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Morphological assessment of preimplantation embryo quality in cattle

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The extensive use of embryo technologies has emphasized the need for assessing embryo quality by morphological techniques, such as transmission electron microscopy, immunocytochemistry for confocal laser scanning microscopy and fluorescence *in situ* hybridization. By a combination of these techniques, it has been possible to demonstrate: (i) that rRNA gene activation, as monitored by embryonic nucleolar development, is comparable in bovine embryos developed *in vivo* and produced *in vitro*, whereas reconstructed nuclear transfer embryos may be deviant, (ii) that generating embryos by both *in vitro* production and reconstruction by nuclear transfer is associated with increased occurrence of apoptosis, in particular in the inner cell mass of blastocysts, and (iii) that these two embryo production techniques are associated with increased occurrence of mixoploidy that is, embryos presenting a large population of normal diploid cells and a small population of abnormal haploid or polyploid cells. It is clear that blastocysts that appear healthy at stereomicroscopy may have subcellular defects. Therefore, the possibility of long-term evaluation *in vitro* of embryos after hatching has been examined. However, whereas embryos developing *in vivo* after hatching present a number of well defined developmental milestones, such as elongation of the trophoblast, formation of hypoblast and epiblast followed by differentiation of endoderm, mesoderm and ectoderm, *in vitro* culture systems for development beyond the blastocyst stage currently allow the embryo to complete only a single milestone, namely hypoblast formation.

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Introduction

With the increased use of embryo technology in both breeding and research, assessment of embryo quality has become a key issue. It is clear that techniques such as embryo production *in vitro* and, in particular, reconstruction by nuclear transfer affect the developmental competence, often referred to as the quality, of embryos (McEvoy *et al.*, 2001). In the long term, development of non-invasive techniques for determining which embryos to transfer and which to discard is an essential goal. However, on a more immediate basis, the use of invasive techniques for assessing, understanding and eliminating the pitfalls inherent to different embryo production techniques is crucial.

Over the past decade, progress has been made with the use of a combination of morphological and molecular techniques for evaluating embryo quality. The morphological approach has been expanded by application of different methods, for example for localization of proteins and nucleic acids by immunocytochemistry and fluorescence *in situ* hybridization (FISH), respectively. Moreover, the optical possibilities of imaging specimens have also improved with the introduction of techniques, such as confocal laser scanning microscopy (CLSM), allowing for optical sectioning of embryos *in toto*. Together, these advances have given rise to the area defined as bioimaging.

In this review, the use of different aspects of bioimaging for the assessment of embryo quality are presented with focus on the use of: (i) transmission electron microscopy (TEM), CLSM immunocytochemistry and FISH for studying the activation of the embryonic genome as evaluated through nucleolar development; (ii) TdT-mediated dUTP nick-end labelling (TUNEL) (detection of DNA degradation) and CLSM assessing the occurrence of apoptosis; (iii) FISH for determining chromosome abnormalities; and (iv) TEM and light microscopy for evaluating embryonic development after hatching.

Nucleolar development

The initial development of mammalian preimplantation embryos is governed by gene transcripts and polypeptides produced by, and stored in, the oocyte during its development (for a review, see Schultz, 1993). However, gradually, the embryonic genome formed at fertilization is activated and assumes control. In cattle, minor activation of the genome is observed already during the first and second cell cycle after fertilization, that is, the one- to two-cell embryo (Plante *et al.*, 1994; Hyttel *et al.*, 1996; Viuff *et al.*, 1996; Memili and First, 2000), and this low level of transcription is followed by a major burst during the fourth cell cycle, that is, the eight-cell embryo (Camous *et al.*, 1986; King *et al.*, 1988; Kopecny *et al.*, 1989).

Adequate numbers of ribosomes are required to translate mRNAs inherited from the oocyte as well as those produced *de novo* from the embryonic genome. Ribosomes are synthesized in the oocyte up to the end of the growth phase at a follicular diameter of about 3 mm (Fair *et al.*, 1995, 1996). Thus, an initial pool of ribosomes is inherited from the oocyte. The synthesis of ribosomes includes transcription of the ribosomal RNA (rRNA) genes, processing of the transcripts and assembly of the product with proteins to the ribosomal subunits. All of these processes occur in the nucleolus, which is the most prominent entity of the nucleus and, therefore, it may serve as a morphological marker for the activation of the embryonic genome (Kopecny and Niemann, 1993). The functional nucleolus contains three main ultrastructural components (for a review, see Wachtler and Stahl, 1993); the two fibrillar components, consisting of the fibrillar centres and the dense fibrillar component, and the granular component. These components of the so-called fibrillo–granular

nucleolus reflect the steps in the biosynthesis of ribosomes according to the following model: the fibrillar centres house the enzymatic apparatus for transcription; the dense fibrillar component carries the nascent unprocessed transcripts; and the granular component represents processed transcripts associated with proteins in the form of pre-ribosomal particles. According to this model, the transcription of the rRNA genes is believed to occur at the interface between the fibrillar centres and the dense fibrillar component (Hozak *et al.*, 1994).

The onset of transcription of the rRNA genes in bovine embryos produced *in vitro* was visualized by using FISH with a probe hybridizing to the rRNA genes and their transcripts (Viuff *et al.*, 1998). The *de novo* synthesized rRNA was detected at the sites of the rRNA genes during the third cell cycle that is, the four-cell embryo stage. As revealed by TEM in a parallel study, this initial activation was not accompanied by formation of functional nucleoli and, thus, by ribosome synthesis (Laurincik *et al.*, 2000). However, during the fourth cell cycle, nucleoli were formed signalling the onset of embryonic ribosome synthesis. The nucleoli developed in so-called nucleolus precursor bodies that are already present in the embryo from the first cell cycle (Fig. 1). Initially, tiny fibrillar centres surrounded by the dense fibrillar component are formed peripherally in the precursors, and later during the cell cycle, the granular component develops in the substance of the precursor resulting in a functional fibrillo–granular nucleolus (Laurincik *et al.*, 2000). The chronology and morphology of nucleolar formation is apparently similar in bovine embryos produced *in vitro* (Laurincik *et al.*, 2000) and developed *in vivo* (Hyttel *et al.*, 2001a). Whether there is the same similarity with respect to the activation of the rRNA genes during the third cell cycle has not been investigated. In contrast, it has been reported that bovine embryos reconstructed by nuclear transfer from embryonic cells displayed a delay in nucleolar formation to the fifth cell cycle (Hyttel *et al.*, 2001b), whereas those reconstructed from granulosa cells displayed premature nucleolar formation during the third cell cycle (Laurincik *et al.*, 2002). In contrast, Baran *et al.* (2002) reported a delay of development of fibrillo–granular nucleoli until the fifth cell cycle in bovine embryos reconstructed from fetal fibroblasts as compared with bovine embryos reconstructed from embryonic cells that exhibited functional nucleoli during the fourth cell cycle.

From the molecular perspective, the nucleolus consists of the rRNA genes and their transcripts associated with proteins that play different roles in rRNA transcription, processing and ribosome assembly. Recently, the allocation of key proteins involved in rRNA transcription (topoisomerase I, RNA polymerase I and upstream binding factor (UBF)), initial rRNA processing (fibrillarin) or later rRNA processing or nucleolar transport (nucleolin (C23) and nucleophosmin (B23)) to the developing nucleolus in bovine embryos was investigated by immunocytochemistry and CLSM (Laurincik *et al.*, 2000; Hyttel *et al.*, 2001a). During the first three cell cycles, some of the nucleolar proteins were localized to small nuclear entities as described by Baran *et al.* (1996). However, during the fourth cell cycle, topoisomerase I, RNA polymerase I, UBF and fibrillarin became localized to clusters of presumptive fibrillar centres and fibrillar component, and nucleolin and nucleophosmin to the periphery of the developing nucleoli in correspondence with the TEM observations referred to above (Fig. 2). The chronology and morphology of protein allocation was apparently similar in bovine embryos produced *in vitro* (Laurincik *et al.*, 2000) and embryos developed *in vivo* (Hyttel *et al.*, 2001a). However, about half of the embryos produced by nuclear transfer from embryonic (Hyttel *et al.*, 2001b) and granulosa cells (Laurincik *et al.*, 2002) lacked allocation of, in particular, UBF to the developing nucleoli even during the fifth cell cycle, that is the tentative 16-cell embryo. Later stages have not been investigated.

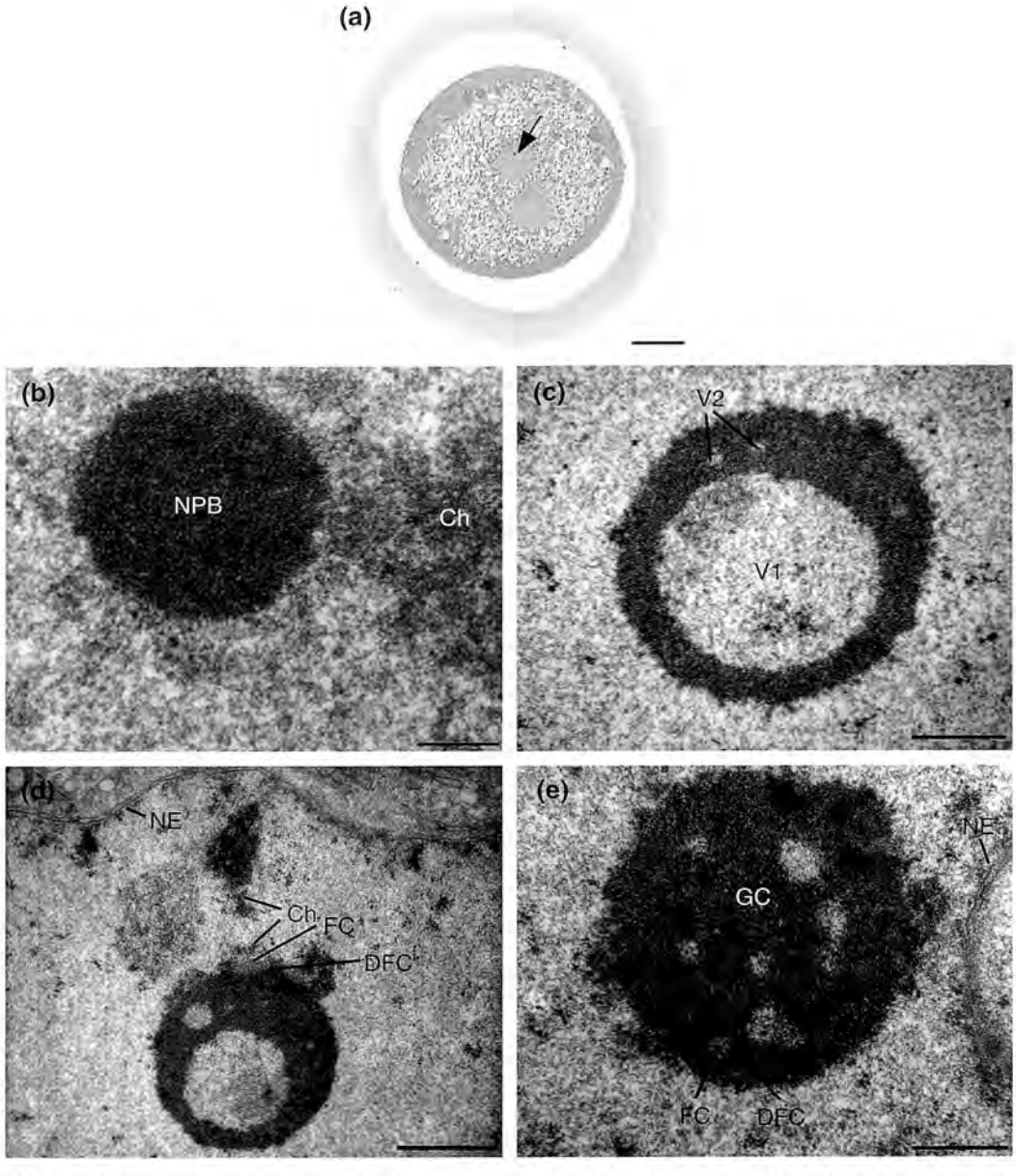

Fig. 1. Light micrograph of (a) a bovine zygote fertilized *in vivo* (first cell cycle) presenting two pronuclei, one of which displays a nucleolus precursor body (arrow). Transmission electron micrographs of nucleolar development in bovine embryos developed *in vivo* during the (b) first, (c) third, (d) early fourth and (e) fifth cell cycle. (b) During the first cell cycle, the spherical nucleolus precursor body (NPB) is associated with chromatin (Ch). (c) During the third cell cycle, the NPB displays a primary eccentrical vacuole (V1) and smaller peripheral secondary vacuoles (V2). (d) During the fourth cell cycle, a fibrillar centre (FC), which is associated with chromatin (Ch) and surrounded by dense fibrillar component (DFC), is seen close to the nuclear envelope (NE). (e) During the fifth cell cycle, a spherical fibrillo–granular nucleolus has developed adjacent to the nuclear envelope (NE) presenting fibrillar centres (FC), dense fibrillar component (DFC) and granular component (GC). Scale bars represent (a) 25 μm, (b) 0.2 μm, (c) 0.5 μm, (d) 1.0 μm and (e) 0.5 μm.

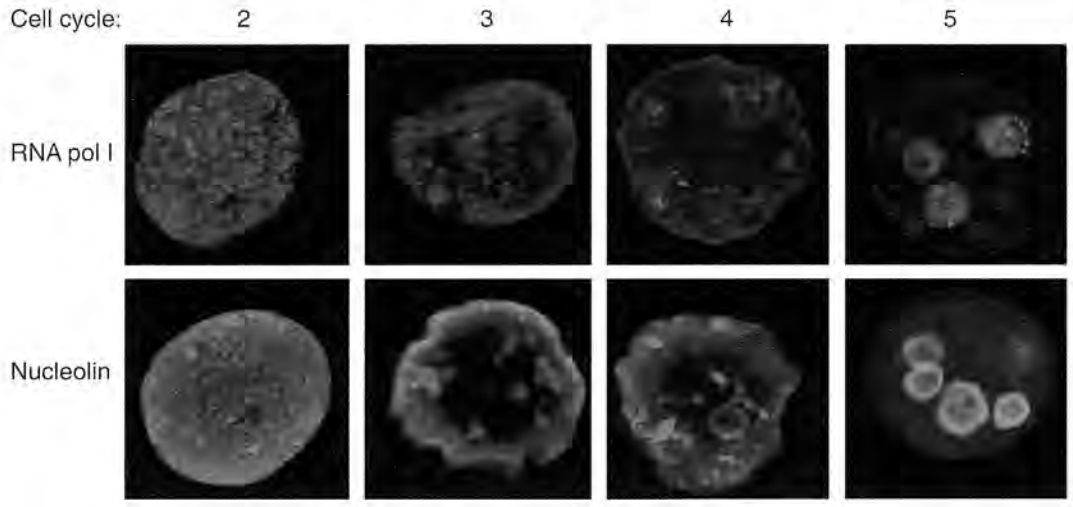

Fig. 2. Confocal laser scanning images of single nuclei from bovine embryos developed *in vivo* during the second, third, fourth and fifth cell cycle. In all images, the DNA has been stained with propidium iodide (red), whereas in the upper panel RNA polymerase I has been immunolocalized with fluorescein isothiocyanate (FITC) (green) and in the lower panel nucleolin has been immunolocalized with FITC (green). Note that RNA polymerase I localize to clusters of foci (presumptive fibrillar centres) during the fourth and, in particular, the fifth cell cycle and that nucleolin during the same cycles localize to the periphery of the developing nucleoli.

Apoptosis

Apoptosis, a regulated form of cell death, has been observed in preimplantation embryos from many mammalian species (for a review, see Hardy, 1997, 1999). This phenomenon has received increasing attention for its potential role in early embryonic loss. In embryos after compaction, there is an inverse relationship between the incidence of cell death and the number of cells in murine (Brison and Schultz, 1997; Devreker and Hardy, 1997), bovine (Byrne *et al.*, 1999) and human embryos (Hardy *et al.*, 1989). Furthermore, the incidence of cell death is higher in blastocysts produced *in vitro* than in blastocysts that are derived *in vivo* in mice (Brison and Schultz, 1997), and it is well documented that culture conditions have a substantial impact on both the number of cells and the cell death in bovine (Byrne *et al.*, 1999; Makarevich and Markkula, 2002), mouse (Brison and Schultz, 1997; Devreker and Hardy, 1997) and rat embryos (Pampfer *et al.*, 1997). Supplementation of culture media with substances that increase the number of cells in the embryo and rates of blastocyst development, such as insulin-like growth factor I (IGF-I), resulted in a decrease incidence of cell death (Makarevich and Markkula, 2002), and the opposite effect can be observed with for example glucose (Moley *et al.*, 1998). Adverse conditions *in vivo* have also been documented to both increase cell death and decrease implantation rates in diabetic rodent models (Pampfer *et al.*, 1997; Moley *et al.*, 1998). Thus, the incidence of cell death can be modulated by environmental conditions both *in vivo* and *in vitro*.

Generally, the two classical types of cell death, namely necrosis and apoptosis, can be distinguished by differences in morphology (Wyllie *et al.*, 1980). However, it is becoming evident that there are several intermediate forms of cell death (Leist and Jaattela, 2001). Necrosis is an accidental form of cell death and is regarded as a cellular response to pathological insults, whereas apoptosis is a much more regulated and suicidal form, which may represent a physiological and controlled pathway for cellular demise.

The typical changes in nuclear morphology associated with apoptosis include chromatin condensation and marginalization as well as nuclear fragmentation by karyorhexis (Wyllie *et al.*, 1980). These changes have been extensively used as markers of cellular death in embryos (for review, see Hardy, 1997). Many molecular elements of the apoptotic process have been identified including activation of a protease family known as caspases as well as extensive internucleosomal DNA fragmentation (Hengartner, 2000). Development of techniques for *in situ* detection of DNA degradation (TUNEL; Gavrieli *et al.*, 1992) has made this parameter available as a molecular marker for assessment of cell death, and it has been used in preimplantation embryos in several species (Jurisicova *et al.*, 1996; Brison and Schultz, 1997; Long *et al.*, 1998; Byrne *et al.*, 1999). However, TUNEL labelling has also been observed in cells undergoing necrotic cell death (Grasl-Kraupp *et al.*, 1995), and it is now evident that it is necessary to use additional markers of apoptosis, for example nuclear morphology, in combination with TUNEL labelling to confirm the apoptotic nature of cell death.

In a recent study, a combination of nuclear morphology and TUNEL labelling was used to estimate the incidence and localization of apoptotic cell death to the trophoblast and inner cell mass (ICM) in bovine blastocysts produced either *in vitro* in a co-culture system and collected on day 7 after insemination, or flushed from superovulated cows (*in vivo*) at day 7 after ovulation (Fig. 3). In this study, 11 blastocysts from each production system were analysed (Gjørret *et al.*, 2001). No difference in the total number of cells could be observed between blastocysts derived *in vivo* and blastocysts produced *in vitro*, but the *in vivo* ICMs contained significantly more cells (102.0 ± 15.2) than *in vitro* ICMs (70.4 ± 6.2). All embryos contained apoptotic cells. An apoptotic index was calculated as the number of apoptotic nuclei × 100, divided by the total number of nuclei. This index was significantly higher in blastocysts produced *in vitro* (10.1 ± 1.4) than in blastocyts developed *in vivo* (6.5 ± 0.8), and this difference was based on a marked significant difference in the apoptotic indices of the ICM (*in vitro*: 19.3 ± 3.6 versus *in vivo*: 7.1 ± 1.2) rather than in the trophoblast (4.5 ± 1.1 versus 4.4 ± 1.4; non-significant; Gjørret *et al.*, 2001).

In a another study, the chronological appearance of the two apoptotic markers that is, nuclear morphology and TUNEL labelling, in two-cell, three- to eight-cell, 9–16-cell, morula and blastocyst stage bovine embryos with normal developmental kinetics either reconstructed by nuclear transfer from serum starved granulosa cells or derived *in vivo* from superovulated cows were investigated (Fig. 3). The first apoptotic nuclei were observed in a six-cell nuclear transfer embryo, and they were seen in all subsequent developmental stages (Gjørret *et al.*, 2002). In contrast, apoptotic nuclei could not be observed until the morula stage in embryos derived *in vivo*. Nuclei displaying only morphological changes, that is, condensation or fragmentation, could be observed in nuclear transfer embryos at all stages investigated, but not before the eight-cell stage in embryos derived *in vivo*. At the morula stage, the nuclear transfer embryos had an apoptotic index of 8.5 ± 2.5, which was significantly higher than the 1.7 ± 0.5 of morulae derived *in vivo* (J. O. Gjørret, H. M. Knijn, S. J. Dieleman, B. Avery, L-I. Larsson and P. Maddox-Hyttel, unpublished). All blastocysts investigated contained at least one apoptotic nucleus, but again the nuclear transfer embryos had significantly higher indices (10.2 ± 1.0) than embryos derived *in vivo* (6.5 ± 0.8). Thus, the nuclear transfer procedure not only increases the incidence of apoptosis, but also disrupts its normal chronological pattern. Despite the fact that apoptosis is not evident until compaction in normal embryos developed *in vivo*, the blastomeres of the early precompaction cleavage stages possess the capacity for apoptosis, as the process can be induced by incubation in staurosporine (Weil *et al.*, 1996; Matwee *et al.*, 2000). The embryo

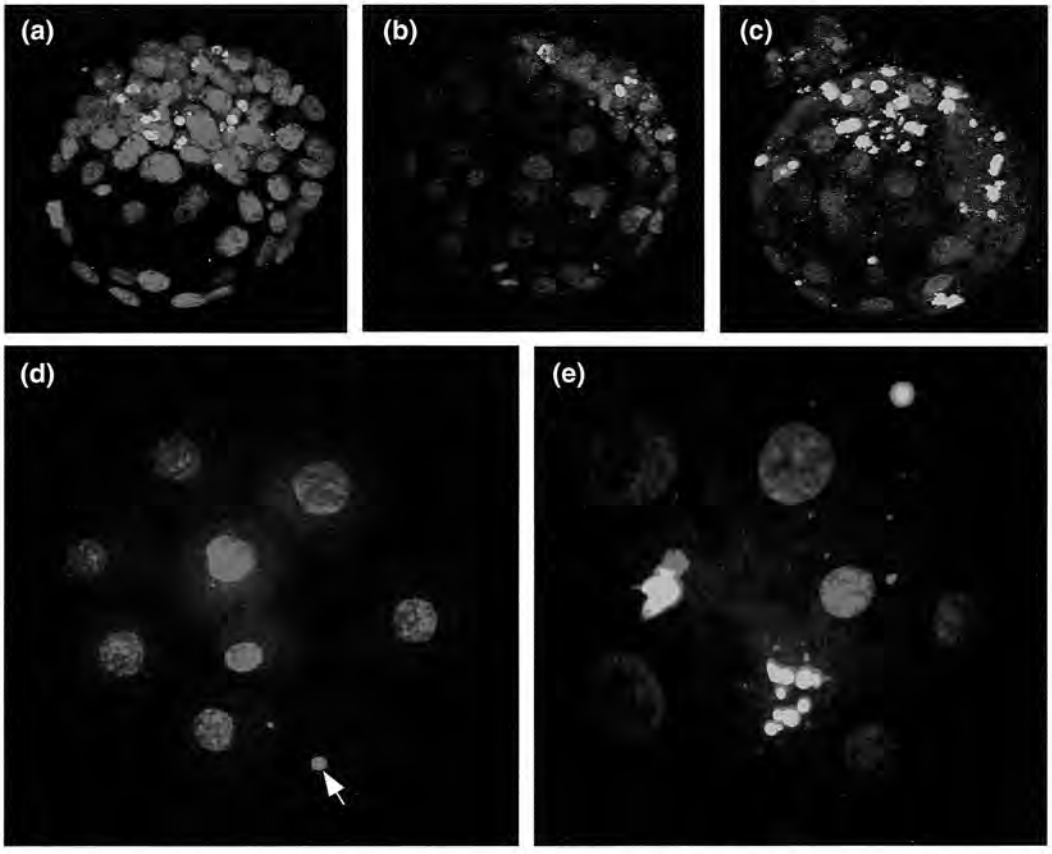

Fig. 3. Confocal laser scanning images of bovine embryos in which DNA has been stained with propidium iodide (red) and DNA fragmentation has been localized with TdT-mediated dUTP nick-end labelling (TUNEL) staining using fluorescein isothiocyanate (FITC) (green) labelled nucleotides. The embryos are (a) *in vivo* developed, (b) *in vitro* produced and (c) somatic nuclear transfer blastocysts and (d) *in vivo* developed and (e) somatic nuclear transfer 8–16-cell stages. Note the sporadic occurrence of apoptotic cells (nuclear condensation combined with positive TUNEL staining) in particular in the inner cell mass of the blastocysts. In addition, note the occurrence of apoptotic cells in the nuclear transfer cleavage stage embryo as opposed to cleavage stage embryo developed *in vivo* in which only a condensed nucleus without TUNEL staining is seen (arrow).

stage-specific onset of the normal apoptotic response *in vivo* may be due to an inadequate expression or activation of certain parts of the apoptotic machinery, or to extensive repression thereof. The accelerated onset of the different apoptotic markers observed in the nuclear transfer embryos indicates that the capacity for apoptosis is partly determined by the (donor) nucleus.

Several roles for apoptosis in preimplantation development have been proposed, mainly concerning elimination of abnormal or unwanted cells (Hardy, 1999; Betts and King, 2001). The observation of a decrease in the number of cells in combination with an increased incidence of apoptosis in the ICM of blastocysts produced *in vitro* indicates that cells are eliminated to a higher degree from this embryonic compartment in particular. This finding may reflect an increase of subcellular abnormalities in this important embryonic compartment provoked by handling *in vitro*.

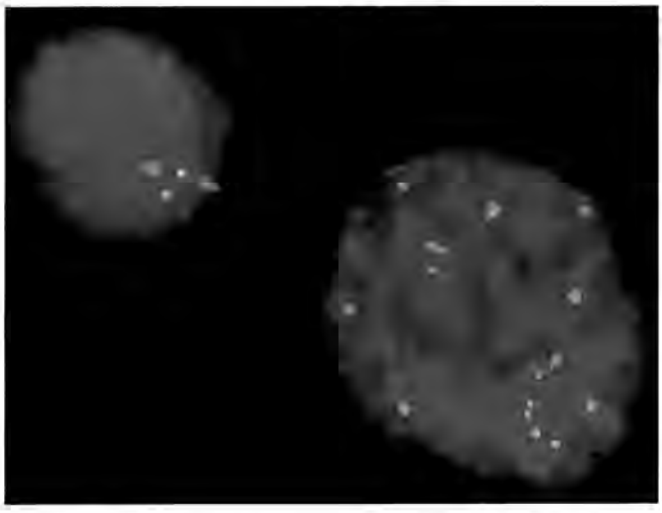

Fig. 4. Epifluorescence images of single nuclei from bovine blastocysts produced *in vitro* that are labelled by fluorescence *in situ* hybridization (FISH) with chromosome specific probes hybridizing to chromosomes 6 (green) and 7 (red). A normal diploid nucleus with two signals from each probe (upper left) and an octaploid nucleus with eight signals from each prope (lower right) are displayed.

Chromosome abnormalities

Embryos consisting only of polyploid or haploid cells are always eliminated in mammals, but karyotyping has clearly documented that single polyploid or haploid cells in otherwise normal diploid embryos are a frequent finding as early as the two-cell stage (for a review, see King, 1990). This finding may not be surprising as polyploid cells are also found normally in many organs of the mammalian body, most prominently in the liver. However, little is known about their biological significance or their lifespan in the adult, the fetus and, in particular, the embryo. Data from experimentally produced diploid–tetraploid mouse embryos indicate that polyploid cells at the morula and blastocyst stage are selected against in the embryo proper resulting in a preferential allocation to the trophoblast (Everett and West, 1998). These data taken together with reports of an inverse relationship between embryo quality and chromosome aberrations (King *et al.*, 1987; Rubes *et al.*, 1988) indicate that polyploid cells are a developmental disadvantage which under normal conditions may be regulated by segregation of aberrant cells to the trophoblast. However, these reports were produced using traditional karyotyping, which *per se* underestimates the frequency of mixoploidy. Therefore, the goal was to generate a more reliable estimate for the normal frequency of mixoploidy and polyploidy in embryos developed *in vivo*, using FISH with two chromosome-specific DNA probes (Fig. 4). The major advantage of this approach compared with karyotyping is that most, if not all, cells of an embryo can be evaluated for errors of ploidy.

The first estimates of chromosome abnormalities in bovine embryos developed *in vivo* generated by FISH analysis revealed that 25% of blastocysts collected on day 7 or day 8 after ovulation were mixoploid, that is, contained a large population of normal diploid cells and a small, in general $< 10\%$, population of haploid or polyploid cells (Viuff *et al.*, 1999). This study included only 28 embryos, but it formed a starting point for further studies that were designed to elucidate at what stage of development the polyploid cells

were formed and what their fate was later in development. The results from our analysis of 256 bovine embryos developed *in vivo* isolated on days 2, 3, 4 and 5 after ovulation revealed an increase in the frequency of mixoploidy (Viuff *et al.*, 2001). Thus, the percentages of mixoploid embryos on days 2, 3, 4 and 5 were 5, 13, 16 and 31%, respectively. The corresponding percentages of polyploidy, that is, embryos in which all cells were polyploid, were 2, 2, 0 and 0%, which were significantly lower. Another important finding was that about 90% of 40 mixoploid embryos contained < 10% aberrant cells. Diploidy–triploidy was the most frequent mixoploid finding (50%), whereas diploidy–haploidy, diploidy–tetraploidy, and diploidy–triploidy–haploidy plus diploidy–triploidy–tetraploidy were observed in 12.5, 25.0 and 12.5% of the embryos, respectively. The polyploid embryos of this study, of which only three were found, were triploid.

The first FISH data on chromosome aberrations in bovine embryos produced *in vitro*, which included 151 blastocysts isolated at days 7–8 after insemination *in vitro*, showed that 72% were mixoploid (Viuff *et al.*, 1999). This was significantly more than in embryos derived *in vivo*. However, again, the frequency of aberrant cells was low in the mixoploid embryos: 83% contained < 10% aberrant cells, 13% contained 11–25%, and only 4% contained > 25% aberrant cells. In a larger study of earlier *in vitro* produced developmental stages it was possible to document an increased rate of mixoploidy at about day 5 (Viuff *et al.*, 2000): of 426 embryos, 22% were mixoploid at day 2, 15% at day 3, 16% at day 4 and 42% at day 5. The corresponding percentages of polyploidy were 5, 13, 3 and 0%, which was markedly more than that seen in embryos developed *in vivo*. In 99 mixoploid embryos, diploidy–triploidy was the most frequent abnormality (65%), whereas diploidy–tetraploidy and diploidy–triploidy–tetraploidy were observed in 11 and 24% of embryos, respectively. Among the polyploid embryos, triploidy was again the most common aberration. In addition to the lack of polyploid embryos at day 5, it was striking to observe that the development of polyploid embryos was significantly ($P < 0.02$) slower than that of the diploid and the mixoploid embryos. In addition, few, if any, polyploid embryos progressed beyond the third cell cycle, whereas the mixoploid embryos seemed to continue development unaffected beyond this stage (Viuff *et al.*, 2001).

In addition, it was demonstrated that there was a significantly lower proportion of polyploid cells in the embryonic disc in comparison with the trophoblast of bovine embryos on both days 7–8 and day 12 after insemination and that the level of polyploidy on days 7–8 was significantly higher than on day 12 (Viuff *et al.*, 2002). Thus, the polyploid cells may be confined to the trophoblast and make little contribution to the embryo itself. However, their functional significance is still unclear.

Thus, there are significant differences in the frequencies of polyploidy and mixoploidy between embryos developed *in vivo* and embryos produced *in vitro*. Therefore, the aim of the following study was to use the occurrence of chromosome abnormalities as a marker for evaluating the effect of performing oocyte maturation versus embryo culture *in vitro*. Oocytes were either matured *in vivo* in superovulated follicles and aspirated under ultrasound guidance or matured *in vitro*, and both categories of oocyte were processed for fertilization and culture *in vitro* (Dielemann *et al.*, 2002). On the basis of the results from embryos developed *in vivo* (Viuff *et al.*, 1999), embryos with < 5% aberrant cells were classified as normal. The preliminary results indicate that significantly more of the blastocysts produced from oocytes matured *in vivo* were normal in comparison with blastocysts produced from oocytes matured *in vitro*. Furthermore, blastocysts from oocytes matured *in vitro* contained fewer cells than blastocysts from oocytes developed *in vivo* (54 versus 96 cells per normal blastocyst). Correspondingly, it has been demonstrated that oocytes matured *in vivo* produce significantly more blastocysts than oocytes matured *in vitro* after *in vitro* fertilization and

subsequent culture (Rizos *et al.*, 2002). Interestingly, the same investigation showed that *in vitro* culture of zygotes fertilized *in vivo* results in the same blastocyst rate as *in vitro* culture of zygotes produced *in vitro*. However, the blastocysts originating from zygotes developed *in vivo* had a higher capacity to tolerate vitrification. Together, these data indicate that maturation of oocytes *in vitro* certainly does affect the development of the embryo to the blastocyst stage with respect to both rate and quality.

Finally, the occurrence of chromosome abnormalities in bovine embryos reconstructed by nuclear transfer from embryonic cells was determined (Booth *et al.*, 2000). The proportion of completely normal embryos was the same as among embryos produced *in vitro*, but among embryos with chromosome aberrations, the nuclear transfer embryos were more severely affected. These abnormalities may be involved in the marked embryonic loss inherent to nuclear transfer.

Embryonic development after hatching

From the data presented here it is clear that a blastocyst, despite having an excellent appearance under the stereomicroscope, may have undetected abnormalities that have an impact on its developmental competence. Therefore, the relevance of using rates of expanded or even hatched blastocysts as end points of evaluation can be questioned. It might be advantageous if further characteristics of embryonic development after hatching *in vitro* could be added to the list of parameters used for assessment of embryonic competence.

The basis for such long-term evaluation *in vitro* of bovine embryos was created by first defining a set of milestones of bovine embryonic development *in vivo* up to day 21 after ovulation and, second, evaluating the capacity of bovine embryos produced *in vitro* to complete these milestones under long-term *in vitro* culture (Vajta *et al.*, 2000, 2001; Hyttel *et al.*, 2002). Bovine embryos developed *in vivo* were collected after slaughter on day 14 and day 21 after ovulation and processed for TEM or immunohistochemistry for germ layer characterization (cytokeratin 8 for potential ectoderm, alpha fetoprotein for potential endoderm and vimentin for potential mesoderm). On day 8 after insemination, embryos produced *in vitro* were transferred to culture on reconstituted collagen matrix in SOFaaci (Holm *et al.*, 1999) supplemented with 50% FCS at 40% O_2, 5% CO_2 and 55% N_2 and processed for TEM at days 8–29 after insemination.

Embryos developing *in vivo* on day 14 were ovoid to filamentous and ranged from about 0.5 to 19.0 mm in total length (Fig. 5). The embryos displayed a complete hypoblast lining of the trophoblast, and an epiblast was inserted into the trophoblast with tight junctions present between adjacent epiblast cells, and between peripheral epiblast and trophoblast cells. In some embryos, the epiblast was more or less covered by foldings of trophoblast in the process of forming the amniotic cavity. Cytokeratin 8 was localized to the trophoblast and the hypoblast underlying the epiblast, alpha fetoprotein to some, but not all, cells of the hypoblast underlying the trophoblast, and vimentin to some, but not all, cells of the epiblast. On day 21, the length of the embryo proper, not the full conceptus, ranged from about 0.6 to 6.0 mm. The developmental stages ranged from embryos presenting a primitive streak and formation of the neural grove to those presenting a neural tube, and up to 14 somites and allantois development. These embryos showed the gradual formation of endoderm, mesoderm and ectoderm as well as differentiation of paraxial, intermediate and latetal plate mesoderm and somites. Cytokeratin 8 was localized to the trophectoderm, the hypoblast and the surface and neural ectoderm, and alpha fetoprotein to the hypoblast, but not the final endoderm, the intensity increasing with development. Vimentin was initially localized to some, but not all, cells positioned particularly in the ventral region of the primitive streak,

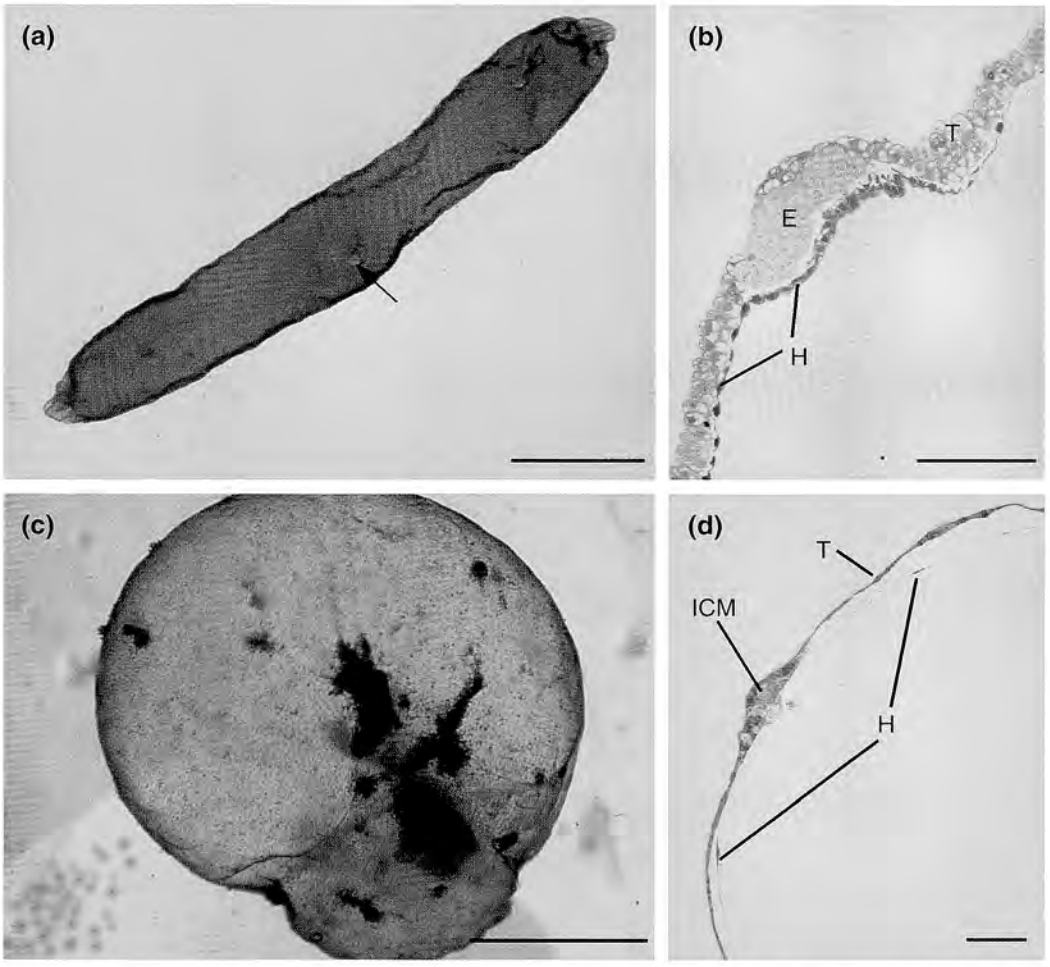

Fig. 5. Stereomicroscope image of (a) a bovine conceptus developed *in vivo* on day 14 after fertilization. Semi-thin Epon section from (b) the region of the embryonic disc of the same embryo displaying trophoblast (T), epiblast (E) and hypoblast (H). Stereomicroscope image of (c) an *in vitro* produced and *in vitro* grown bovine conceptus on day 23 after fertilization. Note the lack of elongation. Semi-thin Epon section of (d) another bovine conceptus grown *in vitro* on day 11 after fertilization displaying trophoblast (T), inner cell mass (ICM) and development of hypoblast (H) from the ICM. Scale bars represent (a,c) 1.0 mm and (b,d) 0.1 mm.

to presumptive final endoderm cells inserted into the upper portion of the hypoblast, and to mesoderm.

Bovine embryos developing *in vitro* remained spherical, and up to day 11 after insemination, half of the embryos displayed hypoblast formation (Fig. 5) and about one third presented abortive establishment of an epiblast by penetration of cells from the ICM mass through the trophoblast. However, in all cases, this process was associated with degeneration of the ICM, and from day 12, the ICM had degenerated in all embryos. From day 14, a 'foot' process gradually developed as a trophoblast outgrowth on the collagen matrix. A compact cell mass, from which several vesicles were formed, was established at the site of the 'foot' process.

The data demonstrate that initial bovine embryonic development after hatching includes well-defined milestones of which only hypoblast formation can be achieved by the present conditions for long-term culture of embryos *in vitro*.

Conclusion

A variety of techniques allow for detection of subcellular deviations, such as aberrant gene activation and increased occurrence of apoptosis and chromosome abnormalities, in biotechnologically manufactured embryos arising from, for example, *in vitro* production or reconstruction by nuclear transfer. With the rapid expansion of the area of bioimaging in which techniques from morphology and molecular biology combine, the coming years will offer a range of exciting possibilities including the potential observation of subcellular function of live embryos by means of multiphoton confocal microscopy.

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