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# Predicting embryo quality: mRNA expression and the preimplantation embryo

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## Abstract

To overcome the low implantation rate (10–20%) following IVF in humans, more than two embryos are commonly replaced, potentially leading to high order multiple pregnancies with associated significantly elevated risks. Selecting the most viable embryos and transferring fewer of them could reduce this risk. Prolonged culture of embryos *in vitro* to the blastocyst stage may expose the embryo to hazards not normally encountered in the female reproductive tract. Recent studies comparing bovine oocyte maturation, fertilization and embryo culture *in vivo* and *in vitro* have demonstrated that the origin of the oocyte is the main factor affecting blastocyst yield, while the post-fertilization culture environment is crucial in determining blastocyst quality, measured in terms of cryotolerance and relative transcript abundance, irrespective of the origin of the oocyte. Production of embryos *in vitro*, particularly when using an extended period of in-vitro culture may predispose the embryo to phenomena such as 'large offspring syndrome', which is probably linked to altered gene expression, particularly of imprinted genes. Post-fertilization culture environment clearly has a profound effect on the relative abundance of gene transcripts within the embryo. Culture under sub-optimal conditions for even one day can lead to perturbations in the pattern of expression.

***Keywords:*** *assisted reproduction, blastocyst, development, embryo culture, gene expression, in-vitro fertilization*

## Introduction

It is now more than 25 years since the birth of the first IVF baby, but success rates are still disappointingly low, with only 1:5 couples going home with a child. The advent of techniques such as intracytoplasmic sperm injection (ICSI) and surgical sperm retrieval has meant that fertilization can now be achieved in almost all couples. However, apparently normal embryos are often replaced into the mother but fail to implant.

In human assisted reproduction treatment a trade-off exists between the risk of multiple pregnancy and the prospects for pregnancy itself. One way of tipping the balance against multiple pregnancies would be to select the most viable embryos for transfer and to transfer fewer of them. Two approaches towards this objective are: to select the fastest cleaving embryos for transfer at day 2 or 3 (Shoukir *et al*., 1997; Lonergan *et al*., 1999; Sakkas *et al*., 2001; Fenwick *et al*., 2002; Salumets *et al*., 2003); or the prolonged culture of embryos *in vitro* for ~5 days to the blastocyst stage, by which time the least viable have succumbed, leaving the most competent for transfer (Gardner *et al*., 1998a,b, 2000).

In humans, blastocyst culture has been reported to substantially increase the implantation rate per embryo transferred (Gardner *et al*., 1998a; Huisman *et al*., 2000; Milki *et al*., 2000) although this has been questioned (Kolibianakis and Devroey, 2002). The safety of prolonged culture in humans has not been firmly established, especially in light of the numerous studies from domestic ruminants of abnormalities apparently associated with perturbations induced by in-vitro culture (Menezo *et al*., 1999; Sinclair *et al*., 2000; Sinclair and Singh, 2004).

Such extended culture allows the embryos to 'select' themselves by growth to the blastocyst stage. In addition, it would help synchronize embryonic stage with the female tract. However, blastocyst formation itself does not fully reflect the viability of the embryo (Tsirigotis, 1998; Jones and Trounson, 1999) and not all blastocysts are of equal quality (Rizos *et al*., 2002b,c, 2003).

Indeed, blastocyst development is only one step along the road to the production of a live offspring and, as pointed out by McEvoy *et al.* (2000), attainment of that stage is more a reflection of past achievement than a guarantee of future ability to implant and give rise to an offspring.

## Embryo production *in vitro*

Generally, for the production of embryos in human assisted reproduction treatment, a matured oocyte is recovered from the pre-ovulatory follicle and inseminated shortly afterwards, and the resulting zygote is cultured for 1 or 2 days before being transferred back to the same donor. By contrast, production of domestic animal embryos *in vitro* is essentially a three-step process involving in-vitro maturation (IVM) of immature oocytes recovered from antral follicles, IVF, and subsequent culture of the in-vitro-derived zygote to the blastocyst stage, at which point they are transferred to surrogate recipients. In terms of efficiency, in cattle approximately 90% of immature oocytes undergo nuclear maturation *in vitro* from prophase I to metaphase II (the stage at which they would be ovulated *in vivo*); and about 80% undergo fertilization and cleave at least once, to the 2-cell stage. However, only 30–40% reach the blastocyst stage. Thus, the major fall-off in development occurs during the last part of the process (in-vitro culture), between the zygote and blastocyst stages, suggesting that post-fertilization embryo culture is the most crucial point in the process in terms of determining blastocyst yield. However, it is known now that this is not the case; there is unequivocal evidence demonstrating that events further back along the developmental axis (i.e., the quality of the oocyte) are crucial in determining the proportion of immature oocytes that form blastocysts and that in fact the post-fertilization culture environment, within certain limits, does not have a major influence on the capacity of the immature oocyte to form a blastocyst (Rizos *et al.*, 2002c, 2003).

There is considerable evidence supporting the notion that the post-fertilization culture environment is crucial in determining the quality of the blastocyst, assessed in terms of cryotolerance and gene expression pattern. For example, by culturing in-vitro-produced bovine zygotes *in vivo* in the ewe oviduct, it is possible to dramatically increase the quality of the resulting blastocysts, measured in terms of cryotolerance, to a level similar to that of totally in-vivo-produced embryos (Galli and Lazzari, 1996; Enright *et al.*, 2000; Rizos *et al.*, 2002c) (**Figure 1a**). Furthermore, in the reciprocal experiment, the culture *in vitro* of in-vivo-produced bovine zygotes results in blastocysts of low cryotolerance (Rizos *et al.*, 2002c) (**Figure 1b**). In other words, the culture of 'poor quality' zygotes, produced by IVM and

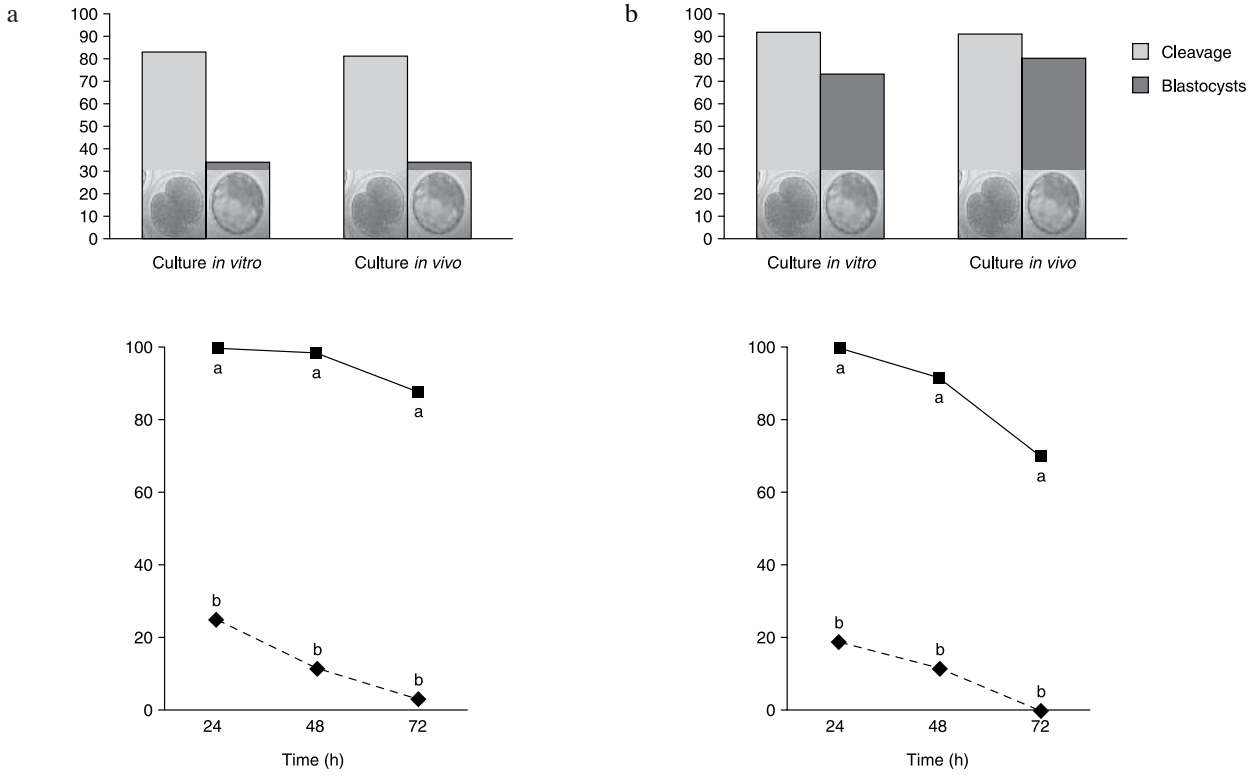


**Figure 1.** Development and survival after cryopreservation of bovine blastocysts produced following (**a**) maturation and fertilization *in vitro* and culture either *in vitro* or *in vivo*, or (**b**) maturation and fertilization *in vivo* and culture either *in vivo* or *in vitro*. Note that blastocyst yield following in-vitro maturation (IVM) and IVF was approximately 35% compared with approximately 80% following in-vivo maturation and fertilization and that this was not affected by culture environment. In addition, note that the survival of in-vivo (solid line) cultured blastocysts after cryopreservation was significantly higher than those cultured *in vitro* (dashed line), irrespective of the origin of the oocyte. Data from Rizos *et al.* (2002c).

IVF, *in vivo* leads to the production of high quality blastocysts (albeit at low frequency), and, conversely, the culture of 'high quality' zygotes, produced by IVM and IVF, *in vitro* leads to the production of poor quality blastocysts (albeit at high frequency).

Apart from the absolute number of blastocysts produced from a given number of oocytes, the quality of in-vitro-produced bovine blastocysts continually lags behind that of blastocysts produced *in vivo*. Compared with their in-vivo counterparts, in-vitro-produced embryos tend to have darker cytoplasm and a lower buoyant density (Pollard and Leibo, 1994) as a consequence of their higher lipid content (Abd El Razek *et al*., 2000), a more fragile zona pellucida (Duby *et al*., 1997), reduced expression of intercellular communicative devices (Boni *et al*., 1999), differences in metabolism (Khurana and Niemann, 2000; Thompson, 2000) and a higher incidence of chromosomal abnormalities (Viuff *et al*., 1999; Slimane *et al*., 2000). In addition, many differences at the ultrastructural level have been reported (Crosier *et al*., 2000, 2001, 2002; Fair *et al*., 2001; Rizos *et al*., 2002a). There are also major differences in gene expression patterns, which will be discussed below.

## Indicators of embryo quality

Preimplantation embryos exhibit an amazing plasticity and tolerance when it comes adapting to the environment in which they are cultured. They are capable of developing in media ranging in composition from simple balanced salt solutions to complex systems involving serum and somatic cells. At least a proportion of the blastocysts that develop in culture are developmentally competent, as evidenced by the fact that live offspring have resulted following transfer.

The primary criterion for embryo selection after human IVF is the morphological appearance based on a combination of cell number and fragmentation (Van Royen *et al*., 2001). In the past few years, the possibilities of viable embryo selection at the early cleavage stages have been improved substantially by the introduction of non-invasive scoring criteria applicable as early as the pronuclear stage and by refining the scoring criteria for cleaving embryos. Among the various approaches are: zygote/pronuclear morphology; kinetics of early cleavage; amino acid turnover; HLA-G; and gene expression profile.

### Zygote/pronuclear morphology

Pronuclear morphology has been used to predict embryo development and chromosomal constitution (Balaban *et al*., 2004); embryos developing from zygotes with a normal pronuclear pattern cleaved faster and formed embryos with better morphology compared with those with abnormal patterns. The morphological parameters for zygote quality include the number of nucleolar precursor bodies and their distribution in the pronuclei (Scott *et al*., 2000; Tesarik *et al*., 2000; Wittemer *et al*., 2000).

### Kinetics of early cleavage

A clear relationship between the time of first cleavage post-insemination *in vitro* and the developmental competence of the embryo has been demonstrated in many domestic species, with those oocytes cleaving earliest after IVF being more likely to reach the blastocyst stage than their later-cleaving counterparts (rhesus monkey: Bavister *et al*., 1983; hamster: McKiernan and Bavister, 1994; buffalo: Totey *et al*., 1996; mouse: Warner *et al*., 1998; cattle: Lonergan *et al*., 1999). In humans, Edwards *et al*. (1984) reported that patients whose embryos cleaved to the 8-cell stage by 55 h post-insemination achieved a pregnancy rate of nearly double that for embryos that reached the same stage after 56 h. Subsequently, many reports have confirmed the usefulness of this phenomenon in selecting human embryos with improved developmental competence (Sakkas *et al*., 1998; Shoukir *et al*., 1998; Bos-Mikich *et al*., 2001; Fenwick *et al*., 2002), where such embryos have been reported to result in higher pregnancy rates after multiple or single (Van Montfoort *et al*., 2004) embryo transfer.

It has been shown that the timing of the first cleavage division in cattle embryos is related to the polyadenylation status of several developmentally important gene transcripts (Brevini-Gandolfi *et al*., 2002). Subsequently, differences in gene expression in the early embryo have been demonstrated that are reflective of differences in developmental competence between early- and late-cleaving zygotes (Lonergan *et al*., 2000; Fair *et al*., 2004a). The factors that control the time of first cleavage are unclear. Although culture conditions can influence the kinetics of early development (Van Langendonckt *et al*., 1997), it is likely that the main factors controlling this parameter are intrinsic to the oocyte (Lonergan *et al*., 1999; Lonergan *et al*., 2000; Brevini-Gandolfi *et al*., 2002), the spermatozoon (Eid and Parrish, 1995; Comizzoli *et al*., 2000; Ward *et al*., 2001), or both.

In mice, a gene controlling the rate of preimplantation cleavage division and subsequent embryo survival (preimplantation embryo development, or Ped) has been identified (Warner *et al*., 1998). The *Ped* gene is located at the Q region of the mouse MHC (Warner *et al*., 1987, 1991). The protein product of the *Ped* gene is the Qa-2 antigen; embryos that express Qa-2 protein cleave at a faster rate than those that do not.

### Amino acid turnover

Non-invasive metabolic profiling can predict the ability of human embryos to develop in culture (Houghton *et al*., 2002); embryos that develop from an early cleavage stage to the blastocyst stage exhibit a different profile of amino acid turnover to those that arrest (Ala, Arg, Gln, Met, Asn). Houghton and colleagues subsequently applied this test retrospectively in a clinical IVF setting and found that the turnover of three amino acids (Asn, Gly, Leu) was significantly correlated with pregnancy and a live birth (Brison *et al*., 2004).

### Human leukocyte antigen-G

One of the potentially more exciting markers of embryo developmental potential is soluble human leukocyte antigen-G (sHLA-G). HLA-G mRNA and protein have been shown to be expressed in a proportion of human IVF embryos (Juriscova *et al*., 1996), and those embryos that secrete the soluble form of HLA-G are apparently more likely to produce pregnancies when transferred back into the uterus than those that do not (Menicucci *et al*., 1999; Fuzzi *et al*., 2002; Sher *et al*., 2004; Noci *et al*., 2005;

Yie *et al*., 2005). This is an important new finding , which has significant implications for the selection of the best embryos for transfer in IVF. The availability of a non-invasive, quantitative assay for a marker of implantation potential (such as sHLA-G) could revolutionize IVF practice by increasing success rates and lowering the risk of multiple pregnancies by reducing the number of embryos that need to be transferred.

The present authors have investigated expression of MHC class I transcription in preimplantation bovine embryos (Fair *et al*., 2004b) using primers that amplify all forms of class I. This study showed that the relative abundance of class I transcripts was higher in early cleaving embryos, compared with late cleaving embryos. A comparison between in-vitro- and in-vivo-cultured embryos showed that the MHC transcription increase was significantly higher in the latter. These results suggest that cattle may have a gene with a similar function to the mouse *Ped* gene mentioned above. However, no sequence data were derived from the PCR-amplified class I fragments, and further investigation is thus required to determine whether classical and/or non-classical class I genes are being up-regulated.

## Gene expression profile

Gene expression has a fundamental role in the co-ordination of homeostatic and metabolic mechanisms throughout life. Precise control of gene expression during the preimplantation phase of development is particularly important; several major developmental events occur during this period, including: (i) the first cleavage division, the timing of which is important (Lonergan *et al*., 1999; see below); (ii) embryonic genome activation, when the embryo transfers from a reliance on maternal RNA derived from the oocyte to expression of its own genome (Memili and First, 2000); (iii) morula compaction, which involves the establishment of the first intimate cell-to-cell contacts in the embryo (Boni *et al*., 1999); and (iv) blastocyst formation, involving the differentiation of two cell types, the trophectoderm and the inner cell mass (Watson, 1992).

The advent of reverse transcription-polymerase chain reaction (RT-PCR) in the late 1980s (Rappolee *et al*., 1988) paved the way for the analysis of expression patterns of individual genes during embryo development. However, many would argue that such a gene-by-gene approach provides too narrow a view of what are complex underlying regulatory networks. Although adequate for quantification of single transcripts, the analysis of multiple genes using this technique is labour-intensive. Depending on their composition, microarrays potentially allow a genome-wide perspective by profiling the expression of thousands of genes simultaneously, and such global gene expression profiles can be used to reveal and characterize the pattern of maternal mRNA degradation and zygotic gene activation, and the effect of modifications to culture environment on the pattern of embryo gene expression (Sharov *et al*., 2003; Bermudez *et al*., 2004; Hamatani *et al*., 2004; Corcoran *et al*., 2005)

There has been some interest in predicting pregnancy outcome from granulosa cell markers of competence. Robert *et al*. (2003) reported that while the presence of LH-R transcripts in bovine granulosa cells is not a key characteristic of a follicle bearing a competent oocyte, a higher proportion of oocytes reach the blastocyst stage when LH-R mRNA is detected in the granulosa cells. McKenzie *et al*. (2004) examined the relationship between human cumulus granulosa cell expression of several genes and subsequent embryo development. Of the genes studied, the expression of three (PTGS2, HAS2 and GREM1) were related to morphological and physiological characteristics.

Due to difficulties in obtaining material, large-scale studies on gene expression in human embryos are scarce (Wells *et al*., 2005). In addition, in some instances the material used (e.g. oocytes that failed to fertilize, retarded embryos) is compromised. Therefore, information derived from animal studies, where numbers of embryos are rarely limiting, can yield valuable information. Nonetheless, there is an increasing number of publications on gene expression in human embryos that further understanding of the regulatory factors involved in early development (e.g., Bermudez *et al*., 2004; Hansis *et al*., 2004; Lindeberg *et al*., 2004).

Analysis of expression patterns of developmentally important genes essential in early development provides a useful tool to assess the normality of the produced embryos and a tool to optimize assisted reproduction technologies. In domestic species, there is a large body of evidence demonstrating that culture media can perturb gene expression in the developing embryo (see review by Wrenzycki *et al*., 2005). This is the case, not only when one compares in-vitro and in-vivo culture systems, but also different in-vitro culture systems (Eckert and Niemann, 1998; Wrenzycki *et al*., 1999; Doherty *et al*., 2000; Wrenzycki *et al*., 2000; Lee *et al*., 2001; Lequarre *et al*., 2001; Natale *et al*., 2001; Wrenzycki *et al*., 2001; Rief *et al*., 2002; Rizos *et al*., 2002b, 2003; Lonergan *et al*., 2003). Rizos *et al*. (2002b) (**Figure 2**) examined the expression of several genes known to be involved in apoptosis (*Bax*), oxidative stress (*Mn-SOD*), gap junction formation (*Cx43*) and differentiation (*LIF* and *LIF-Rβ*) in blastocysts derived from in-vitro matured and fertilized oocytes, which were cultured either *in vitro* in serum-supplemented synthetic oviduct fluid (SOF) or *in vivo* in the ewe oviduct and compared the pattern of expression with that of in-vivo-derived blastocysts. Culture *in vitro* resulted in, among other differences, an elevated abundance of transcripts for *Bax*, as well as reduced expression of the gap junction gene, *Cx43*. In general, bovine blastocysts derived from culture in the sheep oviduct were characterized by an expression pattern that was nearly identical to that of their in-vivo-produced counterparts. Very similar findings were subsequently reported by Lazzari *et al*. (2002). Furthermore, in a comparison of serum-free and serum-supplemented SOF (Rizos *et al*., 2003) (**Figure 3**), the presence of serum during the culture period resulted in a significant increase in the level of expression of *Mn-SOD*, *SOX*, *Bax*, *LIF* and *LIF-Rβ* and a decrease in the relative abundance of transcripts for *Cx43* and *interferon-τ*. Corcoran *et al*. (2005), using cDNA micoarrays, identified previously uncharacterized, differentially expressed, genes involved in cell communication, intracellular signalling and regulation of transcription in bovine blastocysts cultured *in vivo* or *in vitro*; microarray analysis identified 15 gene transcripts that were differentially expressed between blastocysts produced *in vivo* or *in vitro*. Studies on the temporal expression of some of these transcripts have shown that such alterations in mRNA abundance have their origins early during the post-fertilization culture phase (Lonergan *et al*., 2003) (**Figure 4a**, **b**).

The marked aberrations in a proportion of embryos produced *in vitro* demonstrate that current in-vitro production systems

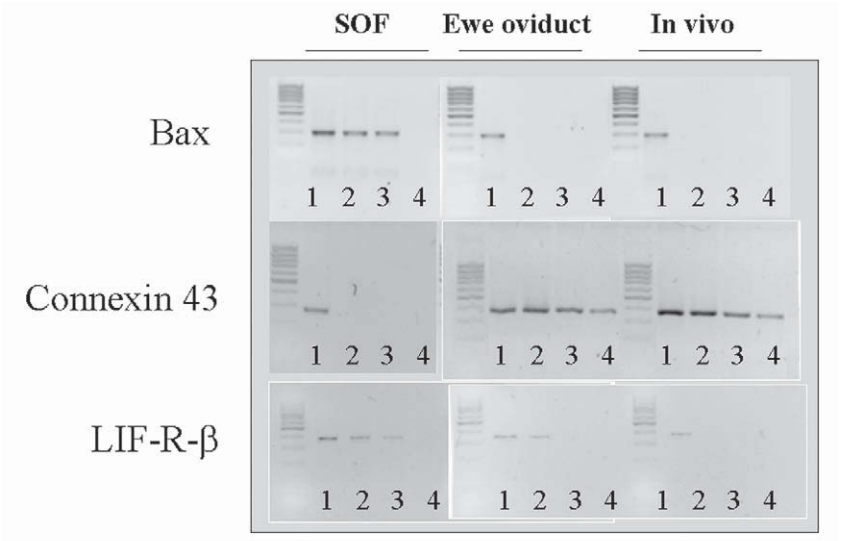


**Figure 2.** Relative transcript abundance for three selected genes in bovine blastocysts produced under different conditions. For each group, mRNA extracted from 10 embryos (lane 1) was sequentially diluted in fivefold steps (lane 2: ×5; lane 3: ×25; lane 4: ×125) and subjected to reverse-transcription polymerase chain reaction. Blastocysts from three sources were used: (i) in-vitro culture in synthetic oviduct fluid (SOF) of in-vitro matured (IVM)/IVF zygotes; (ii) in-vivo culture in the ewe oviduct of IVM/IVF zygotes; or (iii) in-vivo culture, following ovulation induction, artificial insemination and recovery. Bax, *Bos taurus* apoptosis regulator box-α; Cx43, connexin 43; LR-β, bovine leukaemia inhibitory factor-receptor-β. Data from Rizos *et al*. (2002b).

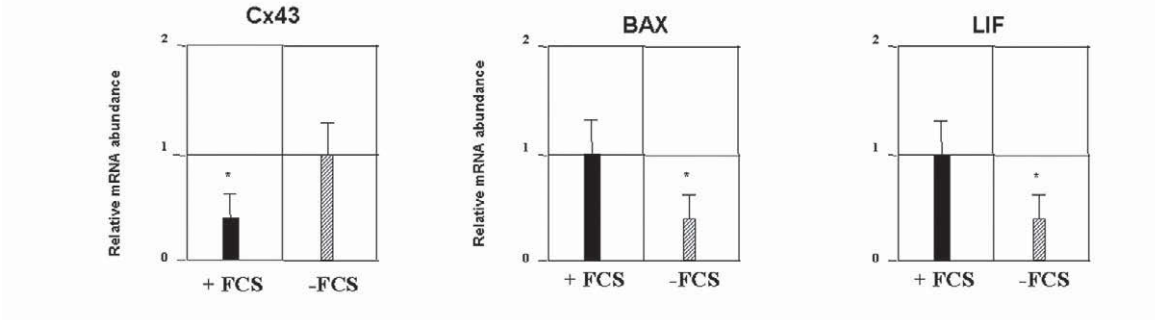


**Figure 3.** Relative abundance of transcripts for *Bos taurus* apoptosis regulator box-α (Bax), connexin 43 (Cx43) and bovine leukaemia inhibitory factor (LIF) in bovine blastocysts produced *in vitro* in synthetic oviduct fluid medium in the presence or absence of fetal calf serum (FCS). An asterisk indicates a significant difference ($P < 0.05$). Data from Rizos *et al*. (2003).

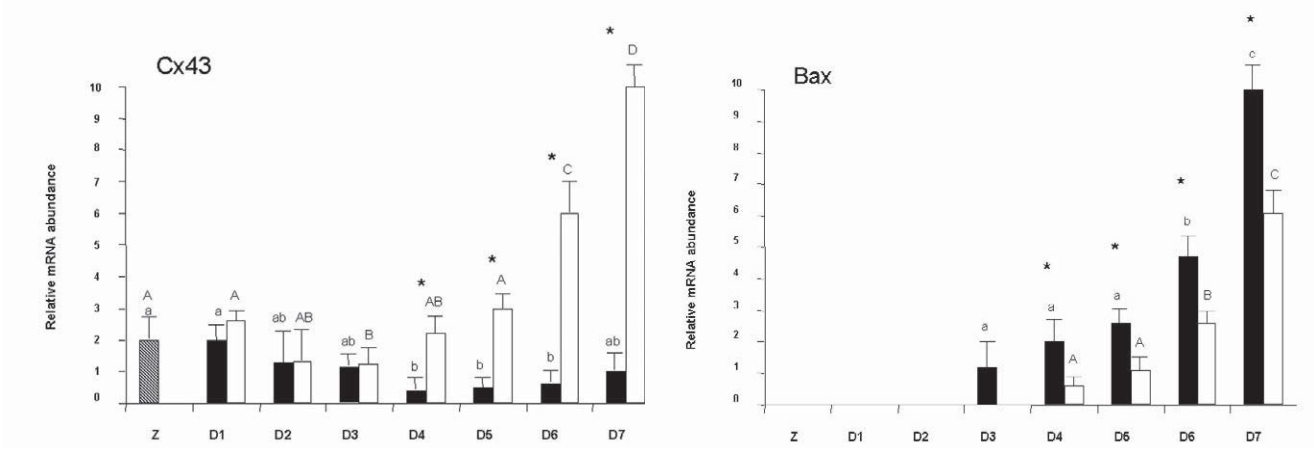


**Figure 4.** Relative abundance of connexin 43 (Cx43) (**a**) and *Bos taurus* apoptosis regulator box-α (Bax) (**b**) transcripts in bovine embryos cultured either *in vitro*, in synthetic oviduct fluid (SOF, black bars) or *in vivo*, in the ewe oviduct (white bars). [abcd] refers to significant differences in relative transcript abundance among in-vitro cultured embryos throughout the early preimplantation period; [ABCD] refers to significant differences in relative transcript abundance among in-vivo cultured embryos throughout the early preimplantation period. An asterisk indicates a significant difference ($P < 0.05$) in relative transcript abundance between in-vitro and in-vivo cultured embryos at a given stage of development. Z: zygote before transfer to treatment group; D1: 2-cell; D2: 4-cell; D3: 8-cell; D4: 16-cell; D5: early morula; D6: compact/late morula; D7: blastocyst. Data from Lonergan *et al.* (2003).

may lead to persistent alterations of gene expression patterns during embryonic and fetal development and may lead to fetal and neonatal abnormalities. The so-called 'large offspring syndrome' in domestic ruminants is characterized by a variety of abnormal phenotypes, including significant increases in birthweight, polyhydramnios, hydrops fetalis, altered organ growth, various placental and skeletal defects, immunological defects and increased perinatal death (for reviews, see Walker *et al.*, 1996; Kruip and den Daas, 1997; Young *et al.*, 1998; Niemann and Wrenzycki, 2000; Sinclair *et al.*, 2000).

Evidence for a direct link between manipulation of the early embryo and epigenetic modifications to DNA leading to altered imprinted gene expression (reviewed by Santos and Dean, 2004) has been established in mice (Doherty *et al.*, 2000; Khosla *et al.*, 2001) and sheep (Young *et al.*, 2001). The expression of imprinted genes appears particularly sensitive to culture conditions; normal maternal monoallelic expression of *H19* is observed after culture in modified simplex optimized medium + amino acids (KSOM/AA), whereas biallelic expression is found after culture in Whitten's medium (WM) (Doherty *et al.*, 2000; Khosla *et al.*, 2001). Rinaudo and Schultz (2004) compared global patterns of gene expression in mouse blastocysts derived from culture in WM or KSOM/AA with that of in-vivo-derived blastocysts using the Affymetrix MOE430A chip. The expression of 114 genes was affected after culture in WM, whereas only 29 were mis-expressed after culture in KSOM/AA. These results are consistent with KSOM/AA supporting better development *in vitro*.

The identification and characterization of the short-term effects of in-vitro culture raises the question about long-term consequences and safety of assisted reproductive technologies. For example, recent reports indicate that in-vitro culture of mouse embryos can have irreversibly long-term consequences of post-natal development, growth, physiology and behaviour in resulting offspring (Ecker *et al.*, 2004; Fernandez-Gonzalez *et al.*, 2004).

Apart from the culture medium used, the conditions of culture can also affect gene expression. Bovine in-vitro-produced embryos respond to changes in oxygen concentrations by altering the expression of glucose transporter-1 (GLUT1) (Harvey *et al.*, 2004), while in mice it has been reported that expression of GLUT1, GLUT3 and vascular endothelial growth factor was significantly increased in embryos cultured under 2% versus 20% oxygen (Kind *et al.*, 2004). In other studies, expression of GLUT1 has been shown to differ between in-vivo- and in-vitro-derived embryos (Wrenzycki *et al.*, 2001).

## Conclusion

To date there have been no reports of abnormally large offspring in humans, despite the experiences in rodents and ruminants. This, as Sinclair and Singh (2004) point out, begs the question of whether human gametes differ so substantially that they are less susceptible to sub-optimal culture environment or whether this is merely a reflection of the relatively slow uptake of techniques such as IVM or blastocyst culture in humans. However, it is well established that human infants conceived following IVF and ICSI are more likely to be born preterm, to be of low birthweight and to be a twin or higher order multiple than spontaneously conceived infants (Olivennes *et al.*, 2002; Helmerhorst *et al.*, 2004; Jackson *et al.*, 2004; Wennerholm and Bergh, 2004). The evidence relating to the risk of birth defects is less clear. The publication of a paper by Hansen *et al.* (2002) reporting a two-fold increased risk of major birth defects in children from IVF and ICSI stimulated much debate (Barlow, 2002; Winston and Hardy, 2002). Hansen *et*

*al*. (2005) carried out a systematic review of 25 papers with data relating to the prevalence of such birth defects; two-thirds of these showed a 25% greater increased risk of birth defects in assisted reproduction infants. From the pooled results the authors suggested that children born after assisted reproduction treatment are at increased risk of birth defects. While the causes of such defects are unclear at present, manipulations of the embryos *in vitro* are probably involved; indeed, reports of epigenetic modifications in genomic imprinting following assisted reproduction treatment in humans have been published (Edwards and Ludwig, 2003; Niemitz and Feinberg, 2004).

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