Advanced reproductive technologies in cattle and buffalo and their impact on breeding

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Introduction

Reproduction lies at the heart of any livestock breeding enterprise and is vital to maintain or increase the number of animals required for production. Assisted reproduction techniques like Artificial Insemination (AI), Multiple Ovulation and Embryo Transfer (MOET) or in vitro embryo production have filled over the years the needs of a sustainable and more competitive livestock industry, particularly in cattle, bringing enormous benefits to the breeders and to people as a whole. A growing interest is emerging for buffaloes in tropical and sub-tropical areas. Buffaloes are in many ways similar to cattle and they live in an environment that has completely different requirements as compared to more traditional cattle breeding areas. Nevertheless the same principles of reproduction technologies that have benefit enormously cattle (both Bos Taurus and Bos Indicus, that is more a tropical animal like buffaloes) are expected to eventually benefit buffaloes as much as they have advanced cattle production systems. At present many, if not all, reproduction techniques well established in cattle have been translated to buffaloes, some on a practical basis like oestrus synchronisation and artificial insemination, others on an experimental basis like the embryo technologies. The latter still remain a research tool and their commercial application is only beginning to emerge being limited by the low efficiency reported in the literature by many investigators. At the forefront of assisted reproduction techniques lies SCNT (somatic cell nuclear transfer) with its many biological limits and its great potential applications especially in the area of genetic engineering. After more than a decade from the birth of the first mammal born by SCNT, more than19 mammalian species have been cloned. SCNT efficiency in farm animals is very low and typically ranges from 1 to 5% (Oback and Wells 2007). Success rate (measured by the birth of live animals) is higher in polytocous species like the pig, because several dozen embryos are transferred at one time thus significantly increasing the number of recipients that become pregnant and deliver live offspring (Vajta et al. 2007).

IN VITRO EMBRYO PRODUCTION

Ovum Pick Up (OPU)

The OPU technique is a non-invasive and repeatable procedure for recovering large numbers of meiotically competent oocytes from antral follicles of live cattle (Galli et al. 2001) and it has been developed in other species as well including horses (Galli et al. 2007, Colleoni et al. 2007) and small ruminants (Cox and Alfaro 2007). For bovine it can now be considered a mature technology (van Wagtendonk-de Leeuw 2006) with many thousand of embryos produced each year (www.iets.org, ET statistics), especially in Bos Indicus cows. Embryo production from ovum pick up oocytes is very variable and affected by age (Galli et al. 2001), season (Takuma et al. 2010, Viana et al. 2010), FSH stimulation (Chaubal et al. 2006, De Roover et al. 2008, Sendag et al. 2008), donor genetics (Merton et al. 2009) and can average 1-3 embryo per session once or twice weekly (Galli et al. 2001). In the buffalo OPU it is still at an experimental stage, the first report of a calf derived from a frozen-thawed embryo produced from OPU oocytes, by in vitro maturation, fertilisation and embryo culture was presented in 1998 by our laboratory. Repeated OPU in buffalo (Gupta et al. 2006) can be performed without sides effects as in cattle with minimal stress to the animal (Petyim et al. 2007).

In vitro maturation (IVM)

Besides OPU on live donors oocytes can also be sourced from abattoirs. In this case there is an extreme variability on the quality and the numbers of oocytes recovered, depending on the type of animal, geographical location, body conditions, season, etc. In vitro maturation is still the most critical step in the success of in vitro embryo production (Galli and Lazzari 2008). We use the same media and conditions both in cattle and buffalo oocytes (Galli et al. 2003). Our results indicate that both oocyte recovery and embryo production are considerably lower in buffalo as compared to average results in cattle (Galli and Lazzari 1996). In both techniques, OPU and recovery from abattoir ovaries, we collect all follicles detectable so the size is usually equal or larger than 1 mm. The meiotic competence of buffalo oocytes has been investigated relative to oocyte and follicle size (Yousaf and Chohan 2003). The majority of oocytes derived from follicles smaller than 4mm was at the initial stages of GV development and had poor IVM rates (about 32%), whereas oocytes from follicles of 4 to 8mm were at the final stages of GV and were more competent for maturation (67- 79% MII). Gasparini et al.

showed (Gasparrini et al. 2008) that the majority of buffalo oocytes accomplish nuclear maturation between 21 and 24 h after the start of in vitro maturation like cattle.

In vitro fertilisation (IVF)

Cattle in vitro fertilisation work quite efficiently although there might be variation with different batch of semen even from the same donor bull associated with aging and or intense semen production activity or genetic background (Comizzoli et al. 2003). The inconsistent quality of buffalo semen used to be in the past an important source of variation. However, in recent years the preparation of frozen semen has improved and fertilisation rates have reached levels similar to those reported in cattle. Bull profiling is used in our laboratory to optimise the sperm concentration required during IVF to maximise the fertilisation rates while minimising polyspermic fertilisation. This profiling even in the case of buffalo oocytes can be performed using cattle oocytes that are easily available in large numbers, therefore avoiding the wasting those of valuable buffalo. Sex sorted semen is now widely use for in vitro fertilisation with satisfactory results (Blondin et al. 2009, Rath et al. 2009, Xu et al. 2009). The use of sexed semen has also been reported for use in vitro fertilisation in buffalo with no difference in blastocyst development and calving after IVF with sexed or non-sexed sperm (Liang et al. 2008, Lu et al. 2007).

In vitro culture (IVC)

In vitro embryo culture covers the period from fertilisation to blastocyst formation and it usually last 6-7 days fro buffalo and 7-8 days for cattle. This stage is required both for a better survival after cryopreservation and for the non surgical transfer into synchronised recipients. Culture conditions can substantially affect not only the pre-implantation development in vitro but also influence the post implantation and post-natal development (Lazzari et al. 2002). In our first successful report of calves obtained from in vitro embryo production, the embryo culture after IVF, from 2-4 cells to blastocyst, was done in surrogate sheep oviducts (Galli et al. 1998, Galli and Lazzari 1996). Later, several in vitro culture methods and media have been reported (Hansen et al. 2010). In buffaloes after an initial trend towards co-culture systems (Nandi et al. 2003) in which oviduct cells in TCM199 supplemented with serum, supported blastocyst development to significantly higher level than cell free systems, there has been a shift, like in cattle, to semi-defined conditions (Gasparrini 2002, Purohit et al. 2005) represented by mSOF supplemented with BSA and aminoacids. Buffalo embryos cultured in vitro develop 12 to 24 h earlier than cattle embryos as it occurs in vivo and generally are frozen on day 6 (day 0: fertilisation). We culture embryos in mSOFaa + BSA (Galli et al. 2001) in 5% CO2, and 5% O2 at 38.5°C . The preferred stage for cryopreservation is the expanding / expanded blastocyst stage that is typical of embryos with high metabolic activity (code 6 and 7 according to the IETS manual). At these stages it is possible to assess the presence / quality / size of the inner cell mass (ICM) of the developing embryo thus increasing the accuracy of selection and the grading of the embryos prior to freezing.

EMBRYO SEXING AND GENOTYPING

Embryo sexing can be performed by amplification of embryo biopsies with several methods: a sex determination assay using primary and multiplex PCR both in cattle and in buffaloes (Appa Rao and Totey 1999, Peura et al. 1991, Peippo et al. 2007, Chrenek et al. 2001) also in association with amplification of selected genes for specific production traits (Peippo et al. 2007). It is envisaged however that sex sorted spermatozoa will supersed embryo sexing by PCR at least for in vitro embryo production. Another technology that is expected to emerge is the genotyping of the embryo for relevant production or fertility markers by transferring the genome wide studies from the whole animal to the embryo biopsy (Huang et al. 2010, MacLeod et al. 2010). The size of embryo biopsy that will not compromise embryo viability should be limited to 10-15 cells at the maximum. With this number of cells the amount of DNA is not sufficient to avoid possible allele drop out during amplification (Roeder et al. 2009). For this reason until more reliable DNA amplification techniques will be available, it will be desirable to expand in culture the number of cells from the embryo biopsies or to derive from it an embryonic like cell line (see below) to overcome this limit.

EMBRYONIC STEM CELLS

Embryonic stem cells are pluripotent cells that originate from the early embryo either from the inner cell mass or from the epiblast. They can be cultured and expanded in vitro in undifferentiated conditions, can be genetically engineered if necessary, and when reintroduced in the embryos can give rise to any cell type including the germ line, except trophoblast. This is today the principal route to make genetically engineered mice (Capecchi 1989). The derivation of farm animal ES cells or epiSC equivalent to those described for the mouse or the human has not been reported yet therefore this route is not available at present in livestock species but it might be so in the near future. However, over the years, from 1981, when mouse ES cells were first discovered, many laboratories have attempted ES cell derivation mainly from cattle, pig and sheep embryos (Galli et al. 1994, Keefer et al. 2007, Notarianni et al. 1991). However, the stemness (staminality) of these cells appeared to be very limited and

most likely they represent trophoblastic cells given their epithelial nature, loss of OCT4 expression and limited differentiation potential (Iwasaki et al. 2000, Keefer et al. 2007, Notarianni et al. 1991). Advances in mouse and, more recently, in hESC culture have demonstrated that a number of different culture conditions can support pluripotency of embryo-derived stem cells (Ying et al. 2003). Other recent protocols, based on the stimulation of the nodal-activin signalling pathways, have been shown to maintain the undifferentiated proliferation of human and mouse epi stem cells (EpiSCs), a novel type of stem cells derived from the epiblast very similar to human ES cells in morphology, growth factors requirement and gene expression (Tesar et al. 2007, Brons et al. 2007). Recently the derivation of pig EpiSC has been reported but ruminant EpiSC are still to be established (Alberio et al. 2010). In an applied perspective embryonic stem cells in farm animals are important for several reasons but the most relevant is to provide a method to introduce precise genetic modification into animals by homologous recombination of ES cells (Lombardo et al. 2007) followed by blastocyst injection for chimera derivation and breeding, or by somatic cell nuclear transfer.

Induced Pluripotent Stem Cells (iPS Cells)

In 2006 a breakthrough study (Takahashi and Yamanaka 2006) demonstrated that viral transduction of a handful of genes (Oct4, Sox2, Klf4 and c-Myc) can reprogram mouse embryonic fibroblasts into ES cell-like cells which carry all the molecular features of true embryo-derived ES cells including the ability to give rise to germ line chimeras. The following year the generation of human iPS cells was achieved (Takahashi et al. 2007, Yu et al. 2007) using slightly modified transduction methods and set of genes. In large animals attempts to derive iPS have been made resulting in a few reports about induced reprogramming of pig fibroblasts (Wu et al. 2009, Esteban et al. 2009, Ezashi et al. 2009). Most likely the lack of robust procedures for the establishment embryo-derived ES cells in large animals represents a major limit also for the development of the iPS technology but potentially can provide a different route to embryonic pluripotent stem cells in livestock.

EMBRYO TRANSFER AND PREGNANCY

Extensive data on pregnancy rate are variable in the published literature both for fresh and frozen embryos. Cattle embryos usually perform satisfactorily even after cryopreservation (Xu et al. 2006) although in some instances as a consequence of in vitro culture some problems are reported (Farin et al. 2010, Lazzari et al. 2002). In buffalo a calving rate of 15-34%, after embryo transfer (ET) of 2 fresh river or river x swamp embryos into sinchronized recipients (Liang et al. 2007), was reported, while 11-15% was obtained for frozen thawed embryos (Liang et al. 2008). In our study (Galli et al. 1998) we obtained on a small number of transfers 33% calving rate (3/9). The most common protocol used today for synchronising the recipients is the Ov-Synch protocol, that does not necessary require estrus detection. However, better results are obtained with the Ov-Synch protocol when there is ovarian activity. A combination of both (CIDR and Ov-Synch) might overcome these limitations (De Rensis et al. 2005). In India, with the buffalo, the efficiency of OPU combined with IVP was found to be higher during the peak breeding season than the low breeding season (Manjunatha et al. 2009).

SOMATIC CELL NUCLEAR TRANSFER (SCNT)

Somatic cell nuclear transfer is an emerging technology with many applications in animal breeding, from multiplying superior genotypes to making genetically engineered animals and genotyping to select the best genomes for breeding. The donor cell origin and differentiation status has not been found to play a major role in term of success rate (Oback 2009), however the genotype of the cells has an important function on the outcome and the role of hybrid vigour has been demonstrated in the mouse (Eggan et al. 2001) and also in the pig (Zhao et al. 2009). The oocyte contains a milieu with all the “ingredients“ required for genome reprogramming, mainly transcription factors that can reset the epigenetic status of the chromatin.

The recipient oocyte is usually used after enucleation at the Met II stage, however other stages including the zygote have been used with minimal improvements (Schurmann et al. 2006). The use of a zona free micromanipulation system has made the procedure simpler (Lagutina et al. 2006, Oback et al. 2003). Embryo aggregation (3 embryos) during in vitro culture has been reported to make up possible individual embryo deficiency in the expression of totipotency genes or cell numbers in the mouse (Boiani et al. 2003, Boiani et al. 2002). The unquestionable proof of complete reprogramming of the genome through SCNT is the birth of viable offspring. However, at present, reprogramming is a stochastic event, taking place immediately after nuclear transfer and in the following days up to the blastocyst stage (Schaetzlein et al. 2004). There is an extensive number of studies looking at the “deviation” of gene expression from the pattern of a fertilised embryo (Smith et al. 2005, Wrenzycki et al. 2004) in order to identify possible markers of correct reprogramming. GeneChip microarrays global gene expression analysis of bovine in vitro fertilized (IVF) and SCNT blastocysts as well as respective donor cell lines to characterize differences in their transcription profiles has been also employed (Beyhan et al. 2007). Gene expression profiles of donor cell lines were significantly different from each other; however, the SCNT and IVF blastocysts displayed surprisingly similar gene expression profiles, suggesting that a major reprogramming activity had been exerted on the somatic nuclei. Despite this remarkable phenomenon, a

small set of genes appears to be aberrantly expressed and may affect critical developmental processes responsible for the failures observed in SCNT embryos.

Post-implantation development

When pre-implantation development has occurred, SCNT embryos still presents some abnormalities of the epigenetic status in the trophoblast lineage causing abnormal placental development, leading to a high rate of embryonic losses and or abortion, while the inner mass cell lineage is relatively normal (Yang et al. 2007, Loi et al. 2006). Failures of SCNT pregnancy are associated with placental abnormalities, such as placentomegaly, reduced vascularisation, hypoplasia of trophoblastic epithelium, and altered basement membrane (Palmieri et al. 2008). The majority of established SCNT pregnancies in cattle are lost between days 30 and 90 of gestation, in association with poorly developed placentomes (Hill et al. 2000). If pregnancy is maintained, placentomes are frequently hypertrophied (Constant et al. 2006). Anomalies appear more extreme in clones produced from somatic cells relative to those produced from embryonic cells (Heyman et al. 2002). Two genes (Mash2 and Hand1) appear to play a critical role in trophoblast development of several species including the cow. The failure of placental development following SCNT is believed to be the major reason of cloning having a low success rate. It is known that many imprinted genes are important for the development of the placenta. It has been shown that even relatively minor manipulations, such as embryo culture (Niemann and Wrenzycki 2000), can affect expression of imprinted genes in mice placenta (Mann et al. 2004). Loss of imprinted expression was associated with a decrease in methylation control regions. Similar observations are described for other ruminants like the sheep (Palmieri et al. 2008) or the buffalo (Shi et al. 2007) that share the same cotyledonary structure of the placenta. Placental failure in bovine cloned pregnancies may originate from abnormal embryo-maternal communication that develops during the peri-implantation period (Bauersachs et al. 2009).

Risk assessment and safety of clones.

The use of cloned animals and their offspring for food production will depend on public acceptance. For these reasons many food safety agencies throughout the world have carried out studies to compare products derived from cloned animals with those of normal animals. Watanabe (Watanabe and Nagai 2008) analyzed 171 clone cattle and 32 offspring, and categorized them according to the following 7 categories: (1) genetic similarities and muzzle prints, (2) hematology and clinical chemistry findings, (3) pathology, (4) growth performance, (5) reproductive performance, (6) meat production performance and (7) milk production performance. No remarkable differences in health status or reproductive performance were found among conventionally bred cattle, somatic cell cloned cattle surviving to adulthood and offspring of somatic cell cloned cattle. Similarities in growth performance and meat quality were observed between nuclear donor cattle and their clones. The physiology of surviving postpubertal cloned bulls and quality of collected semen had equivalent reproductive potential to their original cell donor, with no evidence of any deleterious effects in their progeny (Tecirlioglu et al. 2006, Heyman et al. 2004). Murphey (Murphey et al. 2009) showed that the rate of accumulation of spontaneous mutations was similar in fetuses produced by either natural conception or cloning, indicating that cloned fetuses do not acquire mutations more rapidly than naturally conceived fetuses. These results represent the first direct demonstration that the process of cloning by SCNT does not lead to an increase in the frequency of point mutations.

FINAL CONSIDERATIONS

Assisted reproduction technologies (ARTs) provide a fundamental reproduction tool in monotocous species like cattle and buffalo. In vitro embryo production is complementing MOET schemes for breeding females that otherwise would not produce embryos, like Bos Indicus or buffalo, or for producing large numbers of embryos from donor females that have been pre-screened with genomic tests. Moreover new techniques are being developed to allow the genomic screening of embryos using embryo biopsies obtained by micromanipulation. In this perspective there will be the need to generate even more embryos from few selected genotypes and embryonic stem cells or iPS cells can be a valuable tool in combination with somatic cell nuclear transfer. The latter can already be used to create breeding animals but its implementation by the industry will depend on public acceptance. Finally ARTs require technical improvements and refinements but this will not be sufficient to provide benefits if the public opinion is not correctly informed and made aware of the advantages and the risks associated with the progress of science.

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