Chapter 1

Cryopreservation of Sheep Produced Embryos – Current and Future Perspectives

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Abstract

Due to economical and scientific limitations, sheep embryo reproductive technologies are less commercially applied than in other animal species. However, it is very clear that, in the near future, those techniques are expected to have a central role in animal production as a consequence of genetic and reproductive demands. One drawback is that results obtained after sheep embryo cryopreservation are unattractive for commercial purposes. It is expected that a successful cryopreservation of sheep embryos can push forward all other reproductive biotechnologies in this species, such as multiple ovulation and embryo transfer (MOET), artificial insemination, or *in vitro* production of embryos. This paper tries to discuss the current and future perspectives of cryopreservation of *in vivo*- and *in vitro*-produced sheep embryos concerning advantages and limitations for its practical use and possible solutions for improving methods to allow a higher survival rate of cryopreserved embryos.

Keywords: Sheep, *in vitro*-produced embryos, *in vivo*-produced embryos, cryopreservation

1. Introduction

In the last decade, the production of sheep embryos did not get the improvement researchers would like to announce, and its application is still lower compared to other species [1,2]. It is very clear, however, that these techniques will have a central role in animal production in the next few years as a consequence of genetic and reproductive challenges. In the agricultural context, their use will also follow the needs of a growing demand for agricultural products from the world emerging economies [3].

One of the constraints in this area is sheep embryo cryopreservation. Cryopreservation is of primordial importance to preserve embryos so they can be maintained as genetic reserves or for field application. The use of cryopreserved embryos can facilitate work planning, eliminate distance limitations, and especially reduce sanitary risks [4,5]. Although attempts have been made to understand the effects of freezing on sheep embryos since 1976 [6,7], the results are less than desirable [1,8]. A successful cryopreservation of sheep embryos could push forward the other reproductive biotechnologies in this species, such as MOET, artificial insemination (AI), or *in vitro* production (IVP) of embryos. These latter associated techniques are still expensive, consequently limiting their widespread. However, the improvement in the number of produced embryos as well as the number of cryopreserved embryos resulting in lambs after transfer could increase their use by technicians and farmers.

In sheep, as in other species, embryos may be *in vivo* or *in vitro* produced (IVP). *In vivo* embryo production consists of collecting them directly from the uterus either after natural service or after AI, whereas IVP involves oocyte collection from sheep ovaries followed by laboratorial maturation and fertilization. The advantages and utilization of each technique have been previously discussed [9,10]. Transfer of fresh *in vivo*-derived embryos achieves the highest lambing rates, with reported results of 46.4–66.7% [4], 67.8% [11], 75% [12], and 81.2% [13], whereas fresh IVP embryos have lower results, ranging from 32.8% [12] or 37.5% [11] to 40% [13]. However, after cryopreservation, these rates are even lower. The reported lambing rates after transfer of cryopreserved IVP embryos are not higher than 21.7% [13], 19.4–23.8% [14], or 23–26.6% [11], whereas the results for *in vivo*-derived cryopreserved embryos are 32–36% [4], 50% [15], 60% [16], or 60.1–75.1% [14].

The above results highlight low cryotolerance as the main obstacle on sheep embryo cryopreservation, especially for IVP embryos. In the last 15 years, researchers have tried to understand the reason for the low cryotolerance of IVP embryos and keep on searching for new methods to enhance the results in this species. Both *in vivo* and IVP sheep cryopreserved embryos present ultrastructural damage [17,18] corresponding to the expression of damage to cells during the process. Cryopreserved embryos show less microvilli and lower mitochondria activity compared to fresh ones [17–19]. The lower cryotolerance of IVP blastocysts can be also related to the excessive accumulation of lipids [13,20–23], which can be favored by their culture in serum-containing media [22,24,25]. Likewise, the nature and/or concentration of cryoprotectant(s) and the freezing protocol used are also determinants for embryo cryosurvival [19]. According to Tveden-Nyborg et al. [26], IVP sheep embryos are characterized by a slower developmental rate with detrimental effects in placentation, increasing fetal loss, and lowering lambing rates. Moreover, vitrified/warmed IVP sheep embryos presented decreased concentrations of individual amino acids in the amniotic fluid during the second trimester of gestation possibly due to an impaired placental vasculogenesis and/or to a reduced placental transport [27]. In summary, there are a vast number of variables that could potentially influence the outcome of cryopreservation and more studies are urgently needed to improve its success.

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The present paper briefly reviews the current status of sheep embryo cryopreservation, discussing the main applications of this technique, the limitations of its use, and the progress and future perspectives worldwide.

2. Main applications of sheep cryopreserved embryos

One of the best ways to preserve the genetic resources of endangered populations is through the establishment of germplasm banks in which a long storage of biological material can be achieved. The Food and Agriculture Organization (FAO) has established the minimum number of individuals in each population so they can have a classification of endangered [28]. This classification is the base for choosing the target breeds or species needing to be urgently preserved. Following Rio de Janeiro convention, in 1992, all countries must have a plan of conservation of autochthone genetic resources, also recognizing their qualities concerning adaptation to local conditions as well as their potential use in agricultural production niches [29]. At actual knowledge, cryopreservation is a reliable way of long-term conservation of genetic resources [30]. One of the current alternative strategies for maintenance of some breeds or strains is through embryo cryopreservation, as is now routinely used in mouse strains. For instance, for a long-term maintenance of a colony, cryopreserved embryos have the advantages of saving costs, readiness in distant transport, and health guarantee and also of preventing genetic drift occurrence [31,32]. Germplasm exchanges are nowadays crucial [33] and embryo transfer can have a major role in reducing or eliminating some transmissible diseases in germplasm livestock changes [23,34,35]. For commercial proposes, embryo transfer should be chosen for those reasons in which other cheaper techniques are not as advantageous. For example, Cognié and Baril [35] estimated a 10 times higher cost when comparing it (using either *in vivo* or *in vitro* embryos) with AI, and this represents an elevated cost attending the economic value of the animal species.

On behalf of developing new and improved reproductive-assisted techniques, mainly by research teams, there has always been an interest for research in sheep embryo production. The birth of the first IVP lambs in 1991 [36,37] and the first cloned animal (a lamb) by nuclear transfer in 1997 [38] are only two examples. Based on the International Embryo Transfer Society (IETS) data, a decade ago, Thibier [39] reported 6674 fresh and 2907 frozen sheep embryos transferred in the world in 2003, whereas, for the year 2012, Perry [40] referred 8124 fresh and 4120 frozen embryos, mainly represented by Australia, South America, and South Africa. These numbers, although considered underestimated because of the difficulty in retrieving data, show an evolution on the use of this reproductive biotechnology and, in our opinion, reflect the demands of an emerging global market in this area.

In sheep, as in other species, several methods can be used for *ex situ* conservation of genetic resources, and attention in reproductive cells has been focused in oocyte, spermatozoa, zygote, or embryo cryopreservation [41,42]. In parallel, in the last years, research has been conducted in other methods such as ovary, testicle [42–45], and somatic cell cryopreservation [46,47] as

well as in new methods in embryo technology such as somatic cell nucleus transfer and transgenesis [46,48].

Taking into account the background of other species, we can mention that, for example, in the United States, 79% of all bovine embryos collected in 2011 were cryopreserved [49]. In sheep, cryopreservation of embryos is a crucial developing technique for commercial application, and in our opinion, when good results for cryopreservation of sheep embryos have been attended, it will be the dominant technique of sheep embryo production.

3. Embryo production

3.1. *In vivo* embryo production

Despite recent development at *in vitro* embryo production in small ruminants [8,11,50], *in vivo*-produced embryos still represent the greatest source used for the implementation of breeding and conservation programs of endangered livestock [34,40]. To increase the number of embryos that can be recovered and transferred from each donor female, *in vivo* embryo production is generally associated with multiple ovulation and embryo transfer (MOET) programs. Despite the increased efficiency of *in vivo* embryo production in the last years, there are still some problems to be solved. These problems are mainly related to the great variations observed in the ovarian response, fertilization rates, and embryo quality of hormonally stimulated ewes [9,35,51,52].

3.1.1. Estrus synchronization and superovulation programs

To overcome the reproductive seasonality in this species and to obtain embryos from several females simultaneously while improving superovulatory response, MOET is usually associated with estrus synchronization programs [34]. Estrus synchronization can be performed using an exogenous progestagen administered through the application of an intravaginal sponge or using a controlled internal drug release (CIDR) device [35,53]. During the breeding season, in cycling females, estrus synchronization can also be achieved injecting a luteolytic agent, PGF2α [54]. The application of an intravaginal sponge with synthetic progestogens such as fluorogestone acetate (FGA) or medroxiprogesterone (MAP) for 12 to 14 days is frequently used for estrus synchronization. Although allowing good estrus synchronization, the fertility is usually lower compared to natural estrus [55]. Several reasons have been raised to explain this lower fertility, including (a) the asynchrony between the beginning of estrus and the ovulation time [56], (b) a negative influence on the transport of spermatozoa [57], (c) the reduced expression of estradiol and progesterone receptors in oviduct and endometrial cells, (d) a reduced progesterone secretion by the corpus luteum (CL) [58], (e) an inadequate process of maturation and acquisition of competence by the oocyte [59,60], and (f) the development and lack of ovulation of large persistent follicles [53,59–61] due to altered levels of progesterone after the first 6 days of treatment [62].

Several alternatives have been implemented to overcome the negative effects of long-term treatment with progesterone, including the application of superovulatory treatment starting after natural estrus detection without the use of sponges [63]. The insertion of a second progesterone device to maintain constant levels of progesterone treatment has also been performed [51].

Multiple ovulation is mainly acquired using FSH, although some programs still use equine chorionic gonadotrophin (eCG) alone or associated with FSH [52,64–66]. Despite the increase in the ovulation rate, the number of embryos obtained with eCG is often lower than expected. The long half-life of eCG allows it to be applied in a single administration, which naturally simplifies the process and reduces the associated costs [65]. However, when given in high doses, eCG has some undesirable effects, changing the hormonal profile and therefore conditioning the ovulation and fertilization rates as well as the embryo collection rate and viability [67,68]. Actually, commercial FSH is used in most of the MOET programs. The short half-life of FSH implies, however, a 12-hour interval of repeated administration for a period of 4 days [69]. FSH may be of porcine (pFSH) [66,70] or ovine (oFSH) source [52,66,71]. Usually, the treatment begins 72 hours before the withdrawal of exogenous progesterone. The response to this treatment is dependent on the number of applied doses [72,73] and used protocol (constant or decreasing doses) [51,71,73]. There are also intrinsic factors that may affect the superovulatory response in small ruminants, namely, breed, age, nutrition, and reproductive status, the latter being directly conditioned by the reproductive seasonality in this species [51,66]. In addition, individual differences are of crucial importance in the embryo development process [51].

The genetic influence on the variability of superovulatory response was identified since 1986 [74], and in general, the most prolific breeds have a better response to superovulation treatments [75]. To deal with this variability, a simple protocol for identification of higher responders to superovulation allowing a preselection of ewe donors was recently suggested [76]. Additionally, the influence of seasonality in response to superovulation treatment is obviously more noticeable in breeds located in higher latitudes, mainly affecting the viability of obtained embryos [77,78]. Conversely, in temperate regions, a less pronounced seasonality has been identified without differences in the superovulatory response between animals treated inside or outside the breeding season [66,79–81]. However, embryos collected after the administration of the treatment within the breeding season had higher quality [66,78].

The ovulation rate is directly related to the number of small follicles (2–3 mm) at the onset of treatment [82], but in sheep the number of viable embryos depends on a more restricted population of follicles of 3 mm in diameter [83]. The influence of reproductive seasonality in the efficacy of superovulatory response may also be related to the stage of follicular development during the treatment implementation [78]. According to González-Bulnes et al. [58,84], the presence of a CL at the beginning of FSH treatment may improve the number of viable embryos. Moreover, before the application of the multiple ovulation treatment and to improve the superovulatory response, GnRH agonists or antagonists can also be administered [59]. These latter strategies intend to inhibit the final stage of follicular development [75], suppress-

ing the existence of follicles more than 3 mm [85] and simultaneously implementing the development of small follicles [86].

3.1.2. Oocyte fertilization and embryo recovery

Even controlling the limiting factors associated with superovulation treatments, differences still exist when comparing the total embryo production/total embryos able to be cryopreserved to the number of embryos previewed from the follicles observed in the superovulated ovary. In fact, several failures were described, being the most evident ones associated with failures in the ovulation and fertilization processes [51,87], embryo degeneration [88], and inefficiency of embryo recover methods [9,34].

Anovulation has been described as one of the main reasons for fertilization failures, especially due to the associated higher estradiol concentrations [87], influencing the uterine environment and affecting oocytes [89] and spermatozoa [90] transport. However, other authors described no effect of these anovulatory follicles [91]. Another reason for fertilization failure may be related to asynchrony among ovulations. Although a good synchrony concerning the preovulatory wave of LH has been described, the interval among different ovulations in each female can extend from 6 to 12 hours, with an average of 6 hours [9], impairing the fertilization process. A better synchrony among ovulations can be achieved through the application of GnRH 30 to 36 hours after progestagen removal [87]. Conversely, to overcome the deficient spermatozoa transport, intrauterine insemination at 48 hours after progestagen removal is frequently applied [92,93]. Protocols performing both intrauterine insemination and natural mating were associated with the best fertilization rates compared to the individual use of one of these methods [72,92].

Embryo degeneration can result from a deficient oocyte maturation process or from extrinsic factors that may affect the spermatozoon or the embryo itself. This degeneration is mainly related to changes in the endocrine environment of the oviduct or uterus and to anomalies of luteal function [88,94]. In fact, the number and quality of embryos can be affected by changes in the uterus, namely, modifications in endometrial development and its nutritional competence, due to the superovulatory treatment [88]. It is clearly stated that a normal luteal function assuring high progesterone levels is essential for early embryonic development. Premature luteal regression has been described in superovulated sheep [71,81,95] being more frequent after estrus synchronization with a double treatment of PGF2α [95] and during seasonal anestrus [96].

In sheep, embryo recover is performed at day 6 or 7 (estrus day = day 0), most of the times by laparotomy [35]. This technique allows recovery rates of approximately 80%, but adherences after the second surgery may compromise following recovery rates [35,92]. The use of heparinized saline solution can minimize the occurrence of adherences allowing no changes in recovery rates after two [97] and three [71] successive surgeries. Less invasive techniques, such as laparoscopy, minimize the risk of surgical adherences, offering advantages in animal welfare, but the embryo recovery rates they offer are lower (65% and 70% vs. 80%) [92]. The application of a modified laparoscopic technique allowed repeated recoveries in the same ewe, keeping a recovery rate lower than the one obtained with laparotomy, hence offering no

commercial advantage [98]. In a conservation program, the total number of embryos recovered and frozen after a MOET program is approximately 7 and 6 per superovulated ewe, respectively, associated with an embryo recovery rate between 60 and 70% [66].

3.2. *In vitro* embryo production

In vitro embryo production simulates the natural formation of an embryo and comprises the stages of oocyte maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* embryo culture (IVC). In these steps, the last one is critical for determining the blastocyst yield [99]. As stated also in other species, *in vitro* techniques reduce the rate of produced embryos and, as previously discussed, lead to inferior embryo survival after transfer compared to *in vivo*-derived embryos [23,35,99,100]. Concerning sheep embryo production from abattoir-derived oocytes, Cocero et al. [101] obtained 34.6% and 40.4% blastocyst yield at days 7 and 8, respectively, Romão et al. [50] obtained 42.6–46.9% at day 6, and Cognié et al. [9] obtained 25% at day 8.

In terms of lambing rate, IVP embryo survival is 25% lower compared to *in vivo*-produced embryos mainly due to an increased embryo loss at days 30 to 40 [10]. Other authors [102] have discussed the principal mechanisms underlying embryonic losses, suggesting that the preimplantation period of development is the most sensitive to cellular damage. Specifically, the importance of permanent embryo demise that occurs in the first week of development was also pointed out [103]. In fact, the extent and regulation of cell death during preimplantation development is likely to be critical for later development of the concepts. Therefore, it is of primordial importance to implement tools and culture conditions that can promote better IVP results.

In vitro embryo production is not viewed as a controlled and totally defined technique and in sheep has been associated with the large offspring syndrome (LOS) [20,104], also reported in other species such as bovine [105]. This syndrome is characterized by high birth weights, increased gestation length, frequent dystocia, elevated abortion, perinatal mortality rates, and various morphological deviations [20,106]. These abnormalities seem to be promoted very early in developmental stages, in IVM or IVF [3,104], and apparently can be justified by the use of serum supplementation and coculture with somatic cells [3]. Moreover, these problems could be extended after birth, as higher growing rates were found until weaning age in lambs born from IVP [107]. It is known that IVP embryos have altered morphology compared with *in vivo* derived [18,108]. On the contrary, an increase in the lipid content of IVP was observed compared to *in vivo* bovine morulae and blastocysts regardless of the composition of the medium of culture, although differences also depend on the culture medium used [22]. These changes were further correlated to the lower cryotolerance of IVP embryos as stated before.

Several studies have tried to establish the most successful laboratory method of IVP, including oocyte IVM, IVF, and IVC of embryos, as all these steps are crucial to obtain good results.

3.2.1. Collection of oocytes

The anatomical constraints to access the sheep uterus, mainly by the peculiar anatomy of the cervix, have reduced the chances of an easy collection of oocytes and embryos, although future

interest in new techniques [109] could improve welfare issues and results. There are several techniques of oocyte collection, namely, follicular aspiration or ovary slicing in abattoir-derived ovaries [110,111] and ovum pick-up systems applied in living animals. Ovary slicing technique provides cumulus-oocyte complexes (COCs) of better quality and the number of harvested oocytes collected is higher compared to follicle aspiration [110,112]. Sheep ovum pick-up systems are applied in living animals using minimal invasive procedures such as laparoscopic ovum pick-up (LOPU) technique guided by laparoscopy [113–115]. As referred before, these procedures when used *in vivo* are usually associated with follicular stimulation treatments [116] to raise the number of collected COCs [117], allowing better maturation, fertilization, and *in vitro* development capacities [101,118]. Moreover, the development of these *in vivo* techniques of oocyte collection is important, as abattoir-derived oocytes usually cannot be used in genetic programs because of their unknown sanitary and even genetic status, being applied mainly for research proposes [115].

Oocyte quality seems to be the key factor to achieve high embryo production ratios [9,119]. This quality depends on the competence of the oocyte to complete meiosis, which is affected by the follicle size [35]. It is also clearly established that prepubertal oocytes do not have the developmental potential of those collected from adult ewes [118], although, in the future, the results obtained with juvenile oocyte collection could have potential interest for decreasing half the generation interval in genetic programs [112].

3.2.2. In vitro Maturation (IVM)

Collected oocytes to be fertilized must undergo nuclear and cytoplasmic maturation. During IVM, oocytes must reach nuclear metaphase II stage. Besides nuclear maturation, several modifications in cytoplasmic organelles and compounds need to be accomplished. Oocyte cytoplasmic maturation and metabolism are mandatory, as ATP seems to have an important role in determining oocyte quality by supporting energy requirements for further development [120,121].

IVM is usually performed at 38–39°C in a humidified atmosphere in air and 5% CO_2 [8,18,50, 112]. Tissue Culture Medium (TCM199) supplemented with bicarbonate, pyruvate, LH, FSH, and estradiol (E_2) and with 10% fetal calf serum [10,101] or 10% estrous sheep serum [50,122] is the widest used medium for IVM. Nevertheless, other media and supplementation can be also used. For example, Shabankareh et al. [123] obtained good results using human menopausal serum and Birler et al. [124] showed that, in sheep, SOF medium improves the rate of cleavage compared to TCM199 medium. Also, Guler et al. [125] obtained better results adding follicular fluid to maturation medium or using epidermal growth factor (EGF) conjugated with FSH and E_2 [50]. The positive effect of EGF or other EGF-like ligands in COCs has been shown during IVM [126,127], whereas other researchers [101,128] preferred EGF and cysteamine. Cumulus cells are important to IVM success and subsequent fertilization. These cells and their physical contact with the oocyte are of primordial importance not only for the resumption and progression of nuclear maturation but also for cytoplasmic maturation [129–131].

3.2.3. In vitro Fertilization (IVF)

Fertilization of matured oocytes can be performed with frozen-thawed or fresh semen. Frozen semen is easily available for routine use, and some authors argued that early embryo development is not influenced by the method of semen preservation [132], whereas others demonstrated that fresh semen could improve embryo production rates and quality [50]. Eventually, freezing and thawing disrupt the stability of ram sperm chromatin, reducing its fertilization efficiency [133,134]. On the contrary, cryopreserved semen is also advantageous, as it can be stored in seasonal propitious period. However, when a ram of proven fertility is available, its can be used either fresh or frozen-thawed.

Fertilization is performed with previously capacitated spermatozoa using media containing heparin or sheep serum [10,122] that causes Ca^{2+} influx into the sperm acrosome and consequent capacitation [135]. The choice of the best semen fraction is usually achieved by Percoll gradient centrifugation [10] or swim-up [8,136].

3.2.4. In vitro Culture (IVC)

The goal for this phase is to simulate the events that would occur in the oviduct and uterus, so that putative zygotes obtained after fertilization would undergo optimal development into blastocysts. For this reason, culture can be performed *in vivo* using surrogate oviducts [137]. IVC, on the contrary, is achieved in a controlled atmosphere at 38.5–39°C with 5% O_2, 5% CO_2, and 90% N_2. This medium is usually composed of synthetic oviductal fluid supplemented with amino acids, bovine serum albumin (BSA), and/or serum [112,122], although the use of some media with oviduct cells or fetal fibroblasts was also reported [138]. In this stage, it is important to keep an oxygen low concentration to prevent oxidation [139] and the deleterious effects of reactive oxygen species (ROS). Some low molecular weight thiol compounds are also added to IVM and IVC media, such as cysteamine, β-mercaptoethanol, and cysteines, to increase the synthesis of glutathione (GSH), which in turn reduces the oxidative stress [140]. The embryos spend 6 to 7 days in culture before being transferred in fresh or after cryopreservation.

4. Cryopreservation of sheep embryos

The science of cryobiology aims to preserve cells at low temperatures (-196°C), intending to avoid the negative effects of the process, which could preclude the main objective. For cryopreservation, cells should follow the freezing and warming processes. Cryopreservation of sheep embryos is a difficult task, and practical results are not as encouraging as in cattle mainly due to the reduced embryo cryotolerance in this species. Several reasons have been indicated to justify this fact, mainly cellular [21,22], metabolic [141,142], and biochemical [143] changes. In an ultrastructural evaluation, IVP blastocysts exhibited less microvilli and a less extensive network of intercellular junctions, specifically an apparent lack of desmosomal junctions, a higher number of lipid droplets, and incidence of cellular debris compared to their *in vivo* counterparts [18]. After cryopreservation, both *in vivo* and IVP embryos exhibited

ultrastructural damages that were much more severe in the latter [17,18]. These differences could certainly explain the observed lower cryotolerance of IVP embryos.

Although it seems that very low temperatures will disrupt cell components, the most complicated freezing window is between 15°C and -60°C and especially the step of intracellular ice formation between -5°C and -15°C [144]. The major risks for fracture of the *zona pellucida* or cytoplasm range from -50°C to -150°C [145]. Below -150°C, the risks for embryo damage are lower [146]. In embryo cells of species with high lipid content, as in sheep, the lipid droplets combine with the cytoskeleton, membranes, organelles, and other structures of the cytoplasm. Therefore, when the embryos are cooled below 15°C until -5°C, irreversible and fatal structural damages occur, mostly in the traditional freezing methods [146,147].

Differences in freezing methods were extensively identified depending on the speed of freezing particularly between slow and rapid cooling [23,144,148]. Three major methods have been used to preserve embryos: slow freezing, conventional vitrification or an adaptation of the last one, vitrification in open pulled straws (OPS) [149] or in other specialized embryo vitrification devices (copper or gold electron microscope grids [150] and pipette tips and cryotop [151,152], among others) referred as an ultrarapid vitrification. The major difference among them is the acceleration of the speed of freezing. Whereas slow freezing tries to differentiate the cooling rate in sequential steps of freezing, vitrification increases the freezing speed by reducing volume, thus minimizing ice formation. These techniques were compared using *in vivo*-derived or IVP embryos in sheep [14,52,153] or bovine [154]. Bettencourt et al. [52] described no differences in lambing or embryo survival rates among slow freezing, conventional vitrification, and vitrification in OPS in *in vivo*-derived embryos. Although the achieved results by each method can be relevant, the cost and application in routine field use is also of primordial importance [14,153].

4.1. Slow freezing

The method of slow freezing tries to control the descending cooling event in several steps, regulating extracellular and intracellular water exchange, with a balance between ice crystal formation and structural damage, cryoprotectant toxicity, and osmotic damage [146,155]. The method is possible using programmable freezers with a rapid cooling until -6°C to -7°C, when ice formation is induced (seeding), and then a lower rate of cooling (0.3–1°C/min) causing freezing of extracellular ice and increasing the concentration of extracellular solution and cellular dehydration; at -30°C, straws are then plunged in liquid nitrogen [23,31].

Despite being used by many teams in the world, it is argued that slow freezing has a limited future in embryology, and according to Vajta and Nagy [146], "the rate of advancement in oocyte and embryo cryopreservation will depend on the rate by which embryologists and decision-makers adopt the new approaches".

4.2. Vitrification

Vitrification was found to be interesting in embryo cryopreservation because it reduces cryoinjuries caused by ice formation [156]. In fact, vitrification is based on embryo manipula-

tion into different carrier tools applied to minimize the volume and to submerge the sample quickly into liquid nitrogen, allowing an ultrafast freezing speed, which avoids ice crystal formation, thus eliminating its deleterious effects in the cell [157]. This technique combines the use of small volumes with high concentration of two or more cryoprotectants [158] and it can be more adapted to IVP embryos [149,159,160].

According to several authors, the slow freezing techniques are time consuming and laborious [154], whereas vitrification is simple, rapid, and inexpensive [149,161,162]. Moreover, no publications have demonstrated that the results obtained by vitrification were significantly worse than those obtained by slow freezing [146]. Concerns in safety of vitrification related to transmissible diseases are partially justified, but new methods have been developed to avoid these constraints [163].

Several authors described good results with this cryopreservation technique in sheep. For example, Baril et al. [153] and Bettencourt et al. [52] achieved good field results (50–60% lambing rate) with vitrification of *in vivo*-derived sheep embryos even with direct transfer of cryopreserved embryos, and Folch et al. [4] achieved lambing rates of 32–36%, also in *in vivo*-derived embryos. In IVP embryos, Ptak et al. [164] found no differences in pregnancy rates between fresh and vitrified transferred embryos (47% vs. 42%). However, they achieved significant differences in lambing rates (41% vs. 23%), showing that vitrification of IVP embryos is not yet an optimized technique.

The success of vitrification depends on the stage at which embryos are cryopreserved apart from the used method. In fact, there are different levels of sensitivity to low temperatures depending on the stage of embryo development [165]. Garcia-Garcia et al. [166,167] and Shirazi et al. [168] showed that cryotolerance of IVP embryos to conventional slow freezing or vitrification increased as the developmental stage of embryos progressed perhaps due to the higher cryotolerance of blastocysts compared with early developmental embryos. In fact, Garcia-Garcia et al. [167], using slow freezing, described similar rates of viability between fresh and frozen-thawed embryos (92.5% vs. 83.7%) frozen at the blastocyst stage.

4.2.1. Vitrification in OPS and other specialized embryo devices

To achieve a higher rate of temperature reduction, the OPS method uses thinner, superfine straws, with their ends having half the diameter of the conventional ones. This characteristic enables filling the OPS by capillary action with a volume of approximately 1 μL, different from the conventional straw that contains 5 μL [146,149]. Therefore, this can allow freezing rates of 20,000°C/min [159] (i.e., 10 times higher than in 0.25 mL straws) and thereby overcome some problems of vitrification as toxicity of cryoprotectants or difficulties in the permeability of membranes that can lead to intracellular ice formation and osmotic overswelling [160]. Allowing higher speed of freezing, this type of vitrification is helpful in preventing embryo and *zona pellucida* fracture that occurs at low temperatures, especially with appropriate adjustments of warming parameters [152]. The method was introduced by Vajta et al. [149], and it has been successfully used ever since in several animal species. It is a robust and feasible method for animal embryo vitrification more than other new, but delicate, techniques [146].

Vitrification by OPS is so effective that results in lambing rate of *in vivo*-derived sheep embryos vitrified by this method are persuasive. Green et al. [169] reported higher pregnancy rates when using vitrification by OPS and direct transfer, thus enhancing the field application of this technique. Nevertheless, these good results with OPS vitrification cannot avoid the reduced cryotolerance of IVP embryos. Dattena et al. [16] achieved lambing rates of 60% for *in vivo*-produced embryos vitrified in OPS but only 24% for IVP.

4.3. Cryoprotectants

The role of these substances is to reduce damage to cryopreserved embryos, minimizing ice formation. They usually are classified as permeable and nonpermeable cryoprotectants, being also important in osmotic dehydration [23,146].

The negative effects of cryoprotectants are related to their osmotic and toxic effects and closely dependent on the time of exposure and the concentration used [147,148,159]. The intent to reduce cryoprotectants to a minimum is a goal that has been tried in both slow freezing and vitrification to minimize their deleterious effects. According to Liebermann et al. [158], a balance between the maximization of cooling rate and the minimization of cryoprotectant concentration is the key point for a successful cryopreservation. Mainly in vitrification, where it is necessary to deal with higher concentrations of cryoprotectants, one of the strategies is to use more than one. This strategy reduces individual toxicity of cryoprotectants and also permits adding them in a stepwise equilibration (two or three steps), with increasing concentration or after cooling from 4°C to subzero temperatures when their toxicity is lower [31,146,159].

Ethylene glycol is a frequent choice as a permeable cryoprotectant due to its high penetration rate and low toxicity besides protecting the membranes and cytoplasmic structures of embryo from cryoinjury [19]. However, other cryoprotectants such as acetamide, glycerol, raffinose, and dimethylsulfoxide (DMSO) can be used in several combinations. Ethylene glycol and glycerol [146,170] or ethylene glycol and DMSO [8,18,50,171] are often employed. Varago et al. [172] used dimethylformamide and ethylene glycol to vitrify ovine embryos but concluded that the conventional freezing with ethylene glycol was the most efficient method to cryopreserve ovine embryos.

Mono- and disaccharides, including sucrose, trehalose, glucose, and galactose, can also be added to vitrification media as nonpermeable cryoprotectants, with sucrose being the most commonly used [23,146]. Other substitute substances such as polymers and proteins have been tried to replace the former indicated.

4.4. Embryo warming/thawing

Embryo thawing/warming is also an essential step of the cryopreservation process and a specific procedure should be performed to avoid embryo damage. It comprises the rise of temperature, passing through the above-mentioned critical windows [144–146], and the cryoprotectant removal while maintaining embryo posterior viability. The thawing/warming rate and the protocol used for cryoprotectant removal need to be adjusted to the cooling

procedure and embryo characteristics [23]. Thus, in embryos cryopreserved by slow freezing or vitrification, the best method depends on the specific protocol and cryoprotectants used (time and temperature and cryoprotectant characteristics and concentrations) but also on the origin of the embryo (*in vivo* or IVP derived). For instance, in slow freezing, if the freezing method stops at temperatures of -30°C to -40°C, before plunging in nitrogen, a moderately rapid warming (200–350°C/min) is required to maximize the survival rate but, if freezing reached -60°C or less, warming rate should be slow in the range of 25°C/min [161], although injuries were reported [173].

Warming of vitrified embryos is usually performed directly into a solution at body temperature, although it can be advisable to wait 1–3 seconds in air to avoid fracture damage [146]. Vitrified embryos need to be warmed ultrarapidly in the presence of nonpermeating cryoprotectants (usually sucrose) to dilute and remove the very high levels of intracellular permeating cryoprotectants [161]; this can lead to changes in dynamics of water in the embryo cells that can cause damage due to osmotic overshrinkage [31,160].

Regardless of the method used for embryo cryopreservation, some researchers [1,14,169] obtained promising results after direct transfer of vitrified sheep embryos for field use. These authors reported that the interval between thawing/warming and transfer has a great influence on embryo subsequent viability and valorize the use of direct transfer to spread this technique in field conditions.

4.5. Evaluation of cryopreserved embryos

The aim of embryo production and storage is to give practitioners the possibility of using them as an important resource for improving sheep production worldwide. Nevertheless, the effective use of these techniques depends on the guarantee that these embryos have the necessary quality enabling the reliability of its use. The need for predicting embryo ability to produce an offspring is essential in this context.

Embryo evaluation can use invasive and noninvasive techniques [174]. The last group, in assessing embryos without damage, has been widely used based on morphological changes that occur as the result of the sequential cleavage and this classification can be important in preimplantation embryos and especially in embryos that are being transferred [175]. Ushijima et al. [176] proposed an 11-stage classification for bovine embryos based on the number of cleavages. Embryo stage and quality related to morphology are usually based on the descriptions published by the IETS [177]. This ranking classifies embryos in grades 1 (excellent and good), 2 (fair), and 3 (poor) and become the standard reference worldwide. In cryopreserved embryos, stereomicroscopic evaluation of morphology after thawing has been used for transfer selection [178] to avoid transferring nonviable embryos. Leoni et al. [179] stated that reexpansion of blastocoelic cavity within 8 hours after vitrification/warming can be considered a reliable marker of its quality and developmental potential both *in vitro* and *in vivo*. However, some authors argue that morphological evaluation of thawed embryos is not accurate and the use of direct transfer of embryos can result in an improvement of 7–8% in offspring born [9]. Also, Green et al. [169] found that direct transfer improves the viability of transferred vitrified sheep embryos maybe because the time elongation from warming to transfer has a detrimental

effect on subsequent embryo viability. This fact could suggest that embryo evaluation after embryo warming is dispensable as predictive of success. In fact, it is necessary to find other associated evaluation methods besides those based on embryo morphological evaluation to provide more accurate information. For example, Hernandez-Ledezma et al. [180] showed that good-quality hatched blastocysts produced more trophoblast protein, thus being an indicator of embryo quality before transfer.

Selection of embryos by the commonly used morphological criteria has, as expected, some individual variations because it can be a subjective evaluation. Indeed, studies in *in vivo*-produced ovine morulae and blastocysts have shown that certain abnormalities remain undetected by stereomicroscopy [19].

Invasive methods of embryo evaluation are used mainly for research proposes, validating the above-described techniques with greater accuracy [175]. Embryo postthawing viability and blastocoele reexpansion in different time periods of embryo development have been widely used to determine its feasibility [13,14,22,181]. Although several methods can be performed to predict the success of sheep embryos survival, the ultimate method, the outcome of the *in vivo* transfer to ewes, as the final goal of all the practical applications, cannot be tested after these invasive techniques. Staining methods as propidium iodide and TUNEL assay [182,183] are used for evaluating the number of viable cells. Hosseini et al. [184] established an easy approach for *in vivo*-derived and IVP embryos by evaluating the competence of cell membrane that is maintained in viable cells and can differentiate them from those necrotic or apoptotic, even without other morphological signs of cell death [102].

Ultrastructural evaluation of embryos can predict the damage caused in cell structures by the cryopreservation process and the consequences in their functions [185]. Although this is an expensive and invasive technique, the obtained outputs due to detailed information are of the utmost importance. However, according to Vajta et al. [186], in IVP bovine embryos, relevant ultrastructural changes observed immediately after thawing were progressively restored over a 24-hour period. Thus, it may be important to perform the ultrastructural evaluation of thawed embryos at different time periods.

Research on sheep embryo ultrastructure is limited compared to bovine species. However, recent publications have provided new and enough information allowing its discussion. Bettencourt et al. [187] described the ultrastructure of *in vivo*-produced sheep embryos, and previously, there were also some insights about the effects of cryopreservation in this source of embryos [17,19,188,189]. Concerning IVP sheep embryos, Dalcin et al. [2] revealed its ultrastructure as well as subtle changes in mitochondrial activity or cytoskeletal integrity caused by the cryopreservation process. Later on, Romão et al. [18] performed the ultrastructural characterization of fresh and vitrified *in vivo* and IVP sheep embryos. However, some details about IVP sheep embryos still need to be investigated.

Fresh IVP sheep embryos lacking desmosomal junctions have a reduction in the microvilli, an increase of debris in the periviteline space, and a high amount of lipid drops [17]. According to Romão et al. [18], the ultrastructural observation of these embryos also showed a reduced number of mitochondria and a lower number of mature mitochondria, having, on the contrary,

a larger number of vesicles with light and dense content compared to their *in vivo* counterpart. These lower mitochondria content can be associated with deficient metabolism in IVP embryos.

After thawing/warming, the cryopreserved sheep embryos both *in vivo* and *in vitro* showed signs of ultrastructural injury represented by differences in mitochondria, with presence of more immature ones and lower total number [17,19]. Mitochondria are important for embryo development and metabolism [108,178,190] and thus can be a predictor of the embryo development potential [17,187]. However, in cryopreserved IVP sheep embryos, their function is reduced or absent [2]. Cryopreserved embryos also show cytoskeleton disturbance with actin microfilament disorganization [2,19] that can be caused by cryoprotectants [190]. The ultrastructural evaluation of cryopreserved IVP blastocysts confirmed the presence of more severe cellular damage in these embryos compared to those produced *in vivo* [18], pointing out the need of research to improve IVP sheep embryo quality and cryotolerance.

Having in mind that the evaluation of sheep embryos is important as a prognostic tool for the success rate after embryo transfer, the search for more accurate and practical methods is necessary, especially when dealing with stored cryopreserved embryos.

4.6. Methods of improving the cryopreservation of sheep embryos

Different strategies to increase the cryotolerance of embryos in sheep as in other species were developed mainly by improving the cryopreservation procedures [148], changing the composition of IVC media or other IVP techniques [8,50,192] and/or through the decrease of their lipid content [8,121]. As referred, methods of ultrarapid vitrification have been developed to further increase the freezing speed. However, the ideal device and combination of cryoprotectants have not yet been achieved. Therefore, this technique continues to be a challenge in embryo cryopreservation [148,155]. The number of created devices, such as glass micropipette, solid surface vitrification, cryoloop, microdrop, cryotop, cryotip, electron microscopy grids, or nylon mesh [15,152,155,161,193–195], clearly shows the lack of consensus. On the contrary, recent research pointed out the possibility of a further decrease in the temperature for embryo cryopreservation, from -196°C to -207°C, through the use of slush nitrogen [196], improving embryo cryosurvival at least in the mouse. According to Arav [148], the next evolutionary step in embryo cryopreservation will be to preserve them in the dry state at room temperature, allowing home storage for future use. In this alternative method, vitrification is followed by drying, and embryo storage is done in a dry state, avoiding their maintenance in liquid nitrogen containers and reducing the cost of the entire process.

Attempts were also experienced in IVC conditions, as it was argued that the effect of changing culture conditions could only be seen after embryo cryopreservation and warming. For example, Dattena et al. [192] confirmed higher sheep embryo cryosurvival results when including BSA and hyaluronan during IVC, and Gad et al. [197] realized that altered culture conditions at different time points of the preimplantation period lead to adaptations of the embryos, changing their gene expression and developmental ability. As referred, the initial stages of development are those with higher cryosensitivity, from oocytes to late embryo, with the acquisition of cryotolerance in an intermediate stage of development. Accordingly, Garcia-

Garcia et al. [167] and Lin et al. [198] found differences in the cryotolerance of IVP embryos vitrified by OPS, depending on its developmental stage, with better results in morulae/blastocyst, defining the eight-cell stage as the critical point for acquiring cryotolerance. Therefore, it is not surprising that, in the last years of research, cryopreservation techniques have focused mainly in embryos at morulae and blastocyst stages and less in oocytes or zygotes. In addition, embryos that develop early have better survival rates after cryopreservation, and usually, these are male embryos [199].

Differences in species' cryosensitivity of embryos are responsible for different approaches in their cryopreservation. As indicated before, one of the major concerns in embryo cryopreservation is its lipid content that can hamper the process [13,20–23,200,201], as it happens in species such as sheep or pigs. This phenomenon is more pronounced in IVP embryos [18,202,203] and in those produced in serum-containing media [20,22,24,204]. Serum is useful in oocyte and embryo culture as a source of albumin that balances the osmolality, acting as a free radical scavenger, and also with an additional important nutritive role [205]. However, the fatty acids and lipoproteins of the serum seem to be the source of the cytoplasmic lipids of embryos, hampering embryo quality [20,204,206], albeit the perturbations induced by the presence of serum in sheep embryo culture are higher before rather than after compaction [207].

Conversely, embryo lipid content effect on chilling sensitivity is not totally elucidated at the moment. However, it seems that lipid droplets interact directly with the intermediate filaments of the cytoskeleton, and changes within these organelles during the cryopreservation process may lead to irreversible damages [208,209]. Also, it has been observed that cryopreserved embryos have ultrastructural changes that are visible as degenerated cells, disruption of cell membranes, and mitochondrial injuries, mainly in poor-quality embryos [17–19]. Mitochondrial changes, namely, a reduced total number and an increase in the proportion of immature mitochondria [18,187], have been observed in poor-quality and IVP embryos and were associated with culture in serum-containing medium [210]. These changes have been also associated with inefficient lipid metabolism, with the presence of cytoplasmic vesicles and lipid drops and fewer lysosome-like vesicles [18], compromising ATP production [108,197,204].

Due to the negative effect of embryo lipid content and composition in their chilling sensitivity, attempts have been made to reduce its amount in IVP embryos either by chemical or by physical approaches. Nevertheless, lipids are important in the embryo cell metabolism as a source of energy and are also essential for membrane formation and as intracellular messengers [200,211]. Thus, these strategies should be carefully evaluated. For instance, in cattle, lipolytic agents or chemical delipidators have been successfully applied, increasing the cryotolerance of vitrified embryos (phenazine ethosulfate [212], *trans*-10 *cis*-12 conjugated linoleic acid (CLA) [22,208], and forskolin [213]). These chemicals that regulate metabolism were used to reduce embryo lipid content, inducing smaller lipid droplets and fat indexes, thus improving embryonic cryosurvival [22,208,214]. The addition of CLA during culture of sheep IVP embryos also increased their cryotolerance [8]. Likewise, Nagashima et al. [215] demonstrated that the high lipid content of pig embryos was responsible for their higher chilling sensitivity and also that embryos delipidated by centrifugation and microaspiration

of polarized lipids became more tolerant to chilling. The lipid content was also pointed out as responsible for the chilling and freezing sensitivity of IVP cattle embryos, and when lipid droplets were displaced by ultracentrifugation (mechanical delipidation), their cryosurvival was improved [216,217]. On the contrary, Romão et al. [8] found a reduced viability in IVP sheep embryos submitted to an ultracentrifugation process mostly due to *zona pellucida* and membrane fracture. This problem could be overcome by using cytochalasin D during the ultracentrifugation process.

Cytoskeleton relaxant/stabilizers such as cytochalasin B or D were another approach investigated to improve embryo cryosurvival. Initially, they were used for cattle and pig embryo vitrification to prevent cellular disruption, specifically to the embryonic cytoskeleton during and after cryopreservation [218–220] with either no benefits [221] or with positive results [220,222]. These molecules induce microfilament depolymerization before and during vitrification [219], acting as microfilaments inhibitors, thus preventing actin polymerization and making the plasmatic membrane and cytoskeleton more elastic. Therefore, embryo microfilaments and cytoskeletal architecture were not only destroyed during micromanipulation but also become more resistant to the osmotic stress induced by the exposure and removal of cryoprotectants during the vitrification/warming process [218,220]. Dobrinsky et al. [223] obtained an 82% birth rate in pig vitrified embryos using cytochalasin B. Moreover, the association of two strategies, cytoskeleton relaxants and ultracentrifugation, were successfully attempted by different authors in IVP sheep, cattle, and pig embryos [8,222,224].

In spite of the above-described methods for improving sheep embryo cryosurvival, several limitations persist, impairing their spread as a routine technique. Therefore, possible solutions for improving methods to allow a higher survival rate of cryopreserved embryos should be further investigated.

5. Conclusions and future perspectives

Although sheep embryo technology is less applied than in other animal species (e.g., bovine) due to economical and scientific limitations, it is expected that in the next few years, these techniques will have a central role in sheep production. Cryopreservation is of primordial importance to preserve embryos so that they can be maintained as genetic reserves or for application simultaneously to other reproductive technologies. Vitrification and other ultrarapid vitrification protocols provide nowadays an alternative to slow freezing of sheep embryos. The effective use of these techniques depends on the guarantee that these embryos have enough quality, enabling the reliability of their use. New strategies, allied to emergent technologies, could be implemented to preserve the embryos such as to maintain them in a dry state at room temperature or to warm them with laser pulses. Manipulation of the lipid content of the embryo, one key factor hampering the efficiency of cryopreservation in this species, is a promising strategy. At the moment, *in vivo* and *in vitro* embryo production and cryopreservation remains a challenging technique that is growing worldwide in all species. Continued research to establish the optimal protocol(s) must persist to use embryo production

and cryopreservation as an essential tool in reproductive medicine, guaranteeing superior results that will enable the expansion of clinical/biotechnological services.

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