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Frysnings- och upptiningsgraders effekt på spermiekvaliteten hos kameler

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SUMMARY

The objective of this study was to evaluate the effect of different freezing rates and thawing temperatures on the quality of camel spermatozoa post thawing. Ten ejaculates from five male camels were frozen at five different freezing rates, achieved by placing the straws at specific heights above the surface of liquid nitrogen for different times (4 cm for 15 min; 1 cm for 15 min; 7 cm for 15 min; 7 cm for 5 min + 4 cm for 3 min; 4 cm for 5 min + 1 cm for 3 min) followed by storage in liquid nitrogen. Two thawing temperatures (60°C for 10 sec or 37° for 30 sec) were subsequently tested. The sperm samples were evaluated for total and progressive motility, kinematics, membrane and acrosome integrity and membrane functionality zero, one and two hours post thawing. Camel sperm showed a high tolerance for freezing rates with small differences between the different freezing rates. Significantly ($p<0.05$) better values for sperm characteristics were obtained from the fastest freezing rate compared to a slower rate for total and progressive motility and some kinematic parameters, but no treatment was found to be significantly better for all parameters of sperm quality. However, post thaw sperm quality was better with the faster thawing rate compared to the lower rate. No interactions between freezing and thawing rates were found in the study. In conclusion, according to this study, a fast freezing rate may be more beneficial for camel spermatozoa than slower freezing rates, and the thawing should be conducted at 60°C.

SAMMANFATTNING

Syftet med denna studien var att utvärdera om olika frysningsgrader och upptiningstemperaturer kan påverka kvaliteten hos kryokonserverad kamelsperma efter upptining. Tio ejakulat från fem olika hanar frystes i fem olika hastigheter, hastigheterna bestämdes av avståndet från provet till ytan av flytande kväve (4 cm i 15 min; 1 cm i 15 min; 7 cm i 15 min; 7 cm i 5 min + 4 cm i 3 min; 4 cm i 5 min + 1 cm i 3 min) och kryokonserverades sedan. Två olika upptiningstemperaturer (60°C i 10 sekunder eller 37°C i 30 sekunder) utvärderades sedan. Spermierna evaluerades med avseende på total och progressiv motilitet, kinematiska parametrar, membran- och akrosomintegritet och membranfunktionalitet noll, en och två timmar efter upptining. Kamelsperma visade hög tolerans för olika frysninghastigheter, då det var små skillnader i resultat mellan de olika behandlingarna. Signifikant ($p<0,05$) bättre värden för spermieegenskaper erhölls från den snabbaste frysningshastigheten i jämförelse med en lägre hastighet med avseende på total och progressiv motilitet samt några kinematiska parametrar, men ingen frysningshastighet var signifikant bättre än alla andra hastigheter för någon parameter. Gällande upptiningstemperaturer visade snabb upptining (60°C i 10 sekunder) bättre resultat jämfört med lägre temperatur (37°C i 30 sekunder). Inga interaktioner mellan frys- och upptiningshastigheter påträffades i studien. Sammanfattningsvis, enligt denna studie, kan en snabb nedfrysningshastighet vara fördelaktig för kamelsperma, och upptining av kamelsperma bör utföras vid 60 °C.

CONTENT

INTRODUCTION

The reproductive efficiency of camels in their natural environment is low due to a short breeding season, late age of reaching puberty and a long gestational period. Therefore the use of artificial insemination (AI) and embryo transfer (ET) are becoming more and more important in order to increase their breeding potential. The dromedary camel is significant both as a livestock species and for sporting purposes. In the Middle-East camel racing is an enormous industry; individuals can be very valuable (Spencer et al., 2010). This increases the interest in increasing the number of offspring from desirable male and female genetic combinations.

Assisted reproduction methods such as AI and ET are widely used nowadays in several species. They increase the breeding productivity of a particular male or female and the overall progress rate of genetic improvement (Allen, 2000). Their use in the camel would be to maximize the spread of genetic superior males for racing, and the ability to select the best possible individuals for breeding (Deen et al., 2003).

However, progress in AI and semen preservation has been slow in the camel compared to other livestock species (Bravo et al., 2000). Apart from the difficulties associated with camel reproduction mentioned above, there are also complications associated with handling camel semen, because of the gelatinous nature of the semen and problems with long-term storage of the semen (Skidmore, 2003).

Cryopreservation of semen in any species offers the ability to store semen long term and has some benefits compared to fresh semen for AI. Frozen semen can be transported over long distances without deterioration, there is no contact between animals during mating, the supply of semen is guaranteed when the female is ready for insemination and it allows the semen to be stored until the male has been shown to be free of disease at the time of semen collection. Cryopreservation of semen allows genetic material to be stored for the future and used even after the death of an animal. Semen can be shipped over long distances and so provide access to a greater gene population. It also reduces the transmission of venereal diseases. Camelid sperm are generally considered to be not very tolerant to freezing and thawing procedures (Morton et al., 2010). The freezing methods used for camel semen are modified protocols from other species such as the boar. The post-thaw motility of the semen is reported to be poor, and pregnancy rates obtained with frozen-thawed semen are also low (Bravo et al., 2000; Crichton et al., 2015).

The aim of this study was to evaluate if different freezing rates and thawing temperatures could affect the quality of camel spermatozoa post-thawing and if there were any interactions between the two factors.

LITERATURE REVIEW

The camelus dromedarius

The camelidae family consists of two genera, *Camelus* and *Lama.* Within the genus *Camelus* there is the dromedary camel *(Camelus dromedaries)* with one hump, and the Bactrian camel *(Camelus bactrianus)* with two humps. Both species are also known as Old World camelids (Bravo et al., 2000).

Reproductive anatomy and physiology of the camel

Camels are seasonal breeders with a short breeding season during the cooler months. The testes of the male Camelidae are ovoid in shape and located in the scrotum in a perineal position, as in the pig and dog. The testes are descended at birth, very small in size, but increase in size during puberty, reaching their maximum weight at 10-14 years of age. There are also seasonal changes in the size of the testes, as they become enlarged when the male is sexually active in the breeding season. The penis is fibroelastic with a sigmoid flexure. The apex of the glans penis is a cartilaginous process, which directs the penis through the cervix of the female. The accessory sex glands consists of the prostate and the bulbourethral glands, as camelids do not have vesicular glands (Bravo et al., 2000).

Female camels have a bicornuate uterus with short uterine horns. They are induced ovulators and normally only ovulate when they are mated. When the dominant follicle measures between 1.3 – 1.7 cm in diameter, the camel is ready for mating. The gestation period of a camel is 13 months (Skidmore, 2011). To increase the reproductive productivity of a specific female camel, superovulation can be used. The female camel is then inseminated with fresh semen collected from a male and the embryos are transferred to recipients.

Semen collection

The collection of semen from male camels is performed with a female camel in recumbent position and the male squatting behind (figure 1). An artificial vagina (AV) with a semen vessel attached to the apex is used for the collection. The male camel is first teased with a sexually receptive female so that the olfactory contact will lead to arousal. He is then led up to the sitting female camel and allowed to make a few thrusts before the operator directs the penis into the

AV. The ejaculates usually occurs in fractions and the copulation process is generally between 5 and 10 minutes, but can occasionally last for 20 minutes or longer (Bravo et al., 2000).

Fig. 1. *Collection of semen from a male camel using an AV.*

Semen characteristics

One of the main characteristics of camel semen is its high viscosity. The spermatozoa are entrapped in a viscous seminal plasma that makes handling and estimation of sperm parameters difficult. The gel consists of mucopolysaccharides from secretions from the bulbourethral gland or the prostate, although the degree of viscosity depends on the individual male (Skidmore et al., 2013). Liquefaction of the semen can be achieved by gently pipetting the semen to break down the gel, or by the use of enzymes.

Seminal plasma

Seminal plasma is beneficial to sperm function but detrimental to sperm survival. Seminal plasma contains many factors which helps to retain sperm function (Pérez-Pé et al., 2001), but also other substances that adversely affect sperm motility, and reactive oxygen species (ROS) that can be detrimental to long-term sperm survival (Hammadeh et al., 2008). In vivo, spermatozoa rapidly migrate away from the seminal plasma to reach the site of fertilization. Some parts of the female reproductive tract act as a barrier to the passage of spermatozoa, to create a filtering system so that only viable, motile spermatozoa can proceed through (Suarez, 2007). In in vitro fertilization (IVF) and AI however, this process of natural selection does not occur. Therefore, there is need to remove the seminal plasma and also to select the good quality spermatozoa.

Single layer centrifugation

To remove the seminal plasma from the spermatozoa, the technique of single layer centrifugation (SLC) technique can be used. This technique mimics the selection of good quality spermatozoa in the female reproductive tract by centrifugation through a layer of colloid. The SLC method effectively separates the spermatozoa from the seminal plasma and also selects the spermatozoa with good quality, e.g. motile, viable and with intact DNA (Morrell and Rodriguez-Martinez, 2009).

Artificial insemination

In natural mating in the camel, semen deposition is partly intra-uterine and partly intra-cervical. In AI, the semen is deposited post-cervically. Better pregnancy results may be obtained if semen is deposited at the tip of the uterine horn (deep intrauterine) rather than in the uterine body (Skidmore et al., 2013). A bovine insemination catheter, manually guided transrectally, is used to distribute the semen post cervical, as deep in the uterus as the catheter allows.

In terms of successfully establishing and maintaining pregnancies in camels, AI with fresh semen containing 300 x 10^6 motile spermatozoa has given good results.

Cryopreservation

Cryopreservation is the preservation of biological tissues and cells at cryogenic temperatures (-196°C). At this temperature, cell metabolism is stopped, thus and allowing long-term preservation of the cells (Yeste, 2016). Cryopreservation of semen enables breeders to preserve genetic material for the future, but also facilitates the transport of semen and therefore provides access to a greater population of animals.

Principles of cryoinjury during freezing and thawing

The main problem with freezing and thawing procedures is cryoinjury that occurs at low temperatures, mainly caused by the phase change of intra- and extracellular water at temperature decrease. The most lethal stage during freezing and thawing appears between -15°C and -60°C. At -5°C cells and extracellular medium remains unfrozen, and supercooled. When the temperature decreases from -5°C to -15°C, ice crystals are form in the surrounding medium, but the intracellular contents remain unfrozen and supercooled. Since the chemical potential of water is higher inside the cell (supercooled) than in the extracellular media (frozen), water will flow out of the cell and freeze (Gao and Critser, 2000).

If the cooling rate is too high, the intracellular water does not have time to flow out completely, and when the cell freezes, ice crystals form in the cytoplasm, which causes cryoinjury to the cell (Muldrew and McGann, 1994). If the cooling rate is very slow, the water flows out of the cell, solutes inside the cell are concentrated and supercooling is eliminated. Instead of freezing intracellularly, the cell is dehydrated and the organelles and membrane shrink before they reach the temperature when all components are frozen and solidified. This affects lipid-protein complexes, induces irreversible membrane fusion (Mazur et al., 1972) and can change the

electrolyte balance which can cause the cell to swell beyond its normal isotonic volume and because of that lyse after thawing (Muldrew and McGann, 1994).

For each specific cell type there is an optimal cooling rate that is low enough to avoid intracellular ice formation, but high enough to diminish cryoinjury caused by electrolytes and solute concentrations. Cryoinjury to the spermatozoa caused by freeze-thawing procedures is seen as, for example, reduction in sperm motility, affected acrosome integrity and the fluidity and permeability of the plasma membrane. (Yeste, 2016).

The freezing protocols used for camel semen are modified protocols from other species such as the boar. The post-thaw motility of the camel semen is reported to be poor (Deen et al., 2003), and pregnancy rates obtained with frozen-thawed semen have been low, if any (Bravo et al., 2000).

Cryoprotectants

As previously stated, an optimal freezing rate is used to avoid ice formation inside the cell; however, the formation of the crystals cannot be completely prevented by using only the optimal rate. For this reason, cryoprotectant agents (CPA) are also used when spermatozoa are cryopreserved to diminish stress for the cell. The CPAs may also be toxic for sperm; therefore it is important to use the most suitable concentrations (Okazaki et al., 2009). CPAs are classified in two groups, depending on whether they permeate the cell (permeating CPAs), or not (nonpermeating CPAs) (Yeste, 2016). Egg yolk, sugars and other higher molecular weight compounds are examples of CPAs that perform their effect extracellularly; they are nonpermeating CPAs. They prevent ice formation, as well as stabilizing proteins and cell membranes (Benson et al., 2012). Glycerol and dimethyl sulfoxide are the most commonly used permeating CPAs, and they are able to permeate the cell membrane. These solutes decrease the concentration of electrolytes and the osmotic shrinkage at low temperatures (Gao and Critser, 2000).

Specific objectives

The objective of this study was to evaluate different freezing rates and thawing temperatures for camel spermatozoa, and to determine if there were any interactions between the two factors, that could affect the quality of cryopreserved camel semen.

MATERIALS AND METHODS

Animals and semen collection

Five sexually mature, healthy dromedary camel males from the Camel Reproduction Centre (Dubai, UAE) were used in this study. All the males participating in the study were proven fertile from previous trials. Semen (2 ejaculates/animal) was collected in April using an artificial vagina. Semen samples were immediately transported to the lab and placed in a 37°C water bath. All animal procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Camel Reproduction Centre, UAE.

Media

The medium used initially for semen extension was Tris-Citrate-Fructose Buffer (TCF), containing tris (hydroxymethyl) aminomethane (300 mM), citric acid (94.7 mM), fructose (27.8 mM) at pH 6.9 (Evans and Maxwell, 1987). In addition, bovine serum albumin (0.05%), ethylene diaminetetraacetic acid (10 mM) and 4% egg yolk were added and the solution was filtered using a 0.22 µm filter.

The cryopreservation medium was added in two steps. Fraction A, Green Buffer (GB; IMV Technologies, l'Aigle, France) with 20% egg yolk was added first (Fraction A; FA), followed by Fraction B (FB), which was FA with 6% glycerol added.

Sperm cryopreservation

Extended semen (1:5 v/v in TCF) was gently pipetted to break down the gel at 37°C. The semen was then subjected to SLC as follows: 2 ml of sample were layered on the top of 2 ml of colloid in a 15 ml tube and centrifuged at 300 g for 20 min. Supernatant (semen extender, seminal plasma and colloids) was discarded by aspiration. The resulting sperm pellet was re-suspended in fraction A and examined for motility and concentration to evaluate the recovery, then diluted to a concentration of $150x10^6$ spermatozoa/ml. After cooling to 5°C in a water jacket for 2 h, the samples were further diluted with fraction B (1:1) to achieve a sperm concentration of 100 million spermatozoa/ml and a final glycerol concentration of 3%. The sperm samples were then equilibrated for 0.5 h before loading in 0.5 ml straws and freezing at the 5 different freezing rates shown in Table 1. The temperature decrease was calculated manually every minute by a thermocouple in all the treatments. Finally the straws were plunged into liquid nitrogen for storage. Two straws per ejaculate and per treatment were frozen. An overview of the process in whole is presented in figure 4.

Table 1. *Freezing rates and velocity*

	Freezing rates	
Treatment	Time (minutes) and distance (centimetres) above liquid nitrogen surface	Freezing velocity, °C/min
Treatment 1 (control)	4 cm 15 min	10.58 °C/min
Treatment 2	1 cm 15 min	45.85 °C/min
Treatment 3	7 cm 15 min	7.42 °C/min
Treatment 4	7 cm 5 min + 4 cm 3 min	16.66 + 5.08 °C/min
Treatment 5	4 cm 5 min + 1 cm 3 min	22.98 + 8.24 °C/min

Thawing of samples

From each ejaculate one sample from each treatment (T1-5) were thawed at 37ºC for 30 seconds, and one sample from each treatment at 60°C for 10 seconds. They were assessed for total and progressive motility, kinematic parameters, membrane integrity and functionality, and acrosome integrity 0 and 1 hour after thawing. Total and progressive motility and kinematics were also measured after 2 hours.

Sperm assessment

Motility was measured by means of a computer-assisted semen analysis (CASA) system (CEROS II®; Hamilton Thorne; MA; USA) attached to a Zeiss microscope with a x10 objective and eyepiece, and a warm stage set at 37 °C. For each evaluation, a 3 µL aliquot of the sperm sample was placed in a disposable capillary counting chamber (MicroToolTM, Cytonix, USA) which provides a chamber depth of 20 µm; five fields were analysed (approximately 300 sperm in total) at a frame rate of 30/s. Particles of size 13-101 µm were considered to be spermatozoa. Total and progressive motility and kinematic parameters were recorded. The kinematic parameters were: average path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR) and linearity (LIN). The kinematics were classified as follows: progressive STR 70%, progressive VAP 40 µm/s, slow VAP 20 µm/s, slow VSL 30 µm/s, static VAP 4 µm/s, static VSL 1 µm/s. The CASA settings are shown in appendix 1.

Membrane and acrosome integrity were evaluated with eosin-nigrosin stain (EN). A semen sample was diluted 1:1 with stain solution (5% eosin, 10% nigrosin in 0.1 M citrate solution), smeared on a glass slide and dried on a warm plate (Malo et al., 2016). A total of 100 spermatozoa were assessed per sample and the percentage of intact acrosomes was calculated, using the presence of an intact apical ridge as the criteria for an acrosome-intact spermatozoon.

The percentage of intact plasma membrane was calculated using the colour of the sperm as criteria for an intact plasma membrane. If the spermatozoa did not take up the stain they were considered to have an intact membrane, whereas a purple coloration indicated that the membrane was not intact (figure 2).

Sperm membrane functionality was assessed by the hypo-osmotic swelling test (HOST). 100 µL hypo-osmotic solution (100 mM sucrose) was mixed with 30 µL of semen and incubated at 37˚C for 30 min (Nie and Wenzel, 2001). A total of 100 spermatozoa were assessed per sample and the percentage of functional membranes was calculated, using their swelling characterized by coiled tails indicating an intact plasma membrane. Only tails coiled from the tip (figure 3) was counted as positive, folded tails where the tip was not visible was not counted.

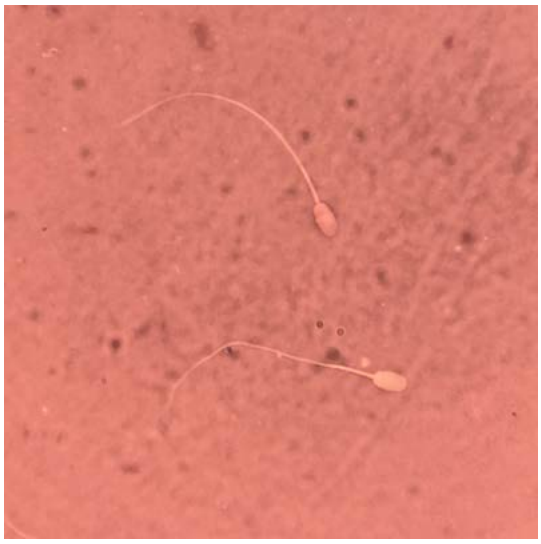

Fig. 2. *Eosin-nigrosin staining.*

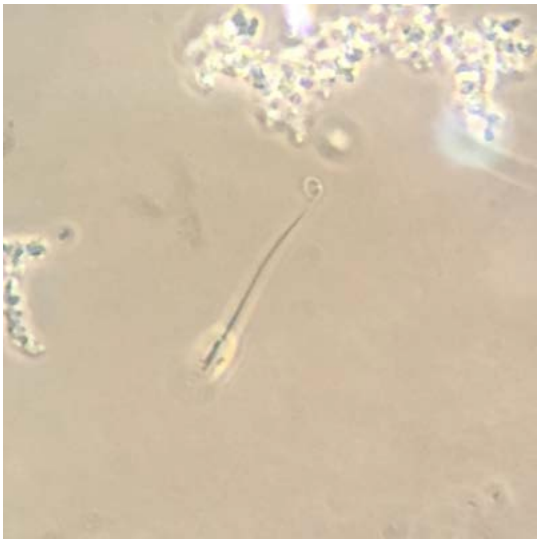

Fig. 3. *Hypo-osmotic swelling test.*

Statistical analyses

Effect of freezing and thawing rates were analysed by generalized linear model (GLM), with means compared by Duncan´s multiple range method setting male as a random effect. Interactions were also calculated with this test. Values were expressed as means ± SEM. All analyses were performed using SPSS 11.0 for Windows. The level of significance was set at $p < 0.05$.

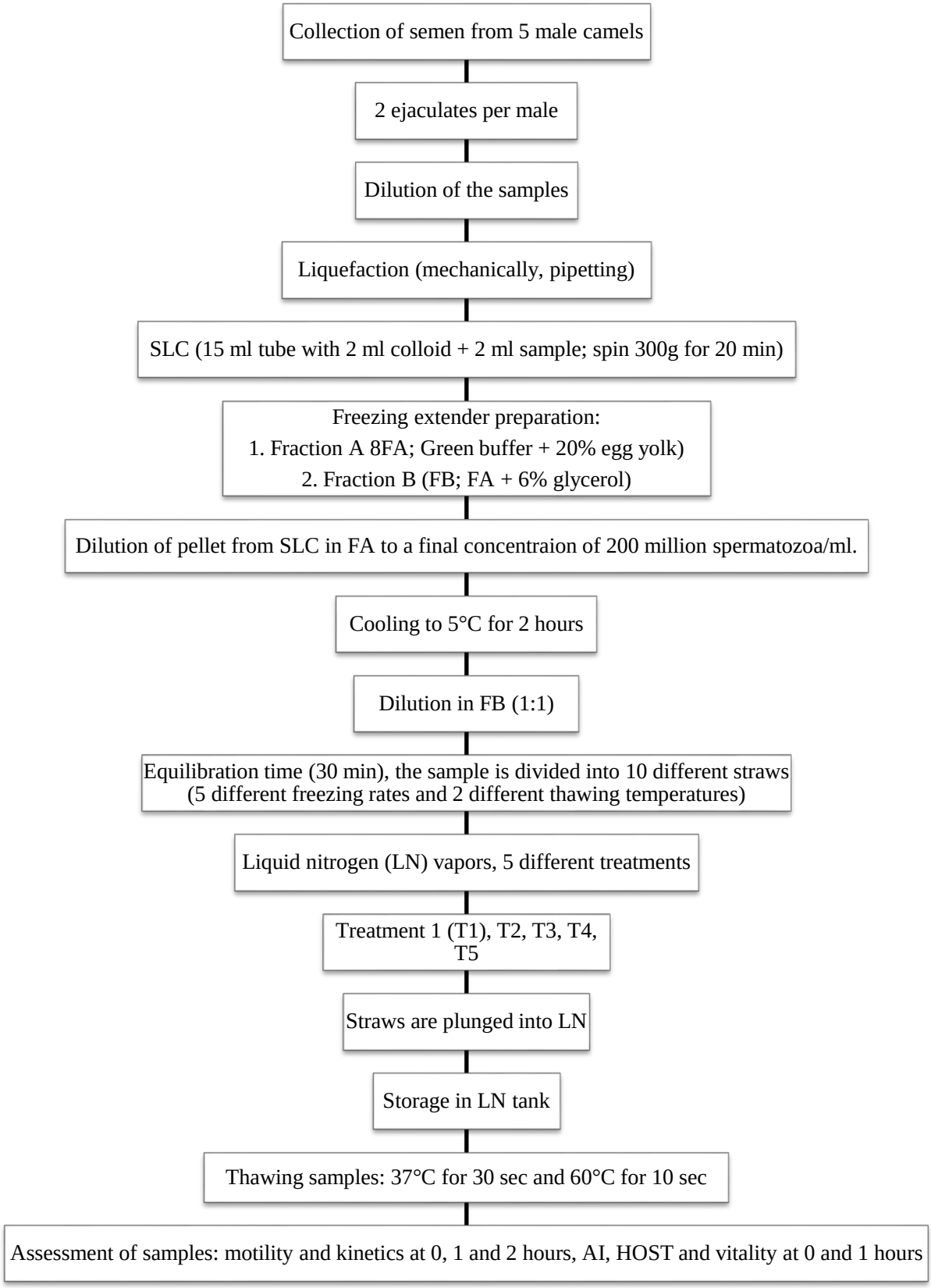

Fig. 4. *Flow chart of the process.*

RESULTS

The of pre-freezing values, freezing rates T1-T5 (0 hours, 1 hour, 2 hours), thawing temperatures 37°C and 60°C (0 hours, 1 hour, 2 hours) and interactions (0 hours, 1 hour, 2 hours) are shown in the following tables. A time sequence for TM and PM is shown in Figure 5 and 6, respectively, and changes in HOST and EN over time are given in Figure 7.

Table 2. *Fresh semen total motility (TM) and progressive motility (PM) of the ejaculates (n=10) from each camel, as well as the post-thaw quality from the control (T1) thawed at 37°C*

Camel	Ejaculate	Fresh semen (% of sperm)		Frozen thawed semen (% of sperm) at 0 h	
		TM	PM	TM	PM
A	1	70.4	44.4	14.4	4.6
	2	60	30	40.9	19.4
B	1	85.4	60	47.4	21.9
	2	81.4	34.9	32.9	12.8
C	1	85.5	63.3	40.6	18.8
	2	80.5	45.5	19.3	7.1
D	1	76.3	41.9	28.8	5.8
	2	86.2	47.3	47.3	18.8
E	1	85.1	50.5	26.2	11.2
	2	80.6	53	36.3	12.4

The variability among individuals and the irregularity in the quality of the ejaculates from the same male, pre-freezing and post-thawing is shown above (table 2).

Values for pre-freezing kinematics are shown in Table 3; post-freezing mean parameters of sperm quality are given in Table 4, 5, 6, 7, 8 and 9. Significant values ($p<0.05$) are marked with a/b.

Table 3. *Mean (±SE) pre-freezing values kinematics (n=10)*

Parameter	Value

TM	79.14 ± 2.64
PM	47.08 ± 3.25
ALH	8.53 ± 0.36
BCF	18.81 ± 0.52
LIN	38.61 ± 0.87
STR	73.42 ± 1.14
VAP	93.13 ± 3.36
VCL	179.03 ± 7.54
VSL	68.27 ± 1.79

Freezing rates

Table 4. *Oh post-thaw sperm quality parameters for different treatments (freezing rates). Values shown are overall means (±SE) for two thawing temperatures (n=10)*

	Values					
Parameter	T1 (control)	T2	T3	T4	T5	P-value
TM	34.99 ± 2.67^{ab}	41.2 ± 2.72^{a}	34.49 ± 2.16^{ab}	30.58 ± 2.01^{b}	33.92 ± 2.5^{ab}	<0.001
PM	14.36 ± 1.53^{b}	18.56 ± 1.69^{a}	15.77 ± 1.25^{ab}	13.9 ± 1.27^{b}	14.41 ± 1.47^{b}	0.006
ALH	7.18 ± 0.15	7.19 ± 0.15	7.16 ± 0.15	6.99 ± 0.14	6.98 ± 0.16	0.066
BCF	18.86 ± 0.33	19.19 ± 0.33	19.29 ± 0.29	19.31 ± 0.3	18.45 ± 0.3	0.355
LIN	34.15 ± 0.5^{b}	35.45 ± 0.41^{a}	35.52 ± 0.47^{a}	34.63 ± 0.41^{ab}	35.01 ± 0.38^{ab}	0.004
STR	67.5 ± 0.68^{b}	69.23 ± 0.77^{a}	69.37 ± 0.59^{a}	69.4 ± 0.58^{a}	68.92 ± 0.63^{ab}	0.008
VAP	63.57 ± 2.18^{ab}	65.6 ± 1.83^{a}	64.04 ± 1.93^{ab}	60.75 ± 1.72^{b}	62.44 ± 2^{ab}	0.006

VCL	129.02 ± 4.33[ab]	131.84 ± 4.03[a]	128.69 ± 3.86[ab]	125.17 ± 3.47[b]	125.78 ± 4.18[ab]	0.002
VSL	43.19 ± 1.69[ab]	45.76 ± 1.64[a]	44.87 ± 1.52[ab]	42.51 ± 1.37[b]	43.22 ± 1.54[ab]	0.009
EN	74.65 ± 1.93	72.15 ± 1.57	76.8 ± 1.51	74.9 ± 2.13	76.1 ± 1.83	0.222
AI	34.65 ± 2.91	37.0 ± 2.89	33.5 ± 3.09	34.05 ± 3.71	34.9 ± 3.34	0.627
HOST	18.7 ± 2.74[a]	15.85 ± 2.96[ab]	15 ± 1.99[b]	14.3 ± 2.5[b]	14.4 ± 2.14[b]	0.036

After 0 h, TM, VAP, VCL and VSL were significantly higher for the T2 freezing rate compared to T4, but there was no significant difference between T2 and the other treatments (T1, 3, 5) regarding these parameters.

Values for PM were significantly higher for T2 than T1, T4, T5, but no significant difference between T2 and T3 was found.

Values for HOST was significantly higher for T1 than T3, T4, T5, but no significant difference between T1 and T2 was found.

Table 5. *1h post-thaw sperm quality parameters for different treatments (freezing rates). Values shown are overall means (±SE) for two thawing temperatures (n=10)*

	Values					
Parameter	T1 (control)	T2	T3	T4	T5	P-value
TM	25.56 ± 3.67[ab]	26.06 ± 3.38[a]	21.65 ± 2.49[ab]	20.6 ± 2.58[b]	22.79 ± 2.99[ab]	0.042
PM	10.34 ± 1.92[ab]	10.95 ± 1.95[a]	7.98 ± 1.23[ab]	7.52 ± 1.22[b]	8.93 ± 1.68[ab]	0.026
ALH	6.84 ± 0.19	6.66 ± 0.26	6.62 ± 0.2	6.48 ± 0.23	6.75 ± 0.17	0.558
BCF	20.04 ± 1.26	21.67 ± 1.31	20.79 ± 0.76	22.43 ± 3.23	21.62 ± 1.29	0.772
LIN	33.26 ± 0.64	33.99 ± 0.76	32.76 ± 0.75	32.72 ± 0.81	31.65 ± 0.86	0.361

STR	66.95 ± 1.03	67.01 ± 0.8	66.75 ± 0.88	65.87 ± 1.36	65.25 ± 1.56	0.598
VAP	56.08 ± 2.53[a]	56.63 ± 2.52[a]	53.24 ± 2.42[ab]	52.2 ± 2.55[b]	54.26 ± 1.9[ab]	0.025
VCL	117.44 ± 5.18	116.4 ± 5.83	112.55 ± 4.72	108.68 ± 5.39	115.34 ± 4.21	0.124
VSL	37.74 ± 2.18[ab]	38.59 ± 2.04[a]	35.93 ± 1.87[ab]	34.69 ± 2.12[b]	35.71 ± 1.89[ab]	0.007
EN	73.89 ± 2.44	71.72 ± 2.35	79.39 ± 1.38	77 ± 1.36	75.61 ± 1.58	0.07
AI	23.61 ± 2.14	22 ± 2.34	26.33 ± 2.5	24.44 ± 2.13	23.06 ± 1.8	0.226
HOST	11.94 ± 1.87	12.22 ± 1.92	11.56 ± 1.71	9.94 ± 1.62	10.11 ± 1.55	0.402

After 1 h, values for TM, PM and VSL was significantly higher for the T2 freezing rate compared to T4, but there was no significant difference between T2 and the other treatments (T1, 3, 5) regarding these parameters.

Table 6. *2h post-thaw sperm quality parameters for different treatments (freezing rates). Values shown are overall means (±SE) for two thawing temperatures (n=10)*

	Values					
Parameter	T1	T2	T3	T4	T5	P-value
TM	11.18 ± 2.44	10.24 ± 2.24	9.18 ± 1.95	8.19 ± 1.86	8.53 ± 1.94	0.141
PM	3.53 ± 1.11[a]	2.52 ± 0.74[ab]	2.3 ± 0.78[b]	2.49 ± 0.66[ab]	2.61 ± 0.79[ab]	0.008
ALH	6.21 ± 0.61	5.84 ± 0.52	6.11 ±0.56	6.92 ± 0.57	6.64 ± 0.49	0.622
BCF	21.28 ± 2	23.85 ± 4.25	22.21 ± 2.32	23.15 ± 2.16	25.2 ± 2.74	0.891
LIN	33.16 ± 3.14	31.22 ± 3.06	28.77 ± 2.92	33.09 ± 2.72	27.95 ± 2.73	0.507
STR	63.2 ± 4.09	61.17 ± 5.2	56.89 ± 4.93	62.26 ± 4.69	56.31 ± 5.22	0.733

VAP	44.37 ± 3.71	42.63 ± 3.85	43.91 ± 3.75	49.07 ± 3.49	51.66 ± 4.34	0.414
VCL	95.99 ± 9.67	91.32 ± 8.22	90.5 ± 8.29	102.15 ± 6.97	110.21 ± 8.84	0.447
VSL	28.7 ± 2.38	29.07 ± 2.63	26.66 ± 2.66	33.94 ± 3.33	30.71 ± 3.68	0.665

After 2 h, T1 was significantly better than T3 for PM, but no significant difference between T1 and the other treatments (T2, 4, 5) was found. The overall sperm quality after 2 hours was low with fewer differences between treatments than at the other time points.

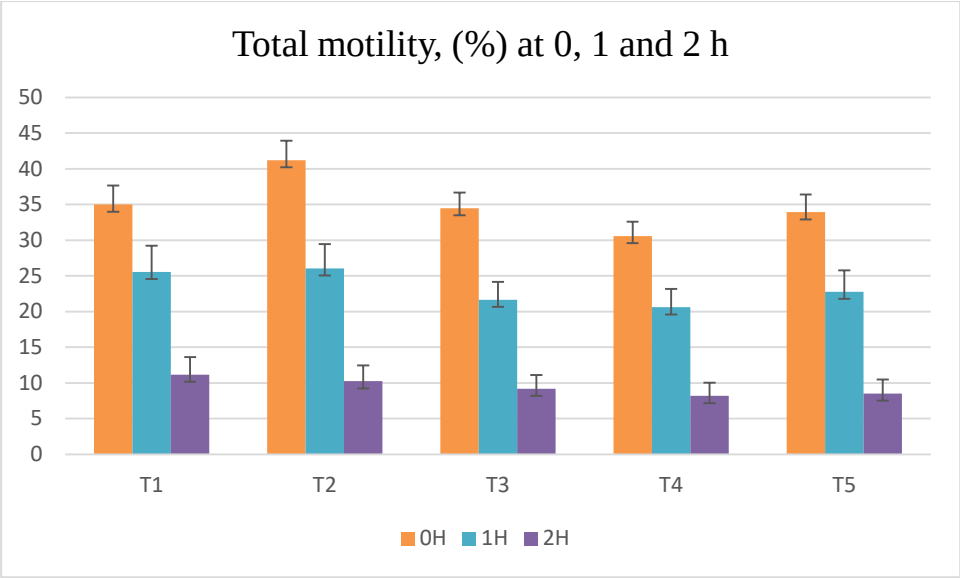

Fig 5. *Bar chart showing the total motility for the five treatments at three time points.*

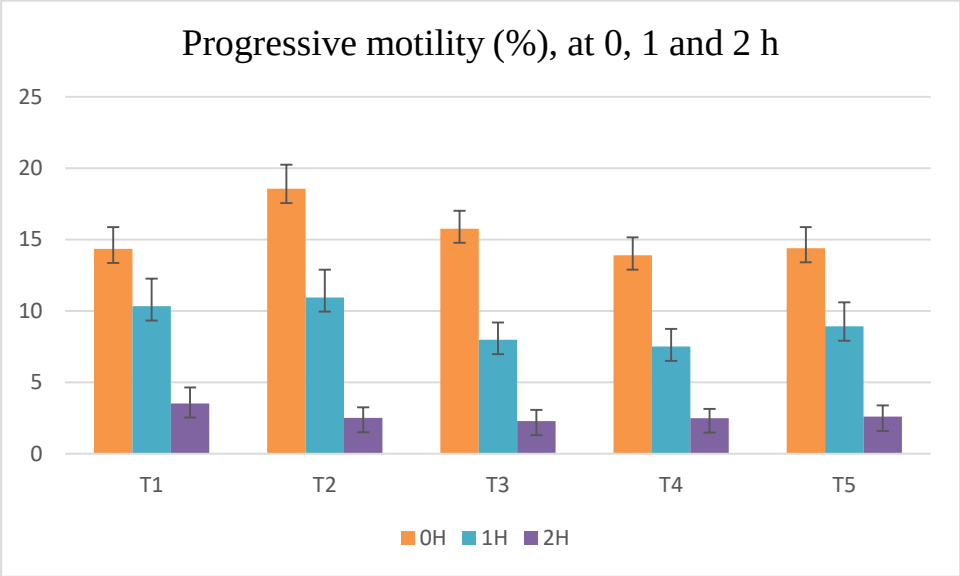

Fig 6. *Bar chart showing progressive motility for the five treatments at three time points.*

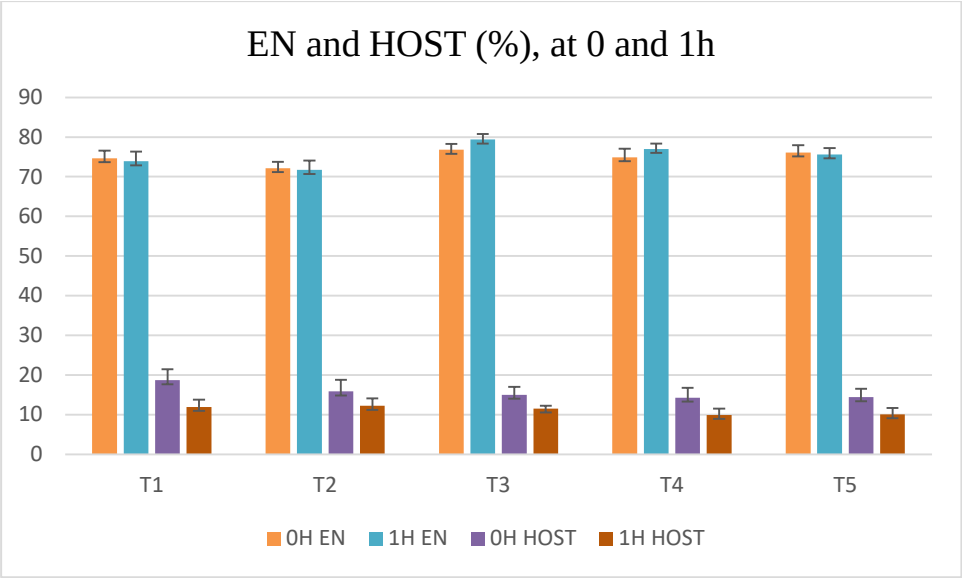

Fig 7. *Bar chart showing values of EN and HOST for the five treatments at two time points.*

Thawing temperatures

The results for EN and HOST for the different thawing temperatures are shown in table 7 and 8, kinematics is shown in table 7, 8 and 9.

Table 7. *Oh post-thaw sperm quality parameters for different thawing temperatures. Values shown are overall means (±SE) for five different freezing rates (n=25)*

	Values		
Parameter	37°C	60°C	P-value
TM	34.23 ± 1.45	36.02 ± 1.75	0.049
PM	14.63 ± 0.81	16.34 ± 1.07	0.059
ALH	7.03 ± 0.08	7.18 ± 0.11	<0.001
BCF	19.03 ± 0.19	19.01 ± 0.21	0.814
LIN	35.1 ± 0.27	34.78 ± 0.29	0.859
STR	68.36 ± 0.34	69.53 ± 0.5	0.117
VAP	62.85 ± 1.11	63.8 ± 1.37	0.192
VCL	126.07 ± 2.17	130.58 ± 2.86	0.021
VSL	43.4 ± 0.9	44.53 ± 1.08	0.157

EN	76.06 ± 0.98	73.53 ± 1.32	0.423
AI	36.67 ± 1.95	32.62 ± 1.99	0.762
HOST	18.04 ± 1.76	12.73 ± 1.04	0.384

At 0h, TM, ALH and VCL were significantly higher for 60°C thawing temperature than 37°C.

Table 8. *1h post-thaw sperm quality parameters for different thawing temperatures. Values shown are overall means (±SE) for five different freezing rates (n=25)*

	Values		
Parameter	37°C	60°C	P-value
TM	21.76 ± 1.7	25.25 ± 2.17	0.075
PM	7.92 ± 0.77	10.64 ± 1.29	0.018
ALH	6.67 ± 0.11	6.68 ± 0.16	0.942
BCF	22.11 ± 1.39	20.32 ± 0.42	0.391
LIN	33.33 ± 0.47	32.31 ± 0.5	0.387
STR	65.79 ± 0.66	67.07 ± 0.8	0.012
VAP	54.15 ± 1.31	54.89 ± 1.76	0.701
VCL	111.9 ± 2.77	116.76 ± 3.7	0.376
VSL	35.98 ± 1.06	37.21 ± 1.52	0.364
EN	75.02 ± 1.13	76.15 ± 1.36	0.310
AI	22.34 ± 1.29	25.83 ± 1.43	0.04
HOST	12.22 ± 1.2	9.83 ± 0.82	0.541

At 1h, PM, STR and AI were significantly higher for 60°C thawing temperature than 37°C.

Table 9. *2h post-thaw sperm quality parameters for different thawing temperatures. Values shown are overall means (±SE) for five different freezing rates (n=25)*

	Values		
Parameter	37°C	60°C	P-value
TM	6.79 ± 0.78	12.99 ± 1.75	0.103
PM	1.74 ± 0.25	3.82 ± 0.71	0.211
ALH	6.8 ± 0.3	5.79 ± 0.39	0.364
BCF	24.29 ± 1.6	21.91 ± 1.95	0.403
LIN	32.32 ± 1.8	29.03 ± 1.85	0.072
STR	60.54 ± 2.62	59.27 ± 3.55	0.853
VAP	49.75 ± 2.07	42.14 ± 2.75	0.207
VCL	104.07 ± 4.74	90.65 ± 5.93	0.401
VSL	31.74 ± 1.84	27.46 ± 1.87	0.218

At 2 h there were no significant differences between the two thawing temperatures.

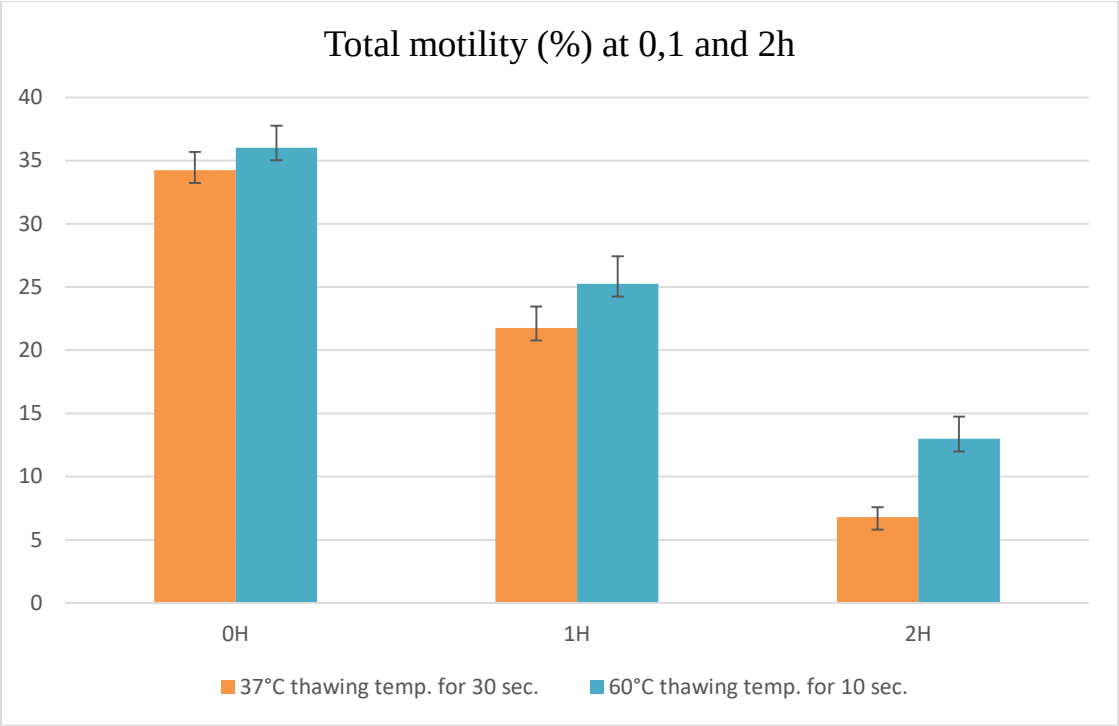

Fig 8. *Bar chart showing total motility for the two thawing temperatures at three time points.*

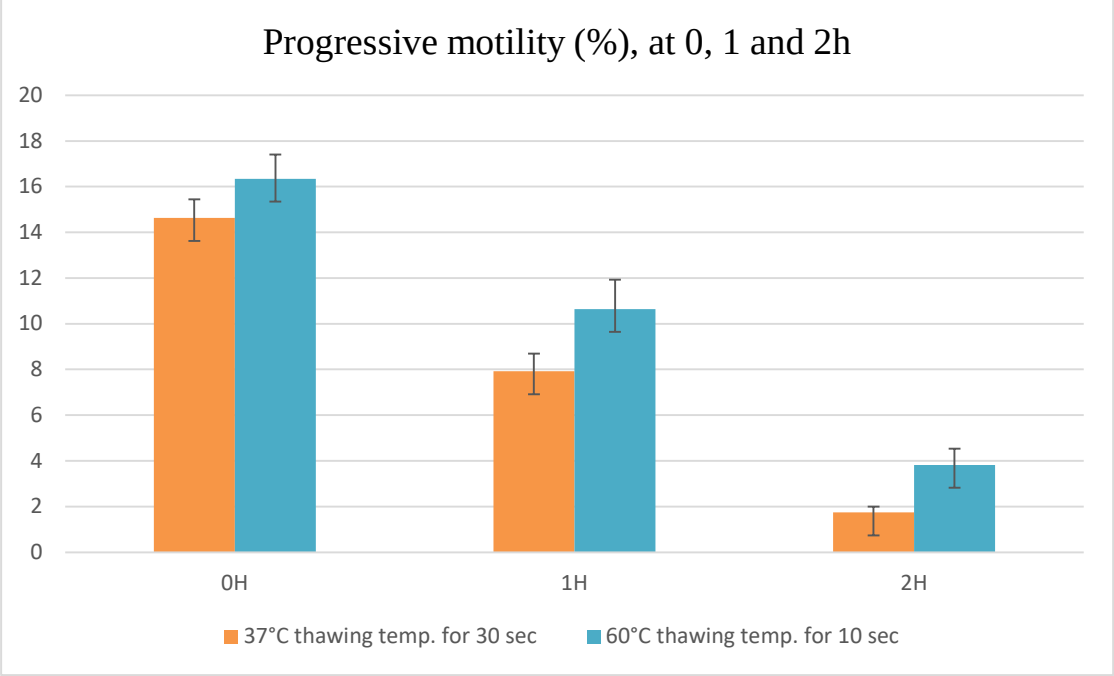

Fig 9. *Bar chart showing progressive motility for the two thawing temperatures at three time points.*

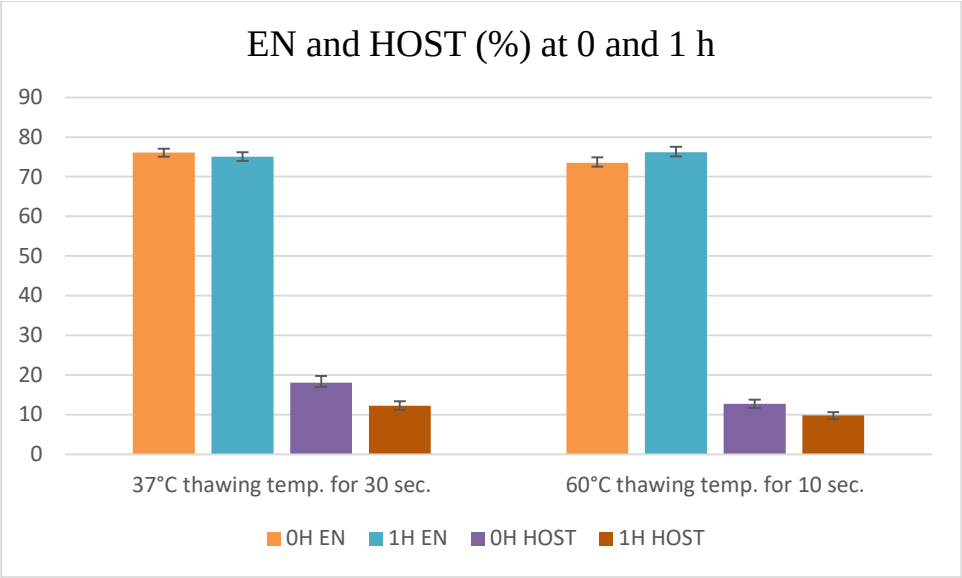

Fig 10. *Bar chart showing EN and HOST values for the two thawing temperatures at two time points.*

Interactions between freezing rates and thawing temperatures

Table 10. *Interactions between freezing rates (T1-5) and thawing temperatures (37°C, 60°C) for 0, 1 and 2h time points*

Time	Parameters											
	TM	PM	ALH	BCF	LIN	STR	VAP	VCL	VSL	EN	AI	HOST
0H	0.994	0.99	0.962	0.835	0.816	0.864	0.986	0.990	0.958	0.741	0.992	0.998
1H	0.999	0.994	0.633	0.754	0.198	0.455	0.997	0.967	1	0.815	0.946	0.998
2H	0.983	0.873	0.521	0.869	0.922	0.999	0.776	0.726	0.885	-	-	-

No interactions between thawing temperatures and freezing rates were found for any parameters; thus the freezing rate and thawing temperature do not influence each other.

DISCUSSION

Cryopreservation of camel semen would be useful in the breeding strategy for this species. However, as stated before attempts to cryopreserve camel semen are complicated for a number of reasons and the post thaw survival of the spermatozoa is low. The protocols used for cryopreservation of camel semen are modified protocols from other species; therefore the aim of this study was to see whether other freezing rates and thawing temperatures would be more optimal for camel spermatozoa. The different freezing rates in this study were achieved by altering the height of the straw containing semen above the surface of the liquid nitrogen. The distance determined the freezing velocity during the freezing phase when the liquid turns into a solid state. Each height above the surface of the liquid nitrogen corresponded to a different freezing velocity.

In this study, camel spermatozoa showed generally a high tolerance for freezing, since the values obtained from the five different treatments showed only small differences for some parameters of sperm quality. Moreover, there was no treatment that was better than all other treatments over all parameters, although some parameters showed significantly better values for some treatments compared to others. A rapid freezing rate could be considered to be beneficial, since the most rapid freezing rate (T2) in this study was associated with the highest total and progressive motility and some of the kinematic parameters, compared to the slower 2-phase freezing (T4), and for some parameters T2 was better than control (T1). However, none of the significantly increased values showed superiority over all other treatments at all time points. Better results from fast freezing rates compared to slower ones correspond to results in similar studies for other species. Madeddu et al., (2016) obtained the greatest sperm motility when using a rapid freezing rates for cryopreserved rooster semen. In another study (Silva et al., 2013) faster freezing rates were observed to be better for semen from collared peccary with regard to sperm motility post thawing.

Fast thawing gave better results compared to lower rates in this study. These results are in correspondence to results from several species, e.g. a study by Eriksson and Rodriguez-Martinez, (2000), who found that faster thawing rates showed better post thawing results with boar spermatozoa compared to slower ones. However, in a study by Muiño et al., (2008), no differences were found between high and low rates for bull semen. Thus it seems that there are considerable differences in “optimal” rates between species.

It was not possible to test the functionality of the samples in this study, which will be the aim of a later study. However a study by Tanghe et al., (2002) showed that single sperm quality tests, such as TM, PM and morphology after thawing, were a reliable basis for predicting in vitro fertilization results for bulls. In contrast, Foote (2003) thought that sperm motility is not sensitive enough as a measure of sperm quality to be used on its own. Similarly, Mocé and Graham (2008), stated that other parameters of sperm quality cannot be used in isolation to predict the fertility of a semen sample; other authors e.g. Holt (2009) suggested different combinations of assays would provide a better indicator of the potential fertility of an ejaculate. Until results are available from fertility trials in camels it is not known which parameter (or

parameters) of sperm quality will be predictive of fertility. By extrapolation from the study by Tanghe et al., (2002) the results in this study; rapid freezing rate and fast thawing rate, should have the best chance of producing functional spermatozoa.

Since dead spermatozoa are not motile, some correlation between viability and motility could be expected. In a study by Bohlooli et al., (2012) on cryopreserved ram sperm, a positive correlation between single sperm quality tests such as membrane integrity (HOST and EN) and progressive motility was found. In our study the values for HOST were low throughout the trial, and there were few similarities between HOST and EN values compared to the values from the CASA. A study on human spermatozoa showed that there was no correlation between HOST and sperm viability in cryopreserved semen, and therefore these assays should not be used to evaluate the viability of cryopreserved sperm (Esteves et al., 1996). The disparity in the results from different assays makes interpretation of our results more difficult. The CASA and the stained samples (EN and HOST) are two different types of assays. The EN and HOST are evaluated manually in a microscope on 100 spermatozoa, whereas the CASA evaluation is computerized and are made on at least 300 spermatozoa, which will increase the accuracy, but the accuracy of the computer values is determined by the settings programmed by humans. Thus, there is always room for human error.

The difference in quality of the semen (table 3) from the males and the ejaculates could also affect the reliability of the results. The number of motile and progressive spermatozoa varied among the males and also between the ejaculates from the same male. Some ejaculates seemed to survive the freezing and thawing procedure better than other, and there was not necessarily a correlation between good pre-freezing quality and good post-thawing quality. Some parameters were consistently and significantly better for the fast freezing rate (T2) and fast thawing temperature (60°C for 10 sec), regardless of the individual and the different sperm qualities. There are other parameters that do not show any significant difference between the treatments and the results from EN and HOST are not always consistent with the results from the CASA. Therefore, the relevance of different sperm assay results for camel spermatozoa should be determined in conjunction with fertility trials to find out what parameters will be predictive of fertility for the camel.

This study included only five males and ten ejaculates in total; a further study could possibly include a larger selection of animals and ejaculates to examine the individual male effect. In addition, the spermatozoa were still in contact with the freezing medium during the evaluations. Glycerol is used as a cryoprotectant in this study, and is toxic to spermatozoa in a temperature dependent manner (Macías García et al., 2012). When inseminated, the spermatozoa swim away from the glycerol and so are not adversely affected by prolonged contact with it. The freezing medium has a high osmolarity, which also adversely affects sperm quality over time. The presence of cryoprotectant during the assessment of the spermatozoa could affect the post-thaw survival in this study. Another way to evaluate the spermatozoa would be to remove the cryoprotectant before assessing sperm quality. The results in this study are sufficiently

interesting to encourage other studies, but ultimately the ability of the thawed spermatozoa to produce pregnancies will be the determining factor in evaluating these techniques.

CONCLUSION

Camel sperm shows a high tolerance for different freezing rates. Rapid freezing protocols (T2; 1 cm above surface) generally showed better sperm quality ($p<0.05$) than 2-phase freezing (T4; 7 cm + 4 cm) for some parameters. However, T2 did not show significantly better values for all parameters compared to any of the other treatments (T1, 3, 4, 5). Moreover, 1 cm height was significantly better than control (4 cm) in terms of PM, LIN and STR at 0H, but again for these parameters there was no significant difference between T2 and the other treatments (T3, 4, 5).

Regarding the thawing rates, post thaw sperm quality is better with a faster thawing rate (60°C for 10 sec) compared to lower rates (37°C for 30 sec).

No interactions between freezing and thawing rates were found in the study. Semen quality decreased after 2 h, not showing any differences among the freezing and thawing rates.

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REFERENCES

Allen, J.S.M.B.W. (2000), "Using modern reproductive technologies such as embryo transfer and artificial insemination to improve the reproductive potential of dromedary camels", *Actes de l'Atelier International Sur Le Chamelon*, Vol. 53 No. 2, pp. 97–100.

Benson, J.D., Woods, E.J., Walters, E.M. and Critser, J.K. (2012), "The cryobiology of spermatozoa", *Theriogenology*, Vol. 78 No. 8, pp. 1682–1699.

Bohlooli, S., Cedden, F., Bozoglu, S., Razzaghzadeh, S. and Pishjang, J. (2012), "Correlation between conventional sperm assay parameters in cryopreserved ram semen", *Ann. Biol. Res*, Vol. 3, pp. 884–889.

Bravo, P.W., Skidmore, J.A. and Zhao, X.X. (2000), "Reproductive aspects and storage of semen in Camelidae", *Animal Reproduction Science*, Vol. 62 No. 1–3, pp. 173–193.

Crichton, E.G., Pukazhenthi, B.S., Billah, M. and Skidmore, J.A. (2015), "Cholesterol addition aids the cryopreservation of dromedary camel (Camelus dromedarius) spermatozoa", *Theriogenology*, Vol. 83 No. 2, pp. 168–174.

Deen, A., Vyas, S. and Sahani, M.. (2003), "Semen collection, cryopreservation and artificial insemination in the dromedary camel", *Animal Reproduction Science*, Vol. 77 No. 3–4, pp. 223–233.

Eriksson, B.M. and Rodriguez-Martinez, H. (2000), "Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws", *Animal Reproduction Science*, Vol. 63 No. 3–4, pp. 205–220.

Esteves, S.C., Sharma, R.K., Thomas, A.J. and Agarwal, A. (1996), "Suitability of the hypo-osmotic swelling test for assessing the viability of cryopreserved sperm", *ResearchGate*, Vol. 66 No. 5, pp. 798–804.

Foote, R.H. (2003), "Fertility estimation: a review of past experience and future prospects", *Animal Reproduction Science*, Vol. 75 No. 1–2, pp. 119–139.

Gao, D. and Critser, J.K. (2000), "Mechanisms of Cryoinjury in Living Cells", *ILAR Journal*, Vol. 41 No. 4, pp. 187–196.

Hammadeh, M.E., Al Hasani, S., Rosenbaum, P., Schmidt, W. and Fischer Hammadeh, C. (2008), "Reactive oxygen species, total antioxidant concentration of seminal plasma and their effect on sperm parameters and outcome of IVF/ICSI patients", *Archives of Gynecology and Obstetrics*, Vol. 277 No. 6, pp. 515–526.

Holt, W.V. (2009), "Is semen analysis useful to predict the odds that the sperm will meet the

egg?", *Reproduction in Domestic Animals = Zuchthygiene*, Vol. 44 Suppl 3, pp. 31–38.

Macías García, B., Ortega Ferrusola, C., Aparicio, I.M., Miró-Morán, A., Morillo Rodriguez, A., Gallardo Bolaños, J.M., González Fernández, L., et al. (2012), "Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential", *Theriogenology*, Vol. 77 No. 7, pp. 1280–1289.

Madeddu, M., Mosca, F., Abdel Sayed, A., Zaniboni, L., Mangiagalli, M.G., Colombo, E. and Cerolini, S. (2016), "Effect of cooling rate on the survival of cryopreserved rooster sperm: Comparison of different distances in the vapor above the surface of the liquid nitrogen", *Animal Reproduction Science*, Vol. 171, pp. 58–64.

Malo, C., Crichton, E.G. and Skidmore, J.A. (2016), "Optimization of the cryopreservation of dromedary camel semen: Cryoprotectants and their concentration and equilibration times", *Cryobiology*, available at:https://doi.org/10.1016/j.cryobiol.2016.11.001.

Mazur, P., Leibo, S.P. and Chu, E.H.Y. (1972), "A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells", *Experimental Cell Research*, Vol. 71 No. 2, pp. 345–355.

Mocé, E. and Graham, J.K. (2008), "In vitro evaluation of sperm quality", *Animal Reproduction Science*, Vol. 105 No. 1–2, pp. 104–118.

Morrell, J.M. and Rodriguez-Martinez, H. (2009), "Biomimetic techniques for improving sperm quality in animal breeding: a review", *The Open Andrology Journal*, Vol. 1, pp. 1–9.

Morton, K.M., Evans, G. and Maxwell, W.M.C. (2010), "Effect of glycerol concentration, Equex STM® supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (Vicugna pacos) sperm", *Theriogenology*, Vol. 74 No. 2, pp. 311–316.

Muiño, R., Rivera, M.M., Rigau, T., Rodriguez-Gil, J.E. and Peña, A.I. (2008), "Effect of different thawing rates on post-thaw sperm viability, kinematic parameters and motile sperm subpopulations structure of bull semen", *Animal Reproduction Science*, Vol. 109 No. 1–4, pp. 50–64.

Muldrew, K. and McGann, L.E. (1994), "The osmotic rupture hypothesis of intracellular freezing injury.", *Biophysical Journal*, Vol. 66 No. 2 Pt 1, p. 532.

Nie, G.J. and Wenzel, J.G. (2001), "Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes", *Theriogenology*, Vol. 55 No. 4, pp. 1005–1018.

Nöthling, J.O. and Shuttleworth, R. (2005), "The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen", *Theriogenology*, Vol. 63 No. 5, pp. 1469–1480.

Okazaki, T., Abe, S. and Shimada, M. (2009), “Improved conception rates in sows inseminated with cryopreserved boar spermatozoa prepared with a more optimal combination of osmolality and glycerol in the freezing extender”, *Animal Science Journal*, Vol. 80 No. 2, pp. 121–129.

Pérez-Pé, R., Cebrián-Pérez, J.A. and Muiño-Blanco, T. (2001), “Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa”, *Theriogenology*, Vol. 56 No. 3, pp. 425–434.

Silva, M.A., Peixoto, G.C.X., Castelo, T.S., Lima, G.L., Silva, A.M., Oliveira, M.F. and Silva, A.R. (2013), “Cryopreservation of collared peccary (Pecari tajacu) semen using different freezing curves, straw sizes, and thawing rates”, *Cryobiology*, Vol. 67 No. 1, pp. 50–55.

Skidmore, J.A. (2003), “The main challenges facing camel reproduction research in the 21st century”, *Reproduction (Cambridge, England) Supplement*, Vol. 61, pp. 37–47.

Skidmore, J.A. (2011), “Reproductive physiology in female Old World Camelids”, *Animal Reproduction Science*, Vol. 124 No. 3–4, pp. 148–154.

Skidmore, J.A., Morton, K.M. and Billah, M. (2013), “Artificial insemination in dromedary camels”, *Animal Reproduction Science*, Vol. 136 No. 3, pp. 178–186.

Spencer, P.B.S., Wilson, K.J. and Tinson, A. (2010), “Parentage testing of racing camels (Camelus dromedarius) using microsatellite DNA typing”, *Animal Genetics*, Vol. 41 No. 6, pp. 662–665.

Suarez, S.S. (2007), “Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction”, *Reproduction, Fertility and Development*, Vol. 19 No. 1, p. 103.

Tanghe, S., Van Soom, A., Sterckx, V., Maes, D. and De Kruif, A. (2002), “Assessment of Different Sperm Quality Parameters to Predict in vitro Fertility of Bulls”, *Reproduction in Domestic Animals*, Vol. 37 No. 3, pp. 127–132.

Yeste, M. (2016), “Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs”, *Theriogenology*, Vol. 85 No. 1, pp. 47–64.

APPENDIX

Appendix 1. *CASA settings*

Calibration	
Objective	0: 10xZeiss
Objective Magnification X	1.22
Objective Magnification Y	1.22
Cell Detection	
Elongation Max (%)	65
Elongation Min (%)	1
Head Brightness Min	75
Head Size Max (μm^2)	101
Head Size Min (μm^2)	13
Tail Brightness Min	101
Chamber	
Capillary Correction	1.3
Chamber Depth (µm)	20
Chamber Type	Capillary
Kinematics	
Progressive STR (%)	70
Progressive VAP (µm/s)	40
Slow VAP (µm/s)	20
Slow VSL (µm/s)	30
Static VAP (µm/s)	4
Static VSL (µm/s)	1
Morph	

DMR Confidence (%)	50
DMR Droplet to tail end Max (µm)	7
DMR Tail Length Max (µm)	20
Droplet Confidence (%)	50
Droplet Distal Distance Min (µm)	4
Droplet Proximal Head Length (µm)	10.5
Min Tail Lenght	0
Tail Bend Angle Averaging Length	5
Tail Bending Angle Rate Min (%)	20
Tail Bent Confidence (%)	50
Tail Coiled Angle Min (°)	180
Tail Coiled Confidence (%)	50
Setup	
Name	CAMEL PROG
Video Capture	
Frame Capture Speed (Hz)	60
Frame Count	30