Vet.Med.J.,Giza. Vol.54, No.1. (2006):29-46.

FREEZABILITY OF CAMEL SEMEN COLLECTED BY "EL-HASSANEIN CAMEL DUMMY" AND DILUTED IN TWO STEPS IN SUCROSE AND/ OR TRIS-BASED EXTENDERS*.

E. E. EL-HASSANEIN

Animal and Poultry Production Division, Desert Research Center, Cairo, Egypt.

Received: 27.9 .2005.

Accepted: 16.10.2005.

SUMMARY

Eight adult male camels were used for semen collection, during rutting season (Nov. 2001- March. 2002), by using the recently invented "El-Hassanein Camel Dummy " at Maryout Research Station of Desert Research Center. Sucrose and Tris and their combinations were used for preparing five extenders for camel semen. Dilution to 150 x 10^6 motile sperms/ml was carried out in two steps. Diluted semen filled in 0.5 ml straws. Three freezing and two thawing rates were applied to evaluate spermatozoa freezability and post-thawing viability. Progressive motility (PPM) and acrosomal integrity (PIA) were evaluated post-dilution and at 0, 2 and 4 hours of post-thawing incubation.

The new collection technique significantly improved the quantity and quality of the delivered semen. The obtained semen volume, ejaculate concentration, progressive motility and acrosomal integrity averaged 15.3 ± 1.15 ml, 810.3 ± 2.21 x 10^6 sperm/ml, 81.6 ± 1.01% and 89.7± 1.94%, respectively.

Dilution of camel spermatozoa in a Sucrose-Tris extender significantly reduced dilution effect on spermatozoa viability and improved their freezability and their post-thawing viability. Higher freezing rates significantly conserved post-thawing viability. Slow and rapid-thawing rates had a relatively comparable effect on post-thawing viability. However, the optimum sperm viability was achieved in semen frozen rapidly (at -140°C for 15 min.) and thawed slowly (at 40°C

* The manuscript was presented to the 9th Inter. Cong. Biotech. Anim. Reprod., Chennai, Dec. 2-4 (2002), India, and published in summary in the proceeding of the congress, pp.: 100.

for 30 sec.) after dilution in an extender composed of: 4.38 % (g/v) sucrose, 1.592 % (g/v) Tris, 0.872 % (g/v) citric acid monohydrate, 20 % (v/v) egg-yolk, 3.5 % (v/v) glycerol, 1000 iu penicillin/ml and 1000 µg streptomycin/ml. Estimated reductions in PPM pre-freezing, post-thawing and after 2 and 4 hours of incubation post-thawing were 5.5, 6.1, 23.3 and 42.5%, respectively. Corresponding reductions in PIA were 5.9, 9.1, 15.7 and 35.3%, respectively.

Consequently, it is concluded that combination of sucrose and Tris in camel semen extender has positive effect on viability of spermatozoa after dilution, freezing and post-thawing incubation.

Keywords: camel; reproduction; semen; collection; dilution; cryopreservation; motility; acrosomal Integrity, freezability, post-thaw survival.

INTRODUCTION

The one-humped (dromedary) camels are characterized by their extremely low fertility (Novoa, 1970) which is considered one of the most important factors affecting their productivity (El-Wishy, 1987). Poor pastoral management systems and harshness of the prevailing environmental conditions in regions where camels are raised, in addition to the advanced age at puberty and limited libido of males adversely affect their reproductive and productive efficiency. Female dromedary, being a seasonal breeder with a long gestation period and reaching to puberty at a very late stage, is likely to produce a few offspring during its breeding life. Normally, only one calf is born every two years, which may be due to short breeding season, together with long lactation periods (Minoia et al., 1992). To increase the number of offspring from genetically valuable female camels in a relatively short breeding period, especially for the racing and milch breeds, a judicious use of artificial insemination (AI) and embryo transfer techniques is required. Development of an AI-system, in concomitant with a successful synchronization of estrus and induction of female's ovulation, is necessary for applying selection programs and rapid genetic improvement of the genetically prestigious camel breeds. The desired results of AI-programs will not be possible without successful semen collection from male camels and successful freezing of semen. Semen collection from camels is the most important step determining the successful use of AI (Salhab, 2001). Unlike other domestic animals, semen collection from male camels is complicated by their natural copulatory behavior, the long duration of copulation and the dripping manner of ejaculation. Recently, a devise has been invented and tested for easy semen collection from dromedary camels (El-Hassanein, 2002). The author lengthily reported the advantages of this modern technique of semen collection from male camels over the traditional ones, i.e. the artificial vagina, in combination with a receptive female, and electroejaculation.

The pre-requisite to the development of a successful AI-program is the development of an optimum freezing protocol for camel semen. Cryopreservation and other related modern reproductive technologies offer many advantages to economic animal production (Greve and Callesen, 2004) and provide an avenue for a long-term storage and retrieval of genetic strains over a long period of time. In addition, the obtainment of cryopreserved camel semen throughout the year increases opportunities of breeding females out of the breeding season. Female dromedary is induced ovulator, demonstrates continuous estrus and being inseminated at any time (Williamson and Payne, 1987). AI in the Bactrian camels has been reported by many authors (Elliot, 1961; Chen et al., 1985, 1990; Zhao et al., 1990, 1994), however, in the dromedaries it is rarely applied (Anouassi et al., 1992; Musa et al., 1992; Deen et al., 2003).

Mixing spermatozoa with an extender allows to preserve the fertility of sperm cells and to increase the total volume so that the proper dose of sperm can therefore be conveniently packaged and used. Preservation of camel semen has been attempted with varying success at different temperatures (Salhab, 2001). Several extenders used for deep freezing of semen of other species have also been adapted to camel semen (Graham et al., 1978; Sieme et al., 1990; Sun et al., 1990; Musa et al., 1992; Willmen et al., 1993; Zhao et al., 1994). The survival rate of camel spermatozoa depends on packaging method, volume of filled semen and freezing and thawing rates (Salhab, 2001). Most of the available data concerning freezing of camel semen used glycerol as cryoprotective agent to protect spermatozoa during freezing process, however, methods of adding glycerol, temperature of adding and the final concentration of glycerol in the extender are scarce (Salhab, 2001).

The objective of this study was to evaluate some extending-freezing protocols for camel semen to obtain acceptable post-thaw sperm viability and survival rates. Sperm viability and survival after dilution in sucrose and/or Tris-based extenders and processed at different freezing and thawing rates have been assessed.

MATERIAL AND METHODS

Eight adult male camels, averaging 7 years old, were used for semen collection during rutting season (November, 2001-March, 2002). Semen collection was carried out at 8:00 PM using the recently invented "El-Hassanein Camel Dummy" (El-Hassanein, 2002) at Maryout Research Station of Desert Research Center. Collected semen was incubated in a water bath (38°C) for a sufficient period to achieve partial liquification and evaluated before its partial extension. Progressive motility (PPM) was estimated by diluting a drop of raw semen in 1 ml of pre-warmed extender and using a phase contrast microscope equipped with a heat-

ing stage adjusted at 40°C. Ejaculate concentration was estimated by using a photometer previously calibrated for measuring camel semen concentration. Percentage of dead and alive sperm was estimated using slide smears according to Salisbury et al. (1985). Percentage of intact acrosomes (PIA) and sperm abnormalities were estimated in samples fixed in 0.2% glutaraldehyde solution and using oil immersion objective (Johnson et al., 1976).

For dilution of camel semen, sucrose-based extender (Zhao et al., 1996) and Tris-based extender (Deen and Sahani, 2000) and combinations of sucrose and Tris were freshly prepared and refrigerated, the night before collection, according to Table (1). Each extender was prepared in two fractions where antibiotics were added to fraction

Table (1): Composition of the sucrose and/or Tris based diluents (D) used in dilution of camel semen.

Constituents /100 ml of double distilled water		D1	D2	D3	D4	D5
Sucrose	(g)	8.76	6.54	4.38	2.22	----
Tris	(g)	------	0.807	1.592	2.377	3.184
Citric acid, monohydrated	(g)	-----	0.442	0.872	1.301	1.743
Egg yolk	(ml)	20	20	20	20	20
Glycerol	(ml)	3.5	3.5	3.5	3.5	3.5
Penicillin	(iu)	100000	100000	100000	100000	100000
Streptomycin	(µg)	100000	100000	100000	100000	100000

"A" and glycerol was included in fraction "B". Fraction "A" of each extender was warmed to 38°C in a water bath before its use in partial extension of semen, while fraction "B" was incubated inside a cooling cabinet adjusted at 4°C until use in completion of extension.

Dilution was carried out to achieve a final concentration of 150 x 10^6 motile sperm/ml. According to the obtained values of ejaculate volume, progressive motility and sperm concentration in raw semen, the estimated volume of fraction "A" of each extender was added slowly to semen with gentle mixing. Semen diluted with fraction "A" was gradually cooled to room temperature (20°C) in a beaker containing warm water (38°C) and completely enveloped with aluminum foil for at least 3 hours. The beaker was then transferred into the cooling cabinet for cooling the partially extended semen to 4°C within 3-4 hours. The estimated volume of fraction "B" of each extender was then added to its counterpart of partially di-

luted semen in a dripping manner with gentle mixing.

Thirty minutes after completion of extension process, precise assessment of PPM and PIA was conducted in samples of the fully diluted semen before filling. Thereafter, filling and sealing of diluted semen in 0.5 ml French straws was carried out inside the cooling cabinet. Filled straws of each extender treatment were allocated into three groups and frozen, according to Sieme et al. (1990) and Bravo et al. (2000b), on nitrogen vapor in the programmable freezing unit at one of the following rates:

a- Slow freezing rate (at -100°C for 30 minutes) - (F1).

b- Medium freezing rate (at -120°C for 20 minutes) - (F2).

c- Rapid freezing rate (at -140°C for 15 minutes) - (F3).

Frozen straws were immediately plunged into liquid nitrogen for long-term storage. Few days later, three straws from each extender-freezing treatment were thawed either at 40°C for 30 seconds or at 65°C for 5 seconds. Thawed straws of each extender-freezing-thawing treatment were pooled and incubated in a water bath (38°C) for four hours. Assessment of PPM and PIA was conducted in frozen-thawed semen at 0, 2 and 4 hours of incubation at 38°C.

Each extender-freezing treatment was repeated twice during the early, median and late weeks of the breeding season. Data were analyzed, after arcsine square root % transformation, using Statgraph program (Version 5).

RESULTS AND DISCUSSION

1) - Semen Physical Characteristics

The average values of raw seminal characteristics are presented in Table (2). Ejaculate volume collected by the camel dummy averaged 15.3 ml and ranged from 3 to 27 ml. This ejaculate volume obviously exceeded the reported values for semen of dromedary camels (2-9 ml) (Abdel-Raouf and El-Naggar, 1976; Tingari et al., 1986; Ismail, 1988; Sieme et al., 1990; Anouassi et al., 1992; Billah and Skidmore, 1992; Musa et al., 1992; Willmen et al., 1993; Badamdorj et al., 2000; Bravo et al., 2000b; Deen et al., 2003). However, Wilson (1984) reported a relatively comparable range of ejaculate volume of 5-22 ml in dromedary camels. These considerable variations might be due to the age, sexual stimulation, method of collection and/or the prevailing conditions in the collection area.

The estimated progressive motility (PPM) in camel ejaculate was 81.6%. Many authors reported lower values of sperm PPM ranging from 30-60% (Sieme et al., 1990; Billah and Skidmore, 1992; Musa et al., 1992; Willmen et al., 1993; Hassan et

al., 1995, Badamdorj et al., 2000). Certainly, the individual motility of camel sperm is greatly affected by the condition of collection and state of liquification (Tibary and Anouassi, 1997), by the type of the rubber constituting the inner liner of AV and the length of time semen stays in contact with the liner (Musa et al., 1992). It has been reported that movement of camelid sperm in undiluted viscous semen is best described as oscillatory, i.e. sperm move back and forth with only 5-10% actively progress forwards (Neely, 1993; Bravo et al., 2000a) and the sperm increase their progressive motility as the ejaculate becomes more liquid (Tibary and Memon, 1999). In the present study, the improvement in sperm motility after collection and partial liquification may be due to the modifications made in the AV to avoid contamination of semen with the inner liner and to receive semen directly into the glass vessel of collection (El-Hassanein, 2002).

Ejaculate concentration averaged 810 x 10^6 sperm/ml. and ranged from 350 to 1500x10^6 sperm/ml. The obtained value of ejaculate concentration was in agreement with that found by Tingari et al. (1986) who reported a mean ejaculate concentration of 800x10^6 in dromedary semen (ranged from 400 to 1300x10^6 sperm/ml). However, many authors reported that ejaculate concentration in dromedary semen collected by AV or electroejaculation ranged from 200 to 400x10^6 sperm/ml (Anouassi et al., 1992; Billah and Skidmore, 1992; Musa et al., 1992; Badamdorj et al., 2000; Bravo et al., 2000b; Deen et al., 2003). It has been reported that the sperm concentration in camelid ejaculates increases with time during a single copulation (Lichtenwalner et al., 1996; Bravo et al., 2002) and decreases with increasing number of ejaculation/day (Bravo et al., 1997a). In the present study, improvement of ejaculate concentration in semen collected by the camel dummy might be due to the observed elongation of copulation duration of males with the dummy to about

Table (2): Average values (means ± s.e.) of raw seminal characteristics of dromedary male camels.

Physical Parameters of Raw Semen	Mean ± S.E	Range
Ejaculate Volume (ml)	15.3 ± 1.15	3-27
Progressive Motility (%)	81.6 ± 1.01	30-90
Concentration (x 10^6 sperm/ml)	810.3 ± 2.21	350-1500
Dead Sperm (%)	6.8 ± 0.89	4-15
Primary Abnormalities (%)	10.9 ± 0.79	6-18
Secondary Abnormalities (%)	12.5 ± 0.83	6-20
Abnormal Acrosomes (%)	10.3 ±0.94	8-14

seven folds of the duration of copulation with an AV in combination with a receptive female (El-Hassanein, 2002).

A noticeable improvement in the other quality parameters of camel semen was also noted in the present study. Percentage of dead sperm and primary, secondary and acrosomal abnormalities in camel ejaculates were 6.8, 10.9, 12.5 and 10.3 %, respectively. The obtained values were relatively lower than the values reported in dromedary semen by many authors (Tingari et al., 1986; Ismail, 1988; Merkt et al., 1990; Musa et al., 1992; Goswami, 2002), who reported that the proportion of dead sperm in dromedary semen is 18-19 %, that of morphologically abnormal sperm is 27-28 % and that of sperm with abnormal acrosomes is 8.0-8.5 %. Ejaculates of fertile male camels usually contain a high number of morphologically normal sperm cells (70-85%) (Chen et al., 1990; Tingari et al., 1986; Willmen et al., 1993; Zhao et al., 1992, 1994) and the proportion of abnormal cells can reach up to 60% in old (> 25 years) males (Tibary and Anouassi, 1997). Recently, El-Hassanein et al. (2004) reported that the percentage of sperm acrosomal, dead and tail abnormalities decreased noticeably in camel ejaculates by the breeding season progress.

Obviously, using a camel dummy as that used in the present study for semen collection successfully improved the quantity and quality of the delivered camel semen (El-Hassanein, 2002). The use of an artificial vagina in combination with a dummy (Garnica et al., 1993; Bravo et al., 1997a,b; Davalos et al., 1999) or half-dummy mount (Lichtenwalner et al., 1996) has yielded the most representative copulation times and mating behavior in South American camelids in addition to improvement of the collected semen composition (Davalos et al., 1999).

2) - Dilution Effect on Sperm Viability

Percent reductions, relative to raw-seminal values, in pre-freezing PPM and PIA of camel spermatozoa diluted in sucrose and/or Tris-based extenders are presented in Table (3). Extender-type significantly ($P< 0.01$) affected the sperm viability after dilution and equilibration. The least reductions in pre-freezing PPM and PIA (5.5 and 5.9 %, respectively) were recorded in semen diluted in D3. Dilution in a sucrose-based extender (D1) or in a Tris-based one (D5) revealed a deleterious effect on the pre-freezing sperm viability where sperm PPM and PIA were reduced by about 19 and 25 %, respectively, from their raw-seminal values.

Reduction in sperm viability after dilution and equilibration may be attributed to sperm sensitivity to cold shock (Watson and Plummer, 1985; Holt, 2000; Watson, 2000) and/or to the toxicity of the cryoprotectant agent, such as glycerol, added to the extender (Watson and Martin, 1975;

Fahy, 1986, Pontbriand et al., 1989; Maxwell and Watson, 1996). In the present study, although semen was incubated at low extension rates for a holding time at room temperature (Pursel et al., 1972) and glycerolated in stepwise manner (Watson, 2000) after a sufficient time of equilibration at 4°C with lipoproteins of yolk, sperm viability was reduced pre-freezing. Combination of sucrose and Tris in camel extenders (D2, D3 and D4) may have led to modulation of phase transition behavior of sperm membrane and improving their resistance to cold shock and glycerol toxicity as compared with dilution in sucrose (D1) or Tris (D5). The optimum buffering capacity during dilution and equilibration was achieved in semen diluted in D3.

Table (3): Average values (mean ± s.e.) of percent reductions, relative to raw seminal values, in sperm PPM and PIA of camel semen after dilution in sucrose and/or Tris-based extenders.

Diluents	PPM			PIA		
	Raw Semen	Pre-freezing Semen	% Reduction	Raw Semen	Pre-freezing Semen	% Reduction
D1	82.0 ± 1.71	66.3 ± 1.50^{b}	1.91^{a}	89.8 ± 1.35	68.2 ± 2.24^{b}	24.1^{a}
D2	82.2 ± 1.58	71.3 ± 1.65^{ab}	13.3^{b}	89.8 ± 1.54	78.7± 1.23^{a}	12.4^{c}
D3	81.2 ± 0.98	76.7 ± 1.28^{a}	5.5^{c}	89.5 ± 1.53	84.2 ± 1.54^{a}	5.9^{d}
D4	81.3 ± 1.15	67.2 ± 1.54^{ab}	17.3^{a}	89.3 ± 1.89	74.2 ± 2.06^{ab}	16.9^{b}
D5	81.2 ± 1.58	65.3 ± 1.65^{b}	19.6^{a}	90.2± 1.28	67.0 ± 1.91^{b}	25.7^{a}
F-test	NS	*	**	NS	**	**

a, b values in the same column, in each block, with different superscripts are significantly different (P< 0.05). NS: Statistically non-significant; * P< 0.05; ** P< 0.01.
PPM: Percent progressive motility; PIA: Percent intact acrosomes.

3) - Sperm Freezability

In the present study, freezability of spermatozoa was evaluated by estimating the recovery rate (%) of sperm viability post-thaw, i.e. calculating the ratio of post-thaw to pre-freezing values of PPM and PIA. Average values of recovery rates of sperm PPM and PIA in frozen-thawed camel semen are presented in Table (4). Spermatozoal freezability was significantly (P< 0.05) affected by the extender-type. The best sperm freezability was recorded in semen diluted in D3, where about 80% of spermatozoa had progressive motility and intact acrosomes post-thaw. On the other hand, dilution in the sucrose (D1) or Tris (D5) revealed the least recovery rates for PPM and PIA post-thaw (about 62 and 70 %, respectively).

Table (4): Average values (mean ± S.E.) of recovery rates (%) of sperm PPM and PIA in frozen-thawed camel semen diluted in sucrose and/or Tris-based extenders.

	Diluents	F1		F2		F3		F-test
		ST	RT	ST	RT	ST	RT	
PPM	D1	54.3 ± 1.05^{d}	51.3 ± 0.98^{d}	63.5 ± 1.24^{bc}	58.7 ± 1.12^{cd}	74.1 ± 1.42^{a}	77.7 ± 1.53^{a}	**
	D2	61.1 ± 1.14^{c}	52.3 ± 0.86^{d}	66.1 ± 1.41^{c}	70.9 ± 1.32^{bc}	77.1 ± 1.34^{ab}	82.1 ± 1.54^{a}	**
	D3	72.6 ± 1.33^{cd}	63.8 ± 1.14^{d}	76.1 ± 1.42^{c}	81.3 ± 1.46^{bc}	93.9 ± 1.67^{a}	89.1 ± 1.53^{ab}	**
	D4	63.3 ± 1.23^{cd}	56.9 ± 1.02^{d}	64.9 ± 1.15^{cd}	68.7 ± 1.44^{bc}	80.7 ± 1.62^{a}	75.1 ± 1.47^{ab}	**
	D5	52.9 ± 0.87^{b}	56.3 ± 0.96^{b}	57.6 ± 1.12^{b}	54.4 ± 0.88^{b}	75.9 ± 1.55^{a}	70.7 ± 1.23^{a}	**
	Mean	60.8 ± 1.07^{bc}	56.1 ± 1.01^{c}	65.6 ± 1.34^{b}	66.8 ± 1.44^{b}	80.3 ± 1.42^{a}	78.9 ± 1.49^{a}	**
	Overall mean	58.5 ± 1.06^{b}		66.2 ± 1.43^{b}		79.6 ± 1.62^{a}		**
PIA	D1	62.1 ± 1.15^{cd}	59.3 ± 0.78^{d}	70.9 ± 1.44^{bc}	74.5 ± 1.32^{ab}	82.1 ± 1.92^{a}	79.5 ± 1.73^{ab}	**
	D2	70.5 ± 1.34^{bc}	65.3 ± 1.46^{c}	76.6 ± 1.71^{ab}	75.6 ± 1.52^{ab}	83.2 ± 1.98^{a}	86.8 ± 1.89^{a}	*
	D3	73.8 ± 1.53^{bc}	68.8 ± 1.54^{c}	76.6 ± 1.52^{bc}	79.8 ± 1.86^{ab}	90.9 ± 2.07^{a}	89.6 ± 2.03^{a}	*
	D4	61.3 ± 1.03^{d}	65.3 ± 1.32^{cd}	74.3 ± 1.35^{abc}	70.9 ± 1.24^{bc}	82.7 ± 1.87^{a}	79.9 ± 1.87^{ab}	**
	D5	59.9 ± 0.87^{e}	63.1 ± 1.26^{de}	65.9 ± 1.32^{cde}	68.3 ± 1.38^{bcde}	75.1 ± 1.51^{abc}	79.9 ± 1.73^{a}	*
	Mean	65.5 ± 1.47^{b}	64.4 ± 1.31^{b}	72.9 ± 1.44^{ab}	73.8 ± 1.34^{b}	82.8 ± 1.82^{a}	83.1 ± 1.79^{a}	*
	Overall mean	64.9 ± 1.36^{b}		73.4 ± 1.43^{ab}		83.0 ± 1.94^{a}		**

a,b values in the same row, in each block, with different superscripts are significantly different ($P<0.05$). NS: Statistically non-significant; * $P<0.05$; ** $P<0.01$.

PPM: Percent progeressive motility; PIA: Percent intact acrosomes.

F1: Freezing at-100°C for 30 minutes; F2: Freezing at-120°C minutes; F3: Freezing at -140°C for 15 minutes.

ST: Slow thawing (at 40°C for 30 seconds); RT: Rapid thawing (at 65°C for second).

Freezing rate significantly (P< 0.01) affected the sperm freezability. Rapid freezing (F3) resulted in improvement of post-thaw recovery rates for PPM and PIA (80 and 83 %, respectively), in contrary to slow freezing (F1) which revealed the least recovery rates for PPM and PIA post-thaw (59 and 65 %, respectively). Unfortunately, scarce data are available concerning the estimated values of post-thaw sperm viability in dromedary camel semen. Recently, Deen et al. (2003) considered freezing of dromedary semen to be successful when achieving at least 20% sperm progressive motility after thawing. However, post-thaw motility in Bactrian camel semen diluted in a sucrose-yolk-glycerol extender containing 3.5 or 7 % glycerol was estimated to be 43 and 64 %, respectively (Chen et al., 1990; Zhao et al., 1994). Similar post-thaw sperm viability (ranged from 15 to 60%) was also reported for alpaca and llama frozen semen (Bravo et al., 2000b).

Evaluation of freezing protocols includes the assessment of sperm viability post-thaw in order to monitor the number of post-thaw surviving sperm (Gravance et al., 1998; Rodriguez-Martinez, 2002) and can provide insights upon their fertilizing capacity (Januskauskas and Zilinskas, 2002). The damage and/or death of sperm during freezing might be due either to formation of intracellular ice crystals as a result of rapid freezing or to exposure to high concentrations of solutes when freezing is too slow (Mazur et al., 1972). Optimal freezing rate therefore is a compromise between rapid and slow freezing to minimize the effects of these two sources of injury. However, post-thaw semen quality and survival of spermatozoa are ascertained to be highly variable from one male to the other even after using the same freezing protocol (Salhab, 2001). A large variation in freezability of camel spermatozoa from individual males have been found (Deen et al., 2003).

Literature on freezability of camel semen and the factors affecting quality of semen after freezing and thawing are rather limited (Salhab, 2001; Deen et al., 2003). However, different extenders that have been adopted in various livestock species were tested for preservation of camel semen with considerable variations in results. Chen et al. (1990) reported a great post-thaw motility in camel semen diluted in sucrose-yolk-glycerol extender. Similarly, Zhao et al. (1994) found that sucrose-yolk-glycerol was superior in preservation of camel semen to other extenders used successfully to freeze bull, stallion, boar, ram and buck semen. However, Sieme et al. (1990) and Musa et al. (1993) reported that extender containing 11 % lactose was suitable for cooling and freezing camel semen. On the contrary, Vyas et al. (1998) found that Tris was superior to 11 % lactose extender. Also, Deen et al. (2003) reported that Tris-yolk-glycerol was successful in protecting camel semen during freezing. In the present study, combination of sucrose and Tris in the extender fol-

lowed by dilution in two-steps and rapid freezing on nitrogen vapor at -140°C improved freezability and post-thaw viability of camel spermatozoa, compared with dilution in sucrose or a Tris-based extender.

4) - Post-thaw Sperm Survival

Cooling, freezing and thawing processes induce capacitation-like changes in the surviving sperm population and these changes cause sperm to have a shorter survival time in the female genital tract compared with their fresh counterpart (Watson, 2000). For this reason, stressing the frozen-thawed semen by long incubation at about 38°C for several hours post-thaw has been applied to evaluate the ability of cryopreserved sperm to traverse the highly viscous cervical mucus (Rodiguez-Martinez, 2002). Survival of frozen-thawed camel spermatozoa during incubation at 38°C post-thaw was assessed by calculating the percent reduction in sperm PPM and PIA, relative to their post-thawing values, after 2 and 4 hours of incubation. The estimated values of percent reduction in sperm PPM and PIA of frozen-thawed camel semen, after 2 and 4 hours incubation post-thaw, are presented in Tables (5) and (6), respectively.

Sperm survival post-thaw was significantly ($P< 0.01$) affected by the used extender-type. Dilution of camel semen in D3 minimized reduction in sperm PPM after 2 and 4 hours incubation post-thaw to about 37 and 62 %, respectively. Corresponding reductions in sperm PIA were 20 and 42 %. Dilution in sucrose (D1) or Tris (D5) revealed a comparable effect on the sperm survival post-thaw, where reduction in sperm PPM after 2 and 4 hours incubation reached up to 57 and 84 %, respectively and sperm PIA was reduced by about 35 and 73 %.

Sperm longevity post-thaw was significantly ($P< 0.01$) affected by the applied freezing rate. Rapid freezing (F3) of camel semen revealed the least reduction, after 2 and 4 hours incubation, for sperm PPM (41 and 68 %, respectively) and sperm PIA (26 and 55 %), compared to the other freezing rates (F1 and F2). Slow and rapid thawing rates revealed a non-significant effect on sperm survival post-thaw. However, a thorough examination of the obtained values revealed that dilution of camel semen in D3 followed by rapid freezing (F3) and slow thawing of frozen semen (ST) obviously improved the sperm survival post-thaw, where sperm PPM after 2 and 4 hours incubation recorded a minimal reductions of about 23 and 43 %, respectively. The corresponding reductions in sperm PIA were 16 and 35 %.

Literature on dromedary camel sperm freezability and survival post-thaw is scarce. However, the viscous nature of camelids semen seems to preclude even mixing of semen with extender and ensures marked heterogeneity of the solution to be cryopreserved which might explain poor post-

Table (5): Average values (mean ± S.E.) of percent reduction, relative to the post-thawing values, in sperm PPM and PIA in frozen-thawed camel semen diluted in sucrose and/or Tris-based extenders, after 2 hours of incubation post- thaw

	Diluents	F1		F2		F3		F-test
		ST	RT	ST	RT	ST	RT	
PPM	D1	60.7 ± 1.05^{ab}	65.5 ± 1.28^{a}	51.7 ± 0.24^{c}	62.5 ± 1.12^{a}	53.3 ± 0.23^{bc}	46.5 ± 0.53^{c}	**
	D2	57.2 ± 0.64^{b}	70.8 ± 1.46^{a}	57.8 ± 0.41^{b}	54.0 ± 0.30^{b}	40.1 ± 0.11^{c}	44.1 ± 0.54^{c}	**
	D3	50.1 ± 0.53^{a}	40.7 ± 0.14^{b}	41.3 ± 0.32^{b}	36.5 ± 0.06^{b}	23.3 ± 0.07^{d}	29.5 ± 0.53^{c}	**
	D4	68.9 ± 1.53^{a}	63.5 ± 1.02^{ab}	50.3 ± 0.25^{c}	59.5 ± 0.87^{b}	36.3 ± 0.22^{e}	43.1 ± 0.47^{d}	**
	D5	61.3 ± 1.21^{ab}	70.3 ± 1.66^{a}	60.4 ± 1.02^{b}	56.4 ± 0.48^{bc}	51.7 ± 0.56^{c}	41.9 ± 0.23^{d}	**
	Mean	59.6 ± 0.87^{ab}	62.2 ± 1.17^{a}	52.3 ± 0.14^{b}	53.8 ± 0.21^{b}	40.9 ± 0.12^{c}	41.0 ± 0.49^{c}	**
	Overall mean	60.9 ± 1.06^{a}		53.0 ± 0.31^{a}		41.0 ± 0.14^{b}		**
PIA	D1	43.1 ± 0.11^{a}	36.1 ± 0.21^{bc}	39.5 ± 0.54^{ab}	34.7 ± 0.07^{bc}	31.5 ± 0.05^{c}	26.9 ± 0.07^{d}	*
	D2	24.3 ± 0.13^{a}	26.7 ± 0.11^{a}	25.1 ± 0.04^{a}	24.3 ± 0.05^{a}	18.1 ± 0.04^{b}	26.1 ± 0.08^{a}	*
	D3	21.9 ± 0.06^{b}	28.1 ± 0.13^{a}	17.0 ± 0.03^{cd}	19.4 ± 0.03^{bc}	15.7 ± 0.04^{d}	19.1 ± 0.03^{bc}	**
	D4	40.6 ± 0.12^{a}	38.6 ± 0.65^{ac}	32.4 ± 0.45^{de}	35.4 ± 0.09^{acd}	29.4 ± 0.07^{e}	33.6 ± 0.31^{cd}	*
	D5	41.1 ± 0.14^{a}	32.3 ± 0.27^{b}	31.3 ± 0.06^{b}	27.1 ± 0.04^{c}	32.1 ± 0.14^{b}	22.7 ± 0.05^{d}	*
	Mean	34.2 ± 0.05^{a}	32.4 ± 0.34^{ab}	29.1 ± 0.04^{bc}	28.2 ± 0.06^{bc}	25.4 ± 0.05^{c}	25.7 ± 0.06^{c}	*
	Overall mean	33.3 ± 0.04^{a}		28.6 ± 0.18^{b}		25.5 ± 0.06^{b}		*

a,b values in the same row, in each block, with different superscripts are significantly different (P<0.05). NS: Statistically non-significant; * P<0.05; **P<0.01.

PPM: Percent progeressive motility; PIA: Percent intact acrosomes.

F1: Freezing at-100°C for 30 minutes; F2: Freezing at-120°C minutes; F3: Freezing at -140°C for 15 minutes.

ST: Slow thawing (at 40°C for 30 seconds); RT: Rapid thawing (at 65°C for 5 second).

Table (6): Average values (mean ± S.E.) of percent relative, relative to the post-thawing values, in sperm PPM and PIA in frozen-thawed camel semen diluted in sucrose and/or Tris-based extenders, after 4 hours of incubation post- thaw

	Diluents	F1		F2		F3		F-test
		ST	RT	ST	RT	ST	RT	
PPM	D1	92.3 ± 1.64^{a}	85.9 ± 1.53^{ab}	85.1 ± 1.43^{abc}	81.7 ± 1.34^{abc}	76.3 ± 1.61^{bc}	74.3 ± 1.23^{c}	*
	D2	90.1 ± 1.87^{ab}	96.7 ± 1.89^{a}	73.4 ± 1.23^{cd}	81.8 ± 1.52^{bc}	65.1 ± 1.25^{d}	68.7 ± 1.32^{d}	**
	D3	85.6 ± 1.56^{a}	93.0 ± 1.57^{a}	45.1 ± 0.89^{c}	58.3 ± 1.04^{b}	42.5 ± 0.63^{c}	48.1 ± 0.23^{c}	**
	D4	88.1 ± 1.78^{a}	93.7 ± 1.64^{a}	71.3 ± 1.32^{c}	84.1 ± 1.53^{ab}	69.7 ± 1.53^{c}	73.3 ± 1.51^{bc}	*
	D5	94.7 ± 1.98^{a}	88.1 ± 1.34^{ab}	87.9 ± 1.41^{ab}	78.5 ± 1.34^{bc}	81.2 ± 1.71^{bc}	75.2 ± 1.43^{c}	*
	Mean	90.2 ± 1.35^{a}	91.5 ± 1.43^{a}	72.6 ± 1.14^{b}	76.9 ± 1.13^{b}	67.0 ± 1.16^{c}	67.9 ± 1.34^{c}	*
	Overall mean	90.8 ± 1.43^{a}		74.7 ± 1.51^{b}		67.5 ± 1.41^{b}		**
PIA	D1	85.7 ± 1.78^{a}	78.5 ± 1.44^{ab}	73.4 ± 1.23^{bc}	65.4 ± 1.12^{cd}	71.3 ± 1.32^{bcd}	63.1 ± 1.12^{d}	*
	D2	63.1 ± 1.14^{a}	65.9 ± 1.32^{a}	51.1 ± 0.67^{c}	59.7 ± 0.98^{ab}	48.4 ± 0.78^{c}	54.0 ± 0.65^{bc}	*
	D3	41.2 ± 0.54^{bc}	50.2 ± 0.42^{a}	37.3 ± 0.23^{cd}	44.5 ± 0.63^{ab}	35.3 ± 0.31^{d}	40.5 ± 0.34^{bcd}	*
	D4	69.3 ± 1.24^{ab}	75.7 ± 1.45^{a}	56.9 ± 0.86^{cd}	61.5 ± 1.04^{bc}	52.1 ± 0.65^{d}	57.7 ± 0.92^{cd}	**
	D5	81.3 ± 1.64^{a}	76.7 ± 1.61^{ab}	71.4 ± 1.12^{abc}	65.2 ± 1.13^{c}	67.3 ± 1.24^{bc}	63.5 ± 1.34^{c}	*
	Mean	68.1 ± 1.35^{ab}	69.4 ± 1.31^{a}	58.0 ± 0.97^{c}	59.3 ± 0.89^{bc}	54.9 ± 076^{c}	55.8 ± 0.72^{c}	*
	Overall mean	68.8 ± 1.24^{a}		58.6 ± 1.02^{b}		55.3 ± 0.85^{b}		*

^{a,b} values in the same row, in each block, with different superscripts are significantly different ($P<0.05$). NS: Statistically non-significant; * $P<0.05$; **$P<0.01$.

PPM: Percent progeressive motility; PIA: Percent intact acrosomes.

F1: Freezing at-100°C for 30 minutes; F2: Freezing at-120°C minutes; F3: Freezing at -140°C for 15 minutes.

ST: Slow thawing (at 40°C for 30 seconds); RT: Rapid thawing (at 65°C for 5 second).

Garnica, J.; Achata, R. and Bravo, P. W. (1993): Physical and biochemical characteristics of alpaca semen. Anim. Reprod. Sci., 32: 85-90.

Goswami, P. (2002): Biometry of camel (C. dromedarius) spermatozoa and their morphological abnormalities. Proc. 9th Inter. Cong. Biotech. Anim. Reprod., Chennai, Dec. 2-4, India.

Graham, E. F; Schmehl, M. K. K.; Evensen, B. K. and Nelson, D. S. (1978): Semen preservation in non- domestic mammals. Syp. Zool. Soc. Land.: 153-173.

Gravance, C. G.; Vishwanath, R.; Pilt, C.; Garner, D. L. and Casey, P. J. (1998): Effects of cryopreservation on bull sperm head morphometry. J. Androl., 19: 704-709.

Greve, T. and Callesen, H. (2004): Integrating new technologies with embryology and animal production. Reprod. Fertil. Dev.,16: 113-122.

Hassan, M. M.; Saeed, M. and Rizwan-ul-Muqtadir (1995): Semen collection by artificial vagina and cryopreservation of camel (C. dromedarius) spermatozoa. Pakistan Vet. J., 15: 105-108.

Holt, W. C. (2000): Basic aspects of frozen storage of semen. Anim. Reprod. Sci., 62: 3-22.

Ismail, S. T. (1988): Reproduction in the male dromedary (C. dromedarius). Theriogenology, 29:1407-1419.

Januskauskas, A. and Zilinskas, H. (2002): Bull semen evaluation post-thaw and relation of semen characteristics to bull's fertility. Vet. Zootech., 17: 1-13.

Johnson, L.; Berndtson, W. and Pickett, B. (1976): An improved method for evaluating acrosomes of bovine spermatozoa. J. Anim. Sci., 42 (4): 951-954.

Lichtenwalner, A. B.; Woods, G. L. and Weber, J. A. (1996): Seminal collection, seminal characteristics and pattern of ejaculation in llamas. Theriogenology, 46: 293-305.

Maxwell, W. M. C. and Watson, P. F. (1996): Recent progress in the preservation of ram semen. Anim. Reprod. Sci., 42: 55-65.

Mazur, P.; Leibo, S. P. and Chu, E. H. Y. (1972): A two-factor hypothesis of freezing injury. Exp. Cell Res., 71: 345-355.

Merkt, H.; Rath, D.; Musa, B. and El-Naggar, M. A. (1990): Reproduction in camels. FAO, Animal Production and Health, paper no. 82.

Minoia, P.; Moslah, M.; Lacalandra, G.M.; Khorchani, T. and Zarilla, A. (1992): Induction of estrus and management of reproduction in the female dromedary camel. Proc. 1st Inter. Camel Conf., 119-123. Edited by W. R. Allen, A. J. Higgins, I. G. Maybew, D. H. Snow and J. F. Wade. R&W Publications (Newmarket) Ltd.

Musa, B.; Sieme, H.; Merkt, H. and Hago, B. E. D. (1992): Artificial insemination in dromedary camels. Proc. 1st Inter camel Conf., Dubai, UAE, PP: 179-182.

Musa, B.; Sieme, H.; Merkt, H; Hago, B. E. D.; Cooper, M. J.; Allen, W. R. and Jochle, W. (1993): Manipulation of reproductive functions in male and female camels. Anim. Reprod. Sci., 33: 289-306.

Neely, D. P. (1993): Reproductive aspects of the male llama. Proc. Hudson-Walker Theriogenology Conf., 4: 31-39.

Novoa, C. (1970): Review. Reproduction in Camelidae. J. Reprod. Fertil., 22: 3-20.

Pontbriand, D.; Howard, J. G.; Schiewe, M. G.; Stuart, L.D. and Wildt, D. E. (1989): Effect of cryopreservative diluent and method of freeze-thawing on survival and acro-

somal integrity of ram spermatozoa. Cryobiology, 26: 341-354.

Pursel, V. G.; Johnson, L. A. and Schulman, L. L. (1972): Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa. J. Anim. Sci., 35: 580-584.

Rodriguez-Martinez, H. (2002): Evaluation of frozen semen. Proc. 9th Int. Cong. Biotech. Anim. Reprod., Dec. 2-4, Chennai, India., pp. 68-79.

Salhab, S. A. (2001): Artificial insemination in camelidae and its Challenges: A review. Proc. 6th Ann. Conf. Anim. Prod. Under Arid Conditions, Reproduction & Production in Camelides, AL-Ain, Nov. 11-13, UAE.

Salisbury, G. W.; VanDemark, N. L. and Lodge, J. R. (1985): Semen evaluation, Indian ed.: Physiology of Reproduction and Artificial Insemination of Cattle. CBS Publishers & Distributors, Delhi, India, pp. 286-328.

Sieme, H.: Merkt, H.; Musa, B., Hago, B. E. O. and Willem, T. (1990): Liquid and deep freeze preservation of camel semen using different extenders and methods. Proc. Workshop "Is it possible to improve the reproductive performance of the camel?"- Paris: 273-284.

Sun, V.; Su, X. S.; Tong, Z. and Kun, Y. (1990): Artificial insemination in Bactrian camel. Acta Vet. Zootech. Suppl., 2: 50-51.

Tibary, A. and Anouassi, A. (1997): Introduction to Camelidae. In: Thereogenolgy in Camelidae, Anatomy, Physiology, Pathology and Artificial Breeding. (A. Tibary and A. Anouassi, eds.), 1st edit., AbuDhabi publishing and publication Comp., UAE., PP.: 1-16.

Tibary, A. and Memon, M. A. (1999): Reproduction in the male South American Camelidae. J. Camel Pract. Res., 6: 235-248.

Tingari, M. D.; El-Manna, M. H.; Rahim, A.T.A., Ahmed, A. K. and Hamad, M. H. (1986): Studies on camel semen. I- Electroejaculation and some aspects of semen characteristics. Anim. Reprod. Sci., 12: 213-222.

Vaughan, J.; Galloway, D. and Hopkins, D. (2003): Storing liquid alpaca semen. In: Artificial Insemination in Alpaca (Lama pacos), Roral Industries Res. Develop. Coop., Australia, pp.: 60-66.

Vyas, S.; Goswami, P.; Rai, A. K. and Khanna, N. M. (1998): Use of Tris and lactose extenders in preservation of camel semen at refrigerated temperature. Indian Vet. J., 75: 810-812.

Watson, P. F. (2000): The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., 60-61: 481-492.

Watson, P. F. and Martin, I. C. A. (1975): Effects of egg yolk, glycerol and the freezing rate on the viability and acrosomal structure of frozen ram spermatozoa. Aust. J. Biol. Sci., 28: 153-159.

Watson, P. F. and Plummer, J. M. (1985): The response of boar sperm membranes to cold shock and cooling. In: Johnson L. A., Larsson K. (eds.), Deep Freezing of Boar Semen, SLU, Uppsala, Sweden, pp.: 113-127.

Williamson, G. and Payne, W. J. A. (1987): An introduction to animal husbandry in the tropics. London. 3rd ed., PP: 484-518.

Willmen, T.; Sieme, H.; Merkt, H.; Saad, F.; Hoppen, H. O. and Waberski, D. (1993): Effects of hormones on semen quality and semen preservation in camels. Reprod. Domestic Anim., 28: 91-96.