Proc. Assoc. Advmt. Anim. Breed. Genet. 18:287-295

CURRENT STATUS AND FUTURE PROSPECTS FOR REPRODUCTIVE TECHNOLOGIES IN SMALL RUMINANTS

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INTRODUCTION

Reproductive technologies such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) have been technically feasible and commercially utilized in small ruminants for many years (reviewed by Betteridge 1981; Evans 1991; Salamon and Maxwell 2000). Sheep and goats are major contributors to the domestic economy of many nations, but neither AI nor MOET has been widely adopted into normal breeding programs as, for example, has been the case for AI in dairy cattle. This paper outlines the costs and benefits of applying reproductive technologies in small ruminants, assesses the current technology and explores potential practical developments. Recent technological developments regarding *in vitro* embryo production, particularly in juveniles, and the incorporation of sexed sperm into artificial breeding programs are described. Reference is made to other livestock species, particularly cattle, where contrast is appropriate, and brief conclusions are drawn on the most likely technologies to be used in the commercial sector in the near future.

THE COSTS AND BENEFITS OF USING REPRODUCTIVE TECHNOLOGIES

The slow commercial adoption of AI and MOET is not because the technologies do not have the potential to facilitate genetic improvement programs; indeed the advantages of AI (Abbott 1994; Windsor and van Bueren 1994; Nicholas 1996) and MOET (Smith 1986; Wray and Goddard, 1994a,b; Nicholas 1996) are well documented. The reasons are the relatively high cost of these technologies compared with the realisable short-term benefits (Windsor and van Bueren 1994). Moreover, in most countries where sheep and goats are maintained in large numbers, the relative value of individual animals is low compared with the cost of the technology. In Australia in the early 1990s, the average cost of breeding sheep to AI with fresh semen (cervical AI) was calculated as 3 times that of natural breeding, and with frozen semen and (laparoscopic) AI about 12 times (McClintock and Nicholas 1991; Abbott 1994); for breeding through MOET the cost was raised over 60 times. However, it may be possible to reduce these costs using new developments from MOET, such as juvenile *in vitro* embryo transfer (JIVET) and mature *in vitro* embryo transfer (MIVET), as these techniques can be applied to very young animals, can yield more embryos per donor than MOET, are repeatable more than once on each donor and, because they rely on *in vitro* fertilisation, can utilise expensive sperm very efficiently. Few commercial wool or prime lamb producers see value in using such technology but ram breeders and studs, which sell a more valuable product (breeding animals), are able to gain from use of expensive artificial breeding. This is particularly the case for advanced MOET technologies which are likely only to be applied at the elite level. Nevertheless, the MOET and AI technologies have been widely applied for the importation and dissemination of genetic material from new breeds, particularly in recent times for improvement of meat production from sheep and goats in Australia.

For sheep AI, fresh semen is simple and effective but there are obvious restrictions on its use over time and distance. In Australia, there has been a small but continuing interest in fresh semen as a cheap alternative to laparoscopic AI, since “in house” labour is used and discounted. More widespread use of valuable sires requires storage of semen. Liquid storage may extend the useful lifetime of semen, but ultimately frozen semen is required to fully

overcome restrictions of time and distance. The cost of using frozen semen successfully has, however, restricted its widespread use, and animal welfare concerns in some countries over use of laparoscopy have compounded the problem. Nevertheless, in situations where animals are of relatively high value, AI is used as the major breeding method. Such a situation occurs in Australia's ram breeding flocks and studs, and the genetic benefits are passed on to subsidiary flocks through sale of rams (Maxwell and Wilson 1990; Abbott 1994).

Similarly, the cost of using MOET in a structured breeding program has restricted its use to research purposes, for the introduction and dissemination of new breeds**,** and to a limited number of high value animals in elite flocks. As in the cattle industry, there is a limited number of examples where MOET has been used in sheep and goats as part of a breeding program leading to increased rates of genetic gain, as opposed to use for entrepreneurial reasons. To our knowledge, only one or two Merino stud flocks have incorporated MOET into a long-term breeding strategy, apparently with some success, but in recent times MIVET and JIVET have been applied on a number of terminal sire breed studs and at least two Merino studs; a number of artificial breeding companies now offer these technologies as part of their services and they are commonly mentioned as available techniques for incorporation in genetic improvement programs (for example, Meat and Livestock Australia 2004).

DEVELOPMENTS IN MOET, JIVET, MIVET AND EMBRYO VITRIFICATION

While the use of MOET in sheep and goats has increased in recent times, there is no evidence of better rates of success from commercial MOET since it was first developed in the 1960s. The only improvements adopted were laparoscopic AI (Killeen and Caffery 1982), which simplified the necessary intrauterine deposition of semen (Trounson and Moore 1974) and allowed the use of frozen semen (Salamon and Maxwell 1995), and pituitary FSH extracts for superovulation (Armstrong and Evans 1984), which overcame some of the problems of using PMSG alone as a superovulatory hormone (Evans and Robinson 1980). A cocktail of PMSG and FSH (Ryan *et al.* 1991), often combined with synthetic gonadotrophin releasing hormone, is commonly used as the most cost-effective regimen in Australia. Even so, choice of superovulatory regime, including various ovine and porcine FSH preparations, treatment schedules and doses, remains one of the more controversial issues in MOET technology. In most commercial situations, the choice of which hormone regime to use in small ruminants remains a matter of personal preference or trial-and-error.

While many factors contribute to the success of MOET, average success rates in properly controlled commercial situations remain disappointingly low, with between 3 and 4 lambs produced from fresh embryos per donor cycle (Maxwell *et al.* 1990; Evans 1991). This in itself does not preclude sufficient numbers of lambs being produced per donor per season, since each donor may undergo several treatment cycles. In this case, surgically-induced adhesions, particularly of the ovary and oviduct, could restrict the number of repeat operations but can be minimised using an embryo recovery technique which does not require handling or exposure of the ovaries and oviducts. Nevertheless, the number of times a donor can be used is limited. Attempts to overcome this problem using laparoscopic embryo recovery techniques (McKelvey *et al.* 1986) have not been widely adopted due to low success rates and the time taken to perform the operation with any degree of success.

A potentially simpler and more productive method of repeated recovery of gametes is by laparoscope-guided oocyte aspiration or ovum pick-up (OPU; reviewed by Tervit 1996; Baldassare *et al.* 2004). The combination of OPU with *in vitro* oocyte maturation (IVM) and fertilisation (IVF), and the *in vitro* culture of the resulting zygotes to transferable embryos, is termed *in vitro* embryo production (IVP) or more recently MIVET. This has been successfully applied to the large-scale production of offspring from transgenic goats (Baldassare and

Karatzas 2004) and is starting to be incorporated into commercial embryo transfer technology for cattle and sheep. MIVET embryos produced with standard IVM/IVF techniques result in normal viable lambs (Crozet *et al.* 1987) and kids (Keskintepe *et al.* 1994), and early problems with unusually large offspring have been largely overcome by use of appropriate culture media *in vitro* (Thompson *et al.* 1995). The efficiency of MIVET has considerably increased in recent years, with OPU repeatable in ewes 3 times or more, yielding 10-15 oocytes per donor per aspiration, with fertilization and blastocyst development rates of 70-80 and 60-80%, respectively (Morton *et al.* 2005). We have also produced lambs from the injection of single sperm into oocytes (intracytoplasmic sperm injection, or ICSI; Catt *et al.* 1996; Gomez *et al.* 1998), though more oocytes/presumptive embryos were lost *in vitro* compared with standard IVF, indicating some subsequent developmental problems associated with this procedure (Gomez *et al.* 1998).

Probably the most promising and exciting of the *in vitro* techniques for adoption into commercial practice is that of MIVET used in conjunction with juvenile donors (JIVET), since a reduction in the generation interval potentially increases the rate of genetic gain in comparison with the use of adult donors (Smith 1986). Early observations that lambs of 4 to 6 weeks of age underwent an unusual natural increase in folliculogenesis (Kennedy *et al.* 1974), which could be further stimulated by exogenous gonadotrophins (Worthington and Kennedy 1979), went unappreciated in a practical sense for many years until the phenomenon was used as a basis for JIVET. This involves superovulation of young calves, lambs, or kids, recovery of oocytes, fertilisation and culture *in vitro,* and transfer to adult surrogates (Armstrong *et al.* 1994; Earl *et al.* 1995). While the viability of prepubertal oocytes is relatively low compared with those obtained from adult animals (O'Brien *et al.* 1996, 1997b), the large number produced can ultimately result in a higher number of offspring than from superovulated adult donors (Armstrong *et al.* 1994). The viability of prepubertal oocytes can also be improved by pre-treatment of the lambs with ovarian steroids (O'Brien *et al.* 1997a). Recent improvements in juvenile donor treatments (Morton 2008) and *in vitro* oocyte maturation (Kelly *et al.* 2008) have brought JIVET close to commercial viability, with limited adoption for both sheep and cattle breeding in Australia. The combination of MIVET or JIVET with sexed sperm provides a powerful technology if single sex offspring are desired (Morton *et al.* 2004a).

One cloud on the JIVET horizon is the finding of alterations in the expression of several developmentally important genes in embryos derived from prepubertal cattle (Oropeza *et al.* 2004) and sheep (Leoni *et al.* 2006). Similarly, we have reported alterations in gene expression in bovine embryos derived from oocytes fertilised *in vitro* with sexed sperm (Morton *et al.* 2007). The implications of these aberrations are yet to be determined but they may contribute to epigenetic effects already reported in the literature (for example, review by Walker *et al.* 2000). On the positive side, two new techniques have great potential for combination with JIVET to enhance the rate of genetic gain. Further reductions in the generation interval could be achieved by incorporating foetal oocytes into IVP systems. However, to date foetal calf oocytes have had lower rates of maturation, fertilization and embryo development than those from adults (Chohan and Hunter 2004). Utilization of gametes from prepubertal males could further dramatically reduce generation interval on the male side. We have been able to initiate spermatogenesis in prepubertal ram lambs by administering PMSG (Morton *et al.* 2004b) and the injection of gametes from prepubertal ram lambs into IVM oocytes from adult and prepubertal lambs has yielded 16–32 cell stage embryos (Morton 2008) but not live offspring.

The most efficient use of MOET often involves cryopreservation of embryos for transport or long-term embryo banking for conservation. We have demonstrated that sheep embryos conventionally frozen for 13 years retained viability (Fogarty *et al.* 1999) indicating that frozen embryos are a safe method of long-term conservation of rare or valuable genetic strains

or breeds. Frozen storage is not routinely used in commercial practice other than for long-distance transport simply because conventional freezing, though successful, is time consuming and expensive. Vitrification of embryos (Rall 1987) is now the method of choice for cryopreservation, as it offers the advantages of simplicity and reduced equipment costs. It is successful for both *in vivo*-produced (Ali and Shelton 1993; Szell and Windsor 1994) and *in vitro*-produced sheep embryos (Evans *et al.* 1999), and for *in vitro* and *in vivo*-produced goat embryos (Traldi *et al.* 1998), though overall success rates in these early reports did not match those of conventional freezing. There has been much improvement in vitrification success over the past 5 years, resulting in commercially applicable methods for cryopreserving embryos in most species (reviewed by Vajta and Kuwayama 2006), including *in vitro* produced porcine oocytes (Liu *et al.* 2008) and embryos (notoriously difficult to freeze because of their high lipid content), even after cloning (Du *et al.* 2008). Vitrification of embryos, whether from MIVET or JIVET, should soon become a simple and routine component of MOET programs.

DEVELOPMENTS IN AI

When performed by experienced operators, AI with fresh ram semen deposited in either the vagina or cervix can usually result in acceptable levels of fertility comparable with that of natural mating (Maxwell and Butler 1984). The methods are simple, can be done "on farm" (Evans and Maxwell 1987) and can make more extensive use of a limited number of superior males than natural mating. However, for fuller appreciation of the benefits of AI, semen is ideally collected and distributed from highly selected males at an AI centre, preferably after careful selection through a sire referencing scheme linked to progeny tests (Windsor and van Bueren 1994; Nicholas 1996). In Australia and other parts of the world, semen is distributed from sheep and goat studs to breeder flocks in other parts of the country, and this requires that semen is stored for transportation, almost exclusively in frozen form, though liquid (chilled) storage is an option that has some advantage in the very short term. Unfortunately for the small ruminant industries, frozen, and to some extent chilled, sperm are not able to penetrate the cervix in sufficient numbers to bring about acceptable rates of fertility in most breeds (Maxwell and Hewitt 1986) and therefore stored semen requires an associated insemination technique which by-passes the cervix. Transcervical insemination is successful in about 60-70% of goats (Leboeuf *et al.* 2000) allowing the commercial utilization of frozen semen (Leboeuf *et al.* 2008). The advent of the laparoscopic AI technique for sheep in 1982 led to an increased use of AI of frozen ram semen in Australia (Evans 1991; Maxwell and Watson 1996) and other parts of the world. Though the proportion of the Australian national flock inseminated in this way is relatively small (1-2 %) it comprises a large proportion (40-50 %) of the ram-breeding ewe population. The small ruminant industries are crying out for improved success rates with cervical AI with frozen or liquid stored semen so that rates of genetic gain can be improved. This can only be achieved through dramatic improvement in semen processing methods or the development of a simple, practical method of non-surgical AI.

LIQUID STORAGE OF SEMEN

Freezing causes cryoinjury, but chilling (to 5°C) has the advantage of reducing the number of sperm deaths. Liquid (chilled) semen can retain acceptable fertility for 24 hours when inseminated via the cervix but thereafter fertility is low unless intrauterine AI is used (Maxwell and Salamon 1993). Since degradation of sperm may involve lipid peroxidation, we have attempted to prolong the life of liquid-stored sperm by addition of antioxidants, specifically superoxide dismutase (SOD) and catalase. In both sheep (Maxwell and Stojanov

1996) and goats (Pomares *et al.* 1995), antioxidants have prolonged the fertilising life of chilled semen to 14 days after intrauterine AI. It seems that chilled semen must be used quickly for cervical AI but laparoscopic AI may allow its utilisation after storage for up to 2 weeks. Liquid storage has been an efficient method for utilising ram semen storage in the past, and remains the method of choice in the New Zealand dairy cattle industry, mainly because of optimum longevity and viability of sperm during storage and after insemination. However, the need for sires to be used nationally or internationally over large numbers of females has meant that, for both sheep and cattle, frozen storage has been the method of choice for utilisation of semen in genetic improvement programs.

FROZEN STORAGE OF SEMEN

It has long been accepted that cervical AI of frozen-thawed semen in sheep cannot consistently produce acceptable fertility, no matter how many motile sperm are inseminated. Despite considerable expense and effort over the past 50 years or more, little progress has been made in developing methods of freezing semen which could result in acceptable fertility after cervical AI (Salamon and Maxwell 1995a). Intrauterine insemination via laparoscopy does, however, result in acceptable or "normal" fertility (Salamon and Maxwell 1995b). Since the advent of this AI method, there has been little progress, and indeed less incentive, to develop better methods of processing semen for cryopreservation. However, recent findings in our laboratory provide hope that frozen-thawed semen may one day be used successfully with cervical AI. The observation that seminal plasma proteins (SPP) could protect sperm through the trauma of flow cytometry (Catt *et al.* 1997) ultimately led us to add them back to diluted frozen-thawed semen, where we found that it arrested the progression of sperm through capacitation-like changes (McPhie *et al.* 1999) and improved motility *in vitro* (Gellatly *et al.* 1999). Ultimately we found that SPP, when added to frozen-thawed ram semen, could produce acceptable levels of fertility in ewes inseminated in the cervix (Maxwell *et al.* 1999), although the results are not consistently high (El-Hajj Ghaoui *et al.* 2007). The active component is a series of proteins that actively repair cryo-damaged sperm membranes (reviewed by Muiño-Blanco *et al.* 2008). Concentrations of these proteins vary with season, breed and among males (reviewed by Maxwell *et al.* 2007).

SEXED SPERM

Sperm sexing by high speed flow cytometry has been one of the most significant new technologies for artificial breeding of livestock developed in the twentieth century. It has been widely applied commercially in dairy cattle, with hundreds of thousands of healthy offspring born to date (Seidel 2009). It should be noted that the rapid adoption of this technology has not necessarily been for the purposes of genetic improvement. AI with sexed sperm has both management and marketing benefits, particularly in the dairy cattle industry, where it is an advantage to produce female replacement heifers from the best cows and male beef progeny from the rest of the herd.

Along with the new sexing technology has come a need to adapt and develop new semen storage and processing methods, so that the sexed sperm can be utilised for AI or for *in vivo* and *in vitro* embryo production. Limitations associated with the sex sorting apparatus (high cost, lack of portability and slow processing rates) have required that sorted sperm are also frozen before use. This has posed particular research challenges, because the sorting process itself subjects sperm to additional stressors, including very high dilution rates, extended time *in vitro*, staining of DNA, centrifugation, and exposure to ultraviolet light, high system pressures and electric charges. The sperm therefore need special protection to survive not only sex sorting but also the freeze-thaw procedures that follow. Despite the apparently harsh treatment of the gametes, the health and

normal reproductive capacity of the large number of offspring born to date attest to the safety of the sex-sorting procedure. Nevertheless, some caution and further research on possible effects of sperm treatments on their DNA are warranted, as increased rates of early and late pregnancy loss have been reported in cattle and pigs, but not sheep, after insemination with sex-sorted frozen-thawed spermatozoa (de Graaf *et al.* 2009) and altered gene expression has been detected in IVP embryos derived from sex-sorted bovine spermatozoa (Morton *et al.* 2007).

Much progress has been made in recent years on preservation of the viability of sperm through the sorting and freeze-thaw processes (reviewed by Maxwell *et al.* 2004; Rath and Johnson 2008), and there have been some unexpected findings, both practical and scientifically interesting, made along the way about the nature and function of ram and bull sperm (reviewed by de Graaf *et al.* 2009). One of these is the discovery that sex-sorting actually selects, apparently by accident, sperm with intact membranes that lack a marker protein for the acrosome reaction (Leahy *et al.* 2008), rendering the selected population more resilient to further processing than unsorted sperm.

In the early 1990s, we demonstrated that fertilisation could be achieved with oviductal insemination of as few as 10,000 sperm in sheep (Maxwell *et al.* 1993), and later obtained high fertilization rates after AI of superovulated ewes with sexed sperm (de Graaf *et al.* 2007a) as a way of maximising the use of their limited numbers. IVF also requires relatively low numbers of sperm and, if sexing is used in conjunction with JIVET, has potential to hasten the rate of genetic gain (Raadsma and Tammen 2005). Therefore, we have produced offspring by IVF with sex sorted-frozen-thawed sperm from *in vitro* matured abattoir-sourced peripubertal lamb oocytes and from oocytes aspirated from hormone-stimulated prepubertal lambs (Morton *et al.* 2004a).

One of the most exciting recent developments in our laboratory has been "reverse" sex-sorting technology for the utilization of frozen ram (Hollinshead *et al.* 2003) and bull semen (Underwood *et al.* 2009a). This allows high purity sorting of frozen-thawed sperm for re-cryopreservation and later use, without a reduction in the fertilizing capacity of the sperm after the two cycles of freezing and thawing. Lambs of predicted sex also have been born after the transfer of both fresh and vitrified IVP embryos, derived from "reverse sorted" ram sperm (O'Brien *et al.* 2004). Furthermore, our work on improved precision in synchronization of ovulation in sheep using GnRH (Reyna *et al.*, 2007) has allowed us to recently confirm the commercial viability of sex-sorted frozen-thawed sperm for artificial insemination in sheep, with a minimum effective AI dose of 1 million sperm (Beilby *et al.* 2009). Furthermore, over a large number of ewes, fertility was not different after insemination by laparoscopy of frozen-thawed (control), sex sorted-frozen-thawed or frozen-thawed-sex sorted-refrozen-thawed ("reverse sorted") sperm (de Graaf *et al.* 2007b). Another recent development has been the application of "reverse sorted" sperm to *in vitro* embryo production in sheep and cattle. In initial studies conducted under commercial conditions in the USA, blastocyst development rates were similar for embryos derived from *Bos indicus* OPU oocytes fertilized with frozen-thawed-sex sorted-refrozen-thawed and control (frozen-thawed) sperm (Underwood *et al.* 2009b). These results demonstrate that frozen-thawed ram and bull sperm can be sex-sorted for either immediate or future use in an IVF system after re-cryopreservation, and point the way to the commercial application of sexed sperm through JIVET or MIVET.

CONCLUDING REMARKS

Major new technologies in embryo production will take time to become commercially viable, though JIVET offers great promise to increase the rate of genetic gain, particularly when combined with sexed sperm and marker-assisted selection in sheep and cattle (Raadsma and Tammen 2005). As far as AI in sheep is concerned, the use of frozen sperm (sexed or unsexed) will be limited in the near future to laparoscopic AI, though our work with seminal

plasma proteins offers the first real prospect of dramatically increasing the use of frozen semen through cervical AI. We have briefly reviewed those technologies which have the most immediate likelihood of application in small ruminant breeding programs. Those omitted - such as somatic cell nuclear transfer (cloning), transgenesis and utilisation of stem cell spermatogonia - are either some years away or, for ethical, animal welfare or epigenetic/safety reasons, are likely to have delayed acceptance by the industry or consumers.

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