

Consequences of manipulating gametes and embryos of ruminant species

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During the past 12 years, ruminants have provided a focus for some significant advances in mammalian reproductive biotechnologies. Lambs were the first offspring generated after nuclear transfer of fetal or adult cells to enucleated oocytes, and many calves of pre-determined gender are today the result of commercialized semen sexing. In 1990, the birth of one calf provided living proof that even 'dead' spermatozoa can be paternal, whereas, more recently, a short-lived gaur calf and viable mouflon lamb represented a novel option for conservation of endangered species. As well as highlights, hazards have emerged, resulting in setbacks or developmental anomalies, such as those associated with the large offspring syndrome which encompasses a range of adverse fetal, placental and post-natal phenomena expressed in ruminants. In this review, the developmental and other consequences of applying manipulative procedures, such as assisted fertilization, semen sexing, cloning and gene transfer, to gametes and embryos from bovine, ovine and caprine species are considered. Although assisted fertilization techniques can overcome mammalian infertility, they also usurp natural gamete selection safeguards, but not always with impunity. In the case of manipulations such as cloning, and to a lesser extent gene transfer, it is evident that nuclear–cytoplasmic interactions and nuclear–mitochondrial DNA interdependences are at least partially damaged or destroyed with a view to reconstruction. Therefore, among surviving zygotes and embryos it is inevitable that the legacy is frequently one of altered genetic, epigenetic or cellular programmes and processes.

Introduction

The application of assisted reproductive technologies (ART) to ruminant species during the past 10 years has, in some cases, at least partly realized the potential attributed to them during the 1980s. In other cases, ART have facilitated advances towards research horizons then considered beyond reach. Transgenic ruminant animal production has been confined mainly to biopharmaceutical agendas at dedicated 'pharming' facilities and research institutions, whereas semen-sexing technology is now available in the UK. Most significantly, nuclear

transfer myths and taboos have been cast aside, thanks to the births, at Roslin, of lambs derived from enucleated oocytes injected with nuclei from somatic fetal or adult cells. In recent years, using some of the technologies in tandem, there has been steady progress towards production, via intracytoplasmic sperm injection (ICSI), of lambs and calves of pre-determined sex (Catt *et al.*, 1996; Hamano *et al.*, 1999) and, via nuclear transfer, of gene-targeted sheep (McCreath *et al.*, 2000). Progress in transgene technology can also influence ruminants indirectly, via plant breeding or modifications to rumen microflora (Robinson and McEvoy, 1999) but, as they do not involve mammalian gamete or embryo manipulation, these advances will not be featured here.

In this review, some of the outcomes and implications, and benefits and limitations, of gamete and embryo manipulation procedures as applied to ruminants will be considered. In so doing, we will not dwell on supporting technologies *per se* (for example, IVF and embryo culture), but instead will consider technologies that use physically disruptive, invasive or otherwise stressful manipulations of gametes or embryos. The importance of *in vitro* environments and their immediate and lasting effects on the development of ruminants, in particular, have been emphasized elsewhere (Young *et al.*, 1998; McEvoy *et al.*, 2000; Sinclair *et al.*, 2000) and must not be underestimated.

Technologies that will be considered in this review include assisted fertilization procedures (notably ICSI), semen sexing, nuclear transfer and transgenic animal production.

Assisted fertilization via gamete manipulation

In vitro assisted fertilization procedures aimed at enhancing embryo production in conditions in which conventional IVF cannot or might not achieve the desired results have been developed primarily for application in humans. There has been less interest in respect of ruminants, partly because it is rarely either feasible or desirable to generate offspring from bulls, rams or bucks with poor fertility or significant semen quality limitations. However, in some circumstances it may be justified, for example when only immature spermatids are available post mortem or, perhaps, to facilitate sperm-mediated gene transfer (SMGT; discussed later) in certain circumstances.

Zona pellucida drilling, partial zona pellucida digestion (PZD), subzonal injection (SUZI) and ICSI are the options available in clinical IVF settings. Of these, ICSI has been most successful and, because the other techniques are quite likely to result in polyspermic fertilization, ICSI has generally superseded them.

ICSI – application to ruminants

The history of ICSI reached its first major milestone when Goto *et al.* (1990) reported the birth of a normal calf after injection of a 'killed' spermatozoon into a bovine oocyte (Table 1). Some years later, a male lamb was born after ICSI of a Y chromosome-bearing ovine spermatozoon (Catt *et al.*, 1996) and, more recently, the same procedure generated caprine blastocysts (Keskintepe *et al.*, 1997).

The usual consequence of ICSI, partly because of its invasive nature, is failure to generate offspring efficiently. For example, in the study reported by Catt *et al.* (1996), only one lamb was born after transfer of 251 oocytes to recipient ewes within 4 h after ICSI. Nevertheless, ICSI is a valuable procedure for generation of embryos in circumstances in which natural breeding or conventional IVF is not an option. Herein, however, lies a concern. The evolutionary forces that have shaped natural breeding practices among ruminants impose stringent selection pressures on the male gamete, and only the very fittest (in terms of maturity, morphology and motility) are permitted to reach the site of fertilization *in vivo*. The fact that ICSI bypasses

Table 1. Summary of studies contributing to the application of intracytoplasmic sperm injection (ICSI) in domestic ruminants

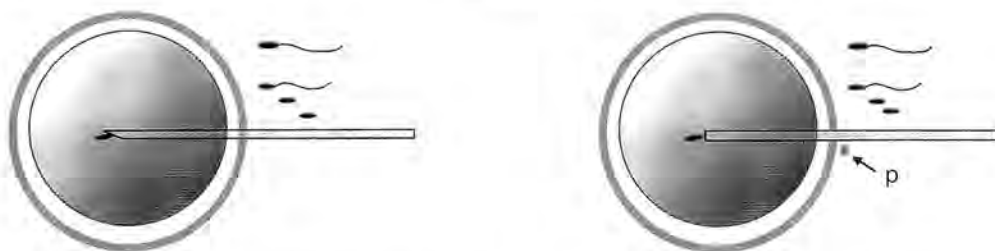
Species	Gamete status	Development	Reference
Cattle	Immobilized and 'killed' spermatozoon	One normal calf	Goto <i>et al.</i> , 1990
	<i>In vitro</i> derived spermatids	Blastocyst	Goto <i>et al.</i> , 1996
	Y-sorted sperm heads	10 normal calves (8 males)	Hamano <i>et al.</i> , 1999
	Lyophilized spermatozoa	Blastocyst	Keskintepe <i>et al.</i> , 2001
	Frozen-thawed immobilized spermatozoa	5 normal calves	Horiuchi <i>et al.</i> , 2002
Sheep	Tail-cut spermatozoa; no oocyte activation treatment	5 normal calves	Wei and Fukui, 2002
	Immobilized Y-sorted spermatozoa	1 normal lamb	Catt <i>et al.</i> , 1996
Goat	Control or acrosome-reacted spermatozoa	2 normal lambs	Gomez <i>et al.</i> , 1998
	Frozen-thawed spermatozoa with broken tails	Blastocyst	Keskintepe <i>et al.</i> , 1997

these selective processes has led some to outline the risks involved and, in effect, question the use of the process (Goto, 1997; Hewitson *et al.*, 2000). Such questioning, particularly in the context of human clinical applications, has sometimes been countered, quite unnecessarily, by pejorative charges of promoting a eugenics agenda. Much more helpfully, some have been willing to engage in constructive debate; a notable and thought-provoking example (including rebuttals) is that between Lamb (1999) and Schlegel (1999). Although the stakes are not nearly so high in the context of ruminant reproduction, it is important to consider whether ICSI is likely to enhance or undermine reproduction (Fig. 1).

As it is a technically demanding and costly procedure, ICSI is unlikely to be used for commercial animal breeding. Horiuchi *et al.* (2002) suggested that ICSI might be used to maximize the progeny from costly semen straws, but we consider this to be improbable. Instead, ICSI is most applicable for conservation biology and genetic salvage, when only 'dead' spermatozoa or immature spermatids are available, or for application in tandem with specialist technologies such as gene transfer. In conservation biology, any risk associated with ICSI is probably outweighed by the threat of extinction or loss of a valued genotype, whereas for genetic salvage, significant investments and the limitations of alternative options could justify ICSI as a route for breeding specialized genotypes. It is possible, for example, that pronuclear microinjection of presumptive zygotes will be superseded to some extent by sperm-mediated gene transfer via ICSI in the near future (see later), not least because it is less technically demanding and, as noted by Perry *et al.* (1999), avoids the need for prior generation of presumptive zygotes.

The outcomes of ICSI, in terms of efficiency of generating embryos or offspring, are influenced by a number of factors including sperm pre-treatments, approaches to oocyte activation and injection-related hazards, such as meiotic spindle disruption (Rho *et al.*, 1998; Hewitson *et al.*, 1999; Wei and Fukui, 1999, 2002). Curiously, sperm viability in the conventional sense is not a significant factor. Immobilized and 'dead' spermatozoa are at least as successful in generating blastocysts or offspring. Indeed, Wei and Fukui (1999) and Yanagimachi (2001) observed that ICSI with immobilized spermatozoa improved fertilization.

A concern about ICSI, particularly when the procedure involves tail-scoring or removal of the tail, is that exposure of the 'damaged' gamete to culture medium may be detrimental to



Method A: One-step procedure with sharply pointed injection needle, to introduce male gamete, with or without sperm tail

Method B: Two-step, pulse-driven procedure with straight-ended needle, first removing zona pellucida plug (p) and then introducing male gamete

	Some ICSI scenarios		
Male gamete category	Dead spermatozoa or immature spermatids	Lyophilised spermatozoa	Spermatozoa carrying 'foreign' DNA
Benefit	Genetic salvage and conservation	More efficient gamete storage	Transgenic animal production
Hazard	Propagate inferior or lethal genotypes	Viability not predetermined	Contaminant DNA uptake

Fig. 1. The advantages and disadvantages of intracytoplasmic sperm injection (ICSI).

the stability of chromosomes exposed by the injury inflicted (Kusakabe *et al.*, 2001), because the ionic composition and other properties of conventional media do not reflect the intracellular milieu of the spermatozoon (Yanagimachi, 2001). Studies with non-ruminants have indicated that damage can be limited by reducing Na^+ and increasing K^+ concentrations (Tateno *et al.*, 2000).

Other possible adverse consequences of ICSI are inadvertent disease transmission (Cummins, 2001) or introduction of unwanted exogenous DNA into injected oocytes after exposure of spermatozoa, especially if damaged, to serum or albumin in conventional media. These are real risks, firstly because serum is considered by some to be desirable for other reasons: for example, it has been suggested that the presence of serum (15% inclusion) reduced the incidence of egg degeneration in the immediate aftermath of bovine and murine ICSI procedures (Keskinetepe and Brackett, 2000). Secondly, although the extent to which extraneous DNA might persist during embryogenesis is unknown, it is precisely such persistence that provides the basis for sperm-mediated gene transfer procedures.

ICSI also risks other consequences relating to DNA persistence, for example when fertilization is achieved by injecting spermatozoa that have damaged nuclear DNA. As reported by Sakkas *et al.* (2000), ICSI with such spermatozoa offers contrasting scenarios. Firstly, paternal nuclear DNA may not be repaired after injection and fertilization may fail to occur. Secondly, DNA repair may be sufficient to ensure that subsequent development is entirely normal. Thirdly, however, partial DNA repair may facilitate fertilization, but lead to later losses or the generation of abnormal offspring. The report of Rho *et al.* (1998) in which ICSI-derived bovine blastocyst transfer resulted in six confirmed pregnancies (including fetal heartbeat detection) at day 49 of gestation but no



Fig. 2. In the 10 years since the first 'semen sexed' calves were produced (by D. Cran and co-workers) at Cambridge in the UK, sperm-based gender preselection technology has advanced to the extent that approximately 50 000 calves have been born worldwide after inseminations with sex-sorted semen, mostly on commercial farms (photograph courtesy of *Farmers Weekly*).

survival to term indicates that ICSI-derived fetuses or their placentae or both were sub-normal.

ICSI – anomalies and opportunities

Whatever the incidence or severity of long-term consequences, there is compelling evidence from elegant primate studies that 'fertilization' events immediately after ICSI are not analogous to those after normal penetration of the oocyte by intact spermatozoa. Some differences are linked to the persistence, during ICSI, of components of the spermatozoon that normally do not enter the cytoplasm after sperm–oocyte fusion. Hewitson *et al.* (1999) noted that the pattern of rhesus monkey sperm decondensation after ICSI was impeded by retention of vesicle-associated membrane protein (VAMP), a constituent of the sperm acrosome, and that migration of oocyte-derived nuclear mitotic apparatus (NuMA) into condensed regions of paternal chromatin was delayed. Such anomalies, they concluded, could mean that ICSI *per se* might be responsible for observed increases in chromosomal aberrations.

One potentially valuable consequence of ICSI is that, in future, preservation of male gametes may not require cryo-storage in liquid nitrogen which, as noted by Kusakabe *et al.* (2001), is costly, subject to failure and carries a contamination risk. Instead, because motility is not essential, cheaper and more transport-friendly drying options, which need only to maintain nuclear integrity of spermatozoa, should become a viable proposition. For example, Keskintepe *et al.* (2001) have demonstrated that ICSI with lyophilized spermatozoa is capable of generating morula–blastocyst-stage bovine embryos (20% yield) *in vitro*.

Finally, as outlined by St John (2002), ICSI shares with both nuclear transfer and cytoplasmic transfer technologies the possibility that mitochondrial inheritance is not exclusively uniparental (Smith *et al.*, 2000), a topic that will be addressed later in this review.

Gender preselection via semen sorting

The facility to predetermine the gender of cattle with approximately 90% accuracy has become a commercial reality in the UK in recent years (Fig. 2), using technologies that separate

Table 2. Application of nuclear transfer for generation of transgenic or non-transgenic goals

Enucleated oocyte source	Donor cell/nucleus	Number of Kids	Outcomes of recorded pregnancies after transfer of reconstructed embryos	Reference
Stimulated donors + <i>in vivo</i> maturation	Fetal somatic cells (transgenic)	3	No pregnancies lost beyond day 40; no postpartum morbidity noted; all 3 transgenic, normal birth weight	Baguisi et al., 1999
Stimulated donors + <i>in vitro</i> maturation	Fetal cells (\pm transgene)	6	No prenatal losses; cotyledons seemed irregular; 50% postnatal loss; 1 transgenic kid survived, but transgene was not expressed; of 5 kids from non-transfected donor cells, only 2 kids survived beyond 3 months	Keefe et al., 2001
Stimulated donors + <i>in vivo</i> maturation	Cumulus cells (NT via fusion)	2	Sole pregnancy (day 30) persisted to term (day 144); twins (2 + 2.2 kg) died soon after birth, from respiratory problems; examinations post mortem indicated organs (heart, kidneys, liver, lungs) were normal	Zou et al., 2001
Stimulated donors + <i>in vivo</i> maturation	Cumulus cell nuclei (NT via injection)	1	Sole pregnancy (day 30) went to term (day 150); kid weighed 3.3 kg (average = 2.4 kg); alive and healthy	Zou et al., 2001
Stimulated donors + <i>in vitro</i> maturation	Fetal fibroblast cells (transfected)	26	16 of 21 pregnancies (day 30) went to term; 17 kids transgenic; 17 survived	Baldassare et al., 2002; Keefe et al., 2002b
Stimulated donors + <i>in vitro</i> maturation	Adult skin fibroblasts (transgenic donor)	3	Both pregnancies (day 30) went to term; all kids transgenic; one survived	Baldassare et al., 2002; Keefe et al., 2002b
Stimulated donors + <i>in vitro</i> maturation	Cumulus-granulosa cells (transgenic donors)	9	7 of 8 pregnancies (day 30) went to term; all 9 kids transgenic; 8 survived	Baldassare et al., 2002; Keefe et al., 2002b
<i>In vivo</i> matured	Various	13	5 died at or soon after birth	Behboodi et al., 2002
<i>In vitro</i> matured	Various	9	1 died after Caesarean section	Behboodi et al., 2002
Ovulating donors + <i>in vivo</i> matured	Female fetal cells (transfected)	3	5 pregnancies (beyond day 50) yielded 3 live births; all 3 kids healthy and transgenic	Chen et al., 2002
Aspirated donors + <i>in vivo</i> matured	Female fetal cells (transfected)	1	3 pregnancies (beyond day 50) yielded 1 live birth; kid healthy and transgenic	Chen et al., 2002
Stimulated donors + <i>in vitro</i> maturation	Adult granulosa cells (frozen-thawed)	7	All 4 pregnancies (day 30) went to term; 1 kid (a twin) died at birth and transgenic	Keefe et al., 2002a
Stimulated donors + <i>in vitro</i> maturation	Fetal fibroblasts (frozen-thawed)	2	Single pregnancy (day 30) went to term; 1 twin died at birth	Keefe et al., 2002a
Stimulated ovaries + <i>in vitro</i> maturation	Fetal cells (transgenic donor)	3	3 pregnancies (day 30) yielded 3 live births; all 3 kids transgenic, alive at 1 year and mated	Reggio et al., 2001
Abattoir ovaries + <i>in vitro</i> maturation	Fetal cells (transgenic donor)	2	2 pregnancies (day 30) yielded 2 live births; both kids transgenic, alive at 1 year and mated	Reggio et al., 2001
Stimulated donors + <i>in vitro</i> maturation	Female fetal cells (+ transgene)	4	Of 6 single pregnancies (day 30), one failed (day 58); 5 young reached term, one died after Caesarean section; all kids transgenic	Reggio et al., 2002
Abattoir ovaries + <i>in vitro</i> maturation	Female fetal cells (+ transgene)	4	Of 4 pregnancies at day 30, one failed at day 127; the other 3 yielded 4 transgenic kids	Reggio et al., 2002

NT: nuclear transfer.

X- and Y-chromosome-bearing spermatozoa on the basis of differences in their DNA content (Johnson, 2000). To date, there is little or no evidence of any attributable adverse effects among offspring born after IVF or AI of cattle or sheep with 'sorted' semen (Cran and Johnson, 1996; Cran *et al.*, 1997; Seidel *et al.*, 1999, 2002). However, semen sorting does reduce sperm viability (Caballero *et al.*, 2002) and, in a recent study, Garner and Suh (2002) showed that mechanical stress, UV laser illumination and exposure to a fluorescent dye with high affinity to DNA all contributed to the demise, after cryopreservation and thawing, of up to two-thirds of sorted spermatozoa. Most damage was attributed to the mechanical stresses imposed, and sire-to-sire variations were also noted.

Nuclear transfer technology

Nuclear transfer in the context of agriculture, where cattle are the primary candidates, is the subject of a separate review in this volume (Wells, this supplement), so emphasis here is on consequences arising either in smaller ruminants (sheep and goat) or from non-agricultural applications, such as the generation of inter-species (Bovidae or Ovidae) hybrids.

Production of nuclear transfer (NT)-derived ruminants is an inefficient process, and most fused 'donor cell–oocyte' couplets fail to generate viable offspring after transfer. Prenatal fetal losses are considerable in sheep (Wells *et al.*, 1997; Wilmut *et al.*, 1997) but, although not always avoided (Reggio *et al.*, 2002), have been less prevalent in goats. In a number of goat NT studies in which pregnancies had been confirmed by detection of fetal heartbeats at day 30 or day 40 of gestation (Table 2), subsequent losses did not occur prenatally (Baguisi *et al.*, 1999; Keefer *et al.*, 2001, 2002a; Reggio *et al.*, 2001; Zou *et al.*, 2001). As a result, Keefer *et al.* (2001) speculated that non-occurrence of prenatal fetal loss was due to avoidance of prolonged *in vitro* culture of NT embryos or, alternatively, to a greater ability of caprine cytoplasts to reprogramme donor nuclei, thereby safeguarding subsequent development *in utero*. Whether goats differ innately from other species in terms of oocyte resilience after the traumas associated with nuclear transfer must remain in doubt until more conclusive evidence becomes available. Moreover, such a scenario is hardly supported by the fact that goats (Table 2) do not appear to differ from sheep or cattle with respect to susceptibility to peripartum or post-natal losses associated with NT-derived offspring.

There is compelling evidence to indicate that, after nuclear transfer, fetal losses are due to significant developmental retardation and placental inadequacies (De Sousa *et al.*, 2001). Deficiencies in chorioallantoic vascularization, although probably correctly viewed as the ultimate cause of pre-term losses, are themselves the result of earlier aberrations in the developmental blueprint of NT-derived embryos. Indeed, the fact that losses can occur at all stages and in various guises, ranging from gross degeneration of preimplantation embryos to sudden post-natal death of apparently normal offspring, confirms that NT procedures are frequently responsible for fundamental and far-reaching disruption of developmental norms. Intuitively, it could hardly be otherwise, given that the reconstructed egg comprises a severely traumatized host cytoplast fused to a donor cell (or nucleus) with which, to a greater or lesser extent depending on its origin, it is virtually incompatible from the outset. Therefore, the more remarkable phenomenon is that, against all the odds, NT sometimes results in the generation of viable offspring. Whether those current odds can be shortened in favour of enhanced efficiencies is a question occupying many minds and underlying recent and ongoing investigations at genetic, epigenetic and cellular developmental levels.

The relevant genetic and epigenetic investigations are primarily concerned with identifying and manipulating mechanisms and circumstances that regulate and programme gene expression and cellular differentiation, not only near the time of NT but also during later stages of

the development of the new individual. Campbell (1999) outlined the effect of the stage of the cell cycle, in both the recipient oocyte and donor nucleus, on maintenance of correct ploidy in the reconstructed egg and on essential reprogramming–remodelling of the transferred nucleus. Although neither is fully understood nor reliably controllable thus far, a key to success is the establishment of a proper ‘dialogue’ between the donor nucleus and recipient cytoplasm (Fulka *et al.*, 2001) to orchestrate normal development in the reconstructed embryo. When circumstances do not permit the necessary choreography, the consequences are manifest as a spectrum of anomalies, ranging from immediate or later deaths to non-lethal developmental aberrations analogous to those of the large offspring syndrome in ruminants (Young *et al.*, 1998; Sinclair *et al.*, 2000; McEvoy *et al.*, 2001; Bertolini and Anderson, 2002). Research investigations, prompted initially by the large offspring syndrome but of relevance to NT outcomes in ruminant species, have considered the effects of epigenetic modifications in the early embryo on later development (Young and Fairburn, 2000). As a result, it has been shown that *in vitro* culture of intact sheep embryos can influence methylation of imprinted genes (Young *et al.*, 2001). Even more recently, Fairburn *et al.* (2002) reported that, for both sheep and cattle, there were increased methylation levels (relative to ‘normal’ embryos) in significant numbers of NT-derived blastocysts. As noted by De Sousa *et al.* (2001), fetal and placental defects later in development may be due to aberrant imprinting of one or more genes in NT-derived sheep embryos. The same authors observed that, although pathologies associated with NT-derived sheep and cattle affected diverse fetal tissues (including heart and liver) as well as placentae, it is possible that these have a common origin, namely embryonic mesoderm. However, as yet, the prospect of a panacea for the developmental defects now associated with nuclear transfer in ruminants seems remote.

Nuclear transfer and mitochondrial DNA inheritance

One reason for suspecting that there may not be a single ‘cure’ for the defects associated with NT-derived embryos is that, despite the ‘clone’ pseudonym, no two cytoplasm–nucleus (or cytoplasm–cell) couplets are identical. For example, recipient cell cytoplasm will differ, as will the extent of heteroplasmy, when donor cells (rather than just nuclei) are fused to the cytoplasm. Therein lies another possible consequence of nuclear transfer, given that the reconstructed egg contains mitochondrial DNA (mtDNA) from two sources. This procedure differs from the normal pattern of mtDNA inheritance which is uniparental and homoplasmic, due both to the demise of paternally derived mitochondria after fertilization and, on the basis of genetic bottleneck theory, the transmission of only a few molecules of maternal mitochondria which subsequently ‘colonize’ the next generation (Smith *et al.*, 2000; Cummins, 2001). Mitochondria undertake crucial functions within the embryo, notably oxidative phosphorylation which relies on a competent electron-transport chain. As emphasized by St John (2002), proper function of the electron-transport chain requires complementary expression of genes encoded by the nuclear genome and mitochondrial genome. Evidently, where such symbiosis is threatened, respiratory deficiency and disease could ensue. Whether mtDNA heteroplasmy, as a result of mixing and persistence of mitochondria from host oocyte and donor cell cytoplasm, constitutes a threat in NT-derived ruminants remains uncertain. Although there is evidence of heteroplasmy among calves generated via nuclear transfer (Hiendleder *et al.*, 1999), tests of some tissues from NT-derived sheep ($n = 10$, including Dolly) revealed no evidence that mitochondria from the somatic donor cells were present (Evans *et al.*, 1999).

Mitochondrial inheritance scenarios are most intriguing when considering NT as a tool to generate interspecific hybrid animals or to conserve endangered species via transfer of

somatic cells to enucleated oocytes of 'near' relatives. Evidently neither could be pursued if heteroplasmy were both inevitable and lethal.

Interspecies nuclear transfer

Smith *et al.* (2000) reported that, in cattle, when Nellore (*Bos indicus*) cells of embryonic origin were introduced into enucleated Holstein (*Bos taurus*) oocytes, *Bos indicus* mtDNA was detectable after fusion (approximately 4%) and at day 50 of gestation (approximately 2%; in one fetus), but was not detectable in the NT-derived live offspring at birth. In a separate bovid interspecies NT programme, Lanza *et al.* (2000) found that transfer of somatic cells from a gaur bull (wild ox; *Bos gaurus*; $2n=58$) into enucleated domestic cattle (*Bos taurus*; $2n=60$) oocytes generated reconstructed eggs, of which 12% became blastocysts, and almost one in five of these generated fetuses after transfer to surrogate recipients. Three fetuses were electively recovered (day 46, $n=1$; day 54, twins) and subsequent analysis of 11 tissue types revealed that mtDNA was exclusively *Bos taurus*, whereas cytogenetic analysis indicated that all three fetuses had *Bos gaurus* nuclear origins. Consequently, the fetuses were genetic chimaeras rather than true *Bos gaurus* individuals.

The most promising success to date was that reported by Loi *et al.* (2001), who generated inter-species embryos by injecting enucleated oocytes of domestic sheep (*Ovis aries*) with granulosa cells collected post mortem from adult female mouflons (*Ovis orientalis musimon*). The ensuing pregnancies ($n=2$) resulted in the live birth of an apparently normal (but 'hybrid') mouflon (mtDNA = *O. aries*; nuclear origin = *O. o. musimon*). Other interspecies ruminant NT studies generated blastocysts after transfer of buffalo (*Bubalus bubalis*) somatic cells to enucleated bovine (*Bos indicus*) oocytes (Saikhun *et al.*, 2002), and a single pregnancy (lost by day 60 of gestation) from a reconstructed embryo generated by transfer of an endangered argali wild sheep (*Ovis ammon*; $2n=56$) somatic cell to an enucleated domestic sheep (*Ovis aries*; $2n=54$) oocyte (White *et al.*, 1999).

Evidently, interspecies nuclear transfer technology could yet provide some valuable opportunities for the rescue, and perhaps the eventual revival, of endangered ruminant species. However, any such NT project proposals should first be evaluated rigorously (Fig. 3), using realistic predictions of probable outcomes. When alternative conservation strategies could suffice, especially if these are simpler or more promising, scientists should resist the greater 'glamour' associated with costly and sometimes contentious cloning programmes. This is because, in terms of conservation, successes will be very short-lived unless NT-derived interspecies offspring can survive to breeding age and beyond (Lee, 2001; Vogel, 2001). The extent to which genotype of available surrogate dams could influence survival *in utero* is an additional consideration (see Lanza *et al.*, 2000; Hammer *et al.*, 2001). Genetic proximity or otherwise of ancestral links, for example among sheep (Hiendleder *et al.*, 2002), may be a key determinant of success or failure.

Transgenic animal production

Almost two decades since Hammer *et al.* (1985) reported the production of transgenic lambs (as well as pigs and rabbits) after gene transfer via pronuclear microinjection, this direct route of transgene delivery, despite its very low efficiency, continues to be used to generate ruminant offspring, and exogenous DNA is randomly integrated into the genome of the individual. Although not necessary in all species, the injection procedure is preceded by routine centrifugation (13 000–15 000 *g*) of the recently fertilized egg if, as in cattle, endogenous lipids would otherwise obscure the pronuclei (Pursel *et al.*, 1989; McEvoy *et al.*, 1992).

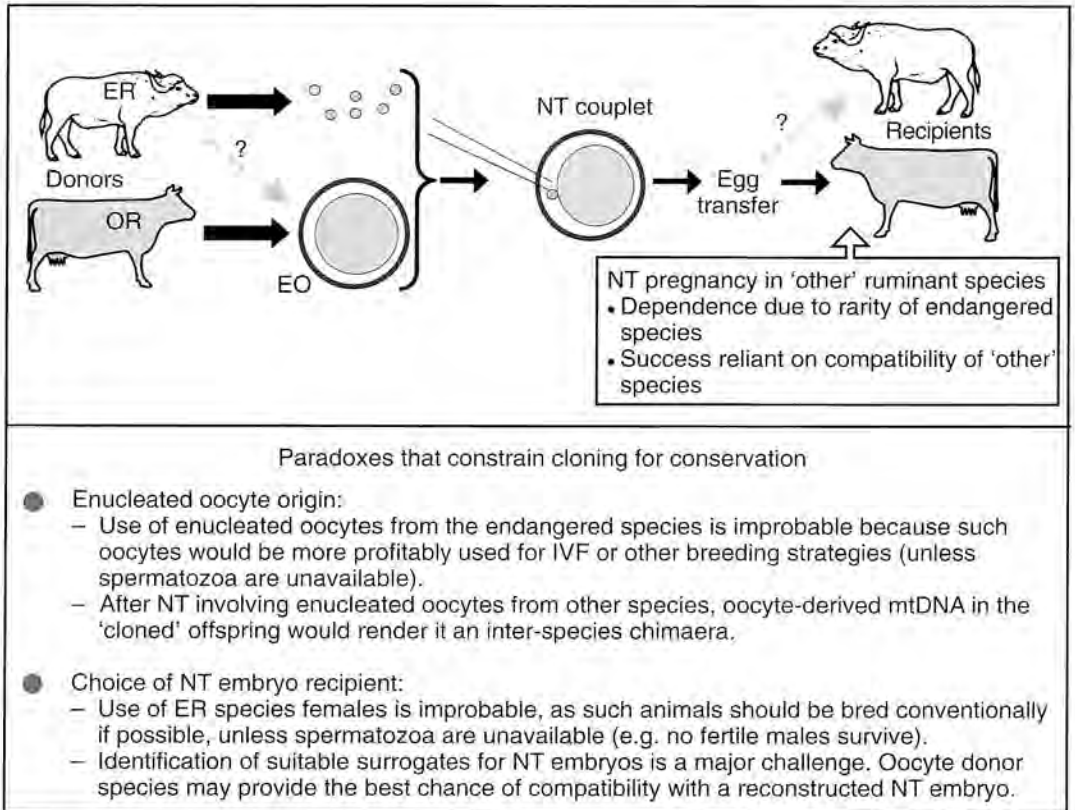


Fig. 3. Consideration of viability of use of nuclear transfer (NT) for conserving endangered ruminants. ER: endangered ruminant; OR: other ruminant; EO: enucleated oocyte; mtDNA: mitochondrial DNA.

Among the consequences of transgenic animal production via pronuclear microinjection, in addition to intended genetic modifications, there have been many unexpected and adverse side-effects, including arthritis, infertility, peptic ulcers and severely reduced body-fat reserves (Pursel *et al.*, 1989). It became apparent that many of these disabilities were due to indiscriminate or inappropriate transgene expression (in terms of developmental stage or tissue or organ site) or knock-on effects. Much subsequent research effort was devoted therefore to refining gene constructs to generate healthy offspring more effectively which would express the transgene not only benignly, but also, if possible, in the desired site- and stage-specific manner. For example, in programmes aimed at modifying milk composition in ruminants, β -lactoglobulin and various casein gene promoter sequences help to ensure specific targeting of expression of associated structural DNA sequences to the mammary gland (Murray and Maga, 1999).

Despite improvements in gene constructs, from the point of view of avoiding detrimental outcomes or sometimes improving transgene regulation in individuals that were genetically transformed, the efficiency of generating transgenic ruminants after micro-injection of recently fertilized eggs has remained very low. Recent reviews confirm that, for cattle, goats and sheep, less than 1% of microinjected eggs transferred to recipients resulted in the births of transgenic calves, kids and lambs (Pinkert and Murray, 1999; Niemann and Kues, 2000; Baldassare *et al.*, 2002). Therefore, it is no surprise that, in the 1980s and 1990s, considerable thought and effort was invested in devising alternative procedures that might hold greater promise for

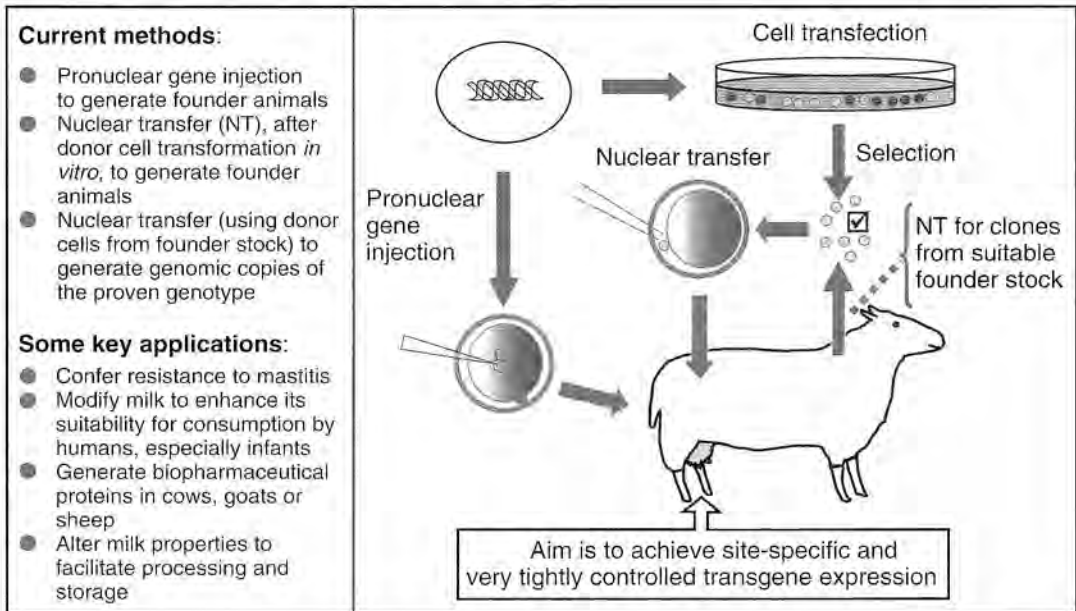


Fig. 4. Gene transfer technology – modification of milk composition or mammary disease resistance in ruminants.

generation and propagation of transgenic animals. The surprise, echoed in doubts expressed elsewhere, was the emergence of two contrasting strategies for transgenic animal production. The first, aimed at *de novo* generation of transgenic individuals, relies on sperm-mediated gene transfer (SMGT) to mammalian oocytes. In theory, the intent here is to use spermatozoa as exogenous DNA vectors during the natural process of egg fertilization; in practice, fertilization in such instances is sometimes achieved via ICSI (Perry *et al.*, 1999). The second strategy, intended for propagation and *de novo* generation of transgenic animals, apparently defies natural processes, involving as it does the transfer of transformed somatic cells or nuclei (including those from adult donors) to enucleated oocytes (Fig. 4).

Sperm-mediated gene transfer

Sperm-mediated gene transfer was first reported more than 30 years ago in rabbits (Brackett *et al.*, 1971), but significant controversy did not arise until Lavitrano *et al.* (1989) indicated production of genetically transformed mice by this route. The incredulity and disbelief that this could happen was reflected in a letter from some of the principal doubters, published later in that year, after failed attempts to repeat this work in a number of other laboratories (Brinster *et al.*, 1989). To their credit, the Italian workers persisted (Wall, 2002) and others also took up the challenge. For many, the report by Perry *et al.* (1999), who used the procedure in tandem with ICSI, provided confirmation that SMGT can be achieved in mammals. Reports relating to SMGT in ruminants are scarce to date but notable. Shemesh *et al.* (2000), using restriction enzyme-mediated integration (REMI), demonstrated that a transgene coding for green fluorescence protein (GFP) was integrated into genomic DNA of bovine spermatozoa which, when used for artificial insemination, resulted in production of transgenic calves. Moreover, after IVF with REMI-transfected spermatozoa, approximately 30% of resultant morulae expressed

the GFP transgene. In a separate study, Rieth *et al.* (2000) demonstrated the production of transgenic bovine embryos after IVF with spermatozoa previously electroporated to enhance DNA uptake. One limitation was that the percentage of eggs developing beyond the 16-cell stage was reduced after IVF with electroporated DNA-treated spermatozoa (27% and 34% for different constructs; 43% for control spermatozoa).

Transgenic ruminants via nuclear transfer

Despite the various limitations of nuclear transfer as applied to ruminant animals (McEvoy *et al.*, 2001), a major justification for pursuing it is that, especially in the context of therapeutic protein production or better understanding of disease, it offers the means whereby precise genetic changes can be achieved in livestock (Wilmut *et al.*, 1999). The key example to date is the report by McCreath *et al.* (2000) which refers to the production of 14 lambs born alive in a targeted gene transfer study. Unfortunately, half of these lambs died within a week and only three survived for more than 3 months. Investigations revealed a high incidence of kidney defects and brain and liver pathologies, but McCreath *et al.* (2000) concluded that some feature of cell treatment or nuclear transfer was more likely to be at fault than gene targeting *per se*. This conclusion was based on the fact that the observed defects mirrored those recorded after nuclear transfer involving the same cells without gene targeting (Schnieke *et al.*, 1997).

Hill *et al.* (1999) studied pathologies in NT-derived transgenic bovine fetuses and calves ($n = 13$) and also concluded that the NT procedure or embryo culture *in vitro* or both procedures contributed to the observed developmental abnormalities. Cibelli *et al.* (1998a) reported the production of three healthy transgenic calves after nuclear transfer of fetal fibroblasts expressing a marker transgene, but significant losses during the first trimester were also a feature. In an alternative procedure, the same authors also generated nine chimaeric transgenic calves after transfer of genetically modified embryonic stem-like cells into day 3 embryos (Cibelli *et al.*, 1998b). These transformed ES-like cells had been generated either conventionally or via NT of transfected fetal fibroblasts into enucleated oocytes. The latter approach gave rise to seven calves after transfer of only ten blastocysts to surrogate recipients. Of these, six expressed the transgene in one or more tissues and all were healthy.

Two studies have investigated the production of transgenic goats after nuclear transfer of genetically transformed cells. In one study by Baguisi *et al.* (1999), three healthy kids were found to be carrying a transgene coding for expression of recombinant human antithrombin III (rhAT) targeted to the mammary gland. Milk from one of the kids was tested and found to contain the gene product. Keefer *et al.* (2001) reported the birth of a female goat after NT of a cell from a transgenic (eGFP) cell line; PCR analysis confirmed the presence of the transgene. In both of these studies of goats, developmental aberrations were less evident than in either cattle or sheep, perhaps reflecting, as discussed earlier, benefits from shorter-term culture *in vitro* or species differences in terms of susceptibility to the various manipulations.

Conclusions

Ruminant animals have been the focus for a range of significant scientific and technical advances via gamete and embryo manipulation in the past decade. Some, particularly semen sexing, promise to influence animal breeding and commercial agriculture significantly. Other advances, such as bio-pharmaceutical protein production by transgenic animals, will be principally or even solely applicable to specialized industries. When widespread application in conventional agriculture is the goal, technical inefficiencies may perhaps be



Fig. 5. Ruminants and assisted reproductive technologies (ART) – key is to produce healthy offspring. Viable offspring from a transgenic nuclear transfer goat (photograph courtesy of R. Godke, Louisiana State University).

accommodated, but tolerance of adverse developmental consequences is very low. However, in other circumstances it may be considered justifiable to use technologies such as nuclear transfer, despite the fact that these are inherently predisposed to triggering a high incidence of developmental defects among resultant offspring. For example, when the generation of even a few healthy transgenic sheep or goats (Fig. 5) could hold the promise of affordable cures for otherwise intractable human diseases, scientists and the public they serve may consider the risks and failures en route to be worthwhile. Nevertheless, there is at all times a duty of damage limitation and a need to avoid unnecessary hazards. As stated by McEvoy *et al.* (2001), culture conditions and constituents associated with physical manipulations need to be made safer, especially in the case of manipulations that, either necessarily or by default, injure or expose spermatozoa, oocytes or embryos. Otherwise, hostile environments will contribute to abnormal development. Simply stated, only when *in vitro* conditions are optimized will adverse consequences of gamete and embryo manipulations be minimized. For procedures that are traumatic, some adverse outcomes will remain inevitable. Nevertheless, further significant achievements, benefits and successes will accrue from the ongoing efforts and expertise invested in reproductive technologies for ruminant species.

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