Challenges in the development of artificial insemination in the dromedary camel

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(Reçu le 14/04/2017; Accepté le 06/06/2017)

Abstract

Artificial insemination (AI) in dromedaries remains challenging. A few AI trials with liquid stored semen have been published but they remain too small in term of number of inseminated females. AI trials with frozen-thawed semen have met with very little success. This papers reviews research performed in the area of dromedary semen collection and preservation with a special emphasize on biological differences compared to other species. These include, the viscous nature of camel ejaculate and the need for it liquefaction, the importance of induction of ovulation and possible effect on fertility and the difficult in reliably obtaining good quality ejaculates. We also present the most recent data regarding behavior of camel semen in various extenders and technique for preservation. Areas where further research is needed are pointed out throughout the manuscript.

Keywords: Artificial insemination, fertility, semen, extenders, dromedary camel

Défis au développement de l'insémination artificielle chez le dromadaire

Résumé

L'insémination artificielle (IA) chez le dromadaire est un vrai défi. Quelques essais d'IA avec du sperme liquide stocké ont été publiés mais le nombre de femelles inséminées et les taux de conception restent relativement faibles. Les essais d'IA avec du sperme congelé et décongelé ont rencontré très peu de succès. Cet article passe en revue les recherches effectuées dans le domaine de la récolte et de la conservation du sperme du dromadaire en insistant particulièrement sur les différences biologiques par rapport aux autres espèces. La nature visqueuse de l'éjaculat du dromadaire et la nécessité de sa liquéfaction, l'importance de l'induction de l'ovulation et son éventuel effet sur la fertilité et la difficulté d'obtenir de façon fiable des éjaculats de bonne qualité. Nous présentons également les données les plus récentes concernant le comportement du sperme du dromadaire vis-à-vis de divers dilueurs et techniques de conservation. Les domaines où d'autres recherches sont nécessaires sont signalés tout au long du manuscrit.

Mots-clés: Insémination artificielle, fertilité, sperme, dilueurs, dromadaire

INTRODUCTION

Artificial insemination (AI) is the single most important technique to insure rapid genetic progress. AI allows more efficient use of genetically superior males, prevention of diseases, elimination of the need for transportation of animals, and elimination of behavioral problems. The need for AI is well illustrated by the high demand for top racing dromedary males. In the 2016-2017 breeding season in the UAE, 47 males bred approximately 27 000 females (3 to 4 mating per male per day). The only way this high demand on top males can be met without jeopardizing conception rate while reducing the risk of disease transmission is through AI (Anouassi, 2017, personal observation).

Artificial insemination in *camelidae* has been reported since the 1960's (Review (Tibary and Anouassi, 1997a; Tibary, 2001)). The first Bactrian camel born by artificial insemination with frozen-thawed semen was reported in 1961(Elliot, 1961). Except for the Bactrian camel, where acceptable pregnancy rates have been achieved, results have been dismal in other camelid species (Chen *et al*., 1984; Chen *et al.*, 1985; Xu *et al*., 1985; Zhao *et al*., 1991; Chen *et al*., 1993; Xu *et al*., 1993). It is not clear if this difference amongst species is due to difference in initial semen quality or in biological and functional properties. Semen collected by artificial vagina from Bactrian camels tend to yield better concentration and motility than observed in dromedaries (Mosaferi *et al*., 2005).

Several reviews on artificial insemination in camelids has been published throughout the years and all conclude poor pregnancy rate particularly with frozen-thawed semen remains (Tibary and Anouassi, 1997a; Bravo *et al*., 2000b; Tibary, 2001; Adams *et al*., 2009; Bravo *et al*., 2013; Skidmore *et al*., 2013). The objectives of the present paper is to provide an updated review on advancement in semen preservation and artificial insemination in the dromedary and discuss challenges and areas of future research.

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SEMEN COLLECTION

Semen collection should be part of a complete breeding soundness examination (BSE) of the male. A protocol for BSE in the male camel was proposed by the authors and includes physical examination, testicular measurements and ultrasonography and semen collection and evaluation (Tibary and Anouassi 1997b, Tibary *et al*., 2014). Unfortunately, most studies pertaining to semen preservation in camels do not provide enough data on the health and reproductive soundness of males used.

Semen has traditionally been collected using either electroejaculation or artificial vaginal (Tibary *et al*., 2014). Although rarely discussed, the method of collection has tremendous effects of the physical and probably biochemical properties of the ejaculated. Most authors would agree that electroejaculation is not a viable procedure for routine semen collection from valuable males. The procedure requires heavy sedation (Detomidine hydrochloride 80 µ/kg BW, IM) (Al-Qarawi *et al*., 2002) or even anesthesia and therefore presents a risk to the life and welfare of the animal. Semen collected by electroejaculation presents large variations in volume and concentration which precludes its use for routine semen collection and cryopreservation. In addition, electroejaculation is not be acceptable in some societies.

Semen collection using an artificial vagina has been described in several previous reviews (Tibary and Anouassi, 1997b). The technique uses a bovine artificial vagina which is modified to provide a narrowing simulating cervical rings which is essential for stimulation of ejaculation in camels (Figure 1). The major challenges encountered with the technique include the need for training of the male and the physical difficulty for the operator due to the position and length of copulation. In addition, this method of collection is not without risk for the operator when aggressive animals are used. In recent years, systems using an artificial vagina inserted under the teaser female (Tibary and Anoaussi, 1997a; Al-Eknah *et al*., 2001) or mounted on a dummy (Ziapour *et al*., 2014) have been proposed with various degrees of success. Several ejaculates obtained by artificial vagina are of poor quality (azoospermic or oligozoospermic) or contaminated with sand and debris from the environment (Figure 2) (Tibary and Anouassi, 1997a). Information on the unsuccessful collection attempts or number of discarded ejaculates is seldom reported in publications. Recently, the dummy technique was reported to provide cleaner ejaculates (Ghoneim *et al*., 2014; Ziapour *et al*., 2014). However, there are very few reports on the conditions required for training males to the dummy. In one trial, none of the males used (n=40) accepted a commercial dummy over a period of 6 months (Anouassi, personal observation). These behavioral limitations can be reduced if males are used exclusively for semen collection which is not practical.

Figure 1: Artificial vaginal for collection of semen. (a) Type of artificial vaginal used for dromedary (b) Coil on short artificial vagina to simulate cervical rings (c) Collection of semen on a receptive female mount

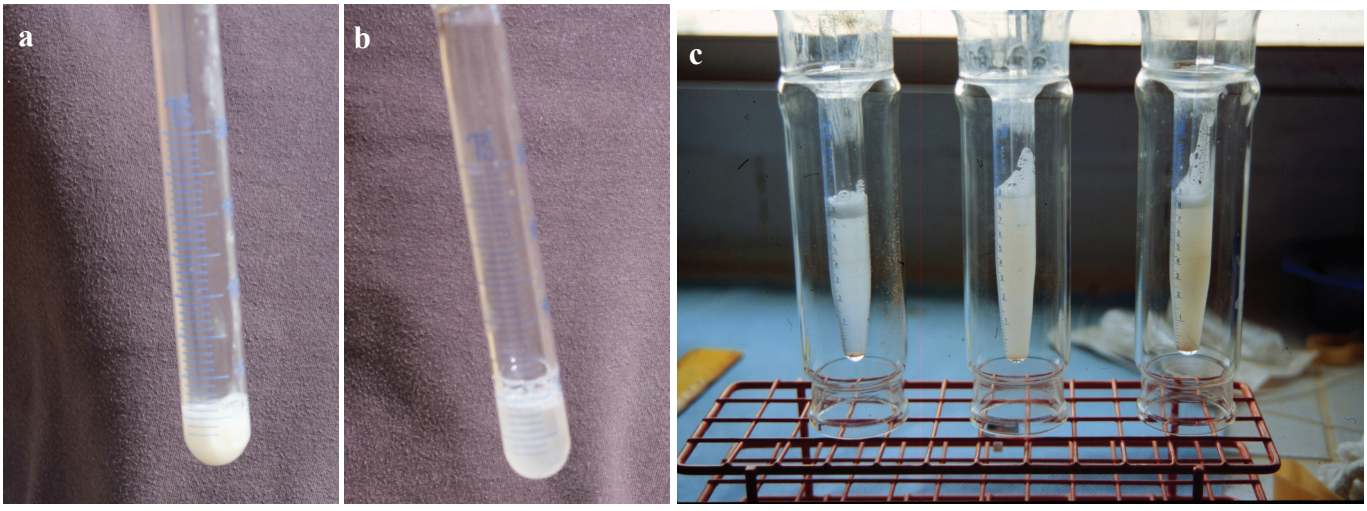

Figure 2: Ejaculate quality variation. (a) Ejaculate with very high concentration and small amount of seminal plasma (Easily liquefied) (b) Ejaculate with poor concentration and mostly seminal plasma (c) Ejaculates with various concentration and contamination with sand. Note the difference in viscosity

An alternative method for semen collection that may be more viable is the use of candoms mounted within the vagina of receptive females (Figure 3). Preliminary results using this technique showed the production of an ejaculate of good quality, with very little seminal plasma, on every attempt without any negative effect on the female (Anouassi, unpublished).

INITIAL EJACULATE QUALITY

Evaluation of initial quality of dromedary ejaculates is a major challenge. The viscous nature of semen does not lend it to a thorough and accurate determination of motility, concentration and morphology. The viscosity of the camelid semen is attributed to seminal plasma which represents 85 to 90 % of the ejaculate (Figure 2). This proportion is highly variable depending on individual males, method of semen collection and length of stimulation (Table 1). Unfortunately most studies do not take this variability into account.

In addition, the viscous material in the ejaculate is not a distinct fraction that can be easily filtered out as in other species but is distributed evenly throughout ejaculation. Viscosity of semen is often estimated by the thread testing technique which we have described in 1997 (Tibary and Anouassi, 1997b) (Figure 4). It is important to note that this test does not measure structural viscosity but rather the rheological properties of seminal plasma (Giuliano *et al*., 2010; Casaretto *et al*., 2012).

Figure 3: Semen collection using a female fitted with a condom. (a) Condom device (left) is inserted in the vaginal of an estrous female and secured with a harness (right) (b) Female with secured intravaginal condom in the breeding pen (c) Male teasing the female with intravaginal condom (d/e) Male breeding within the intravaginal condom

Table 1: Characteristics of the ejaculate in camelids (adapted from Tibary *et al.*, 2014)

Species	Collection method	Volume (ml)	Sperm concentration (million/ml)	Sperm motility (%)	Sperm normal morphology (%)
Camelus bactrianus	AV	2.5-12.5	200-1600	20-80	50-90
	EE	1-12.5	200-600	50-80	50-90
Camelus dromedarius	AV	2-12.5	200-1600	20-80	50-90
	EE	1-9	330-800	20-80	40-70
Lama glama	AV	0.2-8	18	20-80	40-70
	EE	0.3-12.5	20	50-95	50-80
Vicugna pacos	AV	0.4-6	82-250	20-80	45-75
	EE	0.2-12	10-60	20-80	-

AV= artificial vagina, EE= Electroejaculation

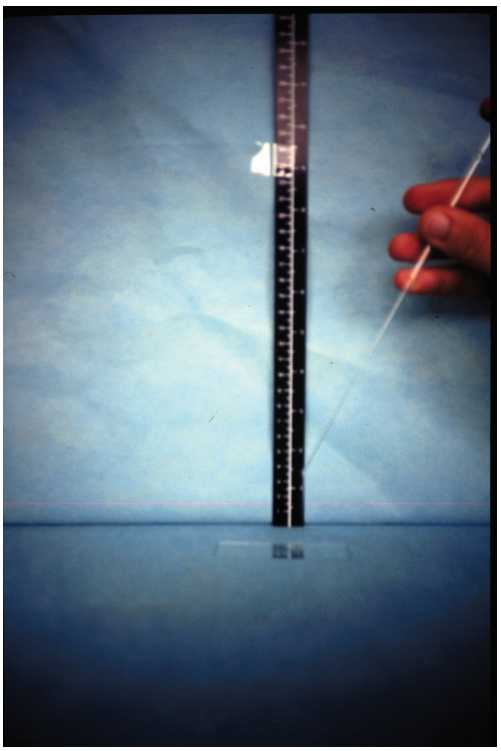

Figure 4: Thread technique for evaluation of ejaculate viscosity

Liquefaction of the ejaculate

Several studies on dromedary semen preservation report spontaneous liquefaction after incubation of the ejaculate at 30 to 35°C for 15 to 30 minutes (Al-Qarawi *et al*., 2002; Wani *et al*., 2008) However, in our experience this is often just a partial liquefaction and it is subject to a high variation amongst males and between ejaculates form the same male. In a recent study, complete liquefaction of dromedary ejaculates took on average 23.9±1.5 hours. This length of time is not practical and would certainly result in loss of quality of semen (Mal *et al*., 2016).

Research on the nature of seminal plasma components contributing to viscosity of ejaculates in camelids have been performed mostly in alpacas. Although camelid seminal plasma contains a high concentration of glycosaminoglycans (mainly keratin), these compounds do not seem to play the major role in the viscosity of semen (Kershaw-Young *et al*., 2013). Treatment of semen with keratinase does not reduce alpaca semen viscosity (Kershaw-Young and Maxwell, 2012; Kershaw-Young *et al*., 2013). Recent studies have shown that alpaca semen viscosity is mostly related to mucin 5B (MUC5B aka MG1), mucin 5AC and apomucin (Kershaw-Young and Maxwell, 2012).

Addition of various proteolytic/mucolytic enzymes (amylase, αchymotrypsin, trypsin, collagenase) to camelid semen was shown to speed up the liquefaction of ejaculates with variable success (Bravo *et al*., 1999; Bravo *et al*., 2000a; Ghoneim et *al*., 2010; El-Bahrawy *et al*., 2015; Monaco *et al*., 2016). Addition of collagenase at 0.5 to 1% to semen seems to provide the best liquefaction without major effect of sperm function. Recently, an approach using papain (1.7 Unit/ml) seems to be promising for dromedary camels although liquefaction was not as fast as in alpacas (1 hour vs 20 to 40 minutes) (Crichton *et al*., 2015; Monaco *et al*., 2016). The efficacy of these treatments varies from one male to another and between ejaculates of the same male. An aspect that is often not disclosed in published papers.

Role of seminal plasma

Seminal plasma is mostly provided by the bulbourethral glands and the prostate as camelids do not possess vesicular glands (i.e. seminal vesicles). A consequence of the absence of the vesicular glands is the low fructose and citric acid concentration in seminal plasma (Wani *et al*., 2011). Seminal plasma contains proteins that are important for sperm function, integrity and fertilizing ability (Kershaw-Young and Maxwell, 2012; Waheed *et al*., 2015).

As stated above, evaluation and processing of camelid ejaculates for cryopreservation requires liquefaction and dilution. These aspects of semen processing may affect sperm function. For instance, the role of viscosity is not fully understood but may be required to prevent loss of sperm from the female tract and protection of spermatozoa (Kershaw-Young and Maxwell, 2012; Kershaw-Young *et al*., 2013). Studies in alpacas have shown that presence of a minimum level of seminal plasma (10%) is needed to maintain motility, acrosome integrity and viability (Kershaw-Young and Maxwell, 2011).

Another aspect of semen processing that may confound the effect of seminal plasma is dilution rate. While several studies proceed with standard dilution of ejaculate in a volume to volume ratio (semen:extender), others attempt to adjust the dilution rate based on sperm concentration. However, the latter is extremely difficult to achieve due to viscosity and variability of concentration in the ejaculate. Sperm concentration varies greatly (80 to 858 million per ml), with total number per ejaculate ranging from 240 to 2576 million (Tibary and Anouassi, 1997a; Morton *et al*., 2011; Tibary *et al*., 2014). Sources of variation of ejaculate volume and total sperm numbers in the dromedary have not been studied and may include individual variation, frequency of collection and technique/time required for semen collection.

Finally, one of the most important discoveries in recent year in camelid reproduction is the presence of a 27kDa homodimer, beta-nerve growth factor (ß-NGF), which is responsible for the induction of ovulation following mating (Ratto *et al*., 2011; Kershaw-Young and Maxwell, 2012; Ratto *et al*., 2012; Kumar *et al*., 2013; Mal *et al*., 2016). It is not yet clear if this factor is involved in other pathways enhancing fertility and how semen processing may affect its function.

Preservation and use of liquid semen

Short-term preservation (i.e. few hours) of camelidae semen has been attempted at different temperatures (25°C, 30°C or 4°C). Several extenders have been used for dilution of freshly collected semen (Dimitropolous 11, Glucose-EDTA, Skim milk, INRA-96, Sodium-citrate-egg yolk, lactose egg yolk, Kenny's equine extender) (An-

ouassi *et al*., 1992; Musa *et al*., 1992; Sieme *et al*., 1993; Tibary and Anouassi, 1997a; Tibary, 2001; Anouassi and Tibary, 2010). Most of these extenders are adapted from other species and contains a buffering system, a source of energy (glucose or fructose), a protein for the protection against cold shock (lipoprotein from egg yolk or casein from milk) and antimicrobials. Studies on the effect of physical and chemical proprieties (i.e. pH, ionic strength and osmotic pressure) of the extender on the motility and fertilizing ability of preserved camelid semen are scarce. A commercial extender has been available for camel semen since the early 90's (Camel Buffer Green®, IMV, L'aigle, France).

In the dromedary and Bactrian camels, semen is diluted initially at a ratio of 1:1 to 1:3 (semen: extender) (Anouassi *et al*., 1992; Musa *et al*., 1992) depending on the concentration of the ejaculate. It is recommended to add the extender to the semen at a temperature of 30 to 35°C. Partial or complete liquefaction can be obtained by thoroughly mixing the extender and semen. Semen has been diluted to achieve a standard concentration of 50 x 10^6/ml (Anouassi *et al*., 1992).

Although some authors recommend keeping semen at 37°C or at room temperature until insemination, this storage temperature is adequate only if AI is performed within a couple of hours of collection (Anouassi *et al.*, 1992). For longer preservation time in a liquid form (up to 48 hours), semen should be cooled to 4 or 5°C. Slow cooling of the semen can be achieved by placing the tube containing extended semen into a water bath at room temperature and placing it in the refrigerator. This system allows cooling of the extended semen to 5°C over one hour. The Equitainer® system used for chilled equine semen is suitable to keep good motility for at least 18 hours and up to 36 hours in Kenny's skim milk, Green buffer® or INRA-96 (Tibary and Anouassi, 1997a). The proper cooling rate for dromedary semen has not been thoroughly studied. One study on Bactrian camels showed that fast cooling rate (55°C/minute) is better than slower cooling rate (0.14°C/min) (Niasari-Naslaji *et al*., 2007).

An early study on dromedary and Bactrian camels reported that the best results in terms of preservation of sperm motility and acrosome integrity are achieved with the commercial bull semen extender Laciphos (a combination of egg yolk and milk extender) (Sieme *et al*., 1993). Several commercial extenders were compared for their suitability for short term preservation of camel semen. These include, Optixcel® (a bovine extender containing liposomes, IMV technologies, France), EquiPlus® (contains highly purified caseinates similar to INRA-96, Minitube, Germany), and tris-citric acid extenders (Biladyl® and Triladyl®, Minitube, Germany). There was no difference in motility, viability and acrosome integrity of dromedary semen preserved at 5°C for 48 hours at a concentration of 100 million spermatozoa in Optixcell®, Green buffer® or Triladyl® (Al-Bulushi *et al*., 2016). However, EquiPlus® did not perform as well. In another study, dilution of dromedary semen 1 to 1 in Tris lactose-egg yolk (2.71%Tris, 1%fructose, 1.4% citric acid, 1% glycine, 3.8% lactose and 80% clarified egg yolk), Tris–tes egg yolk (1.15% Tris, 4.83% tes, 0.4% glucose and 80% clarified egg yolk), or sucrose egg-yolk (8.76% sucrose and 80% clarified egg yolk) liquefied for 60 to 90 minutes at 37°C, maintained good motility and viability for up to 48 hours both at room temperature and at 5°C after dilution of 1 to 3 (Wani *et al*., 2008). Green buffer® was shown to be superior to the Tris-fructose egg yolk for maintenance of chilled dromedary semen for 24 to 48 hours (Waheed *et al*., 2010). Recently, a Tris-based extender called SHOTOR® (tris, 214.6 mM; citric acid, 64.2 mM; glucose, 66.6 mM, and fructose, 49.9 mM; osmolality, 330 mOsm/kg; pH 6.9) was shown to be superior to 10% lactose and 10% sucrose extenders and similar to Green buffer EY for preservation of progressive motility for 24 hours at 4°C in Bactrian camels (Niasari-Naslaji *et al*., 2006).

Artificial insemination trials using fresh and chilled semen

Artificial insemination requires a precise timing after induction of ovulation based on ultrasonographic monitoring of follicular development. A few artificial insemination trials have been reported with fresh or chilled dromedary semen with doses semen varying from 8 to 300 million spermatozoa per insemination. Artificial insemination is generally performed 24 hours after induction of ovulation. Pregnancy rates (PR) have been reported in small trials in camels (n <20) inseminated with fresh semen diluted in lactose 11% - 20% EY (PR=50%) (Anouassi *et al*., 1992), Laciphos® +20% EY (PR=53%), Green Buffer® +20% EY (34-47%) or INRA-96® (PR=34%) (Skidmore et al., 2013). In a recent study, AI performed with fresh dromedary semen extended in Green Buffer®+ 20% EY (150 million motile sperm in 1.5 to 3.5 ml) yielded a pregnancy rate of 73% (n=11) at 25 days. Interestingly the use of a frozen-thawed batch of the same extender resulted in only 27% pregnancy rate (Morton *et al*., 2011).

Pregnancy rate is affected by the number of spermatozoa in the inseminate and the site of insemination. In a study with fresh diluted semen (inseminate volume 1 to 1.25 ml), PR in females inseminated 24 hours after administration of GnRH were not different between deep horn insemination and uterine body insemination when using 150 million spermatozoa (43% vs. 53%). However, reducing the number of spermatozoa to 80 million yielded a PR of 7% for uterine insemination and 40% for deep horn insemination. Further reduction of the dose to 40 million spermatozoa resulted in a PR of 7% and 0% for deep horn and uterine body insemination, respectively (Skidmore and Billah, 2006).

Deep horn deposition of semen using as little as 24 million spermatozoa in Green Buffer®+ 20% EY resulted in PR of 48% and 58.3% when AI was performed at 0 or 24 hours after induction of ovulation, respectively (Anouassi and Tibary, 2010). Deep horn insemination can be performed with an adapted deep horn insemination used in the bovine (Figure 5).

Pregnancy rate drops rapidly when semen is stored at 5°C. Dromedary semen chilled in INRA-96 or Green Buffer® resulted in PR of 17.6% and 0%, respectively (Morton *et al*.,

2010a). In a recent small trial, no embryos were collected from superovulated dromedary camels (n=8) inseminated with chilled semen in Green Buffer® (Tibary, unpublished).

Decrease in sperm viability and fertilizing ability during storage has been attributed to the effect of peroxidation and presence of reactive oxygen species. Addition of catalase to the extender at a rate of 500 IU/ml has been shown to improve camel spermatozoa viability during storage at 5°C. A small fertility trial resulted in 46.5% (6/13), 22.2% (2/9) and 37.5% (3/8) in females inseminated 48 hours after hCG administration with 100 million spermatozoa in non-diluted semen, extended cooled semen without catalase and extended cooled semen with catalase, respectively (Medan *et al*., 2008).

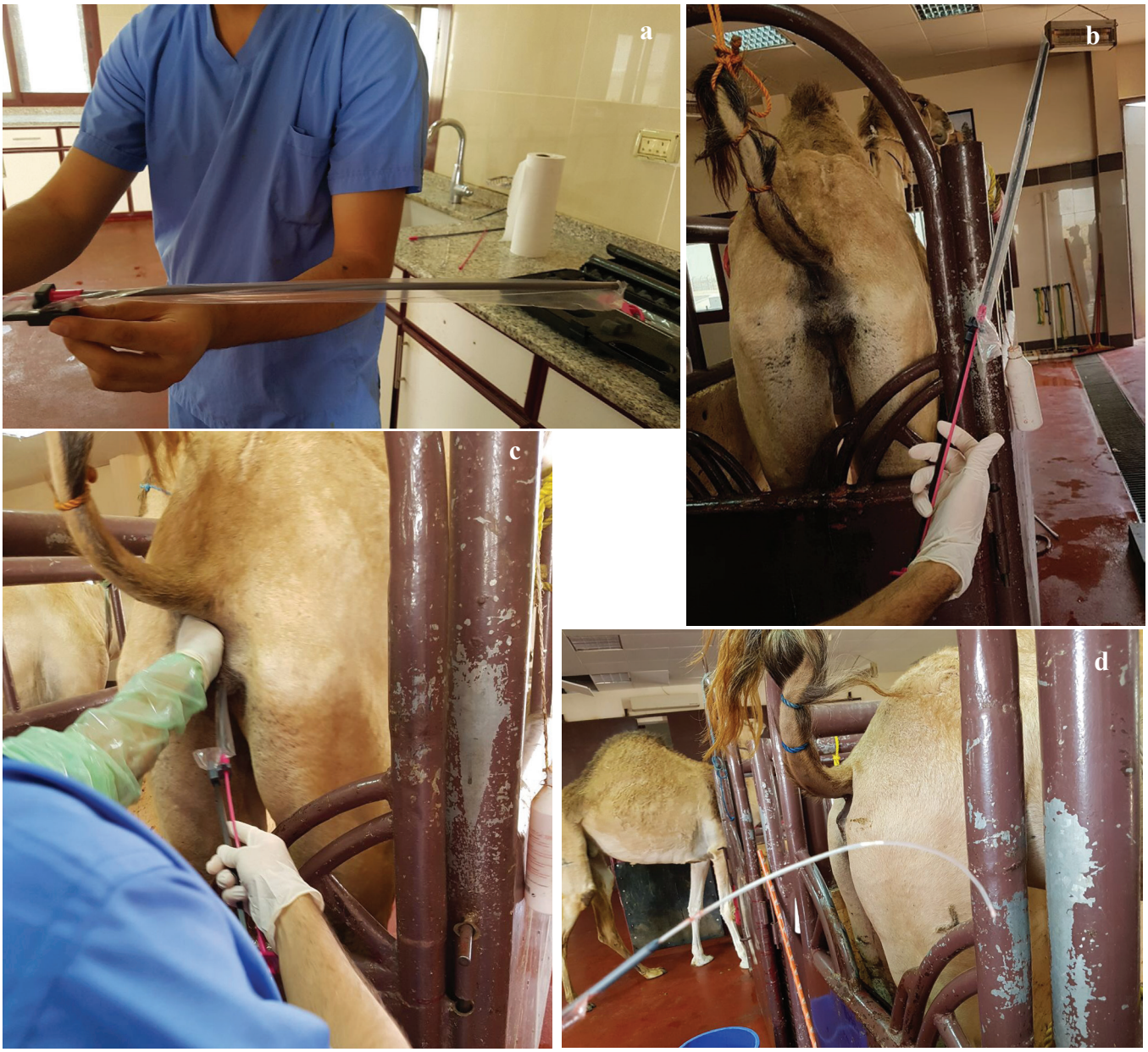

Figure 5: Deep horn insemination gun. (a) Sterile deep horn insemination gun (b) Female prepared for deep horn insemination (c) Insertion of the deep horn insemination gun and transrectal guidance of the flexible catheter (d) Deep horn AI gun after insemination. Note the flexible catheter within the rigid outer catheter

Table 2: Results of artificial insemination with frozen-thawed semen in the Bactrian camel

Extender	Dose	Pregnancy rate	Reference
SYG1 or SYG2	400 x 10^6, 37% motility	86%	(Chen *et al*., 1984; Chen *et al*., 1985)
SGY2	55% motility	100%	
	400 x 10^6, AI twice at 24 hours interval	96% (n=71)	(Zhao *et al*., 1993; Zhao et *al*., 1994; Zhao *et al*., 1996)
	AI once after hCG (1000 IU)	100% (n=10)	
	800 x 10^6 single AI	100% (n=10)	
	400 x 106 single AI	100% (n=5)	
SYG2	400 x106	(96.2%)	(Zhao *et al*., 1993; Zhao *et al*., 1996)

Cryopreservation of semen

Camelid semen has been frozen successfully for over 3 decades (llamas (Graham *et al*., 1978), dromedary (Graham *et al*., 1978), and Bactrian camels (Elliot, 1961). However, insemination trials with frozen-thawed semen have been carried out mainly in the Bactrian camel (Chen *et al*., 1993; Zhao *et al*., 1994; Bravo *et al*., 2000b).

Extenders for cryopreservation

A variety of extenders used for deep-freezing of semen of other species have been adapted to dromedary and Bactrian camel. Several cryopreservation methods with extenders used for bull, ram, dog, stallion and boar semen preservation were compared by assessment of post-thaw motility and morphology. These studies showed that the best extender for freezing dromedary and Bactrian camel semen is a modified boar or stallion technique (Sieme *et al*., 1993). This technique uses two extenders, a cooling extender (80 ml Lactose 11%, 20 ml Egg Yolk) and a freezing extender (95.5 m Cooling extender, 06.0 ml Glycerol, 1.5 ml Orvus paste –Equex). The cooling extender is added to the semen immediately after collection. The freezing extender contains the cooling extender in addition to a cryoprotectant (glycerol) and an emulsifying agent (Orvus paste) that plays a role in the stabilization of the sperm plasma membrane.

In the bacterial camel, comparison of tris-bull extender, sodium citrate-egg yolk extender, ram, stallion and boar extenders with sucrose based extenders (SYG1; 85.5 ml Sucrose 12%, 10.0 ml Egg yolk; 3.5 ml Glycerol and SYG2: 73.0 ml Sucrose 12%, 20.0 ml Egg yolk, 7.0 ml Glycerol) showed a superiority of SYG-2 for post-thaw motility and acrosome integrity (Zhao *et al*., 1994).

Recent studies showed that dromedary semen can be frozen in a variety of extenders Green Buffer® EY-glycerol, INRA + EY (Crichton *et al.*, 2015), Tris-egg yolk-glycerol (Deen and Sahani, 2006). In the Bactrian camel, Tris (3.03%), fructose (1.7%) citric acid (1.2%) (pH7.5) and SHOTOR with 6% glycerol were better than Green buffer® +EY +glycerol (Niasari-Naslaji *et al.,* 2007). Addition of Equex® to SHOTOR® or Green Buffer® did not improve post-thaw quality of Bactrian camel semen (Niasari-Naslaji *et al*., 2008).

Freezing procedure

Freezing procedure depends on the packaging method used (Tibary and Anouassi, 1997a). Semen can be packaged as pellets (Graham *et al*., 1978), in plastic straws with different volumes (0.25 ml, 0.5 ml or 4 ml) (Musa *et al.*, 1992; Willmen *et al.*, 1992; Sieme *et al*., 1993) or ampules (Zhao *et al*., 1994).

Semen pellets are obtained by dropping known volume (0.1 or 0.2 ml) of diluted semen into depression made in dry ice (Graham *et al*., 1978). This technique achieves very high freezing rates and was shown to be superior for some species and processing techniques. However, it is rarely used today, because of the difficulties in labeling the semen and the inability to modify the freezing rate.

Straws are usually frozen by placing them on a rack at known distances above the surface of liquid nitrogen. The volume of the straw has a great influence of the freezing process. The fastest freezing rates are obtained by the use of small volume (0.25 or 0.5 ml). Freezing rates can be modified by modification of the elevation of the straws. More precise freeing rates can be achieved in semen packaged in straws by using computerized freezer that can be programmed to follow a precise freezing curve (Tibary and Anouassi, 1997a).

The freezing procedure used for dromedary and Bactrian camel semen packaged in 4 ml straws (Musa *et al.*, 1992; Willmen *et al.*, 1992; Sieme *et al.*, 1993) can be summarized as follows: raw semen is incubated at 25°C to 30°C until liquefaction followed by dilution with the cooling extender (1 volume semen: 1 volume extender), then cooling to 15°C over a 2.5 hour period. The semen is further diluted using the freezing extender to a concentration of $150x10^6$/ml then cooled to 5°C over a period of 1.5 hour. A final dilution with the freezing extender to a concentration to $100x10^6$/ml precedes packaging in large straws (4 ml). Straws are frozen in liquid nitrogen vapors (2.5 to 4 cm above liquid nitrogen level) for 20 minutes then plunged into liquid nitrogen.

A simplified technique of freezing of semen package in 0.25 or 0.5 ml straws consists of a dilution after liquefaction is complete then cooling to 5°C over one hour. Semen is packaged and maintained at this temperature for 2 hours. The straws are placed on a rack 4 cm above liquid nitrogen surface for 10 minutes then transferred directly into liquid nitrogen (Tibary, 2001). This technique is the most commonly used for dromedary and Bactrian camel semen by various authors with variations in initial cooling rate and equilibration time at 5°C (Deen *et al*., 2003; Niasari-Naslaji *et al*., 2006; Niasari-Naslaji *et al*., 2007; El-Bahrawy, 2010; Crichton *et al*., 2015).

Packaging in 1.5 ml ampules is the most commonly used technique for freezing Bactrian camel semen in China (Zhao *et al*., 1991; Chen *et al*., 1993). Semen is held at 37°C for 10 minutes then cooled step by step from 37°C to 20°C (for 10 minutes), from 20°C to 10°C (for 10 minutes) and from 10°C to 4°C (held for 4 hours). Freezing on a wire grid placed above liquid nitrogen in done four (4) steps: 3 minutes at 3 cm, 2 minutes at 2 cm, 1 minute at 1 cm, then plunging in liquid nitrogen.

A technique using cryovials was described in which semen is cooled from 4 to -15°C at a rate of -1°C per min, -4°C per min from -15 to -60°C, -20°C per min from -60 to -100°C then plunged into liquid nitrogen (Deen *et al.*, 2003).

Thawing rate

Thawing rates vary according to the packaging technique used. Pellets are usually thawed out by dropping them into heated receptacles or by mixing iwith warm thawing extender. Semen frozen in ampules is thawed out by placing them in a water bath set at 45 to 55°C for 30 seconds to 1 minute (Zhao *et al*., 1991; Chen *et al*., 1993). Small straws (0.25 and 0.5 mL) are thawed in a water bath at 35-37°C for 30 to 60 seconds or 40°C for 8 seconds. Large straws

are thawed by continuous agitation in a water bath at 40°C for 50 seconds. Cryovials are thawed out by immersion in 40°C water bath for 2 minutes (Deen *et al*., 2003).

Post-thaw semen quality

Most studies rely on traditional methods for semen evaluation such as post-thaw progressive motility at various intervals during incubation. In recent years, techniques used in for the evaluation of semen in other species have been adapted to camelid semen and include hypoosmotic swelling test (incubation in fructose or sucrose solution of 50 to 100 mOsm at 37°C for 45 minutes (Figure 6), special staining techniques (Isothiocynate-conjugated peanut agglutinin (Morton *et al.,* 2007; 2008), Chlortetracycline staining for spontaneous capacitation (Crichton *et al*., 2015). All these add more rigor in the evaluation of different parameters after cryopreservation but have not been yet been correlated to fertility.

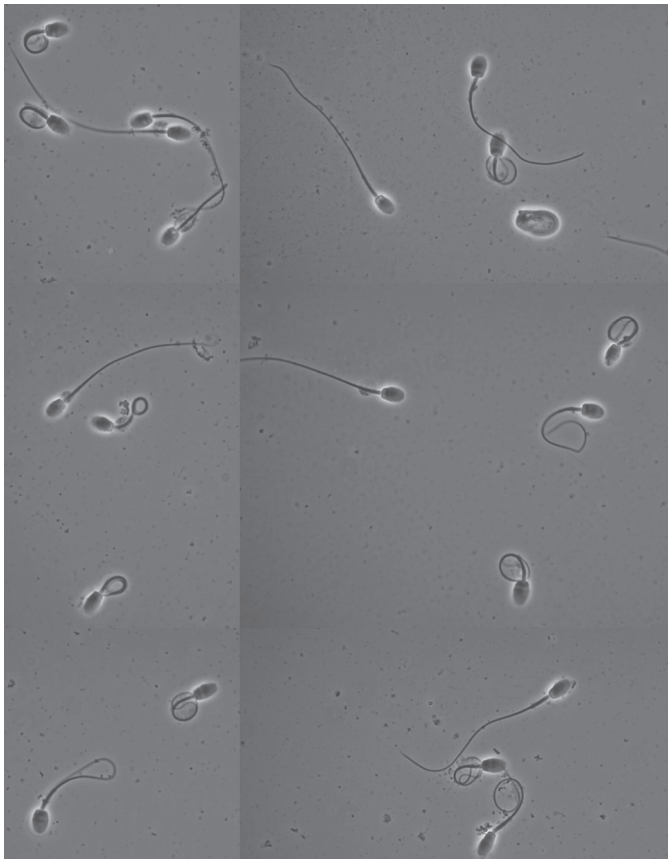

Figure 6: Hypoosmotic swelling test: Spermatozoa with intact membranes present various degrees of swelling and coiling of the tail and mid-piece

Improvement of cryoprotection

Cryoprotection of the spermatozoa is provided by several components in the extender that act to stabilize the sperm membrane and dehydrate the cell in order to avoid formation of large ice crystals. Stabilization of the membrane is provided by lecithin, lipoproteins from the egg yolk, and caseinates from milk. Studies in other species have shown that some detergents may help incorporate these proteins to the cell membrane. Sodium dodecyl sulfate (SDS) (i.e Equex STM® or orvus ES paste) is used for this purpose to enhance the protective effect of egg yolk by breaking down the lipids and making it more accessible to the sperm membrane. This component is often used for stallion semen at very low concentrations (0.5 to 1%). A concentration of 0.5% of SDS did not improve cryopreservation in the Bactrian camel (Niasari-Naslaji *et al*., 2008). The amount of cholesterol in the membrane is also critical for cryoprotection. A recent study showed that addition of Cholesterol-loaded cyclodextrin (1.5 mg/ml) to the extender during liquefaction improves cryotolerance and protects against structural and function damage of dromedary sperm (Crichton *et al*., 2015).

Dehydration and prevention of ice crystal formation is usually provided by strong cryoprotectant which increase significantly the osmotic pressure of the freezing extender. Cryoprotectants used in sperm cryobiology are generally of two types non-penetrating and penetrating. Penetrating cryoprotectant differ by the rate of diffusion into the cell which is dependent on their molecular weight and the temperature at the time if addition.

The most commonly used cryoprotectant is glycerol at concentrations of 3 to 7%. The final concentration of glycerol in the extender and the method of addition to semen have been found to be critical for the survival of spermatozoa and maintenance of its fertilizing ability. Higher concentration 6% are more harmful than lower concentration 3% (Morton *et al*., 2010b). Glycerol is known to induce post-thaw cryocapacitation in several species. There is a lack of studies on the appropriate method of addition of the cryoprotectant (prior to cooling, at cooling or progressively).

A recent study showed that glycerol and ethylene glycol at a concentration of 3 to 6% were equally effective in maintaining motility and acrosome integrity regardless of equilibration times (Malo *et al*., 2017). Amides have been used extensively in the freezing of semen from species, particularly equine, in which glycerol tend to be harmful for fertility. To our knowledge the only amide that has been used in the dromedary camel is methylformamide which resulted in acceptable post-thaw motility and acrosome integrity (Crichton *et al*., 2015).

Artificial insemination trial using cryopreserved sperm

Information on the fertility of frozen-thawed dromedary semen is rudimentary. A few AI trials have been conducted but conception rates are very disappointing (Tibary and Anouassi, 1997a). Bactrian camels are the only camelid species where promising conception rates following AI with frozen-thawed semen were obtained (Table 2). The reason for this discrepancy between Bactrian and dromedary camels is difficult to explain.

It is important to point out that with exception of a few incomplete reports, the disappointing results of AI with frozen-thawed semen is also a problem in South American Camelids (Vaughan *et al*., 2003; Bravo *et al*., 2013).

CONCLUSION

Although studies on preservation of camelid semen have been conducted for over 50 years and despite the intensification of research in this area on the dromedary in the last 15 years, AI with preserved semen in camelid is still far from being optimal except for the Bactrian camel. One can site several reasons for the lack of progress in the development of artificial insemination in camelid and particularly in the dromedary. The first and most important is the lack of rigor in the experimentation on cryopreservation of semen. In the authors experience, several published experiments are not repeatable. The multitude of factors spanning the entire process from semen collection, initial quality analysis, liquefaction procedure, initial dilution, cooling, freezing rates and thawing rate do not make comparison amongst studies possible. Individual animal variation, which has been experienced by the authors, is very seldom reported in publications. It is evident that one of the major hurdles to overcome in semen preservation in camelids is the viscosity of seminal plasma and its role in sperm function. Methods for liquefaction of sperm to allow dilution and incorporation of protecting diluent may interfered later with sperm function despite the quality reported by in vitro assays. Further studies are needed to determine the effect of seminal plasma washing and its replacement on sperm function. Improvements of extenders may be achieved using approaches described in other species such as addition of antioxidants, modification of cryoprotectants and/or their removal prior to insemination and the use of cryoprotecting sugars such as trehalose. Finally, development of in vitro fertilization protocols to test frozen-thawed sperm may shed some light on the effect of processing on sperm function.

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