dieleman SJ** (2002) Developmental rate and cell number of bovine embryos collected from the genital tract at 45 h or 100 h after ovulation and cultured in SOF until day 7. *Proceedings of the 18th scientific meeting of the Association Europeenne de transfert embryonnaire, Maastricht*

**Fokker HW, Vos PLAM, Knijn HM, Van der Weijden GC and Dieleman SJ** (2002) Efficacy of a superovulation procedure with defined LH surge in the bovine. *Proceedings of the 18th scientific meeting of the Association Europeenne de transfert embryonnaire, Maastricht*

**Knijn HM, Gjørret JO, Vos PLAM, Hendriksen PJM, Van der Weijden BC, Maddox-Hyttel P, and Dieleman SJ** (2003) Apoptosis in bovine embryos cultured until day 7 post-ovulation after collection at different times of *in vivo* development. *Theriogenology* **59**: 325

**Vos PLAM, Fokker HW, Knijn HM and Dieleman SJ** (2003) Embryonic development *in vivo* relative to the preovulatory LH surge after superovulation with a controlled LH surge in heifers. *Theriogenology* **59**: 332

**Knijn HM, Wrenzycki C, Hendriksen PJM, Vos PLAM, Zeinstra EC, van der Weijden GC, Niemann H, and Dieleman SJ** (2003) Glucose transporter expression in bovine blastocysts cultured until day 7 post ovulation after collection at different times of *in vivo* development. *Proceedings of the 19th scientific meeting of the Association Europeenne de transfert embryonnaire, Rostock*

**Vos PLAM, de Boer EE, Seinen HJ, Schoevers E, Knijn HM, Dieleman SJ** (2003) Progress of fertilization *in vivo* in oFSH-superstimulated heifers with defined LH surge. *Proceedings of the 19th scientific meeting of the Association Europeenne de transfert embryonnaire, Rostock*

**Knijn HM, Wrenzycki C, Vos PLAM, van der Weijden GC, Niemann H and Dieleman SJ** (2004) Effects of *in vitro* vs *in vivo* culture on expression of embryonic derived gene transcripts involved in apoptosis in single bovine blastocysts. *Reproduction, Fertility and Development* in press

**Merton JS, Gerritsen M, Langenbarg D, Vermeulen ZL, Otter T, E Mullaart, Landman B and Knijn HM** (2004) Effect of cysteamine during *in vitro* maturation on further embryonic development and post thaw survival of IVP bovine embryos. *Reproduction, Fertility and Development* in press