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The use of some assisted reproductive technologies in old world camelids

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ABSTRACT

The use of camels for racing, milking and as show animals is growing in popularity, thus there is increased enthusiasm to breed more of the genetically superior animals. This review highlights recent developments in assisted reproductive techniques in camels, such as embryo transfer and artificial insemination, to ensure more rapid genetic progress.

This paper discusses the difficulties involved in handling the semen due to its high viscosity and ways to reduce it. It also examines methods for short term liquid storage of fresh semen with and without the use of antioxidants to reduce oxidative stress. The widespread use of AI in camels is hindered by the lack of a reliable method for deep freezing and long term storage but various freezing protocols, cryoprotectants and freezing and thawing methods are discussed as well as different insemination techniques.

Embryo transfer requires the donor to be superovulated and the recipients synchronized. This review discusses different protocols used for superovulation of donor animals and the problems involved. It also examines various methods to synchronize recipients, or how to make best use of non-synchronized or non ovulated recipients. Cryopreservation of embryos would greatly improve the wider use of ET and spread of genetics worldwide so methods for slow cooling and new methods of vitrification with promising results are discussed.

1. Introduction

Assisted reproductive technologies provide many advantages to commercial animal reproduction and are used routinely in many domestic species including cattle, sheep, pigs and horses. Techniques such as artificial insemination (AI) and embryo transfer (ET) provide the opportunity to produce more of these genetically superior animals.

The reproductive efficiency of dromedary camels under pastoral conditions can be low partly due to the relatively short breeding season (Chen and Yuen, 1979; Wilson, 1984; Abdel-Rahim and El-Nazier, 1990), late age of reaching puberty (Wilson, 1986) and long gestation period of 13 months. Thus, the introduction of controlled breeding programmes is important; however, there are several problems that have to be considered. For example oestrous behaviour is very variable and often does not relate to follicular development in the ovaries; in some instances the female will accept the male when there is no follicular development or appear to reject him when a mature follicle is present (Skidmore et al., 1995); thus ultrasonography of the ovaries becomes an important tool to determine the stage of the follicular cycle at any given time. Camelids are also induced ovulators, normally only ovulating when mated; therefore alternative methods have been developed to induce ovulation in recipients for ET and AI. In addition, due to the absence of a cyclical corpus luteum (El Wishy, 1987; Skidmore et al., 1995), any regimen of prostaglandin injections cannot be applied. Therefore, alternative methods to synchronize donor and recipient camels have to be investigated. The development of AI in

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camels is also complicated by the difficulty in training males for semen collection and the viscous nature of the semen produced making its handling and assessment very difficult.

This review discusses how these problems have been approached and gives an update on developments in ET and AI in dromedary camels.

2. Artificial insemination

2.1. Semen collection

The preferred method for collection of semen is with an artificial vagina (AV) which has already been discussed in many previous reviews (Tibary and Anouassi, 1997; Skidmore et al., 2013, 2018). This technique uses a modified bull AV with a foam insert to imitate the cervix and an oestrous female as a mount. Due to camels mating in sternal recumbancy sand contamination of the semen sample can occur. In addition, not all males will accept the AV and it is easier to train young males with less experience of natural mating than older, mature, stud males (personal observations). More recently a camel dummy has been designed with an internal AV (El–Hussanein, 2003; 2017; Ziapour et al., 2014). The advantage of which is that there is less sand contamination of the semen samples and less chance of injury to personal collecting the semen, but the males have to be trained to use the dummy and in our experience this was challenging as only one of six males would mount the dummy.

Recent research has shown that the frequency of semen collection (once or twice per week) affects sexual behaviour, libido and semen characteristics. Collecting semen over an 8 week period during the camel breeding season (Al – Bulushi et al., 2018) showed that males collected once a week had greater libido ($P < 0.05$) and greater sperm concentrations compared with males where ejaculates were collected twice weekly ($403 \pm 16 \times 10^6$ spermatozoa/mL vs $261 \pm 18 \times 10^6$ spermatozoa/mL, $P < 0.001$) and a twice weekly collection frequency caused a reduction ($P < 0.001$) in progressive motility.

The problems encountered with handling the semen are most often attributed to the low volume of the ejaculates and the high viscosity of the seminal plasma. Initially this viscosity was thought to be caused by glycosaminoglycans (GAGs) as GAGs were 15 times more abundant in alpaca semen than in ram semen (Kershaw-Young et al., 2012). However, more recent studies have shown it to be related to mucin 5B which is five times more abundant in seminal plasma samples with high viscosity compared with those with low viscosity (Kershaw-Young et al., 2012). The spermatozoa are entrapped within this viscous seminal plasma making it difficult to assess sperm motility until the semen is liquefied. Several studies have reported spontaneous liquefaction of dromedary semen after incubation at 30–37 °C for 15–30 min (Al-Qarawi. et al., 2002; Wani et al., 2008) but this has generally just been partial liquefaction. El-Bahrawy and El- Hassanein (2009) tested different mucolytic agents to reduce viscosity in dromedary camel semen after diluting it 1:3 in Tris – lactose based extender. They found that α-amylase eliminated seminal viscosity and improved sperm motility post dilution (46%) compared with controls (27.5%), however all mucolytic agents had deleterious effects on acrosomal integrity (11.8% detached) after 4 h of incubation at 37 °C. Kershaw- Young and Maxwell (2012) also found that the proteases papain (final conc 0.1 mg/mL) and proteinase K could completely eliminate the viscosity within 20–40 min of treatment. However, agglutination of sperm heads was higher in papain- treated samples than in untreated controls (Monaco et al., 2016). Thus, these enzymatic treatments can also be deleterious to spermatozoa. The viscosity can also be reduced mechanically by a more prolonged method of dilution (1:1–1:5) in a suitable extender (e.g. Tris - Citrate - Fructose) followed by gentle aspiration with a sterile pipette for about 30–60 min (Wani et al., 2008; Malo et al., 2016) with lesser damage to acrosomes. Alternatively, Mosaferi et al. (2005) placed the semen in a conical flask containing a stainless steel clip (Gem Clips., Penguin, China) and floated the flask in a beaker of water at 37 °C over a magnetic stirrer and claimed this removed all viscosity from the semen. A more recent study has also claimed that the viscosity can be reduced by the application of ultrasound (Rateb, 2016).

2.2. Short term liquid storage of camel semen

Numerous studies have sought to find optimal extenders for processing fresh camel semen. Various Tris-and citrate-based extenders containing lactose, fructose or glucose have been shown to support sperm motility during liquefaction (Sieme et al., 1990; Anouassi et al., 1992; Wani et al., 2008) but only Anouassi et al. (1992) attempted AI, with semen diluted in an extender containing 11% lactose and 20% egg yolk, and achieved 5/6 pregnancies in female camels inseminated 24 h after mating to a vasectomized male. A number of commercial extenders have also been found suitable and pregnancy rates of 53% (7/13), 47% (10/21) and 34% have been reported for semen diluted in Laciphos, Green Buffer and INRA 96 (I.M.V. Technologies; L'Aigle, France) respectively (Skidmore et al., 2000; 2006a; Morton et al., 2010).

The ability of EquiPlus, Optixcell, Biladyl, Triladyl and Androhep (Minitube, Germany) to support camel sperm viability and motility after storages at 4 °C for 48 h has also been investigated (Sieme et al., 1990; Al – Bulushi et al., 2016). However, although semen motility could be maintained by Laiciphos (30%), Optixcell, Triladyl and Green buffer (67.3%, 69.1% and 61.7% respectively after 48 h) no insemination results were reported in either of these studies.

In another study with Bactrian camels a Tris – based extender, SHOTOR, was found to be superior to 10% lactose and 10% sucrose extenders for preservation of progressive motility for 24 h at 4 °C (Niasari-Naslaji et al., 2006). In a further study Panahi et al. (2017) investigated supplementing SHOTOR extender with different concentrations of plasma egg yolk (PEY) from six different avian species together with different concentrations of camel skimmed milk (CSM) for chilled preservation of dromedary camel semen. They concluded that 20% pigeon PEY and 20% CSM added to 60% SHOTOR (termed HASHI diluents) provided beneficial effects on sperm viability during chilled storage of dromedary camel sperm. However, none of these studies reported insemination results.

Morton et al. (2010) has shown that conceptions after AI (17.6%) can occur with semen chilled (4 °C) in INRA for 24 h, but this is much lower than the 50%–80% pregnancy rates reported after AI with fresh semen (Anouassi et al., 1992; Skidmore and Billah, 2006a; 2006b). It is thought that prolonged periods of storage are associated with a decline in viability presumably due to accumulation of toxic metabolic products mainly in the form of reactive oxygen species (ROS; Salamon and Maxwell, 2000). This accumulation of ROS leads to oxidative stress which causes damage to sperm membranes and reduces viability and DNA integrity (Aitken et al., 1998). Oxidative stress causes a decline in sperm quality due to the production of hydrogen peroxide. Therefore liquid stored sperm are often supplemented with an antioxidant (such as catalase, cysteine, ascorbic acid) to provide protection against oxidative stress (Roca et al., 2005). Further research is needed in camels to test a wider range of anti-oxidants to lessen oxidative stress and protect sperm membranes and DNA integrity from cold shock.

The variability between studies, for example some studies have sought to wash ejaculates to remove the seminal plasma while others have processed whole semen, and between bulls render conclusive remarks difficult to make regarding which buffer(s), with or without antioxidants, might be optimal and, in final analysis, await larger animal numbers to be tested than are represented in any one study.

2.3. Long term storage of camel sperm

Semen can be preserved long term by deep freezing and storage in liquid nitrogen. Frozen semen can more easily be transported within and between countries with fewer time constraints on getting it inseminated before the camel ovulates. There are many factors that can affect the success of AI with frozen/thawed sperm, namely the extender, the cryoprotectant and the rates of freezing and thawing. There have been many studies on defining the best extenders and cryoprotectants for cryopreserving camel sperm. El-Bahrawy et al. (2006) found that the dilution of dromedary semen with Tris-citrate, Tris-sucrose, Tris –lactose, lactose, skimmed milk extenders with added egg yolk and 2% glycerol revealed similar pre-freeze sperm motilities of between 63.3–68.7%, but Tris-lactose-egg yolk- glycerol extender maintained the highest post – thaw motility (62.3%) and sperm survival (93.2%) compared with the other extenders. In a subsequent study supplementation of the Tris–citrate-egg yolk-glycerol extender with 15 µl/mL amylase enzyme significantly improved sperm post thaw motility (61.6%) and decreased acrosomal damage (10.4%; El-Bahrawy et al., 2012).

Malo et al. (2017a) have compared the cryo-efficiency of four different cryoprotectants (glycerol, ethylene glycol, methylformamide, and dimethyl sulfoxide (DMSO)), all at 3% final concentration, and four different equilibration times, (10 min, 30 min, 1 h, 2 h) on cryopreserving camel sperm diluted in Green Buffer. Glycerol and ethylene glycol provided the best post thaw motility rates (36.8 and 39.5% respectively) compared with methylformamide (14.87%) and DMSO (4.0%) but equilibration times did not have a significant effect. Glycerol and ethylene glycol were then evaluated at four concentrations (1.5, 3, 6, 9%) with 30 min equilibration and results indicated that 3 and 6% glycerol or ethylene glycol offered the best protection (Malo et al., 2017a). In contrast, El-Badry et al. (2017) who also investigated the effects of cryoprotectants glycerol, dimethyl formamide and DMSO, all at 6% final concentration, on freezing and thawing of dromedary camel semen diluted in SHOTOR diluent (Niasari-Naslaji et al., 2006), found that 6% dimethyl formamide was superior to glycerol, DMSO and ethylene glycol in terms of post thaw motility (55.8 vs 47.5, 45.0 and 12.5% respectively), sperm membrane (49.0 vs 39.3, 42.7 and 22.67% respectively), and acrosomal integrities (53.0 vs 57.3, 52.3 and 30.7% respectively). They concluded that camel semen cryopreservation could be enhanced using 6% dimethyl formamide. Amides have been used in a variety of species for freezing of semen and Crichton et al. (2015) found methylformamide (2.5% in INRA-96 + 20% egg yolk) also resulted in acceptable post thaw motility and acrosome integrity of cholesterol-supplemented dromedary camel sperm.

Rates used for cooling and thawing can also dramatically affect the successful outcome of frozen thawed sperm. Niasari-Naslaji et al. (2007) compared a slower cooling rate of an average of 0.14 °C/min with a faster rate of 0.55 °C/ min for deep freezing of Bactrian camel semen and results indicated that progressive forward motility of the spermatozoa cooled at the faster rate was superior (47%) compared with that cooled at the slower rate (31%). Malo et al. (2019) investigated five different freezing rates achieved by placing the straws at either 1 cm, 4 cm or 7 cm above LN for 15 min, or 7 cm for 5 min followed by 4 cm for 3 min (7 + 4), or 4 cm for 5 min followed by 1 cm for 3 min (4 + 1). Motility was significantly higher for the faster freezing rate (1 cm) at 0 h post thaw although at 1 h there were no differences in total motility between 1 cm and 4 cm. However, both were better than the slower freezing rate at 7 cm or the 7 + 4 and 4 + 1 combinations, so it was concluded that a faster freezing rate was beneficial for camel sperm.

2.4. Freezing and thawing methods

Camel ejaculates vary greatly in volume, sperm concentration and viscosity, therefore developing a standard method for freezing and thawing is challenging In our laboratory a recent approach has been to select good quality sperm by passing them through a colloid (single layer centrifugation: Malo et al., 2017b, 2018). Sperm so selected are free of seminal plasma, a possible impediment to the successful access of cryoprotectants during freezing. A two-step freezing method is then followed whereby sperm pellets remaining after centrifugation (300xg, 20 min) are initially suspended in Green Buffer + 20% egg yolk, then cooled to 5 °C over 2 h. Subsequently, Green Buffer containing 6% glycerol is added at a ratio of 1:1. Sperm are then equilibrated for 30 min during which time the semen is loaded into 0.5 ml straws and frozen at 1 cm above liquid nitrogen for 15 min. Semen is subsequently thawed at 60 °C for 10 s. Post thaw motilities average 47% compared with 38% when non selected sperm (whole semen) are frozen by the same method (Malo et al., 2018, 2019).

2.5. Artificial insemination

Camelids are induced ovulators, normally only ovulating after mating. Ovulation therefore needs to be induced before AI. Ovulation can be routinely induced with a single GnRH injection administered intravenously when a follicle of 1.3–1.7 cm is present in the ovaries (Skidmore et al., 1996). One of the most important aspects of insemination in camels is its timing in relation to ovulation and better pregnancy rates have been achieved when insemination of fresh semen is carried out 24 h after GnRH injection (53%, 8/15) compared with insemination at the same time as GnRH injection (36%, 5/14; Skidmore and Billah, 2006b).

Pregnancy rate is also influenced by the number of motile spermatozoa inseminated and the site of deposition of the semen. In AI semen is generally deposited directly into the uterus just cranial to the cervix, however with the relatively short, open cervix during oestrus there can be considerable loss of spermatozoa due to back flow through the cervix. It was therefore proposed that the semen be deposited at the tip of the uterine horn, ipsilateral to the ovary with the mature follicle, thus nearer the uterine tubule junction (UTJ) rather than the body of the uterus. This deep uterine insemination should reduce loss of spermatozoa due to backflow of semen through the cervix and also further reduce the number of spermatozoa required for successful fertilization. Skidmore and Billah (2006a) found that 150×10^6 motile spermatozoa inseminated either at the tip of the uterine horn or in the body of the uterus yielded a pregnancy rate of 53% (8/15), whereas if numbers were reduced to 80×10^6 spermatozoa only 1/14 (7%) became pregnant if semen was inseminated into the body of the uterus compared with 6/15 (40%) when deposited at the UTJ.

Pregnancy rates of around 50–60% can be achieved with fresh semen (Skidmore and Billah, 2006a, 2006b; Anouassi and Tibary, 2010), and there have been reports of some success (22–37%) with cooled (24 h) semen, (Medan et al., 2008; Morton et al., 2010). However, while there is still no standardized method for successful freezing and AI of dromedary camel semen our laboratory has produced several live calves that attest to the eventual potential success of this practice as applied to commercial production. Clearly much work remains to bring consistent success to this aspect of dromedary camel reproduction.

3. Embryo transfer

Since the 1990's there has been increasing interest in ET in camels driven mainly by the camel racing industry but also by camel beauty competitions and a desire to improve the genetics in camel dairy herds. In traditional breeding systems, the dromedary females have a long calving interval of between 18–30 months (Tibary et al., 2005), thus at best only producing two calves in three years. However, using multiple ovulation and embryo transfer (MOET) and optimizing mating plans (i.e. using different males on each MOET cycle) multiple embryos can be produced and transferred in a season thereby greatly increasing the reproductive efficiency and genetic potential of good racing, beauty and dairy animals.

Embryo transfer necessitates that donor camels be superovulated and recipients synchronized. Although good pregnancy rates are achieved with fresh embryos a standardized method for freezing and thawing embryos to achieve equivalent pregnancy rates is still required.

3.1. Superovulation of the donors

Various ovarian stimulation protocols used in ruminants have been adapted for use in dromedary females. These include the use of camel (cFSH; Anouassi and Tibary, 2013), ovine (oFSH) or porcine (pFSH) FSH, equine Chorionic Gonadotrophin, (eCG) or a combination of FSH and eCG (Anouassi and Ali, 1990; McKinnon et al., 1994; Skidmore et al., 2002). Treatment should be initiated when there is limited follicular activity in the ovaries (i.e. no follicle $>$ 0.7 cm in diameter) which can be achieved by pre - treatment with 100 mg progesterone-in-oil (i.m.) injected daily for 8–15 days (McKinnon and Tinson, 1992) or by elimination of the dominant follicle with GnRH and commencing treatment either on Day 4 after ovulation (Day 0 = the day after GnRH injection) at the same time as the start of the new follicular wave (Skidmore and Billah, 2005; Skidmore et al., 2005; Nikjou et al., 2008; Anouassi and Tibary, 2013) or two days after GnRH injection (Ararooti et al., 2017; 2018). Previous examples of these stimulation protocols included a single injection of eCG (1500–6000 IU; Anouassi and Ali, 1990; Cooper et al., 1992; McKinnon and Tinson, 1992; McKinnon et al., 1994) or twice daily injections over 3 days of a total dose of 20 or 30 IU oFSH (Ovagen), but follicular response and embryo recovery using these protocols was poor (Cooper et al., 1992; Skidmore et al., 1992). More recently preferred ovarian stimulation protocols include either the use of pFSH, injected twice daily in decreasing doses for a period of 4–7 days (Anouassi and Tibary, 2013), or a combination of both eCG and pFSH (Skidmore et al., 2002; 2004; 2005). The eCG (2500 IU) is injected on day 4 after ovulation, together with a total dose of 400 mg pFSH administered twice daily in gradually decreasing doses (2 x 80 mg, 2 x 60 mg, 2 x 40 g 2 x 20 mg) over a period of 4 days (from days 4–7 after ovulation), and prostaglandin injected on day 6 after ovulation to induce luteolysis. Follicular response varies between camels but the majority of camels produce between 4–30 + follicles which take between 7–10 days to grow to a mature size of 1.3–1.7 cm in diameter when the donor is ready for mating. There are however a few problems associated with superovulation that have to be addressed. For example, there is always a small proportion of camels (10–20%) that do not respond or only produce follicles that do not grow beyond 0.9 cm in diameter, whereas others (15–20%) will over-stimulate and produce more than 30 + follicles in each ovary (McKinnon et al., 1994; Tibary and Anouassi, 1997; Skidmore et al., 2002; Anouassi and Tibary, 2013). Luteinization of follicles can also sometimes occur before ovulation which may be due to the LH activity of the eCG. In addition some camels will become refractory to superovulation after repeated treatments, perhaps due to producing an antibody response to these hormones.

In a more recent study the use of Human Menopausal Gonadotophin (hMG), which has been used successfully for superovulation in cattle, was tested in camels (Ararooti et al., 2017, 2018). The donors received either, FSH alone (390 mg), eCG (1000 IU, single

injection on Day 0) and FSH (330 mg) or hMG 16.5 ampules (Ararooti et al., 2017) or 17.5 ampules (Ararooti et al., 2018). The FSH and hMG were administered in twice daily injections in gradually decreasing doses over a period of 5.5 days. In the earlier study using the lower dose of hMG (16.5 ampules) the results indicated that there were no significant differences between the total number of corpora lutea among FSH (13.8 ± 2.65), eCG-FSH (15 ± 2.60) and hMG (10.8 ± 2.30) and the number of expanded hatched blastocysts in FSH (5.7 ± 2.32), eCG-FSH (8.8 ± 2.10) and hMG (5.8 ± 2.40) treated donors suggesting all three protocols could be used successfully. In the latter study, however, using a higher dose of hMG (17.5 ampules) the results indicated that although the total number of follicles were greater in the hMG group, the total number of corpora lutea was greater in the FSH group (22.4 ± 2.25) compared with the eCG-FSH (11.6 ± 2.58) and hMG (7 ± 3.19; $P < 0.01$) treated animals. The total number of embryos was also greater in FSH (16.2 ± 2.72) rather than hMG (1.6 ± 1.17; $P < 0.01$) treated donors. These results indicated that the FSH protocol provided a better and more predictable superovulatory response (Ararooti et al., 2018).

3.2. Synchronization of donor and recipients

Pregnancy rates of 60–75% can now be routinely achieved in camels after transfer of fresh Day 7 embryos into synchronized recipients on day 5 or day 6 after ovulation (McKinnon et al., 1994; Skidmore et al., 2002; Anouassi and Tibary, 2013), although the latter authors reported that this can be reduced to 19–44% by day 60 due to early pregnancy loss. If the level of asynchrony between donor and recipient increases to +1 day (a day 8 recipient) or up to 3 days behind (a day 4 recipient) then pregnancy rates reduce to approximately 10% (Skidmore et al., 2002); therefore methods to synchronize the follicular waves of donors and recipients become important.

Synchronization of ovulation between donor and recipient camels has been attempted using several methods. The simplest method is by selection of recipients from a random group of camels. This involves serial ultrasound examination of the ovaries of all the recipient camels and injecting all those with a mature follicle with GnRH 24–48 h after the donor is mated. This method is only feasible when a large number of recipient camels are available.

Alternatively, recipients can be treated daily with progesterone-in-oil (100 mg/ day) for 10–15 days followed by administration of 1500–2500 IU eCG. The eCG treatment is scheduled for the day after the donor receives eCG and should guarantee the presence of mature follicles in the recipient 24–48 h after the donor has ovulated. However, although the progesterone treatment reduced the rate of follicular growth it did not inhibit it completely, so response to the eCG and time taken for the next follicle to reach a mature size was variable (McKinnon et al., 1994). In more recent studies two injections of GnRH given 14 days apart, with the second GnRH scheduled to be administered the day after the donor is mated, has shown promise with ovulation rates of over 80% after the second GnRH injection (Skidmore et al., 2009a; Nikjou et al., 2008).

All these methods can be time consuming and labour intensive so a number of experimental approaches using non-synchronized or non-ovulated recipients have been investigated. For example, if the recipients ovulate too late so that they are only on day 3 or day 4 after ovulation at the time of embryo transfer, pregnancy rates of 50% (ov + 3) and 62% (ov + 4) can be achieved if they are supplemented with progesterone (Skidmore and Billah, 2011). Daily injections of 75 mg progesterone-in-oil are required from 2 days before embryo transfer to day ov + 6, and then the dose is reduced to 50 mg on ov + 7 and further reduced to 25 mg on days ov + 8 and 9. The embryo is then able to maintain the corpus luteum through its maternal recognition of pregnancy signal.

Conversely if the recipients ovulate too early so that they are on days 8, 10 or 12 at the time of embryo transfer, pregnancies can be achieved if the recipients are treated with meclofenamic acid. Meclofenamic acid is a prostaglandin synthetase inhibitor that prevents the luteolytic action of PGF2α and thereby prolongs the lifespan of the CL. Skidmore and Billah (2005) treated camels orally with meclofenamic acid from Day 7 after ovulation until 7 days after embryo transfer and achieved pregnancy rates between 60–80%.

If the recipients do not ovulate at all, pregnancy rates of 44% can be achieved if they are treated with daily injections of 150 mg progesterone-in-oil from 2 days before embryo transfer. However as they would need supplementary progesterone for the entire 13 month gestation period this is not practical. Skidmore and Billah (2011) therefore investigated a method whereby when these recipients were confirmed pregnant on Day 25, they were given a dose of 2000 IU of eCG to induce follicular development in their ovaries, even though they were still receiving progesterone treatment. Ovulation was then induced when a mature follicle of approximately 1.3 cm in diameter had developed in the ovaries (usually 7–10 days after eCG injection) and progesterone treatment was stopped once the CL had developed. Pregnancy rates of 50% were achieved and, as the CL could be maintained by the maternal recognition of pregnancy signal, daily injections of progesterone throughout gestation were not required.

All of these methods described above help relieve the need for tight synchrony between donors and recipients and thereby broaden recipient availability for embryo transfer. Short term preservation of embryos by cooling would also reduce the need for tight synchrony between donors and recipients. Pregnancy rates of 63% (20/32) have been achieved after transfer of cooled embryos that had been kept at 4 °C for 24 h (Skidmore et al., 2002).

3.3. Cryopreservation of embryos

3.3.1. Slow-cooling methods

Reliable methods for the cryopreservation of embryos would greatly facilitate the wider application of ET in camelids without the need to transport live animals. It would also reduce the need to synchronize recipients with donor animals as embryos could be thawed and transferred after timing of natural ovarian cycles. Initial studies investigated the best cryoprotectant to use with camel embryos and of the CPAs tested (glycerol, ethylene glycol, propanediol and DMSO) only those embryos in ethylene glycol survived after 24 h culture (Skidmore and Loskutoff, 1999). Therefore slow-cooling methods of freezing focused on using 1.5 M ethylene glycol

as cryoprotectant. Subsequent studies, using the slow-cooling method of freezing, exposed embryos to 1.5 M ethylene glycol for 10 min before subjecting them to a cooling rate of -0.5 °C / min from -7 °C to -33 °C and then plunging them into liquid nitrogen. After thawing at 32 °C for 2 min and rehydrating in either holding media (HEPES-buffered Tyrodes medium containing sodium lactate + 3 mg/mL BSA + 10% FCS) or holding media containing 0.2 M sucrose for 5 min, pregnancy rates of 33% (4/12) and 37% (7/19) respectively were achieved (Skidmore et al., 2004).

3.3.2. Vitrification

Slow-cooling methods of freezing are time consuming and require specialized equipment so are not practical under field conditions. Vitrification however, is much simpler and quicker so more recent studies on cryopreservation of embryos have focused on vitrification.

The first pregnancies from successful vitrification of dromedary embryos were reported in 2005 by Nowshari et al. (2005) and Skidmore et al. (2005) using two different protocols. Nowshari et al. (2005) achieved 3 pregnancies from 49 camel embryos that had been vitrified/thawed using a simple combination of cryoprotectant and sugars. Embryos were exposed to ethylene glycol (7.0 mol/L) with sucrose (0.5 mol/L) in two steps, transferred to 0.25 mL straws prior to plunging in liquid nitrogen. They were subsequently thawed at 25 °C for 10 s, and rehydrated in 0.5 M sucrose in PBS. Skidmore et al. (2005) used a modified version of Aller et al. (2002) method for vitrifying llama embryos. They exposed embryos to a more complex vitrification solution containing 20% glycerol + 20% ethylene glycol + 0.3 M sucrose + 0.375 M glucose + 3% polyethylene glycol, in three steps, prior to loading into 0.25 mL straws and plunging into liquid nitrogen. Embryos were thawed at 25 °C for 1 min and rehydrated in decreasing doses of sucrose (0.5 M, 0.25 M, 0 M sucrose in PBS + 20% FCS), prior to transfer. Better pregnancy rates (38%; 8/21) were achieved with smaller day 6 embryos ($\leq$350 μm) than with larger day 7 (0%; 0/9) or day 8 embryos (0%; 0/3), as between 50–80% of the bigger embryos were fractured or torn after warming. Better survivability of smaller embryos was also confirmed in studies by Skidmore et al. (2009b) where they examined the effect of cryopreservation on the cytoskeletal integrity of Day 6 and Day 7 embryos. After staining the embryos with diamino-2-phenylindole dihydrochloride (DAPI) to identify dead cells and labeling with Alexa Fluor 488–Phalloidin to assess cytoskeleton integrity, they found that there was a higher proportions of dead cells and degrees of cytoskeleton disruption in cryopreserved embryos > 300 μm in diameter compared with smaller embryos. Six of the 8 embryos with a post thaw-warming Grade III cytoskeleton were Day 7 embryos.

More recently Herrid et al. (2016 and 2017) have achieved better survival rates post thaw (80%) combining ethylene glycol and glycerol (final concentration 3.4 M glycerol + 4.6 M ethylene glycol) as the cryoprotectants and using the open pulled straw (OPS) method of vitrification. However this protocol did not result in any pregnancies. Interestingly, when they used the same vitrification protocol but replaced sucrose in the warming solutions with the same concentrations of galactose (0.5 M - 1 min, 0.25 M- 5 min) promising pregnancy rates of 46.1% were achieved. This could suggest a possible species – specific toxic effect of sucrose on camel embryos. In contrast to Skidmore et al. (2005) however, Herrid et al. (2016) found larger embryos 250–500 μm were more tolerant to vitrification than smaller embryos < 250 μm. This stage dependant sensitivity of the embryos is a major obstacle to a practical application of the vitrification method.

4. Conclusions

Over recent years research has led to many advances in the use of assisted reproductive technologies, such as artificial insemination and embryo transfer, in camels. Semen can be collected using an artificial vagina and the problems involved with handling such viscous semen overcome with a prolonged method of dilution (1:5) and repeated aspiration with a sterile pipette. While better pregnancy rates are achieved if insemination occurs within 1–2 h of collection and 24 h after the camel has received GnRH to induce ovulation, conceptions can occur with semen preserved by cooling for 24–48 h at 4–5 °C. There has also been a big improvement in post thaw viability of cryopreserved camel sperm but further studies are required to improve pregnancy rates following insemination of frozen thawed spermatozoa.

Likewise, significant advances have been made in embryo transfer of dromedary camels. Superovulation protocols using pFSH or pFSH + eCG can reliably produce many follicles in the donor camels and recipients are best synchronized using either progesterone, progesterone + eCG or repeated injections of GnRH given 14 days apart. Ovulated, but non-synchronized recipients can be used if treated orally with meclonfenamic acid or with extra progesterone injections. Those recipients that do not ovulate can be injected with progesterone to maintain the pregnancy, eCG to promote follicle development and then GnRH to induce ovulation and create CL's for maintenance of the pregnancy throughout gestation. Although pregnancy rates of 65–70% can be achieved with fresh embryos, further work is required to improve deep freezing techniques. Recent vitrification studies have reported greater success combining ethylene glycol and glycerol as cryoprotectants and replacing sucrose with galactose in the warming media but further refinements of the protocols are needed before these techniques can be made commercially viable.

Conflict of interest statement

I confirm that there has been no conflict of interest, financial or otherwise, with any other institution whilst carrying out this work.

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