Review

Current status and future direction of cryopreservation of camelid embryos

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ABSTRACT

Over the past 3 decades, and similar to the horse industry, fresh embryo transfer has been widely practiced on large commercial scales in different camelid species, especially the dromedary camel and alpaca. However, the inability to cryopreserve embryos significantly reduces its broader application, and as such limits the capacity to utilize elite genetic resources internationally. In addition, cryopreservation of the semen of camelids is also difficult, suggesting an extreme sensitivity of the germplasm to cooling and freezing. As a result, genetic resources of camelids must continue to be maintained as living collections of animals. Due to concerns over disease outbreaks such as that of the highly pathogenic Middle East Respiratory Syndrome in the Middle East and Asia, there is an urgent need to establish an effective gene banking system for camelid species, especially the camel. The current review compares and summarizes recent progress in the field of camelid embryo cryopreservation, identifying four possible reasons for the slow development of an effective protocol and describing eight future directions to improve the current protocols. At the same time, the results of a recent dromedary camel embryo transfer study which produced a high morphologic integrity and survival rate of Open Pulled Straw-vitrified embryos are also discussed.

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1. Introduction

The camelid family includes dromedary and Bactrian camels, llamas, alpacas, vicunas, and guanacos. The first two are Old World camelids, whereas the last four are known as New World camelids or South American camelids. Camelids are strictly herbivorous animals and have unique reproductive characteristics [1–3]. They are seasonal breeders, the females only ovulate postmating (referred to as induced ovulators), and males produce viscous semen. Interestingly, all of these species have the same number of chromosomes (37 pairs), and interspecies crossbreeding can generate hybrids [4–6].

The reproductive efficiency of camelids is low partly due to the late onset of puberty, the short breeding season, early embryonic loss, and a long gestation period of 13 months. Accordingly, assisted reproduction technologies, such as fresh embryo transfer (ET), have been widely practiced in dromedary camel breeding programs in Middle Eastern countries [7], and in alpaca and llama programs mainly in Australia and South America [8,9]. However, frozen embryos are not used in those commercial operations due to an unacceptably low pregnancy rate [1,10]. The ability to successfully cryopreserve embryos could overcome the spatial and temporal barriers between recipient and donor, and subsequently offer considerable logistical and economic advantage. This is especially important for camelids because recipients need to have certain sized follicles

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(growing follicles) to be ovulated with the treatment of hormones, and this requires a large pool of females from which to select appropriate recipients [11]. In addition, embryo cryopreservation provides an effective means of preserving endangered camelids, such as the vicuna and guanaco. Furthermore, a successful cryopreservation technology would promote the application of other embryo biotechnologies, such as cloning and transgenics, on large commercial scales [12]. Thus, there is a need to compare and summarize recent progress in the field of embryo cryopreservation for camelids to provide researchers with new insight into designing experiments that will lead to more effective cryopreservation protocols for use in ET programs to facilitate a wider application.

To review and cover all results published in the field, literature searches were conducted for each species and each technique using the PubMed database. Key word combinations used were as follows:

I. Camelids, embryo, cryopreservation or freezing,
II. Dromedary, embryo, cryopreservation or freezing,
III. Bactrian, embryo, cryopreservation or freezing,
IV. Alpaca, embryo, cryopreservation or freezing,
V. Ilama, embryo, cryopreservation or freezing.

A total of 22 articles were retrieved and reviewed, and with the current authors' extensive experience in cryopreservation of embryos and stem cells in the human [13], bovine and camel [14–16], several approaches on how to improve the efficacy of the existing protocols for freezing camelid embryos are presented.

1.1. History of cryopreservation of camelid embryos

Two approaches of cryopreservation, slow freezing and vitrification, are most commonly used to maintain functional capacity of animal germplasm during a cooling and warming process. Although cryopreservation of germplasm has been successfully applied in human medicine and to some livestock breeding programs [15,17], the cryopreservation of camelid embryos is in its infancy (Table 1), with the focus on modification of established protocols commonly used for other species [18,27].

Attempts to freeze camelid embryos started in the late 1990s, with the application of ET to dromedary camels [3,18]. As shown in Table 2, a number of pregnancies from

frozen/thawed ETs have been reported in dromedary camels [18] and llamas [8], with two live births from vitrified embryos [10,16] and one from a slow-frozen embryo in dromedary camels [18]. It has been nearly 3 decades since the initial studies, but embryo cryopreservation has still not yet been successfully incorporated into an ET program, a reflection of the difficulties associated with developing an effective procedure for camelids.

Possible reasons for the slow development of an effective cryopreservation protocol for camelids include the following:

I. The lack of zona pellucida in hatched embryos: The permeability of cryoprotective agents (CPAs) during the cooling/warming processes might be influenced by the lack of zona pellucida in hatched embryos [12]. In the current ET practice with dromedary camels, example, hatched embryos are preferably collected on Days 7, 8, or 9 after ovulation with the intention of enhancing the recovery rate. Therefore, the protocols developed for nonhatched embryos in other species are unsuitable for freezing camelid hatched embryos.
II. A much larger variation in embryo size: Embryo size not only differs between donors on Days 6, 7, and 8 but it can also vary substantially between embryos harvested from one animal. Thus, there is a challenge to develop protocols that fit different-sized embryos [27].
III. A great amount of lipids in embryos: Similar to porcine embryos, camelid embryos contain a high concentration of lipids—this has been shown to have an adverse effect on conventional freezing methods [28,29].
IV. The lack of a convenient and reliable evaluation system for embryo quality: The morphologic appearance of cryopreserved embryos does not always correlate to their developmental potential, and so, it is insufficient to assess the outcome of the cryopreservation and to predict ET success [27].

1.2. Slow freezing

The principle of cryopreservation is to use permeating CPAs (e.g., glycerol, propanediol [PROH], DMSO, and ethylene glycol [EG]) and nonpermeating CPAs (e.g., sucrose, glucose, and trehalose) to replace intracellular water from embryos and prevent the formation of ice crystals

Table 1
History of cryopreservation of camelid embryos and its comparison with other domestic species.

Species	Method	Achievement	Years	Reference of first report
Dromedary camel	Slow freezing	Pregnancy/live birth	2002	Skidmore et al. [18]
	Vitrification	Pregnancy/live birth	2005	Skidmore et al. [10] and Nowshari et al. [16]
Lama	Slow freezing			
	Vitrification	Pregnancy	2002	Aller et al. [8]
Bovine	Slow freezing	Live birth	1973	Wilmut and Rowson [19]
	Vitrification	Live birth	1986	Massip et al. [20]
Ovine	Slow freezing	Live birth	1976	Willadsen et al. [21]
	Vitrification	Live birth	1994	Széll et al. [22]
Swine	Slow freezing	Live birth	1989	Hayashi et al. [23]
	Vitrification	Live birth	2000	Dobrinsky et al. [24]
Horse	Slow freezing	Live birth	1982	Yamamoto et al. [25]
	Vitrification	Pregnancy	2005	Eldridge-Panuska et al. [26]

Table 2
The outcome of embryo transfers that resulted in pregnancy or live offspring in different camelids species.

Species	Method	Pregnancy rate (%)	Live birth rate (%)	Reference
Dromedary camel	Slow freezing	14/43 (32.6%)	1/43 (2.3%, unpublished data)[a]	Skidmore et al., 2004 [18]
	Vitrification	2/20 (10%)	1/20 (5%)	Nowshari et al., 2005 [16]
		8/21 (38%)	1/21 (4.8%, unpublished data)[b]	Skidmore et al., 2005 [10]
Llama	Vitrification	2/4 (50%)		Aller et al., 2002 [8]

[a] Only one pregnancy was allowed for full term from 14 pregnant recipients, others were terminated by prostaglandin treatment after the confirmation of pregnancy by ultrasound scanning at 3 mo of the transfer.

[b] Only one pregnancy was allowed for full term from eight pregnant recipients, others were terminated by prostaglandin treatment after the confirmation of pregnancy by ultrasound scanning at 3 mo of the transfer.

during the freezing and thawing process [17,30,31]. In addition, controlled slow freezing rates also facilitate sufficient water to leave the cell during progressive freezing of the extracellular fluid.

Standard slow freezing protocols for mammalian oocytes/embryos usually consist of four distinguishable components:

I. Equilibration media: Single (e.g., glycerol, PROH) or a combination of two (e.g., glycerol + DMSO) permeating CPAs ($\sim$1.5 M), with the addition of a non-permeating CPA ($\sim$1 M) [32].
II. Equilibration time: Embryos are equilibrated in these solutions for 5 to 10 minutes and then frozen at a slow controlled rate ($\sim$0.3 °C/min) until seeding.
III. Seeding temperatures: At $-4.5 \sim -7$ °C, seeding is performed to induce the freezing process and then stepwise cooling. On reaching -35 °C, straws can be plunged directly into liquid nitrogen, or the temperature reduction may be continued but at a faster rate (~ -50 °C/min) to -150 °C before the straw is stored in liquid nitrogen.
IV. Thawing: This includes the direct warming of straws to about $32 \sim 37$ °C in a water bath ($\sim$2 minutes), and then, the embryos undergo rehydration by a sequence of rapid changes through a series of solutions with decreasing concentrations of non-permeating CPAs (e.g., sucrose, $\sim$ 1 M) taking up to 15 minutes [17,31].

Because of the strong dependence of intracellular ice formation (IIF) in mammalian cells and yeast on the temperatures associated with extracellular ice crystallization ("seeding"), the determination of the optimum seeding temperature of a freezing medium is probably the most critical part of an effective protocol [33,34]. However, the importance of this parameter is often neglected in most of the camelid hatched embryo freezing protocols and operators simply use -7 °C, a seeding temperature established for intact blastocysts in cattle and sheep [35].

1.3. Dromedary camels

In the initial phase of developing cryopreservation protocols, slow freezing methods have been used to test the toxicity of standard embryo CPAs to camel embryos. Briefly, the results of those works have shown that camel embryos are sensitive to PROH, DMSO, and glycerol but tolerant to EG [18]. Subsequent studies were performed to determine the minimum exposure time to 1.5-M EG required to achieve cryoprotection and to compare different methods of rehydration with or without sucrose. The highest pregnancy rate (37%) was achieved when the embryos were exposed to EG for 10 minutes, cooled slowly at a rate of -0.5 °C/min to -33 °C before plunging into liquid nitrogen, and then thawed and rehydrated in 0.2-M sucrose in holding media for 5 minutes [18]. In comparison to vitrification, the relatively higher pregnancy rate from slow-frozen embryos may be due to more intact cytoskeleton integrity. Cell death associated with slow freezing was comparable to that of unfrozen control cells, but freezing caused widespread disruption of the actin cytoskeleton, indicating that levels of cell death in an embryo may not be as critical as cytoskeleton integrity for embryo survival and implantation. It appears that slow freezing maintains better cytoskeleton integrity of embryos compared with vitrification [36].

1.4. Other camelids

There has only been one report of the other camelid species (specifically llama), undergoing embryo cryopreservation by a slow freezing method [37]. It is interesting to note a sensitivity of llama trophoblastic vesicles to the different CPAs, with there being no difference in embryo survival rate in either 10% EG or propylene glycol after 24 hours culture, but then only those in EG were able to progressively expand in culture. This result confirms our own previous finding that EG is the most effective CPA for freezing camelid embryos [18,27].

1.5. Vitrification

Vitrification is currently widely used for cryopreservation of human and bovine oocytes and embryos because of its simplicity of use and efficacy [38]. A combination of higher concentrations of CPAs and an increased cooling/warming rate reduces ice crystal formation and thus improves the survival of biological material. Although current vitrification methods differ considerably in technical detail between laboratories or clinics, four basic components are similar in all disciplines:

I. Equilibration and vitrification media: 7.5% (v/v) EG and DMSO, and 15% to 16% (v/v) EG and DMSO plus sucrose, respectively.

II. Equilibration and vitrification temperature: Room temperature (22 °C–27 °C) or mammalian body temperature (37 °C) are used, with a principle of the higher the temperature, the faster the loading rate.

III. Exposure duration: Embryos are exposed to the equilibration media for times ranging from 3 to 15 minutes, and then vitrification is achieved within 40 to 60 seconds.

IV. Warming: Direct warming of carrier tools in holding media with sucrose ($\sim$1 M) at 37 °C ($\sim$2 minutes), and then, embryos are equilibrated sequentially in a series of solutions with decreasing concentrations of sucrose, preferably at room temperature (22 °C–25 °C) for up to 15 minutes to ensure complete rehydration.

There are different types of carrier tools that can be used in vitrification, but Open Pulled Straw (OPS) is the most commonly used tool in embryo vitrification of livestock species because of low cost and ease of use [15,30].

1.6. Dromedary camels

The first study of embryo vitrification and transfer using the OPS method failed to produce a pregnancy [10]. However, the use of 0.25-mL French straws along with a complicated equilibration media (20% glycerol + 20% EG + 0.3-M sucrose + 0.375-M glucose + 3% polyethylene glycol) in three steps did produce reasonable viabilities for Day 6, 7, and 8 embryos. The embryo age/size appeared to be an influencing factor for viability, with the smaller Day 6 embryos being more resistant to cryoinjuries. The transfer of the smaller embryos resulted in a 38% pregnancy rate (8/21), whereas no pregnancies were achieved with the larger Day 7 or 8 embryos [10]. In 2005, the first birth of a live calf was reported from the transfer of 20 vitrified embryos (5% success), using high concentrations of EG (7.0 mol/L) and sucrose (0.5 mol/L) [27].

Recently, to develop a more effective cryopreservation procedure for camels, the current authors have attempted to modify a protocol originally designed for human oocytes and embryos [15,39], to make it suitable for freezing camel hatched embryos. After a thorough examination of different parameters, an optimized protocol (7.5% EG + 0.25 M sucrose for 1 minute, a second equilibration solution 15% EG + 0.5 M sucrose for 2 minutes, followed by two drops of 30% EG + 1 M sucrose vitrification solution for 20 seconds each) was established [27]. Unfortunately, despite a high survival rate (91%) as judged by the morphologic appearance of embryos after warming and in culture, the transfer of 18 vitrified embryos (Day 7 or 8) into six recipients (three each) during the breeding season resulted in no pregnancy (unpublished data). In a subsequent experiment, 10 vitrified embryos were transferred into five recipients (two each), who had received 75 mg progesterone-in-oil (intramuscularly) for 3 days before transfer and for another 14 days after transfer when the pregnancy could be identified by ultrasonography. This exogenous progesterone was injected in case the frozen/thawed embryos did not produce enough maternal recognition of pregnancy

signal to maintain the CL and prevent luteolysis. However, there was still no pregnancies, apparently indicating a total lack of developmental competence of those vitrified embryos (unpublished data). The frustrating results of these transfers imply that the current protocol still needs further modification, and importantly, that the morphological integrity of frozen embryos cannot be used to predict the success of ET outcome [27].

Similar to our observations that most vitrified hatched embryos exhibited normal morphology and expansion in culture, and then become dormant in culture, it is interesting to note that pig morulae and early blastocysts, from which intracellular lipids had been removed by centrifugation and micromanipulation before subsequent vitrification, were able to develop in culture, but then became dormant at the blastocyst stage [27]. However, the transfer of those embryos produced healthy offspring with a high rate of success (9/11, 82%). Therefore, it is reasonable to speculate that removal of lipids from embryos before cryopreservation may be vital not only for survival but most importantly to retain developmental competence [29].

1.7. Llama

Similar to the results in dromedary camels [27], although the morphology and re-expansion of OPS-vitrified embryos in 40% EG was acceptable, transfers of such embryos did not result in pregnancy. The high content of intracellular lipids in embryos may be a reason for low survival rate [28]. In 2002, the first pregnancy was reported from embryos vitrified in French straws with a three-step equilibration in 20% glycerol + 20% EG + 0.3 M sucrose + 0.375 M glucose + 3% polyethylene glycol [8]. However, the birth of live offspring is not reported.

1.8. Future directions

In comparison to slow controlled-rate freezing, vitrification is a simple and advantageous technique, thus becoming the dominant approach for cryopreservation of reproductive cells [15,17,27,29]. Recent achievements with *in vitro* survival of hatched blastocysts from the dromedary camel after vitrification are promising [27]. However, more work is required to clarify the reasons for there being different requirements for *in vitro* and *in vivo* development. To optimize further parameters, the following procedures or techniques might be considered.

I. Cytoskeleton stabilization: Cytoskeleton-stabilizing agents, such as the cytochalasins (cyto-b), have been used in pig embryo vitrification to prevent cytoskeletal disruption during and after cryopreservation. The landmark success of live piglet production from embryos frozen by stabilized vitrification shows the importance of the cytoskeleton structure for retaining developmental competence of embryos [29]. However, the effect of cyto-b on the outcome of vitrification appears to be dependent on the developmental stage of embryos (e.g., hatched embryos in

pig), with a narrow size range (e.g., 325–375 μm). This feature may limit its broader application in vitrification, but it can be circumvented by culturing early stage embryos *in vitro* to meet these requirements [29]. In a preliminary experiment, Day 7 camel embryos (n = 36) were recovered and cultured with or without pretreatment with 7.5-μg/mL cyto-b. Eleven embryos were vitrified after cyto-b treatment (French straw method, [10]), and 16 embryos were not frozen but used to determine if prefreezing treatment alone caused cell damage. Without freezing, cyto-b did not affect cell viability as 12/16 (75%) transferred embryos resulted in pregnancies. However, after cryopreservation, 0/11 (0%) embryos pretreated with cyto-b and 3/9 (33%) without cyto-b pretreatment resulted in a pregnancy (unpublished data). It is obvious that cyto-b treatment does not have any lasting detrimental effects on embryo developmental competence, but that the treatment also does not provide protection for the embryo cytoskeleton during vitrification. A similar effect of cyto-b treatment on embryos has also been observed in an equine study [40].

II. Development of modified carrier tools: Carrier tools that are better adapted to the larger size and special structure of zona-free camel embryos may improve results. Although a direct comparison of OPS straws versus 0.25 mL French straws was not carried out [10], it appears that a relatively larger vitrification volume is beneficial for embryo survival (Vajta G, personal communication).

III. Delipidation: Removal of intracellular lipids by centrifugation and subsequent micromanipulation has been shown to be effective in the vitrification of pig morulae and early blastocysts [29]. When undertaking ET of camelids, for example, in dromedary camels, the uterus is flushed 7 to 8 days after mating to enhance the recovery rate; however, embryos are hatched at this stage. Without the protection of the zonae pellucidae, it would be difficult to centrifuge hatched blastocysts and to perform delipation. But it may be possible to collect expanded blastocysts from Day 6 donors, with two flushes at intervals of 12 hours. Collection and cryopreservation of unhatched embryos will have to be established if international exportation and transportation is intended, as current regulations allow only pathogen-free embryos with an intact zona pellucida to be exported [41].

IV. Embryo size: To overcome the problems associated with the large variation of embryo size from superovulated donors, the selection of a superovulation protocol appears to be an option for producing uniform embryos. For example, superovulation of dromedary camels with two injections of FSH dissolved in hyaluronan solution produces more similar sized embryos compared to other protocols [42].

V. A novel nonpenetrating CPA, carboxylated ε-poly-L-Lysin (PLL): A Japanese group has recently reported that the addition of PLL in vitrification solutions significantly improved the survival rate of mouse oocytes and embryos [43]. Unfortunately, this product is not commercially available. The authors are currently collaborating with this group to see if PLL can improve the survival rate of dromedary camel embryos after freezing.

VI. Automation of freezing procedures: Inconsistencies in the manual handling of samples and differences between individual embryologists can only be eliminated by full automation of the established procedure—an unavoidable task of the next decade applicable to cryopreservation for all species including humans, and for all procedures in assisted reproduction [44].

VII. Synchrony between donor and recipient: Apart from the quality of embryos, the optimal pregnancy rates of ET may depend on various other factors, such as the preparation of the recipient females. The degree of ovulation synchrony between donor and recipient appears to be of paramount importance for the success of an ET program. In the dromedary camel, for example, the pregnancy rate was highest (67%) when embryos were transferred into the recipients that had ovulated 24 hours after the donor, whereas the pregnancy rates dropped sharply to less than 10% if embryos were transferred to the recipients that had ovulated 72 hours behind the donor [45].

VIII. Inhibition of luoteolysis in recipient females: Due to the lack of the cyclical CL and a relatively short luteal lifespan of only 8–10 days in camelids, prolongation of the lifespan of the CL appears to be beneficial for establishing pregnancy by allowing more time for the embryos to secrete the important maternal recognition of pregnancy signal to the mother. The oral administration of the prostaglandin synthetase inhibitor, meclofenamic acid, can prevent both the luteolytic action of exogenous PGF2α and the normal increase in periphereal plasma PMSG concentrations in late diestrus, thereby prolonging the luteal phase and improving the pregnancy rate of ET [46].

On current evidence, we will continue to focus on vitrification of dromedary camel embryos with the previously mentioned approaches and believe that a breakthrough of embryo cryopreservation in the dromedary camel will lead to success in other camelid species.

References

[1] Skidmore JA. Reproductive physiology in female Old World camelids. Anim Reprod Sci 2011;124:148–54.

[2] Dong C. Biology of the Alpaca. Beijing, China: China Press; 2015.

[3] Anouassi A, Tibary A. Development of a large commercial camel embryo transfer program: 20 years of scientific research. Anim Reprod Sci 2012;145:211–22.

[4] Niasari-Naslaji A, Nikjou D, Skidmore JA, Moghiseh A, Mostafaey M, Razavi K, et al. Interspecies embryo transfer in camelids: the birth of the first Bactrian camel calves (*Camelus bactrianus*) from dromedary camels (*Camelus dromedarius*). Reprod Fertil Dev 2009;21:333–7.

[5] Skidmore JA, Billah M, Short RV, Allen WR. Assisted reproductive techniques for hybridization of camelids. Reprod Fertil Dev 2001; 13:647–52.

[6] Skidmore JA, Billah M, Binns M, Short RV, Allen WR. Hybridizing Old and New World camelids: *Camelus dromedarius* × *Lama guanicoe*. Pro Biol Sci 1999;266:649–56.

[7] Skidmore JA. The main challenges facing camel reproduction research in the 21st century. Reprod Suppl 2003;61:37–47. Review.

[8] Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Successful transfer of vitrified llama (*Lama glama*) embryos. Anim Reprod Sci 2002;73: 121–7.

[9] Sumar JB. Embryo transfer in domestic South American camelids. Anim Reprod Sci 2013;136:170–7. Review.

[10] Skidmore JA, Billah M, Loskutoff NM. Comparison of two different methods for the vitrification of hatched blastocysts from the dromedary camel (*Camelus dromedaries*). Reprod Fertil Dev 2005; 17:523–7.

[11] Skidmore JA, Morton KM, Billah M. Unique strategies to control reproduction in camels. Soc Reprod Fertil Suppl 2010;67:467–74. Review.

[12] Wani NA, Wernery U, Hassan FA, Wernery R, Skidmore JA. Production of the first cloned camel by somatic cell nuclear transfer. Biol Reprod 2010;82:373–9.

[13] Vajta G, Rienzi L, Ubaldi FM. Open versus closed systems for vitrification of human oocytes and embryos. Reprod Biomed Online 2015;30:325–33. Review.

[14] Redden E, Davey R, Borjigin U, Hutton K, Hinch G, Hope S, et al. Large quantity cryopreservation of bovine testicular cells and its effect on enrichment of type A spermatogonia. Cryobiology 2009; 58:190–5.

[15] Vajta G. Vitrification in human and domestic animal embryology: work in progress. Reprod Fertil Dev 2013;25:719–27. Review.

[16] Nowshari MA, Ali SA, Saleem S. Offspring resulting from transfer of cryopreserved embryos in camel (*Camelus dromedarius*). Theriogenology 2005;63:2513–22.

[17] Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. Hum Reprod Update 2012;18:536–54.

[18] Skidmore JA, Billah M, Loskutoff NM. Developmental competence *in vitro* and *in vivo* of cryopreserved, hatched blastocysts from the dromedary camel (*Camelus dromedarius*). Reprod Fertil Dev 2004; 16:605–9.

[19] Wilmut I, Rowson LE. Experiments on the low-temperature preservation of cow embryos. Vet Rec 1973;92:686–90.

[20] Massip A, Van Der Zwalmen P, Scheffen B, Ectors F. Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo Letters 1986;7:270–3.

[21] Willadsen SM, Polge C, Rowson LEA, Moor RM. Deep freezing of sheep embryos. J Reprod Fertil 1976;46:151–4.

[22] Széll A, Windsor DP. Survival of vitrified sheep embryos in vitro and in vivo. Theriogenology 1994;42:881–9.

[23] Hayashi S, Kobayashi K, Mizuno J, Saitoh K, Hirano S. Birth of piglets from frozen embryos. Vet Rec 1989;125:43–4.

[24] Dobrinsky JR, Pursel VG, Long CR, Johnson LA. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. Biol Reprod 2000;62:564–70.

[25] Yamamoto Y, Oguri N, Tsutsumi Y, Hachinohe Y. Experiments in the freezing and storage of equine embryos. J Reprod Fertil Suppl 1982; 32:399–403.

[26] Eldridge-Panuska WD, di Brienza VC, Seidel Jr GE, Squires EL, Carnevale EM. Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. Theriogenology 2005; 63:1308–19.

[27] Herrid M, Billah M, Malo C, Skidmore JA. Optimization of a vitrification protocol for hatched blastocysts from the dromedary camel (*Camelus dromedarius*). Theriogenology 2016;85: 585–90.

[28] Von Baer A, Del Campo M. Vitrification and cold storage of llama (*Lama glama*) hatched blastocyst. Theriogenology 2002;57:489. (Abstract).

[29] Dobrinsky JR. Advancements in cryopreservation of domestic animal embryos. Theriogenology 2002;57:285–302.

[30] Edgar DH, Karani J, Gook DA. Increasing dehydration of human cleavage-stage embryos prior to slow cooling significantly increases cryosurvival. Reprod Biomed Online 2009;19:521–5.

[31] Fasano G, Fontenelle N, Vannin AS, Biramane J, Devreker F, Englert Y, et al. A randomized controlled trial comparing two vitrification methods versus slow-freezing for cryopreservation of human cleavage stage embryos. J Assist Reprod Genet 2014;31:241–7.

[32] Saragusty J, Arav A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction 2011; 141:1–19.

[33] Nakamura T, Takagi H, Shima J. Effects of ice-seeding temperature and intracellular trehalose contents on survival of frozen Saccharomyces cerevisiae cells. Cryobiology 2009;58:170–4.

[34] Trad FS, Toner M, Biggers JD. Effects of cryoprotectants and ice-seeding temperature on intracellular freezing and survival of human oocytes. Hum Reprod 1999;14:1569–77.

[35] Van Wagtendonk-De Leeuw AM, Den Daas JH, Kruip TA, Rall WF. Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. Cryobiology 1995; 32:157–67.

[36] Skidmore JA, Schoevers E, Stout TA. Effect of different methods of cryopreservation on the cytoskeletal integrity of dromedary camel (*Camelus dromedarius*) embryos. Anim Reprod Sci 2009;113:196–204.

[37] Palasz A, Adams G, Brogliatti G, Mapletoft R. Effect of day of collection and of permeating cryoprotectants on llamas (*Lama glama*) embryos and trophoblastic vesicles. Theriogenology 2000; 53:341.

[38] Vajta G. Vitrification of the oocytes and embryos of domestic animals. Anim Reprod Sci 2000;60-61:357–64. Review.

[39] Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod Biomed Online 2005;11: 608–14.

[40] Tharasanit T, Colenbrander B, Stout TA. Effect of cryopreservation on the cellular integrity of equine embryos. Reproduction 2005;129: 789–98.

[41] Stringfellow DA, Seidel Sarah M. International Embryo Transfer Society. Manual of the International Embryo Transfer Society: a procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures. Savory, IL: International Embryo Transfer Society; 2007.

[42] Manjunattha BM, Al-Hosni A, Al-Bulushi S. Superovulation of dromedary camel with two injections of FSH dissolved in hyauronan solution. The proceeding of Satellite Meeting on Camelid Reproduction. Tours, France: ICAR; 2016.

[43] Watanabe H, Kohaya N, Kamoshita M, Fujiwara K, Matsumura K, Hyon SH, et al. Efficient production of live offspring from mouse oocytes vitrified with a novel cryoprotective agent, carboxylated ε-poly-L-lysine. PLoS One 2013;8:e83613.

[44] Zhang X, Liu J, Huang G. Efficiency of an automatic dehydrated carrier for the vitrification of human embryos derived from three pronuclei fertilized zygotes. Reprod Biomed Online 2015;30:144–9.

[45] Skidmore JA, Billah M. Embryo transfer in the dromedary camel (*Camelus dromedarius*) using non-ovulated and ovulated, asynchronous progesterone-treated recipients. Reprod Fertil Dev 2011; 23:438–43.

[46] Skidmore JA, Billah M. Embryo transfer in the dromedary camel (*Camelus dromedarius*) using asynchronous,meclofenamic acid-treated recipients. Reprod Fertil Dev 2005;17:417–21.