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Review

Current status and future direction of cryopreservation of camelid embryos

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

Table 1

| 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. [18] and Nowshari et al. [16] |
| Lama            | Slow freezing | Pregnancy/live birth | 2002  | Aller et al. [8]                          |
| Bovine          | Slow freezing | Live birth           | 1973  | Wilmut and Rowson [19]                    |
|                 | Vitrification | Live birth           | 1986  | Maspup 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]              |

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...
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 (~1.5 M), with the addition of a non-permeating CPA (~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 (~0.3 °C/min) until seeding.

III. Seeding temperatures: At ~4.5 ~ 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 ~ 37 °C in a water bath (~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, ~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.

| Species          | Method      | Pregnancy rate (%) | Live birth rate (%) | Reference                        |
|------------------|-------------|--------------------|---------------------|----------------------------------|
| **Dromedary camel** | Slow freezing | 14/43 (32.6%)      | 1/43 (2.3%, unpublished data)* | 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)** | 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.

*Reference: 1. Skidmore et al., 2004 [18]  2. Nowshari et al., 2005 [16]  3. Aller et al., 2002 [8]  4. M. Herrid et al. / Theriogenology 89 (2017) 20–25
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 (~1 M) at 37 °C (~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 + 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.

1. 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
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 camels, 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 zona 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 luteolysis in recipient females: Due to the lack of the cyclical CL and a relatively short luteal lifespan of only 8–10 days in camels, 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 peripheral 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.

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