Cryo-preservation of sperm and embryos in small ruminants

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Abstract

Cryo-preservation of sperm and embryos is an important biotechnology for preservation and propagation of genetics. The aim of this paper is to review established procedures and recent advances in sheep, goats and some wild small ruminants. The use of frozen-thawed semen is more common in goat than in sheep. This is primarily due to the need for laparoscopic insemination in ewes. The major difference between sheep and goat is in the behavior seminal plasma towards egg yolk proteins. Recently, research focused on development of new approaches to improve freezing extenders by eliminating animal products such as egg-yolk and milk from extenders and their replacement by lecithins from vegetal origin, testing new cryo-protectant and reducing the effects of oxidative stress. The effect of these factors can be tested now more rigorously through the use of various morphological and function techniques such as fluorescent stains, hypoosmotic stress and computerized semen analysis to detect DNA stability, membrane integrity and motion parameters. Small ruminant embryos have been cryopreserved by the slow-cooling technique, which is being slowly replaced by vitrification. In sheep, morulae and early blastocyst are more suitable for freezing. Whereas in goat, expanded blastocysts and hatched blastocyst produce better results. Pregnancy rates after transfer of cryopreservation sheep and goat embryos yields acceptable results when management of recipients and transfer techniques are performed adequately.

Keywords: Freezing, sperm, embryos, insemination, fertility, extenders.

Cryoconservation du sperme et des embryons chez les petits ruminants

Résumé

La congélation du sperme et des embryons est une biotechnologie importante pour la conservation et propagation de la génétique. L'objectif de cet article est discuter les techniques de congélation établies ainsi que les acquisitions récentes dans ce domaine chez les ovins, caprins et petits ruminant sauvages. L'utilisation du sperme congelé en production est plus développée chez les caprins que chez les ovins. Ceci est principalement dû au besoin de l'utilisation de l'insémination par laparoscopie chez les brebis. La plus grande différence entre les caprins et les ovins est le comportement du plasma séminal vis as vis des protéines du jaune d'œuf. Récemment, les efforts de recherches se sont penchés principalement sur le développement de nouvelles approches pour l'amélioration des dilueurs de congélation. Les axes les plus importants étant le remplacement des protéines animales (jaune d'œuf et lait) par les lécithines végétales, l'utilisation de cryoprotcteurs autres que le glycérol, et la réduction des effets de l'oxydation des lipides. L'introduction de méthodes d'analyses de la morphologie et fonction des spermatozoïdes, telles que les techniques de fluorescence, les tests hypopsmotiques et l'évaluation numérique de la mobilité, permet une évaluation plus rigoureuse des effets de ces facteurs sur la congélabilité du sperme La conservation des embryons des petits ruminants par vitrification se développe de plus en plus et pourrait remplacer les techniques lentes, plus laborieuses et coûteuses. Chez les ovins, les morulas et blastocystes semblent être plus résistants à la congélation. Par contre chez les caprins, les blastocystes en expansion et éclos sont plus résistants à la congélation que les morulas et jeunes blastocyste. Les taux de gestation obtenus après transfert d'embryons congelés sont très intéressants surtout quand la gestion des receveuses est adéquate et les techniques de transfert sont maîtrisées.

Mots-clés: Congélation, sperme, embryons, insémination, fertilité, dilueurs.

INTRODUCTION

Cryopreservation of sperm and embryos of small ruminants is an important reproductive biotechnology for the preservation of rare or local breeds and the propagation of genetic material from imported high performing individual animals. Another advantage of these technologies is the introduction in a flock or herd of new genetics without breaching biosecurity measures if donor animals are submitted to rigorous veterinary inspection and disease testing. The objective of this paper is to review the general principles of sperm and embryo cryopreservation and discuss new research aimed at improvement of these technologies in small ruminants.

SEMEN CRYOPRESERVATION

Although sperm preservation is considered common practice in both sheep and goat, new improvements have been introduced mostly to simplify the technique for field use. Improvement in post-thaw quality aims to bypass laparoscopic insemination particularly in sheep. Advances in cryopreservation of small ruminant sperm include development of new extenders to eliminate animal products such as milk or egg yolk. Differences in sperm cryopreservation exist between sheep and goat. Goat sperm is known for the toxic reaction to high levels of egg yolk in the extender.

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Cryopreservation of ejaculated sperm

One of the primary conditions for successful cryopreservation of sperm is the initial quality of the ejaculate. Rams and bucks that are candidates for semen cryopreservation and use in artificial insemination programs should undergo a thorough breeding soundness examination (BSE) and disease testing prior to enrollment. Cryopreservation causes biological and functional changes that can affect sperm viability and fertility. These changes occur primarily at the membrane levels and are due to ice crystal formation and changes in membrane permeability. A significant proportion (20 to 30%) of cryopreserved spermatozoa lose their fertilizing ability after thawing. Therefore, only ejaculates of excellent quality (>90% motility and >70% normal morphology) should be used for cryopreservation (Dorado *et al.*, 2010). Principles for BSE in rams and bucks are described in detail elsewhere in the present publication (Tibary et *al.*, 2018). It is important to take into account seasonal effects on initial quality of the ejaculate and seminal plasma proteins and their interaction with membrane stability and ability to withstand freezing and thawing (Goularte *et al.*, 2014; Sobrinho et *al.*, 2014; Wang *et al.*, 2015). Melatonin treatment of bucks improved fresh semen quality but not cryopreservation (Gallego-Calvo *et al.*, 2015).

Cryopreservation of small ruminant sperm has been practiced for decades. Several extenders have been developed and are either egg yolk (EY) or skim milk based (Table 1) (Cseh *et al.*, 2012; Kucuk *et al.*, 2014; Emamverdi *et al.*, 2015). The main buffers used are citric acid and Tris. Zwitterionic buffer have also been used successfully for small ruminant semen preservation (Tuli and Holtz, 1992). Egg yolk low-density lipoproteins (lecithin and phosohatidylcholines) protect the sperm membrane during the freezing and thawing processes. A major difference between ram and buck semen is their behavior toward egg yolk, which tends to coagulate in presence of seminal plasma from bucks. This effect has been attributed to the presence of a specific lipase belonging to the pancreatic lipase-related protein 2 family that is secreted by the bulbourethral glands (egg yolk coagulating enzyme or BUSgp60) (Cseh *et al.*, 2012). Goat ejaculates are washed by centrifugation (700 x g for 15 minutes) in PBS or extender without egg yolk prior to dilution with egg yolk containing extenders (Batista *et al.*, 2014). Alternately, semen can be diluted with extenders containing very low level of egg yolk (2 to 3%) (Shamsuddin et *al.*, 2000; Bispo *et al.*, 2011; Anand and Yadav, 2016). Other researchers have used up to 12% egg yolk without washing (Cabrera *et al.*, 2005).

Following dilution, semen is slowly cooled to 5°C for a minimum of 1.5 to 2 hours, loaded in straws and frozen in liquid nitrogen vapors (Cseh *et al.*, 2012). The final dilution of semen prior to cryopreservation is generally between 100 and 200 million sperm per mL. Increasing the concentration above 400 million per mL results in a decrease in post-thaw quality (Alvarez *et al.*, 2012a).

There are few studies on the optimal freezing rate for ram and buck sperm. The optimal freezing rate should avoid intracellular ice formation, cell shrinkage and long exposure to high osmotic and ionic strength. Freezing rates commonly used for small ruminant sperm cryopreservation range from -10 to -100°C/min. In a recent study on ram semen, the best freezing rate was determined to be -40°C/minute when semen was diluted in Tris-glucose EY and cooled at 4°C for 2 hours in 0.25 mL straws (Fang *et al.*, 2016). Although protocols for cryopreservation of ram and goat spermatozoa have been successfully used for several decades, introduction of technologies such as sex-sorted semen requires improvement of cryopreservation (Bathgate *et al.*, 2013; Quan *et al.*, 2015). Factors that have been studied recently include the type and method of addition of cryoprotectant (Silva et *al.*, 2012b; Pelufo et *al.*, 2015), cooling rate and equilibration time (Ahmad et *al.*, 2015) and individual male variability. Cryoprotectants are generally added in a single step or in two steps (after cooling). Cooling rates and equilibration times from 2 to 8 hours have been tested (Ahmad *et al.*, 2015). Equilibration time of 2 hours seems to be sufficient for most protocols (da Silva *et al.*, 2014). Equilibrated semen is placed in 0.25 or 0.5 mL straws and cooled at a rate of 3°C/min from 5°C to -8°C and at 25°C /minute from -8 to -120°C.

Individual male variation exists in the ability of sperm to withstand freezing. This variation may be linked to differences in protein content of seminal plasma and membrane stability (Moura et *al.*, 2010; Romon et *al.*, 2013). These individual variations are illustrated by difference in changes in sperm head volume during the freezing process (Romon *et al.*, 2013). Genetic variation may also affect cryopreservation of semen. Recently, the heat shock protein 70 (HSP70) gene has been suspected to have an effect on semen quality and possibly freezing ability in Boer goats under tropical conditions (Nikbin *et al.*, 2014).

Table 1: Extenders used for cryopreservation of small ruminant sperm

Extender	References
TRIS–glucose–citric acid glucose or fructose and egg yolk	(Forouzanfar *et al.*, 2010; Naing et *al.*, 2010; Salmani *et al.*, 2014; Ustuner *et al.*, 2014; Abdi-Benemar *et al.*, 2015)
TES-tris fructose egg yolk glycerol	(Alvarez *et al.*, 2012b)
Soybean lecithins	(Forouzanfar *et al.*, 2010; Najafi *et al.*, 2014b; Salmani et *al.*, 2014; Ustuner *et al.*, 2014)
Andromed®	(Nordstoga *et al.*, 2010a; Nordstoga *et al.*, 2011; Jimenez-Rabadan *et al.*, 2012)
Biladyl®	(Jimenez-Rabadan *et al.*, 2012)
Bioxcell®	(Sariozkan *et al.*, 2010)
Optixcell®	(Stewart *et al.*, 2016)
Triladyl®	(Rekha *et al.*, 2016)

New developments in sperm cryobiology

Replacement of egg yolk

As stated above, addition of egg yolk to extenders provides cryoprotection to the sperm through its low-density lipoprotein. Egg yolk from different species has been tested resulting in some differences (Kulaksiz *et al.*, 2010). Egg yolk, as a membrane protective agent in semen extenders, has become progressively undesirable because of its anti-

genic proprieties (Ustuner *et al.*, 2014) and the reluctance of some countries to import semen frozen with egg yolk containing extenders for biosecurity reasons. This led to its replacement by lecithins from vegetal origin such as soybean (Del Valle *et al.*, 2013; Emamverdi *et al.*, 2013; Vidal *et al.*, 2013; Salmani *et al.*, 2014; Bohlool et *al.*, 2015; Chelucci *et al.*, 2015; Sharafi *et al.*, 2015; Masoudi *et al.*, 2016a; Toker *et al.*, 2016). The combination of soy milk and glycerol has been used successfully for cryopreservation of ram sperm (Jerez *et al.*, 2016). The use of soy extender (Bioxcell®) for goat semen has been shown to remove the need for washing of ejaculates prior to dilution and freezing (Sariozkan *et al.*, 2010; Roof *et al.*, 2012).

Addition of antioxidants

Sperm membrane has a high concentration of polyunsaturated fatty acids and is extremely sensitive to oxidative stress during cryopreservation (Del Olmo *et al.*, 2015; Amidi *et al.*, 2016; Toker *et al.*, 2016). Research has focused, in the last decade, on improvement in cryopreservation of ram and buck semen through addition of various antioxidants to extenders with variable results. Enzymatic antioxidants such as glutathione (GSH), superoxide dismutase (SOD) and catalase (Silva *et al.*, 2011; Santiani *et al.*, 2014) have been used in various concentrations to improve post-thaw sperm quality. In rams, addition of SOD, GSH and MNTBAP (a superoxide-dismutase mimetic) seems to reduce acrosomal damage (see review by Budai *et al.*, 2014). Addition of Trolox and catalase to tris-egg yolk extender significantly reduced lipid-peroxidation in ram semen (Maia *et al.*, 2010; Sicherle *et al.*, 2011; Silva *et al.*, 2013; Camara *et al.*, 2016). Trolox was also beneficial for preservation of sperm of plasma membrane and mitochondrial sheath integrity of goat sperm frozen in a skim milk based extender (Soares *et al.*, 2015).

Other antioxidants (zinc, tocopherol, albumin, glutathione, taurine, hypotaurine, carnitine, carotenoids, cysteine, urate and prostasomes) (Mata-Campuzano *et al.*, 2011; Mata-Campuzano *et al.*, 2012; Budai *et al.*, 2014; Mata-Campuzano *et al.*, 2015) have also been tested in small ruminant sperm cryopreservation with variable results (Budai *et al.*, 2014). Oxidized gluthatione (Camara et *al.*, 2011; Razliqi *et al.*, 2015; Hazarika *et al.*, 2016), cysteamine (Najafi *et al.*, 2014a; Alcay *et al.*, 2016), methionine (Tuncer *et al.*, 2010; Alcay *et al.*, 2016), catalase, cysteamine (Bucak *et al.*, 2007), Butylated hydroxytoluene (BHT) (Naijian et *al.*, 2013; Alcay *et al.*, 2016), ergothioneine (Coyan *et al.*, 2011; Najafi *et al.*, 2014a), hypotaurine (Bucak *et al.*, 2013), taurine (Bucak et *al.*, 2007) and trehalose (Bucak *et al.*, 2007; Tuncer et *al.*, 2010; Najafi *et al.*, 2013; Tuncer *et al.*, 2013; Bohlool *et al.*, 2015) have all been used as antioxidants and reactive oxygen species scavengers. Resveratrol and quercetin have also been shown to improve post-thaw viability and mitochondrial membrane potential (Silva *et al.*, 2012a). Dithiothreitol, a protamine disulfide bond reducing agent, has been shown to have a positive effect on post-thaw motility of ram semen (Baspinar *et al.*, 2011). All these approaches proved to be beneficial in increasing viability, post-thaw motility and acrosome integrity. However, the true effect of some these antioxidants on sperm viability and post-thaw fertility remains controversial. In one study, addition of cysteamine to electroejaculated ram semen was detrimental to cryosurvival while addition of iodixanol was beneficial (Cirit *et al.*, 2013). Glutathione addition had no beneficial effect on buck semen extended in soybean lecithin containing extender (Salmani *et al.*, 2013). In one study on ram semen, no advantage was found with addition of gluthatione, superoxide dismutase or catalase (Camara *et al.*, 2011). Supplementation of extender with vitamin C has been shown to improve post-thaw buck sperm quality (Gangwar *et al.*, 2015). Other studies have shown little benefit or negative effect of some antioxidants on post thaw sperm quality (Yildiz *et al.*, 2015; Zhandi and Sharafi, 2015). The effects of antioxidants on post-thaw sperm quality merits however further studies to elucidate the interaction between specific extender and concentration of antioxidants (Mata-Campuzano *et al.*, 2015; Zhandi and Sharafi, 2015).

Another approach to reduce reactive oxygen species due lipid peroxidation in semen is through nutrition. Addition of fish oil to ram diet has been shown to improve sperm quality and *in vitro* fertilization results (Behzad *et al.*, 2014; Esmaeili *et al.*, 2014; Masoudi *et al.*, 2016b). Semen quality was also shown to be improved in rams supplemented orally with vitamins E and C (Memon *et al.*, 2013; Cofre-Narbona *et al.*, 2016). Dietary supplementation with n-3 polyunsaturated fatty acid of rams had limited effects on the quality of liquid stored semen (Towhidi *et al.*, 2013; Fair *et al.*, 2014).

Alternative cryoprotectants

Glycerol is the most commonly used cryoprotectant for sperm. However, exposure to high levels of glycerol is toxic and can be detrimental to sperm function and viability in utero. Therefore, research efforts have focused on alternative cryoprotectants. In goats, dimethylsulfoxide (DMSO) and ethylene glycol (EG) did not present any added advantage in sperm cryopreservation (Buyukleblebici *et al.*, 2014). In rams, EG at concentrations of 3 or 5% was beneficial but not as good as glycerol (Silva *et al.*, 2012b). DMSO was ineffective in ram semen preservation (Bezerra *et al.*, 2011; Moustacas *et al.*, 2011). In rams, propanediol, sucrose and trehalose did not perform as well as glycerol (Nur *et al.*, 2010). However, trehalose supplementation to extenders improved cryopreservation of Boer semen in Tris-citric acid extender (Naing *et al.*, 2010). Amino acids (L-glutamine and L-proline) have been used successfully to improve cryoprotection and reduce lipid peroxidation (Farshad and Hosseini, 2013; Mehr and Noori, 2013; Sangeeta *et al.*, 2015). Addition of cholesterol-loaded cyclodextrin may allow reduction of the concentration of glycerol to 3% (Moce *et al.*, 2010; Awad, 2011; Konyali *et al.*, 2013; Moce *et al.*, 2014; Motamedi-Mojdehi *et al.*, 2014).

Counteracting capacitation-like changes

One of the major effects of cryopreservation on sperm is induction of capacitation-like changes (i.e. cryocapacita-

tion). Several studies have shown that semen collected by electroejaculation (i.e. higher seminal plasma content) has better cryotolerance than semen collected by artificial vagina (Jimenez-Rabadan *et al.*, 2013; Ledesma *et al.*, 2015). However other studies did not show any difference between the two methods of collection while others have shown that AV collected semen from bucks has better post-thaw motility (Jimenez-Rabadan *et al.*, 2012). Addition of seminal plasma to cryopreserved sperm was found to improve fertility in ewes inseminated transcervically (Maxwell *et al.*, 1999). Some proteins that are provided by seminal plasma such as spermadhesins and SPINK3 (Serine Protease Inhibitor Kazal Type 3 or caltrin) interfere with the capacitation pathways by either stabilizing the membrane or inhibiting calcium transport (Maxwell *et al.*, 2007; Zalazar *et al.*, 2016). SPINK3 was found in high concentration in seminal plasma from rams with good freezing ability spermatozoa (Rickard *et al.*, 2016). This protein is known to modulate calcium increase and to downregulate the signal transduction pathway leading to capacitation. Addition of heterologous recombinant SPINK3 to the freezing extender or after thawing was shown to improve post-thaw progressive motility and reduce acrosomal loss (Zalazar *et al.*, 2016). Post-thaw addition of seminal plasma was also shown to reverse cryopreservation damage (Bernardini *et al.*, 2011). There is a seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa (Leahy *et al.*, 2010). Despite these studies, the effect of addition of seminal plasma to cryopreserved sperm remains controversial (Prado *et al.*, 2013; Rovegno *et al.*, 2013).

Other additives

Fruits juices from pineapple and orange (Daramola *et al.*, 2016b), coconut water (Daramola *et al.*, 2016a), honey (Jerez-Ebensperger *et al.*, 2015) as well as herbal antioxidants (Mascaro *et al.*, 2013; Zanganeh *et al.*, 2013; Baghshahi *et al.*, 2014) have been used with various degrees of success.

Cryopreservation of semen from other small ruminants

Commercial extenders developed for the cryopreservation of ram and buck semen have been used successfully for the cryopreservation of semen from farmed cervids (i.e. elk, white-tailed deer) and wild small ruminants (i.e. Bighorn sheep, roe deer, mouflon...). Semen is generally collected by electroejaculation with or without ultrasound guided rectal massage under general anesthesia. In farmed white tail deer, soybean (Andromed®) and liposome-based (Optixcell®) extenders were found to be superior to Tris-based extenders (Biladyl® or Triladyl®) (Stewart *et al.*, 2016). Electroejaculated semen from captive roe deer (*Capreolus capreolus*) showed better post-thaw motility and acrosome integrity when from frozen in Berliner Cryomedium® (TES-Tris-fructose-lactose-EY) compared to Tris-citric acid-glucose or TES-Tris-glucose (Prieto-Pablos *et al.*, 2016). Similar extenders have been used successfully for the cryopreservation of semen from endangered gazelles (*Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*) (Garde *et al.*, 2003; Garde *et al.*, 2008), ibex (*Capra pyrenaica*) (Santiago-Moreno et *al.*, 2009b; Coloma *et al.*, 2010a; Coloma *et al.*, 2010b; Coloma *et al.*, 2011; Pradiee *et al.*, 2015), chamois (*Rupicapra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia sahariensis*) (Santiago-Moreno et *al.*, 2013; Pradiee *et al.*, 2016). In our laboratory, electroejaculated semen from Bighorn sheep (*Ovis canadensis*) has been successfully cryopreserved in ram semen extenders and used for artificial insemination (Rodriguez *et al.*, 2009; Subramaniam *et al.*, 2014).

Cryopreservation of epididymal sperm

Post-mortem cryopreservation of *cauda epididymis* sperm and its use for artificial insemination is an important area of research. Optimization of the technique allows salvage of genetics from terminal animals or from rare domestic and wild small ruminants (Vasquez *et al.*, 2013). Epididymal sperm can be harvested from severely injured valuable males or wildlife and cryopreserved. Testes can be cooled to 5°C and stored for up to 48 hours before processing (Turri *et al.*, 2014). This technique has been used successfully in a variety of species including the ibex (Santiago-Moreno *et al.*, 2006a; Santiago-Moreno et *al.*, 2006b; Santiago-Moreno *et al.*, 2007; Santiago-Moreno *et al.*, 2008; Santiago-Moreno et *al.*, 2009a; Fernández-Santos *et al.*, 2011; Pradiee *et al.*, 2014; López-Saucedo *et al.*, 2015), Iberian red deer, roe deer, catabrian chamois (Martinez-Pastor et *al.*, 2005; Martínez *et al.*, 2008), and Bighorn sheep (Rodriguez *et al.*, 2010; Hansen *et al.*, 2012; Campbell *et al.*, 2014) (Figure 1).

Advances in method of evaluation of cryopreserved sperm and relationships with fertility

Method to evaluate post-thaw semen quality has been a subject of research for several decades. Post-thaw progressive motility is often used as the primary criterion. Velocity and linearity determined by computer assisted sperm analysis (CASA) seems to be a more reliable indicator of quality and correlates with fertility (Furstoss *et al.*, 2010; Del Olmo *et al.*, 2013). In addition to motility, evaluation of membrane integrity, viability and functional tests have become more and more commonly used to objectively evaluate sperm quality. Viability or membrane integrity can be determined with eosin-nigrosin staining (Rekha *et al.*, 2016). However, fluorescence techniques such as SYBR-14 and prodium iodide offer a more repeatable measure. SYBR-14 is used as a membrane-permeable DNA intercalating agent with maximum emission of 516 nm (green) and readily stains all nuclei. Propidium iodide is used as an intercalating agent with maximum emission of 617 nm (red), but it only stains the nucleus if the sperm plasma membrane is damaged which makes it a useful counter stain for dead cells (Yaniz *et al.*, 2013; Stewart *et al.*, 2016) (Figure 2). Membrane integrity can also be evaluated with carboxyflurescien diacetate and propidium iodide (Soares *et al.*, 2015).

Membrane integrity can also be assessed by the hypo-osmotic swelling test (HOST). The HOST solution is prepared using a mixture of fructose (9 g/L) and sodium citrate (4.9 g/L) (Alcay *et al.*, 2016; Quan *et al.*, 2016; Rekha *et al.*,

2016) or a 100 mOsm solution of sucrose. Semen (20 µl) is added to the solution (200 µl) and incubated for 30 to 60 minutes at 37ºC. Spermatozoa (200) are evaluated under the microscope (400×). Sperm with swollen membranes (curled tails) are considered to have an intact membrane (Figure 3).

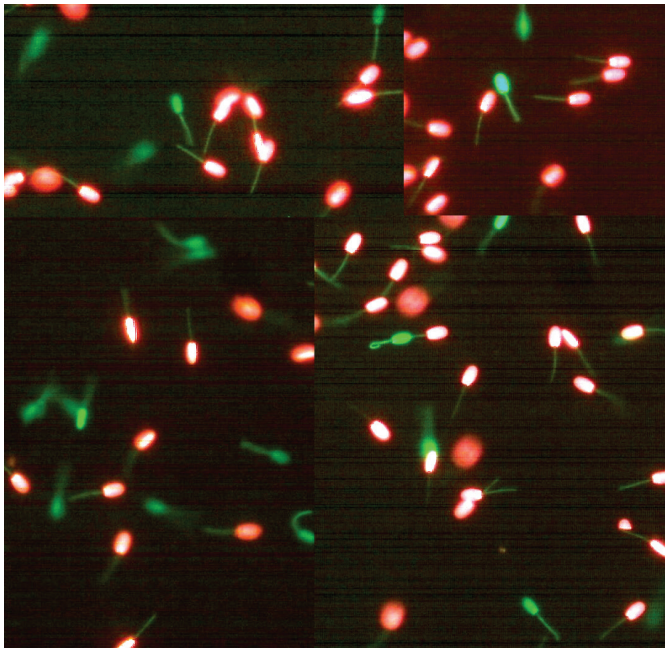

Figure 2: SBYR-14 and propidium iodide viability staining: Red sperm (stained with PI) have damaged plasma membrane, Green sperm (SYBR-14 stained are viable).

Acrosome status can be assessed with Chlortetracyclin staining (Najafi *et al.*, 2014b) or by a combination of Fluorescein isothiocyanate-conjugate peanut (*Pisum sativum*) agglutinin (FITC-PNA) and PI. This stain has a maximum emission of 521 nm (green) and targets the inner leaflet of the outer acrosomal membrane identifying damages acrosomes. PI is used as a counterstain to allow for simultaneous sperm viability and condition of acrosome (Soares *et al.*, 2015; Alcay *et al.*, 2016; Stewart *et al.*, 2016) (Figure 4).

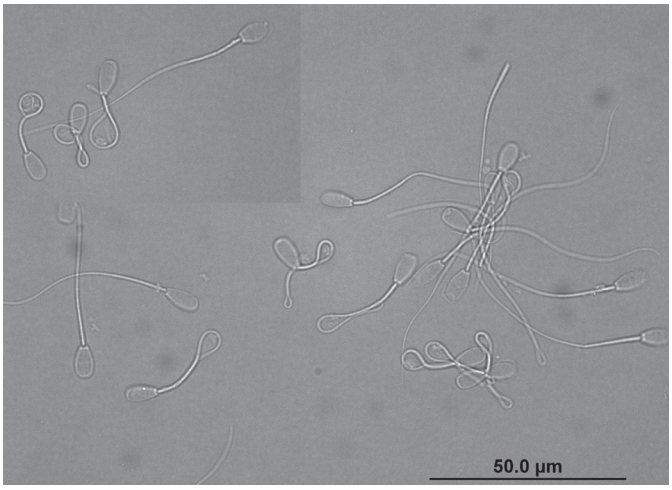

Figure 3: Hypoosmotic swelling test: sperm with damaged membranes do not swell (straight), normal sperm shows various coiling of the tail and mid-piece due to swelling.

Figure 1: Collection of epididymal sperm from a) big horn sheep (Ovis Canadensis) and endangered species in North America. b-c) Dissection of the testis and removal of the cauda epididymis from a deceased male, d) the tail of epididymis is washed from blood with extender, e) mincing and floating of epididymal tissue in semen freezing extender

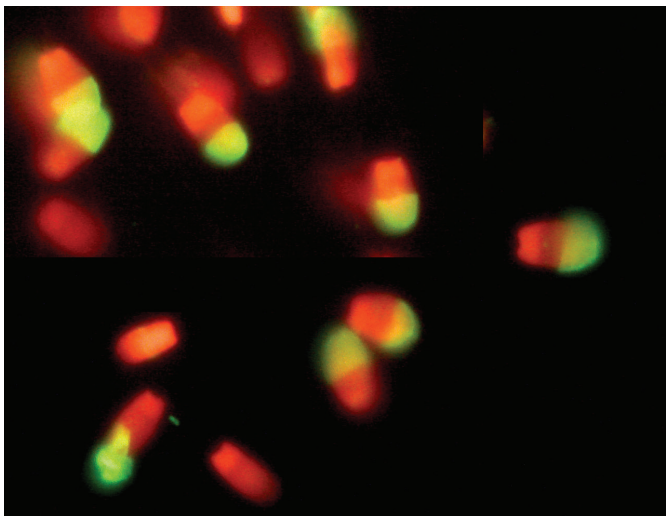

Figure 4: FITC-PNA damaged acrosome (green fluorescence).

Sperm mitochondrial status can be assessed using mitochondrial stain rhodamine l23 or 5,5′,6,6′tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). For staining, 300 µL of semen is mixed with 2.5 µL of 1.53 mM solution of JC-1 in DMSO and 2.5 µL of 0.2 % solution of PI in distilled water. The sample is gently mixed and incubated at 37°C in dark for 20 minutes. JC-1 aggregates inside the functional mitochondria. Highly functional mitochondria are more penetrable by the stain and glow yellow-orange compared to green fluorescing weak functioning mitochondria (Figure 5).

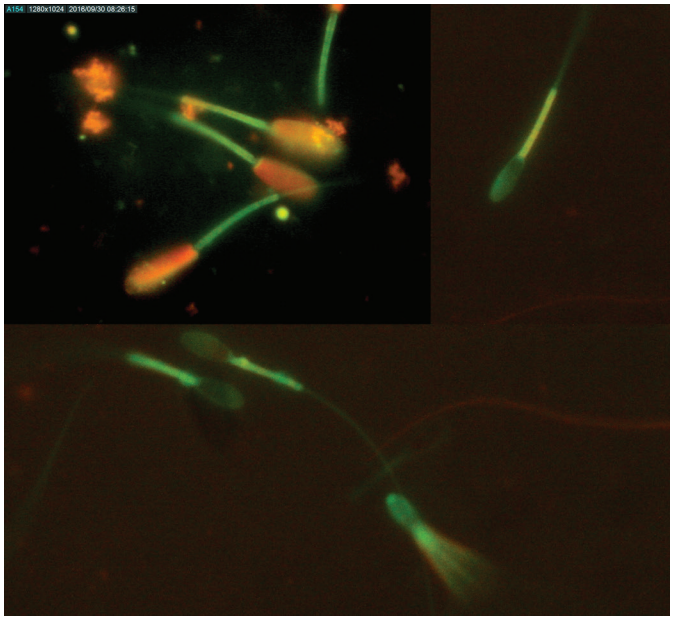

Figure 5: Mitochondrial activity assessed with JC-1. Sperm with midpiece yellow-orange fluorescence have highly functional mitochondria. Green fluorescence denotes weak activity

Another sperm quality test that has gained a lot of interest is sperm chromatin structure assay (SCSA) using acridine orange stained sample analyzed by flow cytometry under 488 nm argon excitation laser. Acridine orange is a cell permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA (normal) and red when bound to single stranded DNA (abnormal) (Stewart *et al.*, 2016).

Sperm death can be determined using apoptosis detection kits. One such kits uses PI and Annexin V. PI can only penetrate membrane of necrotic cells to stain nucleus. PI positive population, dead; Annexin V-FITC positive and PI negative population, early apoptotic; both Annexin V-FITC and PI positive population, late apoptotic; both Annexin V-FITC and PI negative population, normal (Quan *et al.*, 2016). *In situ* cell death detection can also be determined enzymatically using a Terminal deoxynucleotydyl transferease dUTP nick end labeling (TUNEL) assay to detect DNA fragmentation (Alcay *et al.*, 2016). Apoptotic cells can also be identified by mitochondrial membrane potential using specific stains such as MitoLight® (5,5_,6,6_-tetrachloro-1,1_,3,3_-tetraethylbenzimidazolylcarbocyanine chloride) that differentially stains mitochondria of healthy cells red while it remains as monomers in the cytoplasm and fluoresces green (Quan *et al.*, 2016).

FERTILITY

Pregnancy rates using frozen-thawed semen vary from 50 to 70% in goats. Several factors, including breed, season, male, dose of semen, method of synchronization and flock/herd management (Anel *et al.*, 2005; Arrebola *et al.*, 2014). Artificial insemination is performed at a fixed time following synchronization or 12 hours after onset of estrus. In goats, most breeds can be inseminated by transcervical catheterization except for very small breeds where laparoscopic insemination is required in order to deposit semen *in utero* (Nordstoga *et al.*, 2010b).

In sheep, the main challenge in artificial insemination with frozen-thawed semen has been the difficulty in placing semen *in utero* via transcervical catheterization because of the morphology of the cervix (Moura *et al.*, 2011; Richardson *et al.*, 2011; Robinson *et al.*, 2011). Traditionally, ewes are inseminated with frozen-thawed seem via laparoscopy (Masoudi *et al.*, 2017). Laparoscopic insemination does not seem to be affected by side of insemination (ipsilateral *vs* contralateral to ovulation) (Anakkul *et al.*, 2014). In recent years, transcervical or vaginal inseminations gained more popularity. Several treatments have been tested to improve pregnancy rate following intracervical insemination. These include the use of estrogens, relaxin, oxytocin (IM) (Bartlewski and Candappa, 2015), topical application of PGE_2 on the cervix (Horta *et al.*, 2010; Barbas *et al.*, 2013; Bartlewski and Candappa, 2015) and treatment with carazolol (a beta adrenergic blocking agent) (Gündüz *et al.*, 2010). However, these treatments result in only modest improvement of pregnancy rates or sometimes a reduced fertility. Deposition of semen in the vaginal fornix can result in pregnancy although the overall conception rates are generally 10 to 20% lower than with intracervical insemination (Richardson *et al.*, 2012a). A recent small study on vaginal insemination using a dose of 400 million spermatozoa per insemination resulted in a 50 % conception rate (Richardson *et al.*, 2012a). Despite a reduced pregnancy rate with vaginal insemination, the technique is more attractive given the lesser cost and animal welfare issues compared to laparoscopic insemination (Paulenz *et al.*, 2007). However, excellent semen quality is required with vaginal insemination (Paulenz *et al.*, 2007; Richardson *et al.*, 2012b). In sheep, epididymal sperm produced equal pregnancy rates

as semen collected with artificial vagina. However, semen collected by electroejaculation produced lower pregnancy rates (Alvarez *et al.*, 2012b).

CRYOPRESERVATION OF EMBRYOS

Embryo cryopreservation has been used in small ruminants since the late 1970's. There are two methods of embryo cryopreservation; the conventional slow method and vitrification (Massip, 2001). The first step in embryo cryopreservation is selection of excellent quality embryos (Grade I or II) (Dalcin *et al.*, 2013) (Figure 6). The main recent advances are in the development of simple procedures of vitrification making the process cheaper and faster for field use.

Cryopreservation of embryos by the slow cooling methods

The conventional method for embryo cryopreservation is often referred to as a slow-freezing method because it involves several steps which include, dehydration and equilibration with cryoprotectant, slow cooling to the seeding point, seeding and holding then further cooling at a slow rate prior to plunging the embryos in liquid nitrogen (Youngs, 2011).

Cryoprotectant addition and equilibration

The cryoprotectant can be added in a single step or in multiple steps with increasing concentration. Embryos are equilibrated in each solution for 5 to 10 minutes depending on the cryoprotectant used. The most common cryoprotectants used are glycerol of ethylene glycol. After equilibration, the embryos (2 per straw) are loaded in 0.25 mL straws (Youngs, 2011).

Initial cooling and seeding

The slow embryo freezing methods requires the use of a controlled rate biological freezer (Figure 7). These devices are designed to cool the embryos at a rate of 1°C/min from room temperature to the seeding point (-6 to -6.5°C). Seeding is the process of induction of ice crystal

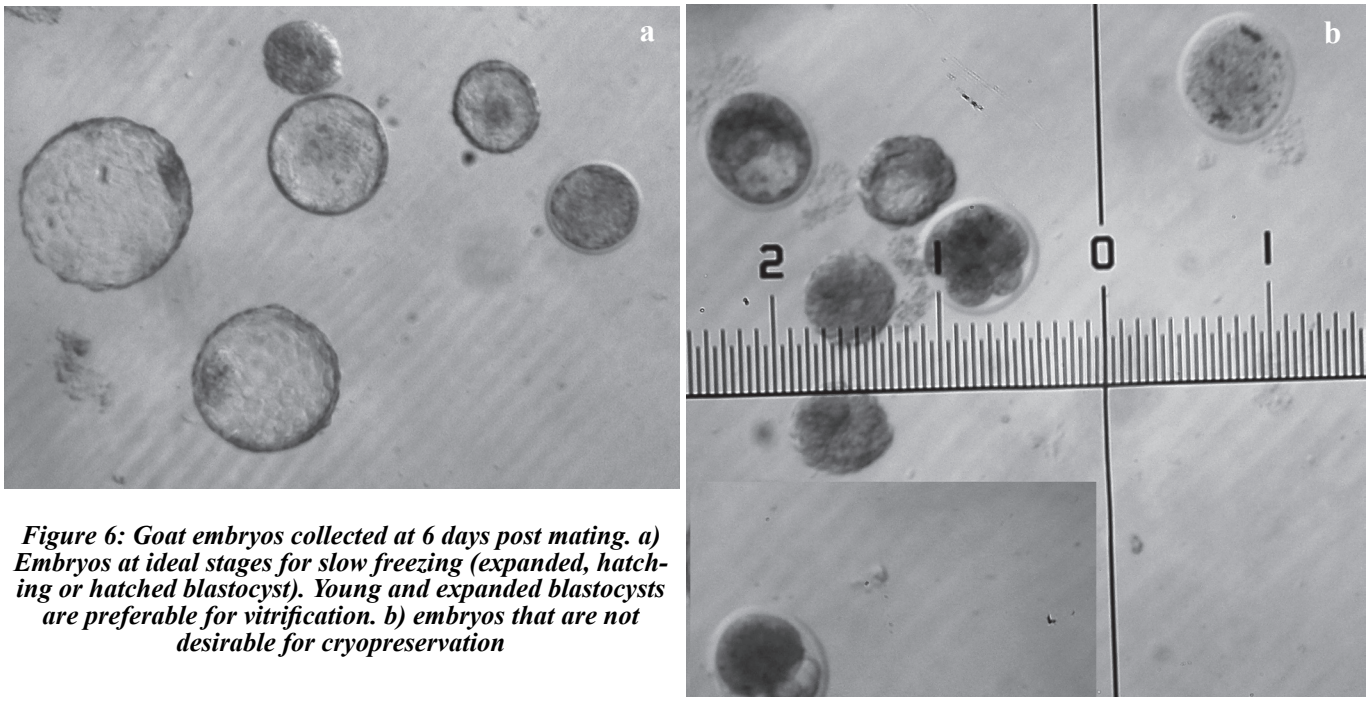

Figure 6: Goat embryos collected at 6 days post mating. a) Embryos at ideal stages for slow freezing (expanded, hatching or hatched blastocyst). Young and expanded blastocysts are preferable for vitrification. b) embryos that are not desirable for cryopreservation

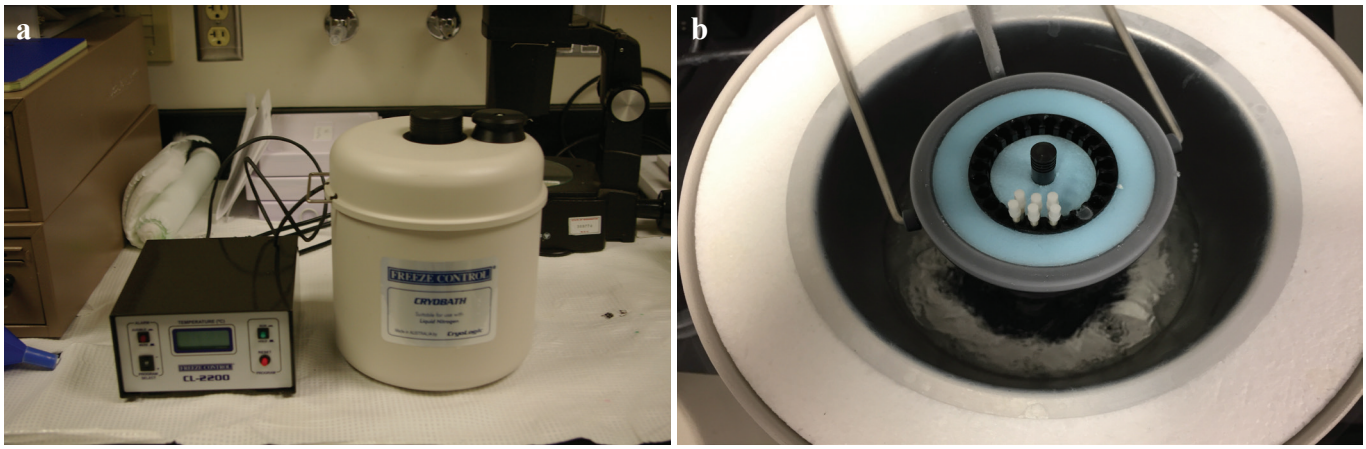

Figure 7: Controlled rate embryo freezer used by the authors. This freezer allows for 4 different controlled freezing curves based on a liquid nitrogen bath (cryochamber). a) complete unit with controller box (left) and cryogenic chamber (right). b) straws loaded in the cryochamber

formation. This is achieved by touching the meniscus of the medium in the straw with very cold small tweezers or cotton-tipped stick that have been placed in liquid nitrogen (Figure 8). The formation of ice crystal in the medium surrounding the embryo results in further dehydration of the embryo. The straw is held at the seeding temperature for approximately 10 minutes before resuming the cooling process (Youngs, 2011).

Final cooling and storage in liquid nitrogen

After holding at seeding temperature, the straws must be cooled at a rate of 0.5°C/min to approximately -34 or -35°C where they are held for approximately 10 minutes prior to direct plunging in liquid nitrogen (-196°C) then transferred to a liquid nitrogen tank for storage (Youngs, 2011).

Thawing of embryos frozen with the conventional method

Cryopreserved embryos must be thawed following a strictly defined method. Straws are usually removed from the liquid nitrogen tank and held at room temperature for 3 to 5 seconds. This would reduce the risk of cracking the zona pellucida and explosion of the straws. Straws are then placed in a water bath at 35 to 38°C for 25 to 30 seconds. The embryos are removed from the straw and placed in a rehydration (cryoprotectant removal) medium prior to transfer. The process of cryoprotectant removal or rehydration is performed in a stepwise or single step fashion. The stepwise technique requires embryos to be placed successively in media with lower concentrations of cryoprotectant (1.5, 1.0 and 0.5 Molar) for 10 minutes per step. In the single step technique, the embryos are placed in a 1.0 Molar solution of sucrose (a non-penetrating sugar) for 10 minutes (Youngs, 2011).

In recent years, the trend has been to freeze embryos using the direct transfer method which does not require removing the embryos from the straws. The direct transfer method requires the use of ethylene glycol as the cryoprotectant. Because ethylene glycol is a small molecule, it diffuses quickly out of the embryo upon transfer to the recipient. It is very important to minimize the time from thawing to transfer into the uterus when using this method. In sheep, a one-step addition of 1.5 M ethylene glycol followed by the one step removal in 1 M sucrose after controlled freezing and thawing yielded very high survival rate (>70%) (McGinnis *et al.*, 1993).

In goats, the maximum survival of frozen-thawed embryos is attained with expanded, hatching or hatched blastocysts (Li *et al.*, 1990) (Figure 8).

Embryo vitrification

Vitrification is an ultrafast fast method for freezing (2500°C/min) resulting in "glass" (i.e. vitrous state) instead of ice crystal formation. The technique was first described for mammalian embryos in 1985 (Rall and Fahy, 1985). This avoidance of intracellular and extracellular ice crystal formation reduces cellular damage and improves embryo viability (Depaz *et al.*, 1994). In addition, this technique does not require any sophisticated equipment, which reduces the cost of cryopreservation of embryos. Vitrification uses media containing high concentration of permeating cryoprotective agents. Embryos are placed in a first medium of 2 Molar of the cryoprotectant. Following equilibration, the embryos are placed immediately in a medium with much higher concentration of cryoprotectant (e.g 7 Molar) for a very short period of time (30 to 45 second) then they are plunged in liquid nitrogen (Massip, 2001). Several vitrification protocols have been reported for sheep and goat embryos. There seems to be an important difference between species in terms of behavior of embryos towards the vitrification medium (goats (El-Gayar and Holtz, 2001; Al Ahmad *et al.*, 2012; Araujo-Lemos *et al.*, 2015); sheep (Ali and Shelton, 1993; Bettencourt *et al.*, 2009; de Araujo-Lemos *et al.*, 2014; Santos Neto *et al.*, 2015)).

In sheep, vitrification of embryos was reported to be as efficient as conventional freezing (Depaz *et al.*, 1994; Martinez and Matkovic, 1998; Baril *et al.*, 2001; Isachenko *et al.*, 2003; Bettencourt *et al.*, 2009; Green *et al.*, 2009). Several improvements of the vitrification protocol have been achieved in recent years resulting in improved viability of embryos. There is a general agreement amongst authors that ovine embryos are more resistant to cryodamage than those of other species (Bettencourt *et al.*, 2009).

A recently described protocol resulted in good survival of embryos. Embryos are placed in a basal solution of

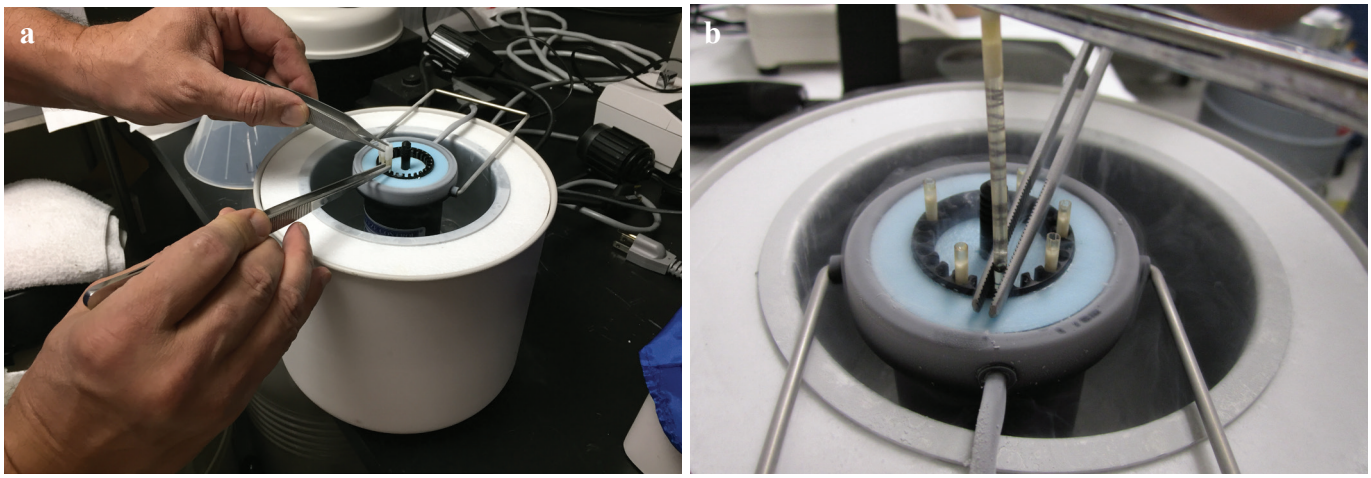

Figure 8: Seeding process. a) The straws containing embryos are raised. b) The medium meniscus is touched with very cold tweezers to initiate ice crystal formation

Hepes-TCM 199 supplemented with 20% Fetal Bovine Serum (FBS) for 5 minutes then transferred into a medium containing 10 % ethylene glycol (EG) and 10 % dimethylformamide (DMF) for 1 minute and moved to a solution of 20 % EG, 20% DMF and 0.5M sucrose and frozen using the open pulled straw technique by plunging in liquid nitrogen (Araujo-Lemos *et al.*, 2015).

Sheep and goat embryos have been vitrified successfully using a standard simple commercial protocol used for bovine embryo vitrification. Embryos are placed in a commercial holding medium (Syngro®) until vitrification. The commercial vitrification protocol (Bovipro®, Minitube, Germany) consisted of a basic medium supplemented with 20 % FBS. The embryos are placed for 5 minutes in BM+10% glycerol then transferred into BM + 20 % glycerol +20 % EG for 5 minutes. Finally, the embryos are placed in the vitrification solution (BM+25 % glycerol+ 25 % EG) for 30 seconds. Embryos are loaded in 1 µl of vitrification medium in the tip of a micropipette and then placed in a 3.6 ml cryotube filled with liquid nitrogen. For thawing, the micropipette tips are warmed between the thumb and the middle finger for 10 seconds and the embryos are immersed in BM at 25°C in three dilution steps for 5 minutes each (12.5 % G+12.5 % EG+ 0.5 M sucrose, 0.5 M sucrose and 0.25 M sucrose). The embryos are then placed in BM solution for 5 minutes prior to transfer (Gibbons *et al.*, 2011).

The stage of the embryo is very important. In sheep, morulas and blastocysts have similar survival rates while day 2 embryos have lower survival rate after vitrification (Santos Neto *et al.*, 2015). In goats, blastocysts seem to tolerate vitrification better than morulae and hatched blastocysts (Al Yacoub *et al.*, 2010).

Factors affecting pregnancy rate of frozen-thawed embryos

Several factors may impact the success of embryo cryopreservation and need to be further evaluated. These include method of production of embryos (*in vivo vs in vitro*), stage of embryo development, breed, age and diet of the donor. Vitrification protocols have been hindered by the toxicity of cryoprotectants. Research efforts have been centered on testing new, less toxic cryoprotectants. Pregnancy rates from vitrified embryos have improved to reach levels that are very comparable to transfer of fresh embryos.

Pregnancy rates following transfer of cryopreserved embryos vary between 38-73% for slow freezing and between 52-79% for vitrification and are not significantly different from those obtained with fresh embryos (50 to 90%) (Baril *et al.*, 2001; Guignot *et al.*, 2006; Hong *et al.*, 2007; Green *et al.*, 2009; Gibbons *et al.*, 2011). There is however an interaction between method of freezing and cryoprotectant used and some vitrification techniques may produce lower pregnancy rates than conventional slow freezing (Varago *et al.*, 2014). In sheep, direct transfer of vitrified embryos resulted in higher pregnancy rates than for embryos frozen by the slow method (Green *et al.*, 2009).

Several factors may affect results of embryo cryopreservation. *In vitro* produced embryos have lower survival rates than *in vivo* produced embryos (Zhu *et al.*, 2001; Martinez *et al.*, 2006; Bhat *et al.*, 2015). Pregnancy and birth rates achieved with *in vitro* produced cryopreserved embryos is also affected by method of culture during production (Mara *et al.*, 2015). The poor survival of *in vitro* produced embryos may be due to epigenetic alterations (Nieddu *et al.*, 2015; Romão *et al.*, 2015). Also, in goats more advanced stages of development (expanded and hatched blastocysts) survive cryopreservation better than morulas (Gibbons *et al.*, 2011). The effect of breed on embryo ability to withstand cryopreservation merits further studies (Fair *et al.*, 2006). Some the breed effect may be due to differences in lipid content of oocytes and embryos (Romão *et al.*, 2016).

CONCLUSION

Sperm and embryo cryopreservation are established techniques in small ruminants. Incorporation of these biotechnologies in a strategy for the preservation of genetic diversity of small ruminants and propagation of elite sires and dams is a reality. Improvements in sperm preservation through better extenders and reduction of oxidative stress may render the need for laparoscopic artificial insemination in sheep absolute and expand the use of the technique in the field. The development of new more objective methods for evaluation of cryopreserved semen allowed a better understanding of factors affecting post-thaw semen quality. Embryo preservation by vitrification produces similar results as conventional slow methods and reduces the cost of investment in specialized equipment. Further research is needed to refine vitrification techniques for use in the field for direct transfer.

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