

REVIEW

Embryo biotechnologies in sheep: Achievements and new improvements

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Abstract

To date, large-scale use of multiple ovulation and embryo transfer (MOET) programmes in ovine species is limited due to unpredictable results and high costs of hormonal stimulation and treatment. Therefore, even if considered reliable, they are not fully applicable in large-scale systems. More recently, the new prospects offered by in vitro embryo production (IVEP) through collection of oocytes post-mortem or by repeated ovum pick-up from live females suggested an alternative to MOET programmes and may be more extensively used, moving from the exclusive research in the laboratory to field application. The possibility to perform oocytes recovery from juvenile lambs to obtain embryos (JIVET) offers the great advantage to significantly reduce the generation interval, speeding the rate of genetic improvement. Although in the past decades several studies implemented novel protocols to enhance embryo production in sheep, the conditions of every single stage of IVEP can significantly affect embryo yield and successful transfer into the recipients. Moreover, the recent progresses on embryo production and freezing technologies might allow wider propagation of valuable genes in small ruminants populations and may be used for constitution of flocks without risks of disease. In addition, they can give a substantial contribution in preserving endangered breeds. The new era of gene editing might offer innovative perspectives in sheep breeding, but the application of such novel techniques implies involvement of specialized operators and is limited by relatively high costs for embryo manipulation and molecular biology analysis.

KEYWORDS

ewe, IVEP, LOPU, oocyte competence, recipient, transfer

1 | INTRODUCTION

Assisted reproductive technologies (ARTs) play a critical role in increasing the dissemination of superior genotypes, reducing generation interval and enhancing productivity. In small ruminants,

ARTs encounter limited commercial application compared with the bovine species, although the interest towards these techniques is constantly increasing. According to the International Embryo Transfer Society (IETS) Data Retrieval Report, the total sheep embryo production both in vivo derived (IVD) and in vitro produced

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(IVP) increased by almost 20% between 2018 and 2019. For the same time interval, the report indicated a significant increase in fresh IVP embryo transfers, while no transfer of frozen ones was performed. The production of IVD embryos by multiple ovulation and embryo transfer protocol (MOET) is confined to defined geographical areas (USA, Brazil, Australia), while in Europe, it accounts to approximately 1,100 embryos, most of which are transferred fresh (IETS, Data Retrieval Report, Viana, 2019). The efficiency of this technique is still debated, and several limiting factors reduce its applicability in large-scale breeding programmes. The most critical factors are the high costs of this procedure and the unpredictable results, depending on the reproductive status of donor animals, the high variability of superovulatory response to hormonal stimulation, fertilization failures, early luteal regression, and the need of surgery for gametes and embryos collection and transfer (González-Bulnes et al., 2004).

More recently, IVEP advances might represent an alternative system to MOET, moving from exclusive research in the laboratory to the field context. The recent improvements of embryo production and freezing technologies might allow wider propagation of valuable genes in small ruminant populations and may be employed for the constitution of flocks without risks of disease. In addition, they can provide a substantial contribution in creating efficient cryobanks for preservation of embryos of high genetic value and from endangered breeds. The aim of this review was therefore to provide an overview of current IVEP procedures focusing on factors that can significantly affect the efficiency of this method in the ewe (Figure 1).

2 | IN VITRO EMBRYO PRODUCTION

In vitro embryo production consists basically in (i) collecting oocytes, (ii) submitting them to in vitro maturation (IVM) under specific conditions; (iii) in vitro fertilization (IVF) with selected sperm; and (iv) in vitro culture (IVC) of presumptive embryos at different stages of development. Every undertaken step is crucial for the final success, and during the past decades, many experiments have been devoted to establishing the appropriate conditions. The overall efficiency of in vitro embryo production, in terms of blastocyst rates, ranges between 15% and 79% (Zhu et al., 2018) with significant differences between experiments and laboratory procedures, depending on the oocyte source (i.e. abattoir or in vivo derived), age of donor, culture conditions, reproductive status and genetic background (Zhu et al., 2018).

2.1 | Oocyte sources

For in vitro embryo production, oocytes can be collected either post-mortem from slaughtered animals or in vivo by laparotomy or laparoscopy.

2.1.1 | Post-mortem

Numerous oocytes are commonly retrieved post-mortem from abattoirs by gently slicing the ovaries, puncturing follicles or by follicular

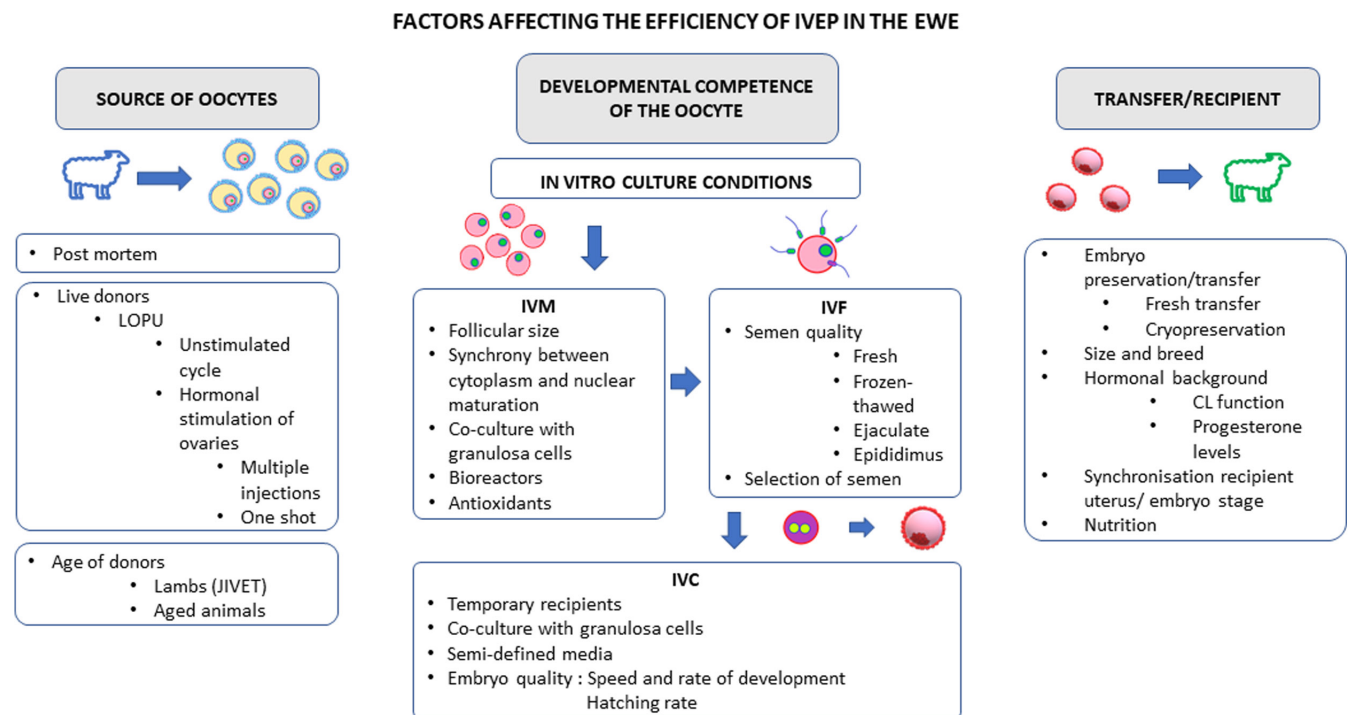


FIGURE 1 Factors affecting the efficiency of IVEP in sheep

fluid aspiration. The large number of oocytes collected represents a valuable tool to assess the best IVEP conditions. In small ruminants, oocyte retrieval yields an average of 5–6 oocytes/ovary and collection techniques can affect their quality (Majeed et al., 2019; Wang et al., 2007). Based on our experience, slicing gently the ovarian tissue better preserves the structure of cumulus-oocyte complexes (COCs), while aspiration may cause loss in integrity of cumulus layers for excessive mechanical strain (Rodríguez et al., 2006). Post-mortem oocyte recovery presents several limitations, such as unknown stage of oestrous cycle as well as age and sanitary and nutritional status of slaughtered animals, that might affect the quantity and quality of IVP embryos. However, it provides the advantage of rescuing oocytes from animals of high genetic value and to generate viable offspring (de Souza-Fabjan et al., 2014).

2.1.2 | In vivo

Alternatively, in programmes of genetic improvement, oocyte collection for IVEP can be performed on selected live animal, increasing the number of embryos from the same valuable female. For the purpose, oocytes can be collected by laparoscopic ovum pick-up (LOPU), firstly described in the ovine species in 1974 by Snyder and Dukelow (Snyder & Dukelow, 1974) and successively optimized by few research groups (Baldassarre, 2021; Wiczorek et al., 2020). Through laparoscopy, this technique allows visualization of ovaries and oocyte collection by follicle aspiration. However, the number and dimensions of follicles in unstimulated females may hamper a satisfactory aspiration and oocyte retrieval. For this reason, LOPU requires an ovarian stimulation by multiple FSH injections in order to enhance the follicular growth and number as well as the competence of collected oocytes. (Alberio et al., 2002; Ptak et al., 1999). Recently, a protocol of a 'one shot' stimulation has been proposed in which FSH is delivered in one administration, dissolved in 0.5% hyaluronic acid solution (MAP-5): It works as a slow-releasing factor, leading to collection rates similar to classic protocols (Baldassarre et al., 1996). Oocyte recovery rates range from 35% (Alberio et al., 2002) to approximately 90% (Baldassarre et al., 2007; Cox & Alfaro, 2007) with an average 14 oocytes/donor in goats and 10 oocytes/donor in sheep and a range of 2.6–5 transferrable embryos/recipient (Baldassarre, 2021; Cox & Alfaro, 2007). Laparoscopic ovum pick-up can be safely repeated on the same animal up to 9–10 times in a year without compromising the number of aspirated follicles, oocyte recovery rates and quality of collected COCs (Teixeira et al., 2011). Moreover, this technique can include donors that are not eligible for embryo production by MOET, such as aged females of high genetic value (Baldassarre et al., 2007) and pre-pubertal animals (Kelly et al., 2005).

2.1.3 | Age of donors

The age of animals selected as oocyte donors can affect the developmental competence of oocytes collected for IVEP. It is known

that adult animals reach the reproductive maturity when ovarian dynamics are under a consolidated hormonal control (Padmanabhan & Cardoso, 2020). Optimal oocyte donors are 2/3-year-old animals, while older females showed altered follicle function with a decrease in dominance effects, inhibin and E_2 secretion (Berlinguer et al., 2012; Gonzalez-Bulnes et al., 2004).

On the contrary, IVP embryos can derive from oocytes collected from juvenile donors enhancing the genetic gain by reducing generation interval. Oocytes are usually collected from living unstimulated or stimulated pre-pubertal donors of 3–4 weeks to 5–6 months of age (Kelly et al., 2005; Morton et al., 2005) or from slaughtered animals. Moreover, ovaries from pre-pubertal females have a large number of small antral follicles from which several oocytes can be retrieved.

Donors are hormonal stimulated to increase the follicular size, facilitating oocyte retrieval and developmental competence (O'Brien et al., 1997), but a high proportion of them show low response to the stimulatory treatment (Ptak et al., 1999). The number of collected oocytes is highly variable ranging from 10 up to 200 oocytes/donor. This variability is a consequence of a variation on waves of follicular growth and atresia, especially in young lambs (30–40 days). The IVM rates of oocytes collected from stimulated and unstimulated pre-pubertal donors are similar to those derived from adult ewes, but a slight delay in meiotic progression (Leoni et al., 2015) has been reported as well as lower blastocyst rates compared with adult animals (15/25% versus 30/60%). No differences in blastocyst development were found between stimulated and unstimulated lambs (O'Brien et al., 1997). Such a reduced developmental potential is related to morphological and functional alterations (Ledda et al., 2001; Leoni et al., 2007; O'Brien et al., 1996; Ying et al., 2021). In the perspective of exploiting the large JIVET potential, the availability of predictive markers on good developmental ability has been investigated. Among others, high levels of anti-Müllerian hormone (AMH) can be associated to antral follicle count (Torres-Rovira et al., 2014) and to higher blastocyst rates, suggesting that its concentrations might be used to select successful donors for JIVET (McGrice et al., 2020).

2.2 | In vitro maturation

Throughout folliculogenesis, the oocyte undergoes a series of progressive modifications that lead to the acquisition of developmental competence. Not all the oocytes selected for IVEP acquire complete nuclear and cytoplasmic maturation essential to be fertilized and to develop to the blastocyst stage (Mermillod et al., 1999). These differences in term of developmental potential could depend on follicular size since larger follicles contain more competent oocytes (Cognie et al., 1998). Oocytes collected from small and medium-size follicles exhibit an impaired cytoplasmic maturation, although they can mature in vitro in presence of gonadotrophins.

Other factors can influence the oocyte developmental competence, such as reproductive seasonality, age of donors, nutritional status and in vitro culture conditions. The effects of these factors

have been recently reported (Paramio & Izquierdo, 2016). Regarding the culture conditions, it should be noticed that despite the large number of studies the protocols for IVM in small ruminants are those designed almost 40 years ago, performed nowadays with only slight modifications (Paramio & Izquierdo, 2016; de Souza-Fabjan et al., 2014; Zhu et al., 2018). The most used maturation media is TCM199, supplemented with serum of different origins, hormones and amino acids; incubation conditions are generally 5% CO₂ at 38–39°C for 24–27 hr [as reviewed by (Zhu et al., 2018)].

Many strategies have been proposed to improve developmental competence of IVM oocytes tackling the asynchrony between nuclear and cytoplasmic maturation, improving the selection of the oocyte and evaluating the beneficial effects of factors added to the culture medium. Indeed, once the oocyte is removed from the follicular microenvironment (mimicking an ovulation), there is a meiosis resumption (Pincus & Enzmann, 1935) that is accompanied by concurrent cytoplasm maturation. Meiosis resumption seems mediated by a decrease in cAMP that can affect the developmental competence (Eppig et al., 1994). Therefore, one approach proposed to delay meiotic maturation of oocytes for 6–12 hr prior IVM, thus allowing full growth and cytoplasm maturation. For this purpose, roscovitine has been observed to reversibly promote meiosis arrest without negative effects on subsequent resumption although no increase in blastocyst rates has been reported (Crocomo et al., 2016). More recently, another strategy to increase oocyte and embryo developmental competence has been the control of nuclear maturation by the use of a simulated physiological oocyte maturation (SPOM) system (Rose et al., 2013). The latter consists of using cAMP modulators to simulate the maturation mechanisms and enhance synchrony between nuclear and cytoplasmic maturation (Leal et al., 2020).

Alternatively, attempts to improve cytoplasmic maturation and control of meiotic resumption have been performed by supplementation of specific paracrine factors. The addition of exogenous oocyte-secreted growth factors (OSFs) released by fully grown oocytes can positively influence granulosa cells by improving the functional coupling between oocytes–cumulus cells (Gilchrist et al., 2008).

Furthermore, it has been recently reported that pre-IVM exposure of granulosa cells to factors such as natriuretic peptides improved developmental competence of oocytes collected from small follicles resulting in increased blastocyst rates (Rouhollahi Varnosfaderani et al., 2020). Although promising, these results need further investigations. Recently, in the attempt of increasing the paracrine factors produced during IVM by concentrating them in small volumes, a novel technique has been proposed (Ledda et al., 2016), based on oocyte maturation in a small microreactor formed by coating a small amount of IVM medium with a superhydrophobic powder. Interesting results were recently obtained by intra-follicular oocyte transfer in adult ewes, in which the injection of 20–25 oocytes into a pre-ovulatory follicle led to embryo production, suggesting that exploiting physiological conditions of follicular microenvironment may help maturation (Falchi et al., 2022).

Improvements on IVM oocytes have also been obtained by addition of antioxidant molecules in the culture medium. Significant

results have been obtained especially in oocytes derived from pre-pubertal donors where the presence of nanoparticles of cerium oxide enhanced blastocyst development (Ariu et al., 2017).

The possibility to select more competent oocytes by a non-invasive method such as brilliant cresyl blue staining (BCB) is an interesting perspective. The principle of this procedure lays on the production of higher levels of glucose-6-phosphate dehydrogenase (G6PDH) in growing oocytes compared with fully grown ones. Since BCB dye is reduced by G6PDH activity, the oocytes can be distinguished based on the amount of G6PDH between those stained in bright blue that completed their growth and those unstained that are growing. Oocytes selected through the BCB staining produced more blastocysts and of better quality (Catalá et al., 2011). However, due to different staining and methodology procedures, this technique needs to be fully validated before considering it a discriminating system to evaluate developmental capacity (Opiela & Kątska-Książkiewicz, 2013).

Finally, to meet the need of non-invasive selection systems to estimate the developmental competence of collected oocytes, a new platform based on Raman technology has been proposed and experimented (Bogliolo et al., 2012, 2015). Unfortunately, although promising, these evaluation systems require expensive instruments and high skill competences (Bogliolo et al., 2020).

2.3 | In vitro fertilization

The success of IVF depends on several factors: male individual variability, sperm morphological and functional parameters (motility, integrity of membranes), origin of sperm (ejaculation versus epididymal collection), storage (fresh versus frozen-thawed) and seasonal variations. Moreover, spermatozoa derived from rams with similar motility parameters showed a different ability to fertilize in vitro, possibly as a consequence of the capacitating sensitivity during sperm preparation (Fukui et al., 1988). Before co-incubation with IVM oocytes, ejaculates undergo seminal plasma removal and dilution with specific extenders that are designed to discriminate the fittest spermatozoa, excluding dead or hypomotile ones and debris that may negatively affect the fertilizing ability. Several techniques are routinely used to achieve sperm selection including density gradient centrifugation (Garcia-Alvarez et al., 2010), swim up (Grasa et al., 2004) or magnetic-activated cell sorting (Gil et al., 2013). After selection, sperm capacitation in vitro is induced through incubation with several capacitating agents such as heparin, hypotaurine and oestrus sheep serum (ESS). In sheep, basic conditions for in vitro fertilization are co-incubation of spermatozoa and oocytes in synthetic oviductal fluid (SOF) medium supplemented with ESS for 16–24 hr at 38–39°C (Zhu et al., 2018) under low oxygen tension (5% O₂) or 5% CO₂ in air. High-quality ram semen when regularly prepared for IVF (Leoni et al., 2007) should guarantee in both conditions IVF rates of approximately 75% [reviewed by (Zhu et al., 2018)]. Alternatively to IVF, fertilization of IVP can be achieved by intracytoplasmic

sperm injection (ICSI), which consists of introducing a single spermatozoon into the ooplasm of a matured oocyte. The use of this technique can offer several advantages: avoiding polyspermy fertilization; allowing the selection of the best male germ cells and the use of sex-sorted spermatozoa; and producing viable offspring from non-motile or subfertile spermatozoa (Kaneko, 2016). However, different problems have limited the efficacy of ICSI. Among them: fertilization rates are lower compared with results obtained by co-incubation of viable sperm with matured oocytes; oocytes need to be activated as the sole sperm injection is not sufficient to determine high rates of activated oocytes. To avoid these problems, treating sperm cells with detergents before ICSI slightly improved the spontaneous activation and blastocyst rate (Anzalone et al., 2018).

2.4 | In vitro embryo culture

Following IVF, presumptive zygotes can be cultured in vitro for 2–3 days and then transferred into the oviduct of recipients (Wang et al., 2003) or more commonly cultured in vitro for 6–8 days up to the blastocyst stage (Anzalone et al., 2018). In the ovine species, several embryo culture protocols have been tested over the past decades. Basically, three main methods can be summarized: (a) culture of zygotes in temporary recipients; (b) in vitro co-culture with oviductal cells (Gandolfi & Moor, 1987; Gardner et al., 1994; Walker et al., 1992); and (c) culture in semi defined media (Gardner et al., 1994; Walker et al., 1992).

Transfer of early embryos to a transient recipient resulted indeed in optimal embryo quality, comparable to levels obtained in vivo, due to the embryonic support derived by the optimal conditions provided by the physiological environment in the oviducts. However, it involved the use of a higher number of animals and it is technically challenging.

The idea of embryo co-culture with oviduct cells was experimented in sheep by Gandolfi and Moor over 30 years ago (Gandolfi & Moor, 1987). Cultured oviduct cells seem to support embryo development up to the blastocyst stage through the release of potentially 'embryotropic' factors into the culture medium and/or acting as scavenger of toxic factors during IVC (Watson et al., 1994). Recently, following the same approach, 3D systems of oviductal cells have been developed opening new perspectives to better understand fertilization and early embryo development stages and to provide new tools for enhancing in vitro embryo production (Ferraz et al., 2017).

Other attempts to enhance embryo IVC have been based on development of culture medium comparable to oviductal fluid composition. The medium widely used for ovine IVC consists of SOF supplemented with amino acids and serum (generally bovine serum albumin). Culture conditions do not change during the 6/7 days of culture and are performed in 5% of O₂, 5% CO₂ and 90% Nitrogen. Sequential media, which should respond to the different metabolic requests of the embryos during the pre-implantation stages, have

been tested but did not indicate significant improvements in terms of blastocyst rates, while hatching rates were negatively affected (Garcia-Garcia et al., 2007; Ledda et al., 1995).

Different criteria have been proposed to estimate in vitro the quality of IVC culture systems in sheep although the best assessment remains pregnancy rates and number of offspring after transfer of IVP embryos into recipients. One of these criteria is to evaluate the cleavage rates and speed of development at 24 and 30 hr. According to our experiences, the presence of more than 50% of 2-cell embryos is a good indicator of oocyte developmental competence. Embryos that cleaved within the first 24 hr produce more blastocyst having higher quality compared with those that cleaved later (Leoni et al., 2006). Several embryos with slow cleavage arrest their development at different pre-implantation stages, usually before the genomic activation (8–16 cell stage) or at compacted morula stage.

Another criterion to evaluate the quality of IVC systems in sheep is to quantify the in vitro hatching rate. High hatching rates are usually observed in embryos that develop faster, and it is correlated with the total cell numbers (Coello et al., 2017).

Although significant progress has been achieved in sheep embryo IVC, it has been reported that culture conditions in some circumstances can determine alterations in foetal development and in viability of derived offspring, compromising its application in modern breeding protocols. Oversize lambs, characterized by aberrant growth of foetus (Large Offspring Syndrome – LOS), have been obtained after transfer of IVP embryos cultured in medium supplemented with serum (Young et al., 1998). Inappropriate IVC conditions may also induce defects in the placenta and absent or reduced signs of birth (Holm et al., 1996). These alterations can be the result of suboptimal embryo culture conditions that may cause epigenetic changes altering gene expression of imprinted and non-imprinted genes functionally important for the early embryonic stages (Rizos et al., 2002).

2.5 | Cryopreservation of IVP embryos

Recent improvements of in vitro embryo production and advances in cryopreservation technologies might allow wider propagation of valuable genes in sheep populations and facilitate the distribution of superior genotypes with a consequent reduction of shipping costs of live animals. In addition, these technologies might substantially contribute to preserve endangered species or breeds.

To date, despite the improvements achieved, the application of this technique in large scale is still relatively low (Baril et al., 2001). The successful application of embryo cryopreservation and transfer of IVEP in small ruminants is largely dependent on the efficiency of techniques used.

The first cryopreserved ovine embryo was obtained by a slow freezing technique (Willadsen et al., 1976), a technique that is being gradually replaced by the vitrification method, more efficient in terms of in vitro produced embryos. The viability rate of vitrified in vitro derived morulae and blastocysts in sheep is significantly higher

compared with embryos cryopreserved by slow freezing techniques (Martínez et al., 2006; Shirazi et al., 2010).

The other reason for success of vitrification compared with slow freezing is that it does not need special equipment, it reduces labour time, and it is cost effective, thus more adequate to routine use in field (Vajta & Kuwayama, 2006).

The effectiveness of vitrification of IVP embryos depends on many factors such as the embryonic developmental stage, devices used, volume and cooling rate (Dos Santos-Neto et al., 2017). Earlier embryonic stages in sheep, such as pre-compacted and compacted morulae, are more sensitive than blastocysts to vitrification procedures (Naitana et al., 1997). Similar differences were observed between early and expanded IVP blastocysts (Ledda et al., 2019).

The efficacy of embryo vitrification can be estimated in vitro by recording embryo morphokinetics after post-warming culture. Blastocoel re-expansion and hatching after vitrification/warming can be considered as predictive indicators of subsequent development (Leoni et al., 2008). Vitrification methods provided good pregnancy rates (ranging between 30%–55%) following the transfer of in vitro produced blastocyst (Dos Santos-Neto et al., 2017).

Several methods and devices have been proposed to improve and simplify embryo cryopreservation methodologies in ovine. The 0.25-ml straw (Naitana et al., 1997) and the open pulled straw (OPS) have been successfully used to vitrify in vitro produced morulae and blastocysts (Dattena et al., 2004). Many of these methods require the release of warmed embryos in washing solution for cryoprotectant removal and the successive loading in new devices for their transfer. When using vitrification technology in field conditions, it would be more feasible to cryopreserve embryos, warm them and dilute in simple systems without the need of specialized equipment and laboratory skills. For this reason, previous studies have proposed in-straw systems that allow the direct transfer of cryopreserved embryos into recipient animals (Baril et al., 2001; Green et al., 2009). These systems in most of the cases involved more than one device and were developed mainly to perform in-straw warming and dilution (Morató & Mogas, 2014).

Recently, we suggested the use of a new system 'E.Vit' in which embryo vitrification, warming and dilution can be performed within a straw. Post-warming results showed high survival rates and embryo quality that could be predictive of subsequent development (Ledda et al., 2019). This method reduces the need for equipment, technical skills and embryo handling and can facilitate direct embryo transfer to the uterus.

3 | EMBRYO TRANSFER

3.1 | Factors that can be relevant for a successful transfer

3.1.1 | Number, stage and morphology of transferred embryos

Transferring multiple embryos to reduce the number of recipient ewes is economically more sustainable. However, it has been

reported that uterine crowding in nulliparous recipients, receiving more than one embryo, can cause an increased frequency of pregnancy losses (Anderson et al., 1979). Embryo loss mainly occurs prior to Day 14 of gestation with 6% losses before Day 4, and 12% loss between Days 4 and 14 of gestation (O'Connell et al., 2016). Naqvi et al. (2006) found that the incidence of embryonic mortality up to Day 40 of gestation was reduced when multiple embryos were transferred, with a 38.1% and 28.6% viable offspring rate when 3 embryos or 2 embryos per recipient were transferred into the ipsilateral horn, respectively (Naqvi et al., 2006). Higher pregnancy rates (55.2%) were reported in Hungarian Merino ewes following transfer of two embryos per recipient, compared with 45.6% obtained by single transfer (Cseh & Seregi, 1993). Conversely, other available data have shown that doubling the number of embryos transferred resulted in a decrease in the number of newborn lambs (Land & Wilmut, 1977). Transferred IVP embryos are usually 2–4 per recipient; single transfers are not recommended since IVP embryos may have a reduced number of cells and consequently produce smaller amounts of luteotrophic or anti-luteolytic signalling molecules necessary for pregnancy maintenance.

A positive correlation was found between developmental stage of transferred embryos and pregnancy rates (Alabart et al., 2003). Usually, embryos are transferred at late morula, early blastocyst or expanded blastocyst stages. Transfer at earlier or later stages results in no significant advantage or reduced viability to term. In fact, while in vivo-derived embryos at the stage of late morulae and expanded blastocyst resulted in comparable pregnancy rates, lower pregnancy rates were obtained transferring hatched embryos (Hasler, 1998). Embryos transferred at earlier stages (2–3.5-days post-fertilization) led to acceptable pregnancy rates (Alabart et al., 2003) but without a practical advantage, since viability does not change compared with later embryonic stages. In IVP embryos, which are more stage-sensitive compared with in vivo-derived ones, higher conception rates were achieved following transfer of expanded blastocysts compared with morulae or earlier stages (Naitana et al., 1996).

A correlation between embryo morphology and pregnancy rates has been discussed for many years. However, the absence of precise non-invasive evaluation tools reduces an effective estimation of embryo quality grading before transfer and in most cases quality scores are the result of embryologist experience (Steer et al., 1992). Without a precise visual morphological scoring (Betteridge & Rieger, 1993) for in vitro produced embryos, timing of development is considered a predictive marker for embryo quality. Reduced viability related to poor quality grade resulted in slower rates of cleavage (Leoni et al., 2007) and in reduced pregnancy rates after transfer (Hasler, 1998).

3.1.2 | Recipients

Transfer of embryos into suitable recipients is the final step in programmes of embryo production for genetic improvement. Technically, the embryos are commonly transferred into the uterine horn ipsilateral to the CL by laparotomic (Tervit, 1996; Tervit &

Havik, 1976) or laparoscopic (Schiewe et al., 1984; Shin et al., 2008) techniques. In both cases, it requires sedation or a proper anaesthesia. Only few modifications to these two transfer methods have been attempted up to now (Li et al., 2008). Recently, a non-surgical transcervical technique has been experimented for embryo transfer in sheep and goat. This technique has the great advantage to be more suitable in field applications since it does neither require anaesthesia nor expensive equipment (Fonseca et al., 2019; Santos et al., 2020). Despite the promising results, its applicability in ewes of different breeds and ages still remains to be tested since transcervical passage of catheters is limited by the anatomy of the cervix and strictly depends on breed and number of parturitions (Falchi et al., 2012; Kershaw et al., 2005).

The selection of recipients requires special attention since an unfitting physiological status may hamper the success of embryo transfer programmes. Reliable criteria to assess recipient suitability for ET programmes have been targeted by many research groups.

Corpus luteum and progesterone

In order to get the highest viability of transferred embryos, an optimal uterine environment should be provided establishing a correct hormonal support and an appropriate synchrony between donor and recipient. These conditions are relevant when fresh and especially cryopreserved IVP embryos are transferred. In this context, the recipients should have a fully functional corpus luteum (CL) that produces sufficient levels of progesterone to sustain pregnancy. The CL in the recipients can be estimated considering its size and blood perfusion by ultrasonography, since morphological and echogenic characteristics are related to plasma concentrations of progesterone (Gonzalez de Bulnes et al., 2000). In sheep, plasma progesterone concentration in recipient animals is related to the number of CLs (Ashworth et al., 1989). However, limited knowledge is available regarding the correlation between embryo survival and number of CLs in this species.

Synchrony between the embryo and the uterine environment in the recipient is of primary importance for the transfer success since it has been demonstrated that an asynchrony of just 3 days has deleterious effects on embryo survival (Wilmut & Sales, 1981). Current protocols of synchronization are based on the use of synthetic progestogens implants that control ovarian function and create a suitable uterine environment for embryo development and survival.

Size and breed

The effect of the size and breed of recipients on embryo survival was studied by Naqvi et al. (2006). They investigated developmental competence, birth and survival of lambs after transfer of two or three embryos of a small prolific breed into large size non-prolific recipient ewes. They found that embryos derived from prolific sheep developed to term at a higher proportion when transferred into the uterine environment of larger non-prolific sheep, providing more space for embryos to develop (Naqvi et al., 2006). The effects of recipient and embryo genotype on growing performance and

survivability of offspring were investigated using inter-breed embryo transfer. The results showed that recipient breed did not affect survival and weaning performance of lambs from the prolific breed (Emsen et al., 2012).

Nutritional status

In the ovine, nutritional management can significantly affect reproductive performances and undernutrition induces changes in the sensitivity of the uterus to steroid hormones at early stages of pregnancy altering uterine environment and hampering embryo survival (Abecia et al., 2006). Undernutrition in recipient ewes increases the levels of non-esterified fatty acids (NEFAs) as a result of fat reserves mobilization and decreases the levels of insulin, compared with animals fed with maintenance diet. Both alterations in NEFAs and insulin levels are associated with low progesterone in the first phases of pregnancy establishment and embryo mortality (de Brun et al., 2016).

4 | EMBRYO MANIPULATION, CLONING AND GENE EDITING

Over the past 50 years or more, embryo manipulation has been considered a system to enhance the genetic gain in small ruminants and especially sheep embryos have been used to prove the feasibility of different techniques. Embryo manipulation has been performed for different purposes such as to increase the number of identical individuals of high genetic value or for genomic selection by genetic screening of pre-implantation embryos. Identical individuals were firstly obtained by generating monozygotic sheep twins (Willadsen, 1979) through the blastomere separation technique or by bisecting blastocysts (Széll & Hudson, 1991). Later, the same target of increasing the number of identical subjects was obtained by cloning, using the nuclear transplantation technique. This technology captured the major interest and evidenced the most significant progress.

The increase in cloning efficiency is the results of advances in nuclear transplantation technology such as enucleation procedures; extensive use of in vitro matured oocytes; cells reprogramming using less differentiated nuclei donor; and improvements of cell cycle synchronization between the recipient cytoplasm and the transplanted nucleus. Currently, by staining the metaphase plate chromatin with fluorescent probes, a 100% enucleation success rate can be obtained after the removal of a small portion of oocyte cytoplasm (Iuso et al., 2013). In vitro matured oocytes are used for nuclear transfer in sheep with results similar to in vivo produced oocytes (Yuan et al., 2019). The synchronization between the recipient cytoplasm and the transplanted nucleus represented the real and significant improvement in sheep cloning by nuclear transfer and resulted in the extraordinary success of the birth of Dolly in 1996 (Campbell et al., 1996).

In line with this, following the idea that reprogramming is largely depending on the best synchronization between the recipient cytoplasm and the nuclear donor cells, an interesting alternative for

cell donor preparation has been proposed (Czernik et al., 2016; Iuso et al., 2015). The idea is based on the possibility to replace the histones with protamines in sheep fibroblasts, uploaded by human protamine 1 (hPrm1) gene in cell culture. Improvements in nuclear reprogramming have been reported also by employing histone deacetylase inhibitors or impeding Xist expression (Czernik et al., 2019).

Another interesting aspect in establishing modern breeding programmes is the possibility to perform a genetic screening for the genomic selection of fresh or cryopreserved pre-implantation embryos in vitro produced. The latter requires micromanipulation and collection of a sufficient number of cells through biopsy of each embryo. Recovered cells are then subjected to genomic estimation by molecular biology analysis to predict the possible improvements in animal production. The method can also be used to prevent the transfer of embryos that are carrier of known recessive lethal genetic defects or other genetically transmitted diseases. Nowadays, most of the studies concerning sheep are related to biopsy techniques optimization, estimating the effects of biopsy methods on subsequent post-manipulation viability of pre-implanted embryos at different stages of development (Leoni et al., 2000; Naitana et al., 1996). Finally, another future approach might be considering the possibility of generating genetically modified founders. Currently, gene editing in sheep using CRISPR/Cas9 systems have attracted a great deal of attention with the idea of producing viable genetic modified subjects, improving breeding and disease resistance (Vilarino et al., 2018).

5 | CONCLUSIONS

Despite the significant increase in efficiency in ovine species, the development of ARTs is still producing fluctuating results that limit their practical and extensive application in the modern breeding system. The production of in vitro embryos from oocytes of prepubertal and adult animals still requires further development and needs to define and consolidate IVEP methodologies. Techniques related to the creation of genetic cryobanks of embryos are constantly improving. More advanced technologies such as ICSI, cloning and embryos gene editing remain at an experimental stage. The application of ARTs could bring benefits in areas of the world that promote sheep rearing leading to an improved production of local breeds and safeguarding biodiversity.

ACKNOWLEDGEMENT

Open Access Funding provided by Università degli Studi di Sassari within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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How to cite this article: Falchi, L., Ledda, S., & Zedda, M. T. (2022). Embryo biotechnologies in sheep: Achievements and new improvements. *Reproduction in Domestic Animals*, 57(Suppl. 5), 22–33. <https://doi.org/10.1111/rda.14127>