ET-Technologies in Small Ruminants

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Abstract

In the last decades small ruminants have become increasingly important, and nowadays sheep and goat are continuously increasing in the number of breeds and their geographic distribution. An important feature of small ruminants is that they can live and produce on land that is unfavorable for other forms of agriculture. The increase in small ruminant breeding has been supported more recently by the development and improvement of assisted reproductive technologies (ARTs). However, while some ARTs have reached widespread application, including estrus induction, estrus synchronization, and artificial insemination, other ARTs, such as superovulation and embryo transfer, in vitro embryo production, and embryo cryopreservation, are only rarely used. Multiple ovulation and embryo transfer (MOET) programs in small ruminants are usually restricted to few countries and still remain experimental. The success of this technique is unpredictable due to many limiting factors that contribute to the overall results, such as the reproductive seasonality with a long, naturally occurring anestrus period, high variability of the superovulatory response, fertilization failures, and the need of surgery for collection and transfer of gametes and embryos. However recent progress in better understanding of the follicular wave patterns, the elucidation of follicular dominance, and the integration of this information into superovulation treatments are instrumental in predicting good responders and reducing variability. Protocols that control follicular dominance have been developed to allow the initiation of precise hyperstimulation protocols which are designed to recruit and stimulate a homogeneous pool of small follicles that are gonadotrophin respon-

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sive, thereby enhancing superovulatory response and embryo yields. Significant improvements in the development of nonsurgical techniques are paving the way to reducing stress and costs of donors and recipient management, indicating the possible repeated use of individual donors. In addition, the progress with IVP embryos generated from adult and juvenile animals, combined with the genomic analysis of economically productive tracts, is opening new perspectives and could be instrumental for improving MOET programs in small ruminants.

6.1 Introduction

In the last decades small ruminants have become increasingly important, and nowadays sheep and goat breeding plays a crucial, economic and social role, as shown by the continuous increase in the number of breeds and their geographic distribution. An important feature of small ruminants is that they can live and produce on land that is unfavorable for other forms of agriculture.

According to FAO (faostat.fao.org, 2013), the number of sheep and goats in the world was 1169 and 996 million, respectively. Sheep and goats are shown to have a global distribution with emphasis in Africa and America with 848 and 929 million, respectively, whereas in Asia, Europe, and Oceania, 173 million sheep and 67 million goats were held. The global economic value of sheep and goat milk was 5.6 and 6.4 billion USD and for meat it was 37 and 25 billion, respectively. International sheep meat trade is limited (around 7% of the total production), and the bulk of this trade consists of export from the southern hemisphere (New Zealand has 47% and Australia has 36% of the total) to the European Union, North Asia, the Middle East, and North America. In many parts of the world, particularly in temperate regions, meat from sheep and goats is the most consumed product, and its importance as source of high-quality protein is steadily increasing.

The increase in small ruminant breeding has been supported in the last decades by the development and improvement of assisted reproductive technologies (Armstrong and Evans 1983; Loi et al. 1998). The control of reproduction and its modulation is an efficient tool for achieving genetic progress in these productive species.

While some assisted reproductive technologies (ARTs) have reached widespread application, including estrus induction, estrus synchronization, and artificial insemination, other ARTs, such as superovulation and embryo transfer, in vitro embryo production, and embryo cryopreservation, are only rarely used compared to cattle. Multiple ovulation and embryo transfer (MOET) programs in small ruminants are usually restricted to few countries and still remain experimental, even if this technique is considered an efficient and provides a low-cost option to exporting genetic material across international boundaries. However, the success of this technique is rather unpredictable due to many factors that contribute to the overall results, and many practitioners consider MOET one of the most frustrating ART in small ruminants.

The main limitation to field application in small ruminants is the reproductive seasonality with a long, naturally occurring anestrus period, high variability of the superovulatory response, fertilization failures, and the need of surgery for collection and transfer of gametes and embryos (reviewed by Cognié 1999; Cognié et al. 2003). This unpredictability combined with high costs of the pharmacological stimulation treatments have prevented large-scale use of MOET in sheep and goats, and up to now this technique is considered as being not enough robust to be applicable in large-scale breeding systems.

New prospects offered by in vitro embryo production (IVP) and repeated ovum pick-up from live adult and juvenile female donors are suggesting that IVP technology can be used as an alternative system to MOET programs, thus moving this technology from the research status in the laboratory to the field (Cognié et al. 2004; Paramio and Izquierdo 2014). Recent improvements of embryo production and freezing technologies could allow a wider propagation of valuable genetics in small ruminant populations and could also be used for establishing flocks without risk of disease transmission. In addition, they can make a substantial contribution to the preservation of endangered species or breeds.

The aim of this review is to provide an overview of some recent developments in MOET programs in small ruminants, updating recent information regarding estrus synchronization methods, follicular wave synchronization, and/or ovulation induction techniques during superovulatory treatments in ewes, as well as embryo collection and transfer techniques. The possibility offered by the generation of in vitro-produced embryos obtained from selected adult and juvenile donors will be also discussed with regard to the possibility offered by these new techniques to accelerate genetic progression of highly selected valuable animals.

6.2 Management of Reproductive Activity and Control of the Ovarian Cycle in Donor and Recipient Females

Sheep and goats are characterized by seasonal cycles of reproduction, consisting of a breeding season (which usually begins in late summer or early autumn in response to decreasing day length and ends in the late winter or early spring in response to increasing day length) and an anovulatory period (which covers the late spring to midsummer), which are separated by transition periods.

The breeding season is composed of a succession of sexual cycles (named estrous cycles since they are characterized by sexual receptivity (named estrus from the Latin word *estruus*), in the period preceding ovulation. The estrous cycles in small ruminants average 17 days in sheep and 21 days in goats and include the follicular phase and the luteal phase. The objective of the ovarian cycle is the development of a follicle able to ovulate and release an oocyte competent to be fertilized and able to develop in a viable embryo and, afterward, the maintenance of a corpus luteum competent for maintaining pregnancy.

Hence, the adequate management of reproductive activity and the ovarian cycle is indispensable in both donor and recipient females involved in MOET programs. The

main objective is to render the ovarian follicular population responsive to the gonadotrophin treatments in a healthy and large number and, as in the case of in vivo embryo production, to control the timing of ovulation in donor females. The precise control of the timing of ovulation and the availability of corpora lutea competent for maintaining pregnancy are the main objectives in recipient females. Several methods have been proposed to regulate seasonality and control ovarian activity in small ruminants.

6.2.1 Administration of Progesterone and Analogues (Progestagens)

The most widely used methods for synchronization of estrous cycle and ovulation are based on the administration of progesterone or its analogues (progestagens; the most common being fluorogestone acetate and medroxyprogesterone acetate). These treatments simulate the action of natural progesterone produced by the corpus luteum during the luteal phase of the cycle and allow control of LH secretion from the pituitary gland and thus prevent occurrence of ovulation. Removal of the substances leads to the appearance of a follicular phase with the growth of a preovulatory follicle and the occurrence of estrus and ovulation. The first successful protocol was developed in the early 1960s for sheep and consisted of intravaginally inserted sponges impregnated with progestagens (Robinson et al. 1967). The treatment was found to be equally effective for inducing ovulation in both the breeding season and the anovulatory period, with a high degree of synchronization in females treated at the same time. Thereafter, the method was found to be useful also for estrus synchronization in goats (Ritar et al. 1984). An alternative to intravaginal sponges is the controlled internal drug release (CIDR) dispenser, which is made with an inert silicone elastomer usually impregnated with natural progesterone (Welch et al. 1984).

The use of either progesterone, fluorogestone acetate, or medroxyprogesterone acetate seems not to affect superovulatory yields (Bartlewski et al. 2015); conversely, the protocol of administration seems to have a determinant effect (Gonzalez-Bulnes et al. 2004b).

Protocols for the administration of progesterone and progestagens aim to exceed the life span of the corpus luteum in the ovary and last for 14–16 days in sheep and goats, respectively. However, plasma concentrations rise during the first 48 h after insertion (Robinson et al. 1967), and, at the end of the treatment, the levels may even be too low for suppressing LH secretion effectively (Kojima et al. 1992) which in turn may lead to inadequate follicular growth with the appearance of persistent large estrogenic follicles (Johnson et al. 1996; Leyva et al. 1998; Viñoles et al. 1999). In superovulatory treatments, the appearance of persistent large follicles has a dramatic negative effects on oocyte and embryo yields (Gonzalez-Bulnes et al. 2004b), and low plasma levels of progesterone/progestagens during the superovulatory treatment could be avoided by the use of two CIDRs/sponges from early onward (Thompson et al. 1990; Dingwall et al. 1994).

However, the use of long-term treatments and high doses has been associated with alterations in final follicle growth (Gonzalez-Bulnes et al. 2005), in patterns of

the LH release (Scaramuzzi et al. 1988; Gordon 1975; Menchaca and Rubianes 2004), in the quality of ovulations (Killian et al. 1985; Gonzalez-Bulnes et al. 2005; Viñoles et al. 2001), and/or in sperm transport and survival in the female reproductive tract (Hawk and Conley 1971). An alternative would be the use of short-term treatments (6-day length), which would avoid the abovementioned shortcomings caused by the use of long-term and high-dose treatments (Ungerfeld and Rubianes 1999; Menchaca and Rubianes 2004; Letelier et al. 2009). However, a 6-day treatment period is shorter than the half-life of a possible corpus luteum in the ovaries; thus it is necessary to apply a single dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or its analogues for inducing regression of the corpus luteum.

6.2.2 Administration of Prostaglandins and Analogues (Prostanoids)

The objective of the administration of $PGF_{2\alpha}$ or its analogue (prostanoids) is to eliminate the corpus luteum and, in consequence, to induce growth of a follicular phase with ovulation (Abecia et al. 2012). Treatments with prostaglandin are therefore only effective in cycling animals with a functional corpus luteum. In association with the short-term treatment with progesterone/progestagens, the goal is to remove the corpus luteum, to allow the appearance of the follicular phase. Treatment with $PGF_{2\alpha}$ alone for estrus synchronization in a group of females requires two injections 9–10 days apart, thereby assuring that nearly all animals will be in midluteal phase at second $PGF_{2\alpha}$ dose and thus will respond with estrus behavior and ovulation. This treatment is effective in synchronizing estrus, but its practical application has been limited by reduction in fertility when compared to progestagen sponges (Killian et al. 1985; Scaramuzzi et al. 1988). However, most of the animals treated at 9–10 days intervals are in the midluteal phase of the estrous cycle, which coincides with a follicular wave with reduced fertility. Treatments during the early luteal phase (achieved by two doses of $PGF_{2\alpha}$ 5–6 days apart) may be an adequate alternative for synchronizing estrus (Contreras-Solis et al. 2009a, b).

$PGF_{2\alpha}$-based treatments were implemented in MOET protocols by Mayorga et al. (2011) who showed that it is possible to produce high enough numbers of transferable embryos during natural estrus induced by $PGF_{2\alpha}$ without the use of progestagen sponges.

6.2.3 Use of Melatonin

The discovery of the melatonin function in photoperiod-dependent breeding animals opened up new ways to control reproduction in these species, by inducing changes in the function of the photoperiod and the annual pattern of reproduction. Administration of melatonin could simulate the females during the reproductive season, but the effectivity of the synchronization obtained by this treatment and efficacy in increasing the superovulation response is still under debate. In fact, it has

been shown (McEvoy et al. 1998) that a melatonin treatment of embryo donor and recipient ewes during anestrus affects their endocrine status, but not the ovulation rate, embryo survival, or pregnancy. On the other hand, Zhang et al. (2013) reported that the number of corpora lutea in ewes with subcutaneous 40 or 80 mg melatonin implants was significantly higher than that in the control group ($p < 0.05$). Similarly, the number of recovered embryos from ewes having received subcutaneous 40 or 80 mg melatonin implants was higher than in the control group ($p < 0.05$). After transfer of embryos collected from 40 to 80 mg melatonin-treated donors, pregnancy and birth rates were significantly increased compared to control ewes.

Melatonin implants inserted 3 months prior to the superovulatory treatment in aged high-prolificacy Rasa Aragonesa ewes (Forcada et al. 2006) did not improve the superovulation rate but was associated with the recovery of embryos with a better viability compared to controls and an increase in the number of blastocysts. These blastocysts were also more viable after cryopreservation. Moreover, a melatonin treatment reduced the number of nonviable (degenerate and retarded) embryos.

6.3 Induction of Superovulation by Exogenous Gonadotrophin Treatments

The superovulatory gonadotrophin treatment aims to increase the number of follicles growing to the preovulatory stage and ultimately yields a higher ovulation rate. Administration of gonadotrophins is concurrent with the last days of a progestative treatment to avoid premature ovulations and to synchronize ovulations. The first gonadotrophin protocols consisted of a single high dose of equine chorionic gonadotrophin (eCG). However, such protocol was associated with high variability and a high inconsistency of the ovulatory response in successive treatments (Cognié 1999).

Superovulatory protocols have mainly been based on multiple doses of FSH, administered twice daily, due to the short half-life of the hormone. A superovulatory treatment induces the growth of a high number of follicles, but the supply of large amounts of exogenous gonadotrophins necessary to achieve a superovulatory response may be associated with detrimental effects. There is evidence showing that the response to superovulatory treatments is associated with alterations in follicular development, oocyte maturation, and/or ovulation failures similar to other ruminants (Rubianes et al. 1997). Thus the number of transferable embryos obtained after a superovulatory treatment can sometimes be disappointingly low (Cognié 1999). The main causes of the decrease in viability of embryos collected from superovulated ewes can be related to alterations in follicular-oocyte competence, changes in the periovulatory and preimplantation endocrine patterns, and decreased intrinsic developmental capacity of the embryos and/or negative effects from the uterine environment (Gonzalez-Bulnes et al. 2004b). Some of these alterations are common to all superovulatory treatments, but some factors like the source of the gonadotrophin preparation, its purity, and the way of administration affect the final outcome.

Source and purity of the gonadotrophin preparations have been identified as main factors affecting the ovulatory response mainly due to the variable LH contents

(Lindsell et al. 1986). Some researchers have employed a recombinant follicle-stimulating hormone agonist (Rutigliano et al. 2014) for avoiding the presence of variable contamination with LH. High LH contents stimulate a higher number of follicles to grow, but such follicles regress during the treatment or are unable to ovulate (Rubianes et al. 1995; González-Bulnes et al. 2000a), which are possibly related to saturation of the LH receptors in theca and/or granulosa cells as described in cattle (Boland et al. 1991). These observations have favored the use of highly purified gonadotrophins, but one has to keep in mind that very low amounts of LH at the end of treatment may also induce lower ovulation rates and a higher incidence of fertilization failures (Picton et al. 1990; Cognié 1999). These aberrations may be reduced by inducing ovulation via appropriate drugs (i.e., GnRH; Menchaca et al. 2010).

The protocol of administration of gonadotrophins is also critical for the ovulatory response. Some protocols use constant dosages instead of decreasing dosage regimens (step-down). However, mean ovulation rate and mean numbers of recovered and viable embryos are usually higher in the step-down approach, which is closer to the physiological situation in which FSH secretion decreases during non-stimulated follicular phases (Gonzalez-Bulnes et al. 2004b). However, administration of high doses of nonphysiological FSH may be associated with the above limitations; the use of lower doses of FSH, although yielding lower ovulatory rates, favors embryo viability and is compatible with the application of repetitive treatments (Bruno-Galarraga et al. 2014).

The complexity of treatments with several dose of FSH has favored research on the use of combined treatments such as single eCG/FSH shots for in vitro embryo production (Gibbons et al. 2007; Forcada et al. 2011). However, this protocol yielded a low number of transferable embryos when applied in vivo (Cueto et al. 2011).

6.4 Individual and Ovarian Factors Affecting Superovulatory Response

The number of transferable embryos obtained after a superovulatory treatment of donor females, in small ruminants like in other species, is characterized by high individual variability, which is actually a limiting factor in MOET programs. The number of transferable embryos is dependent on the follicular growth, the ovulation, and the viability of the embryos collected in response to the hormonal treatment for inducing a superovulatory response (Gonzalez-Bulnes et al. 2004b; Menchaca et al. 2010).

Intensive research activities developed during the past decade have identified several features that affect the ovarian status at the onset of the superovulatory treatment.

Briefly, the ovulation rate is positively related to the number of small gonadotrophin-responsive follicles (2–3 mm in size) at the first gonadotrophin dose, in both sheep (Brebion et al. 1990; González-Bulnes et al. 2000a) and goats (Gonzalez-Bulnes et al. 2003a). However, the total number of embryos and their viability are closer related to the category of follicles 3 mm in size in sheep and 4 mm

in goats; a higher number of smaller follicles usually correlates with more degenerated embryos. This finding may indicate that these follicles can grow and ovulate in response to the gonadotrophin treatment, but are not sufficiently matured to develop into a viable embryo because the recruitment might have required more time to complete maturation prior to exposure to a preovulatory LH surge and ovulation. On the other hand, follicles larger than 3 mm in diameter might be in an adequate stage of development to support growth and release of a healthy oocyte. This hypothesis is supported when considering that such follicles are the main source of estradiol and inhibin A (Gonzalez-Bulnes et al. 2003b, 2004a), which are two well-known markers of the follicular status (Ireland and Roche 1983; Campbell et al. 1995).

The final number of transferable embryos is affected by the presence or absence of a large follicle (Gonzalez-Bulnes et al. 2002a, 2003a). This effect is thought to be related to the dominant effects, since the presence of a large follicle at the first gonadotrophin injection (or two in case of codominance effects; Veiga-Lopez et al. 2006a) determines both the number of corpora lutea and the total number of recovered embryo derived from small follicles, 2–3 mm in diameter (Veiga-Lopez et al. 2005). In the absence of a large follicle, ovulation rate and the number of total embryos are related to the number of follicles 3–5 mm in diameter, suggesting that dominant follicles impair the development of gonadotrophin-dependent follicles (4–5 mm in size). Such dominance effects are primarily systemic, but there are also local effects, exerted by direct action, which are independent from systemic pathways through FSH modulation, on neighboring follicles (Gonzalez-Bulnes and Veiga-Lopez 2008).

Moreover, there is evidence that the presence of large follicles modulates the timing of the preovulatory LH surge and ovulation ultimately inducing a shorter period for final maturation and ovulation of smaller follicles (Veiga-Lopez et al. 2006a, 2008a). Some of the subordinate follicles may even grow to preovulatory size, but ovulation is disturbed or impeded (Veiga-Lopez et al. 2006b). The persistency of these follicles beyond the ovulation period contributes to decreased embryo yields by affecting rates of fertilization and viability in the oocytes from other follicles.

The presence or absence of a functional corpus luteum at the time of superovulation induction has a significant effect on oocyte and embryo yields. In the breeding season, the presence of a corpus luteum (CL) at the beginning of the progestagen treatment and its persistency at the start of a subsequent gonadotrophin treatment affect the final number of transferable embryos, likely by interaction with the dominant follicles. Ewes bearing a CL at the first gonadotrophin injection have a lower rate of degenerated embryos and show fewer deleterious effects resulting from the presence of a dominant follicle (Gonzalez-Bulnes et al. 2002b, 2005).

6.5 Strategies for Selection and Preparation of Donor Females

The effects of the ovarian status on the superovulatory response after the gonadotrophin treatment suggest (for ethical, technical, and economic reasons) the possibility of selecting females in adequate conditions prior to treatment and/or attempting to

defining adequate ovarian status. Moreover, the high individual variability in the response to superovulatory treatments is also associated with a high intraindividual repeatability in response to successive superovulatory treatments (Bari et al. 2001; Ptak et al. 2003; Bruno-Galarraga et al. 2014), which suggest the possibility of applying predictive measures for preselection of ewes with high ovulatory responses.

A predictive evaluation of the superovulatory response may be attempted by evaluating the ovarian status directly by ovarian imaging (ultrasonography) or indirectly by hormonal analyses. The use of high-resolution ultrasonography (probes with a frequency of 7.5 or higher) is useful for determining the presence or absence of large follicles and corpora lutea and the number of gonadotrophin-responsive follicles and their growth during gonadotrophin treatment (Gonzalez-Bulnes et al. 2002c, 2004b). The use of Doppler ultrasonography for examination of follicle blood flow on the final day of the superovulatory treatment appears to be predictive with regard to number and percentage of unfertilized oocytes (Oliveira et al. 2014).

Hormonal assays are a major tool in the evaluation of follicular hormones, including estradiol, inhibin, and, more recently, anti-mullerian hormone (AMH). The high correlation observed between the growth pattern of follicles yielding viable oocytes and the plasma profile inhibin A, rather than with E2, favors inhibin A measurement for surveillance of ovarian functionality in stimulated cycles of sheep (Gonzalez-Bulnes et al. 2002a; Veiga-Lopez et al. 2008b) and goats (Gonzalez-Bulnes et al. 2004c). Measurement of AMH levels is predictive of the follicular pool (Lahoz et al. 2014; Torres-Rovira et al. 2014) and therefore of the ovarian response to FSH stimulation. High numbers of oocytes are collected from lambs with high level of AMH, and after in vitro fertilization and culture development to blastocysts, the number of oocytes is higher from these animals than those derived from lambs with low levels of AMH (McGrice et al. 2016). Moreover, the measurement of AMH in lambs is promising for discriminating high and low responders (Torres-Rovira et al. 2014).

Preselection of ewes with high ovulatory responses may also be performed via exogenous FSH ovarian reserve tests (EFORTs). EFORTs are based on the administration of a single-shot treatment and the evaluation of subsequent follicular development (Torres-Rovira et al. 2014). A single eCG dose has been reported to be a useful tool to discriminate populations of prolific carriers from populations of non-prolific carriers in adult ewes (Kelly et al. 1983) and in prepubertal ewe lambs (Davis and Johnstone 1985; Gootwine et al. 1989, 1993). The use of a single-shot FSH/eCG is practical and cost-efficient for choosing donors with putatively high ovarian responses for a cost-efficient eCG treatment (Bruno-Galarraga et al. 2015).

Another approach for optimizing superovulatory yields in a group of females is the preparation of adequate ovarian conditions as determined by the presence of corpora lutea, the absence of large follicles, and/or a high number of gonadotrophin-responsive follicles. The presence of corpora lutea may be induced by pre-synchronization of the cycle with two prostaglandin doses and starting the progestagen treatment in the early luteal phase. The follicular status may be modified via direct ablation of the follicle (Gonzalez-Bulnes et al. 2004b), or the use of

Day-0 protocol, in which the superovulatory treatments initiated soon after the previous ovulation (Rubianes and Menchaca 2003) and/or by using GnRH analogues.

Administration of the GnRH antagonist or agonist analogues has a double effect and eliminates a dominant follicle and in parallel increases the recruitment of gonadotrophin-responsive follicles. Treatment with a GnRH agonist suppresses secretion of LH pulses during treatment, after an initial short stimulatory "flare effect," and thereby blocks follicle development beyond 3 mm (McNeilly and Fraser 1987). On the other hand, GnRH antagonists produce an immediate effect, without a desensitization period, by competitive blockade of the GnRH receptors, causing a rapid decline of FSH and LH levels in serum, and the loss of follicles larger than

Table 6.1 Hormonal treatments management of reproductive activity and control of the ovarian cycle in donor and recipient females

Compound	Donor/ recipient	Aim	Effect	Administration
Progesterone or analogues (progestagens)	Both	Induction and synchronization of ovulations and estrous cycles	To simulate endogenous corpus luteum	CIDR or intravaginal sponges
Prostaglandins or analogues (prostanoids)	Both	Induction and synchronization of ovulations and estrous cycles	To cause lysis of endogenous corpus luteum	Intramuscular injection (single/ double dose)
Melatonin	Both	Induction of breeding season activity	To raise melatonin levels for mimicking reproductive season	Subcutaneous implants
Equine chorionic gonadotrophin (eCG)	Both	Induction of follicular growth and ovulation	To raise endogenous levels of FSH and LH	Intramuscular injection (single dose)
Follicle-stimulating hormone (FSH)	Donor	Induction of follicular growth	To raise endogenous levels of FSH	Intramuscular injections (multiple dose)
FSH/eCG	Donor	Induction of follicular growth	To raise endogenous levels of FSH	Intramuscular injection (single dose)
GnRH analogues (agonists or antagonists)	Donor	Suppression of dominant follicles and stimulation of follicular growth	To decrease endogenous levels of FSH and mainly LH	Intramuscular injections (multiple dose)
GnRH or GnRH agonist	Donor	Induction of ovulation	To increase endogenous levels of LH	Intramuscular injection (single dose)
LH	Donor	Induction of ovulation	To increase endogenous levels of LH	Intramuscular injection (single dose)

3 mm in ewes (Campbell et al. 1998). A GnRH antagonist pretreatment is a good option to increase efficiency of superovulatory protocols in sheep (Brebion et al. 1990; Cognié 1999; Cognié et al. 2003) and goats (Cognié et al. 2003; Gonzalez-Bulnes et al. 2004d), albeit daily doses of GnRH represent a time-consuming procedure. In contrast, injection of a single dose of 1.5 mg of GnRH antagonist in sheep suppresses the effects of follicular dominance, thus allowing a significant increase (usually more than twofold) in the mean number of gonadotrophin-responsive follicles 2–3 mm in size, which grow to preovulatory size in response to the administration of exogenous FSH (Lopez-Alonso et al. 2005a, b). However, negative consequences of a high number of smaller follicles (2–3 mm) on oocyte maturation and fertilization and degeneration rates must be taken into account (Cognié et al. 2003; Gonzalez-Bulnes et al. 2004c, Gonzalez-Añover et al. 2004; Berlinguer et al. 2006) (Table 6.1).

6.6 Embryo Recovery and Transfer: Surgical and Nonsurgical Methods

Currently, embryo collection and transfer in small ruminants can be performed by surgical (Loi et al. 1998; Lehloenya and Greyling 2009; Torres and Sevellec 1987; Bruno-Galarraga et al. 2014), laparoscopic (McKelvey et al. 1986; Flores-Foxworth et al. 1992), or transcervical methods (Nagashima et al. 1987; Pereira et al. 1998; Fonseca et al. 2013, 2014).

Laparotomy—Surgical techniques for embryo collection and transfer in small ruminants, with exposure of the reproductive tract, are now used on a global scale. Laparotomy allows exact counting of the number of corpora lutea and visual inspection of the reproductive organs. The percentage of embryos recovered with this technique ranges between 40% and 80% according to the flushing methodology used, volume and number of washing attempts of the uterine horns, and operator skills and experience. Since the 1930s, when the first experiments were performed (Warwick et al. 1934), it remained in use even though it implies more risk for the treated animals as it requires general anesthesia, which in turn involves the need for animal fasting, drug administration, and surgical intervention. A consequence of this approach is that the donor can develop adhesions that can involve the ovaries, oviducts, and uterus and usually is associated with reduction in embryo recovery rates (Lehloenya and Greyling 2009). Hence, the number of collections per female is usually limited to two or three (Torres and Sevellec 1987). Other limitations are the relatively high costs of equipment and the stress of the animals due the manipulation of the exteriorized reproductive tract. Transfer of embryos by surgical laparotomy follows the same procedure used for collection: the embryos (generally one or two per recipient if in vivo-produced embryos are used, or higher numbers if in vitro-produced embryos are employed) are transferred in the exposed uterine tract with the aid of a small catheter (Tomcat catheter or similar) into the uterine horn, 2–3 cm from the uterine-oviductal junction.

Laparoscopy—Laparoscopy is an alternative technique to recover and transfer embryos from goats and sheep. It leads to fewer adhesions, and, therefore, a donor could be repeatedly used for collections for up to seven times (Flores-Foxworth et al. 1992). However, this method still requires special equipment and skilled personnel. Regardless of the good efficiency (Schiewe et al. 1984), this technique has not been extensively adopted worldwide (Schiewe et al. 1984). Limiting factors are the required refined ability of operators associated with the relatively expensive equipment to perform embryo recoveries. Both laparoscopic and laparotomy techniques are associated with prolonged fasting of donors that are usually maintained under general anesthesia. Transfer of embryos can be also performed by endoscopical examination of the uterine horn (to the horn ipsilateral to the corpus luteum). Embryos are placed in an insemination straw (0.25 ml) and are inserted in a modified insemination gun equipped in the terminal part with an 18 needle that, after penetrating the uterine wall, can facilitate the release of the embryos inside the uterine lumen.

Transcervical procedures—Recovery of embryos from sheep and goats by nonsurgical procedures (NSER) has been developed in the 1980s (Lin et al. 1979) and recently has received renewed interest. The technique is less invasive and needs a simpler anesthetic protocol (epidural block and local cervical anesthesia) than laparotomy and laparoscopy. Moreover, animals may remain in a standing position under sedation. Nonsurgical collection and transfer have been reported first in goats, in which, due to the anatomical configuration of the reproductive tract, it is easier to pass the cervical plicas compared to sheep. When using the nonsurgical technique, the cervix is clipped with nontraumatic forceps that allow traction, and a catheter is inserted through the cervix to reach the desired uterine horn (Fonseca et al. 2014). A different type of catheter can be used (usually with one or two ways) to flush the reproductive tract with different volumes of collection medium. Recovery rates range from 60% to 100%, depending on the animal and the operator skills, and are not very different from the rates obtained by laparotomy or laparoscopy. Future studies have to reveal if the technique can be used in different breeds of small ruminants and, in particular, in animals with reduced size and weight.

The absence of adhesions when using NSER is a major advantage and allows successive collections in contrast to laparotomy or laparoscopy. On the other hand, the difficulty of introducing a catheter through the cervix, mainly in sheep, and the missing option of rectal manipulation of the tract are main obstacles of NSER procedures. Transfer of embryos is performed in a similar way and a device containing the embryo is coupled to a catheter to release the embryo into the uterus. A comparison revealed that nonsurgical embryo transfer usually results in a recovery rate similar to that of surgical techniques (Fonseca et al. 2014; Zambrini et al. 2014, 2015) with pregnancy and birth rates of around 50% (Fonseca et al. 2014). Studies with higher numbers of animals could show if the technique can be used successfully in different breeds, in particular, in animals with reduced size and weight (Table 6.2).

Table 6.2 Methods of embryo recovery in sheep and goats and their efficiency

Method	Required anesthesia	Repeatability	Amount of flushed medium (ml)	Range of recovered embryos	Embryos collected/N. ovulations	Average embryos recovered
Laparotomy	Yes	1–4 times	40–60	0–30	40–100	5–12
Laparoscopy	Yes	1–8 times	40–60	0–15	0–85	3–8
Transcervical	No	1–>15 times	40–1200	0–18	0–90	4–10

6.7 Factors That Can Be Relevant for Pregnancy Success

The full realization of the potential of embryo transfer procedures in small ruminants depends on optimizing the number of progeny born from females with high genetic merits. Pregnancy rates after ET in sheep and goats vary from 29% to 75% and are affected by synchronization protocol and superovulatory response but are also directly related to the viability of the transferred embryos.

Several factors play a critical role in determining embryonic and fetal losses in the ewe. Bolet et al. (1986) suggested that these losses could be caused by at least three components: (a) paternal influences, related to the quality of semen; (b) the female, due to the quality of the ova and uterine environment; or (c) the embryo itself. There are conflicting reports on embryo survival in sheep ranging from unaffected survival rates (Armstrong and Evans 1983) to increased (Cseh and Seregi 1993) or decreased survival rates (Mutiga 1991).

Several other factors need to be included: the maternal effect of the recipient, the number and the developmental stage of the embryos that were transferred, and the method of embryo production (e.g., in vivo versus in vitro). Eventually, embryo storage and its manipulation could affect the success of as well the age of donors and culture conditions for in vitro-produced embryos (Thompson et al. 1995; Holm et al. 1996; Ptak et al. 1999; Dattena et al. 2000; Naitana et al. 1996).

6.7.1 Maternal Effect

Factors related to both embryos and recipients have been suggested to affect survival of the transferred embryos in sheep and goats, including the stage of embryo development, embryo quality, the number of corpora lutea, and age and parity of the recipients (Donaldson 1985; Alabart et al., 1995; Thompson et al. 1995; Armstrong and Evans 1983).

The term “maternal effect” indicates an influence of the dam on its offspring, either from genetic or environmental causes. Embryo transfer technology enables experimental investigation of embryo-maternal communication providing unique opportunities to study the genetic control of embryonic survival and growth.

Among the possible factors, progesterone levels have been found to play a vital role in early embryo development, implantation, and establishment of pregnancy. The plasma progesterone concentration in recipient animals is related to the number of ovulations or corpora lutea in sheep (Ashworth et al. 1989). While in goats embryo survival usually is higher with increased numbers of corpora lutea (Armstrong and Evans 1983) and plasma progesterone concentrations, little information is available for sheep. We have observed that pregnancy rates after embryo transfer were higher in ewes with more than one CL compared to animals with only one CL (data not published—personal observation). On the other hand, it is known that at least one embryo must be present in the uterine lumen by day 12.5 post-estrus to prevent luteolysis (Moore 1985). The bidirectional communication between endometrium and embryos is critical to determine the role of the uterine environment. In a large-scale study with records from 11,369 animals, the effects of age, weight, and sire on embryo and fetal survival in sheep were investigated (Shorten et al. 2013). The author concluded that, from a genetic point of view, the dam's ability to maintain a pregnancy is significantly higher than the effects of embryo competence. Therefore, a selection of dams based on their maternal performance could provide effective means to improve embryonic survival.

Moreover, Cumming et al. (1975) reported that embryonic survival from breeding to days 26–30 was greater in crossbred than in Merino twin-ovulating ewes, but did not differ between breeds of single-ovulating ewes. Naqvi et al. (2006, 2007) investigated developmental competence, birth, and survival of Garole (small-sized) lambs after transfer of two or three embryos into large-sized 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 higher-body-sized non-prolific sheep, which provided more space for embryo development than small-sized Garole ewes. They also observed that the monotocous character of recipients was not a limiting factor for pregnancy success when two or three embryos had been transferred.

6.7.2 Number of Embryos Transferred

There is an economic incentive on transferring multiple embryos to reduce the number of recipient ewes or does. However, Anderson et al. (1979) reported that uterine crowding can cause an increased frequency of pregnancy losses in nulliparous recipients receiving more than one embryo.

In sheep, embryonic and fetal mortality leads to large economic losses. Embryonic and fetal losses are estimated to 30% (Bolet et al. 1986). Most embryonic losses have been reported to occur before day 18 (Hulet et al. 1956; Moore et al. 1960; Quinlivan 1966). Complete losses from day 18 to lambing were estimated to 9.4% (Hulet et al. 1956), and fetal losses from day 30 to term were only 1–5% (Quinlivan 1966). More recently O'Connell et al. (2016) reported that embryo loss mainly occurred prior to day 14 of gestation with 6% losses before day 4 and 12% loss between days 4 and 14 of gestation. It has been reported that embryonic losses increased with an increasing ovulation rate (Kleemann and Walker 2005).

Naqvi et al. (2007) found that the incidence of embryonic mortality up to day 40 of gestation was reduced when the number of transferred embryos had been increased. Embryo survival up to 40 days of gestation and up to term was 38.1% when three embryos had been transferred per ewe which was higher than after transfer of two embryos per ewe (28.6%). In the same study, all embryos were transferred to the ipsilateral uterine horn. It has also been reported that transfer of two embryos to the ipsilateral or both uterine horns does not influence survival of the embryos (Torres and Sevellec 1987), due to migration of embryos during early stages of development. A higher pregnancy rate of 55.2% has been reported in Hungarian Merino ewes following transfer of two embryos per recipient, compared to 45.6% in case of single-embryo transfers (Cseh and Seregi 1993). Mutiga (1991) reported that transfers of multiple embryos in tropical sheep increased the number of lambs born per pregnant ewes. Pregnancy rate was significantly higher after transfer of embryos pairs (64%) than single (39%) embryos in in vitro-produced embryos (Brown and Radziewic 1998). Contrary to this result, data from embryo transfer studies (Land and Wilmut 1977) have shown that doubling the number of embryos transferred resulted in a decrease of the number of lambs born.

Armstrong and Evans (1983) indicated that embryo survival can be observed in twins when embryos had been placed into the same oviduct, which suggests that synergism between embryos influences each other's survival upon transfer in the goat. A possible explanation for such cooperation includes enhanced luteotrophic or anti-luteolytic gender actions resulting in improved luteal maintenance in recipients or enhanced signaling to the endometrium involved in the process of implantation (placental attachment). Whatever the explanation, the finding has important implications by enabling the embryo-carrying capacity of the recipient pool of goats to be doubled.

In addition, transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-t and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis.

6.7.3 Development Stage and Grade of Embryos

Morphological evaluation of the developmental stage takes into account age and quality of the embryo. Embryonic stages and quality are usually based on the descriptions published by the International Embryo Transfer Society (IETS) (Stringfellow and Seidel 1998).

In embryo transfer, programs in small ruminants and cattle, higher fertility rates were obtained when the transferred embryos were in a more advanced development stage (Alabart et al. 2003). These findings agree with previous work conducted by Moore and Shelton (1962) in which an increased embryonic survival was observed with an increased age of the transferred embryos.

Embryo age largely corresponds to the stage of development. Based on a large number of fresh in vivo-derived embryo transfers, it was shown that

embryonic stages ranging from late morulae to expanded blastocysts result in comparable pregnancy rates, whereas after hatching lower pregnancy rates can be expected (Hasler 1998). It has also been reported that when the embryos were recovered at early stages, in vitro embryo culture until the blastocyst stage might provide advantages over traditional protocols by allowing transfer of embryos into a synchronized uterine environment. Moreover, during culture, there is the possibility to select only those embryos that have demonstrated the potential for continued development under embryonic genomic control (Johnson et al. 2007).

Transfer or recovery of embryos has been also performed by transferring at early stages (2–3.5 days post fertilization) with acceptable results (Alabart et al. 2003). Technically the method implies transfers into the oviduct when the embryos are prior to the 8-cell stage and transfers to the uterus with embryos beyond the 8-/16-cell stage. Practical advantages are usually not associated with embryo transfers at early stages, and viability is not changed compared to later embryonic stages (Ishwar and Menon 1996). In contrast, as IVP embryos are more stage sensitive than are in vivo-derived embryos, higher conception rates were achieved following transfer of expanded blastocysts compared to morulae or earlier stages (Lamb 2005; Naitana et al. 1996).

A correlation between embryo morphology and pregnancy rates has been discussed for many years (Steer et al. 1992). Within each embryonic stage, morphological quality is also closely associated with pregnancy rate, as reported in a number of studies (Donaldson 1985; Hasler 2001; Lindner and Wright 1983). Farin et al. (1995) showed that agreement among six experienced embryo evaluators was higher for in vivo-derived embryos compared to their in vitro-derived counterparts. In addition, there was a relatively high degree of agreement when evaluating excellent and degenerated (poor) embryos, but a lower agreement relative to good and fair embryo viability categories (Lindner and Wright 1983).

Although many comprehensive morphological descriptions have been published (Shea 1981; Lindner and Wright 1983), individual variation in embryo grades and quality ratings is still prevalent. It is not surprising that the individual embryologist is found to account for significant variation in the embryo's quality grading. Conversely, the embryologist has less influence on the developmental scores, suggesting that this trait is easier to describe. Quality evaluation is further hampered by loose or degenerate cells in the embryo that are often more difficult to see in the blastocyst than in the morula. No practical method to replace the visual morphological scoring method has been found so far (Betteridge and Rieger 1993). Evaluation of embryo quality is even more challenging when embryos have been produced in vitro. Timing of development is considered as predictive marker of embryo quality in these embryos. Reduced viability due to poorer quality grade resulted in slower rates of development (Walker et al. 1996; Leoni et al. 2007) and reduced pregnancy rates after transfer (Shea 1981; Lindner and Wright 1983; Hasler 1998) (Fig. 6.1).

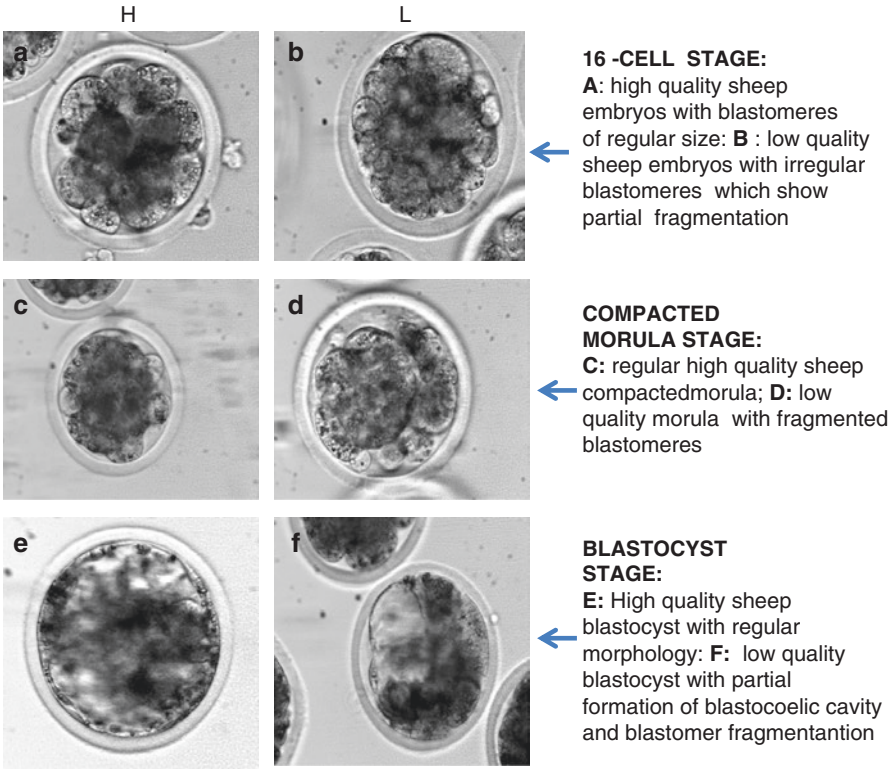

Fig. 6.1 Morphological evaluation of high-quality (H) and low-quality (L) embryos recovered from superovulated ewes. *Cell stage*: (**a**) High quality sheep embryos with blastomeres of regular size: (**b**) low quality sheep embryos with irregular blastomeres which show partial fragmentation. Compacted morula stage: (**c**) regular high quality sheep compacted morula; (**d**) low quality morula with fragmented blastomeres. Blastocyst stage: (**e**) High quality sheep blastocyst with regular morphology: (**f**) low quality blastocyst with partial formation of blastocoelic cavity and blastomer fragmentantion

6.7.4 Donor Effect on Embryo Quality

A large proportion of the variability in embryo development and quality has been attributed to the donor animal. The background for this variation cannot be fully explained, as indicated by the relatively low repeatability for both embryo stage and quality grade in the bovine (Callesen et al. 1995). Probably, donor hormone levels during the preovulatory period may affect fertilization and early embryonic development. In cattle the causes of the variation between donors (i.e., donor breed and parity, insemination bull, year and season) were insufficient and could only partially explain the variability (Callesen et al. 1995). With regard to gonadotrophin regimes, embryo quality seems closely related to the type and dosage of the stimulating

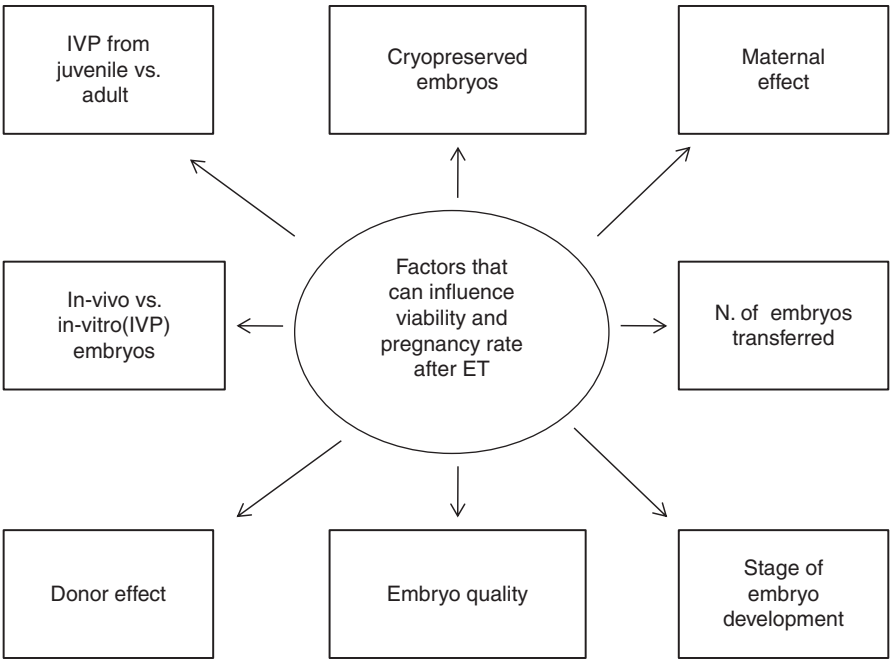

Fig. 6.2 Factors that can be relevant for pregnancy success

hormones during the superovulatory treatment. It has been shown that different FSH regimes can affect the developmental capacity and cryotolerance of ovine embryos derived from oocytes collected by ovum pick-up of donor sheep (Berlinguer et al. 2004). In particular the dosage regimes of FSH influence the developmental capacity of recovered oocytes to develop to blastocysts in vitro and their cryotolerance after vitrification procedures. Embryo quality is also significantly affected by the stimulating hormones during the superovulatory treatment. Often a high superovulatory response is followed by reduced fertilization rates and reduced embryo quality. A superovulatory treatment of ewes with eCG/FSH increased the ovarian responses compared with FSH alone, but the embryos showed reduced viability rates after vitrification (Leoni et al. 2001) (Fig. 6.2).

6.7.5 Source of Embryos: In Vivo vs. In Vitro Embryos

One of the solutions to overcome the relatively low efficiency of MOET programs is to produce and transfer in vitro-produced embryos (IVEP). The IVEP procedure does not require superovulation because oocytes are recovered directly from the follicle in hormonally or unstimulated females. IVEP can also be used in non-fertile females and pregnant, lactating, and even slaughtered females. Moreover, embryos can be produced in vitro from oocytes of prepubertal females with a technology

called "juvenile in vitro embryo transfer" (JIVET) that is compatible with reduced generation intervals and concomitantly increased genetic gain. Thus, in a JIVET scheme using oocytes obtained from 3- to 4-week-old females, it is possible to increase the rate of genetic gain by approximately 5% (reviewed by Morton 2008). In practical terms, animals of high genetic merit are selected, and their oocytes are collected from live individuals (adult or juvenile) by surgical procedures or from slaughtered animals. These techniques are compatible with the production of a high number of cheap embryos, but several limitations currently prevent a more widespread application.

The collection of cumulus-oocyte complex (COCs) from living small ruminants implies a laparotomy or laparoscopy. COCs collection via laparotomy would prevent the reuse of the same donors in repeated collections, while oocyte collection can be performed repeatedly by laparoscopy ovum pick-up (LOPU) in the same animals. Furthermore, several studies indicate that the viability of in vitro-produced embryos in small ruminants is lower compared to their in vivo-produced counterparts (Cognié et al. 2003, 2004). This low viability is observed irrespective of the embryonic stage, age of donors, and technique used to obtain the oocytes (from slaughterhouse or LOPU).

6.7.6 IVP from Adult Animals

IVP embryos are usually produced from oocytes collected from slaughtered animals or from in vivo by laparoscopy and oocyte ovum pick-up (LOPU). These oocytes can be in vitro matured, fertilized, and cultured up to blastocysts. Success rates are high in all these steps with rates of IVM and cleavage being around 90% and 75%, respectively, and blastocyst rates at 30–50%, to some extent dependent on age, genetic background, nutritional management, and culture conditions. Comparative studies in sheep and goats have shown that the two species can generate embryos with similar rates of development and viability (Cox and Alfaro 2007). Hormonally stimulated ewes and goats have been subjected nine to ten times to oocyte collection by laparoscopic-guided follicular puncture. The success rates after IVM, IVF, and IVC were similar to those obtained with oocytes derived from abattoir ovaries.

Similar results have been found by Cocero et al. (2011) who showed that the development of IVP embryos up to the blastocyst stage was not different between slaughterhouse and laparoscopic ovum pick-up-derived oocytes in sheep. In goats, oocytes derived from abattoir ovaries had different oocyte maturation kinetics and a higher percentage of development up to the blastocyst stage compared to oocytes isolated laparoscopic ovum pick-up. No differences were observed in the number of blastocysts per cleaved IVP embryos (Souza-Fabjan et al. 2014). Differences have been observed when oocytes were recovered by LOPU after different stimulation regimens that can influence the quality of the derived oocytes and subsequent development in vitro (Berlinguer et al. 2004). At present, the percentage of blastocysts that can be produced from adult sheep ranges between 30% and 60% with pregnancy rates of 30–60% which is lower than after transfer of in vivo-produced

embryos (60–80%). The reduced viability of in vitro-produced embryos becomes also more evident if the number of embryos transferred to recipients that can develop to term is considered. In fact, ewes that receive two or more IVP embryos often develop a single pregnancy and yield one offspring and only rarely carry twins (Papadopoulos et al. 2002). Similar data have been reported by Dattena et al. (2000) which showed a lambing rate from in vitro-produced and freshly transferred embryos of 40% (20 lambs/50 blastocysts transferred), which was significantly lower when compared to the 81.2% of in vivo-derived blastocysts (32 transferred fresh, 26 lambs born).

This reduced viability leads to embryonic losses mainly at 20–25 days of gestation, while prior to this there is no a significant reduction in the development of transferred embryos (14- and 25-day-old embryos, personal observation). The elevated embryonic and fetal losses of IVP embryos in this period could be related to alterations in angiogenesis in IVP embryos compared to in vivo embryos (Reynolds et al. 2015). An aberrant placental angiogenesis is thought to interfere with embryonic and fetal development. An increase in fetal weight is observed in IVP embryos, and consequently the reduced trophic supply of the altered placenta can interfere with regular conceptus development.

6.7.7 IVP Embryos from Juvenile Donors

The use of juvenile donors in embryo transfer (ET) programs offers considerable potential for accelerated genetic gain in domestic livestock through reduced generation intervals. This possibility has been investigated in the last years and factors such as donor selection, oocyte collection methods, and hormone stimulation methods designed to produce maximum yields of viable oocytes for young age donors have been studied. Overall the rates of juvenile ovine IVP embryos are significantly lower compared to embryos derived from adult dams and far away from that of in vivo-recovered embryos. Due to the presence of a large population of antral follicles, high numbers of oocytes can be collected from each donor, with the possibility to generate an average of eight to ten pregnancies from 6–8-week-old lambs (Armstrong et al. 1997).

The production of viable IVP embryos has become possible in younger lambs (4 weeks old) subjected to different hormonal stimulations (Ledda et al. 1997; Ptak et al. 1999). Results indicate that the number of oocytes recovered increased in lambs stimulated by hormones, while blastocyst quality seemed to be equivalent in hormonally treated and non-stimulated animals. Overall, results confirmed the reduced viability of embryos derived from juvenile animals with increased fetal losses after embryo transfer. The reduced viability seems to be related to morphological and metabolic changes observed in oocytes derived from prepubertal animals compared to their adult counterparts (Ledda et al. 2001; Leoni et al. 2015). Similar results have been observed in goats. Comparing the IVP embryos from prepubertal and adult animals, the developmental competence was primarily related to

the size of follicles and oocyte diameter. Thus, oocytes derived from the largest follicle had a diameter comparable to that of adult oocytes performed in a similar way when subjected to IVM, IVF, and IVC (Paramio and Izquierdo 2014; Romaguera et al. 2011). To increase the efficiency of IVP, embryos derived from prepubertal animals, research has been undertaken to optimize donor selection and hormonal stimulation methods to reduce the variability and increase the proportion of donors responding to hormonal stimulation and to increase oocyte developmental competence. Recent improvements to JIVET, resulting from a modified hormonal stimulation regime, have eliminated the failure of donors to respond to hormonal stimulation and increased both number and developmental competence of oocytes harvested from very young prepubertal lambs (Kelly et al. 2005). This increased efficiency has facilitated incorporation of other reproductive technologies such as sperm sexing with JIVET, resulting in the birth of lambs of predetermined sex from prepubertal lambs (Morton 2008).

To respond to the increasing interest in the generation of embryos from juvenile donors, several other strategies have been explored to improve the efficiency of the JIVET system. As the technique is based on the large number of developmentally competent oocytes collected per single animal, predictive markers of the potential follicular population have been investigated, to select the best responding animals for hormonal stimulation. The concentration of AMH in lambs during the first weeks after parturition has been found to be a good predictive marker of the antral follicle population (Torres-Rovira et al. 2014; Kelly et al. 2016) that correlates well with success to hormonal stimulation. Lambs that were 3 weeks of age with high level of AMH yielded the largest number of oocytes with highest development to blastocyst when cultured in vitro. The number of antral follicles in prepubertal ewes is affected by specific gestational environmental conditions. The proportion of blastocysts calculated, as a percentage of cleaved embryos from total cumulus-oocyte complexes collected, was higher ($p < 0.05$) in females born with a female co-twin compared with those born with a male co-twin. These results indicate an enhancing effect of the female co-twin on oocyte development. Taking this into consideration could allow to selecting lambs for a JIVET program based on litter size and sex of the co-twin. In prepubertal goats and sheep, the possibility to select oocytes with high developmental competence prior to maturation has been investigated by noninvasive systems. Immature oocytes from adult and prepubertal donors can be differentially stained by Brilliant Cresyl Blue (BCB) which indicates differences in glucose-6-phosphate dehydrogenase (G6PDH) activity. The G6PDH amount is higher in growing oocytes, while it is low in fully grown oocytes, which are the ones that most frequently yield viable offspring. Oocytes selected by the BCB stain produced more blastocysts in vitro. The blastocysts were also of better quality compared to the pool of unselected oocytes. However, due to different staining and technical protocols, the BCB approach needs to be validated before it can be used for evaluating developmental capacity of ovine and capacity oocytes (Opiela and Kątska-Książkiewicz 2013) (Fig. 6.3).

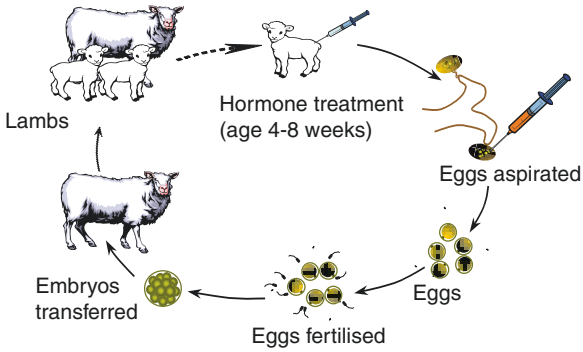

Fig. 6.3 Workflow of in vitro embryo production (IVP) form juvenile donors and embryo transfer (JIVET)

The figure was kindly provided by J. Kelly

6.8 Embryo Cryopreservation and Transfer

A successful MOET program usually includes the possibility for freezing the embryos prior to transfer to synchronized recipients. Cryopreservation has become an integral part of the commercial embryo transfer industry, but application in small ruminants is based on relatively few studies (Boundy et al. 1985; Ishwar and Menon 1996), and the freezing process needs to be constantly improved and simplified (McGinnis et al. 1993; Vajta 2000). From the practical viewpoint, embryo freezing has many advantages: (1) freezing of embryos obtained from females with high genetic value facilitates distribution of superior genetics from dams, which accelerates the rate of genetic improvement; (2) embryo cryopreservation facilitates international trade of valuable genetic stock which is a financially feasible and safe alternative to live animal transport. Data on the success rates of embryo freezing protocols in small ruminants are relatively scarce compared to cattle. The first lambs from frozen/thawed embryos were born in 1976 (Willadsen et al. 1976), and lambs from vitrified embryos were born in 1990 (Széll et al. 1990).

Slow-freezing protocols require a biological freezer and need more time to be completed. The ultra-rapid technique, such as vitrification, is time and cost effective, since it does not require any special equipment, and is, therefore, well adapted to routine field use (Baril et al. 2001). Sheep and goat embryos are able to survive both slow-freezing and vitrification procedures (Martinez et al. 1998). Comparisons between the different techniques are mainly based on lambing rates after embryo transfer. However, selection of embryos for transfer is based on the stereomicroscopic evaluation of embryo morphology after thawing (Abe et al. 2002) in accordance to the guidelines of the International Embryo Transfer Society (Stringfellow and Seidel 1998).

This selection step can be somewhat subjective as has been demonstrated by ultrastructural studies of vitrified in vitro- and in vivo-produced bovine blastocysts (Vajta 2000) and in controlled slow frozen in vivo-produced ovine morulae and

blastocysts (Cocero et al. 2002), which have shown that certain abnormalities remain undetected in the stereomicroscopic analysis.

Vitrification of embryos is most likely the technique that will be used in the future (Fahy and Rall 2007) and different devices and systems have been proposed, varying with regard to type and concentrations of the cryoprotectant. The 0.25 ml straw (Naitana et al. 1997) or the open-pulled straw (OPS) has been used for successful freezing of ovine morulae and blastocysts produced in vivo (Baril et al. 2001; Dattena et al. 2004; Martinez et al. 2006) or in vitro (Dattena et al. 2004).

Baril et al. (2001) reported a 50% embryo survival rate and a high pregnancy rate to term (72%) after direct transfer of vitrified ovine embryos which was similar to the results to the stepwise dilution method (72% and 60%, respectively). No differences were found between vitrified embryos transferred after in vitro removal of the cryoprotectant or directly after thawing in terms of lambing (67% vs. 75%, respectively) and embryo survival rates (lambs born/embryos transferred; 49% vs. 53%, respectively). However, the viability depends on the origin of embryos, and differences were found in the survival rates between embryos produced in vivo and embryos derived from IVP techniques. In fact, the viability is not significantly reduced after freezing of in vivo-produced embryos (70–90%), which it is significantly lower for cryopreserved IVP embryos (30–40%). Post-thaw viability is also reduced in IVP embryos generated from prepubertal oocytes.

6.9 Recipient Females

Transfer of the embryos to suitable recipients is the final step in a MOET program. The conditions of the recipient females (breed, age, nutrition and health, and reproductive status) were described as main limiting factors for the success of embryo transfer programs (Moore et al. 1959). Other variables include the aptitude of the recipient to maintain the pregnancy and the degree of synchronization between donors and recipients (Rowson and Moor 1966). Nowadays the selection of the most suitable recipients remains critical, and finding reliable criteria for ultimate recipient is a major focus of research. Selection of recipients is primarily based on direct observations either by laparoscopy or laparotomy of the corpora lutea (size, number, and vascularization of the structure) and the evaluation of uterine tone and morphology (Torres and Sevellec 1987). These approaches have the limitation of an invasive handling, which may interfere with pregnancy rates.

Alternatively the corpora lutea can be evaluated by transrectal ultrasonography, which allows to visualizing if the recipient has ovulated and evaluation of the quality of the luteal tissue, since morphological and echogenic characteristics of the corpus luteum are related to concentrations of progesterone in plasma and are reliable factors for determining luteal function in small ruminants (González-Bulnes et al. 2000b).

Conclusion

The current state of the art in multiple ovulation and embryo transfer (MOET) technology in small ruminants is steadily improving and could become one of the most applicable tools for the development of a future-oriented genetic program. New findings on the follicular wave patterns in small ruminants, the elucidation of follicular dominance, and the integration of this information into superovulation treatments are instrumental in predicting good responders and reducing variability. Protocols that control follicular dominance have been designed to allow the initiation of superstimulation precisely at the beginning of a follicular wave. These new approaches are based on the pretreatment with a gonadotrophin-releasing hormone (GnRH) antagonist prior to the FSH treatments to avoid follicular dominance and the administration of somatotropin or melatonin to improve oocyte quality and competence. These protocols will provide a rather homogeneous pool of small follicles that are gonadotrophin responsive, thereby enhancing the superovulatory response and embryo yields with a reduction of the incidence of unovulated follicles and early regression of corpora lutea. Significant improvements in the development of nonsurgical techniques are paving the way to reducing stress and costs of donors and recipient management, indicating the possible repeated use of individual donors. In addition, the progress with IVP embryos generated from adult and juvenile animals, combined with the genomic analysis of economically productive tracts, is opening new perspectives and could be instrumental for improving MOET programs in small ruminants.

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