Retrograde flushing collection and freezing of dromedary camel epididymal spermatozoa with seminal plasma

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ABSTRACT

The objectives of this study were to describe the parameters of dromedary camel epididymal spermatozoa collected by retrograde flushing (RF) technique and to evaluate the freezability of the collected sperm, diluted with and without the supplementation of seminal plasma (SP). Two experiments were conducted: in Experiment 1, ES were recovered within 6–8 h after castration; selected samples were diluted with a Tris-citrate egg-yolk glycerolated buffer and frozen. In Experiment 2, epididymides were stored for 24 h at 4 °C before RF and semen samples were frozen after dilution with a Tris-lactose egg-yolk glycerolated extender with and without 15% SP. In Experiment 1, eight semen samples were obtained from ten epididymides with a mean of 500×10^6 total spermatozoa recovered, per flushed epididymis. Mean post-thaw motility and progressive motility were 75 and 17%, respectively. In Experiment 2, 15 samples were collected, out of the 18 epididymides (mean number of collected spermatozoa: 700×10^6), and 13 of these samples were of excellent quality. Post-thaw parameters were not satisfactory but the supplementation of the freezing medium with 15% SP improved the progressive motility and kinematic parameters of the spermatozoa.

INTRODUCTION

The recovery of epididymal spermatozoa (ES) provides an alternative source of germoplasm for genetic conservation in case of events that preclude the reproductive life of males of high genetic value. Testes and epididymides could be stored at 4 °C for many hours, until it will be possible to collect and process the semen samples (Monteiro et al., 2011).

Given the peculiarities that limit the obtainment of suitable ejaculates from dromedary camel (DC) bulls (male aggressiveness, long mating time, failure of ejaculating with the

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artificial vagina, contamination of ejaculates), the collection of ES of adequate amount and good quality (i.e. from neutering or slaughterhouses material) could also help in the development of semen preservation and artificial insemination protocols (Monaco et al., 2016). Besides, ES could be used for overcoming the limitations imposed by the viscosity of the seminal plasma (SP) and as an experimental model for understanding the role of SP within semen cryopreservation protocols.

DC ES have been used for *in vitro* fertilisation and *in vitro* production of embryos and were obtained, in low number, through 'cutting' of the *cauda epididymis* (Khatir and Anouassi, 2006; Waheed et al., 2011). Despite the fact that ES have been collected with retrograde flushing (RF) in many animal species, in the DC this technique has been described only in two studies (Abdoon et al., 2013; Turri et al., 2013). However, other than sperm viability and total motility, the number of recovered sperm and other sperm parameters have not been described in detail. The objectives of this study were, therefore: (a) to describe the RF technique and the results of its application in the DC species; (b) to evaluate the freezability of collected ES and (c) to evaluate the effect of the addition of 15% SP to the freezing extender, on post-thaw semen parameters of the collected samples.

MATERIALS AND METHODS

Two separate experiments were performed in 2015–2016 and 2017–2019, respectively. The testicles used for the experiments were waste material resulting from ordinary castrations performed to prevent aggressive and restless behaviour of DC bulls assigned to tourism activities. In Experiment 1, RF was performed on 10 epididymides, within 6–8 h after neutering, whereas in Experiment 2, RF was performed on 18 epididymides, 24 h after neutering. Neutering was performed at Fuerteventura Oasispark; semen samples were collected and frozen at the University of Las Palmas Gran Canaria (Spain), whereas sperm kinematic parameters were evaluated at the University of Bari Aldo Moro (Italy).

Experiment 1

In this experiment, ten testes with annexed epididymides, obtained from five male camels (5 years of age), were transported at 4 °C and, 6–8 h after surgery, the epididymides with annexed deferent ducts were isolated. After the deferent duct was cannulated with a blunted needle (25 G; 5×16 mm), the epididymal tails were separated from the body, using a scalpel, proximally to the separation body/tail (considering the epididymis regions described by Fouchécourt et al. (2000) in stallions, the cut was made approximately in the region n°8).

RF was performed with a syringe filled with Tris-citrate buffer (Tris 247 mM; citric acid 87 mM; cephalexin 0.1%) kept at room temperature (Bruemmer, 2006; Turri et al., 2013). Each sample collected from one epididymis was

considered as an experimental unit. The obtained samples ($n = 8$) were evaluated for volume, sperm concentration, total and progressive motility, viability and morphology. The sperm concentration was assessed with a Neubauer haemocytometer, after diluting an aliquot of semen 1:100 with 0.5% formaldehyde solution. Total and progressive motility were assessed using a phase-contrast microscope, by two experienced operators: ten fields and a minimum of 400 spermatozoa were evaluated on sperm aliquots diluted to $15–30 \times 10^6$ spermatozoa $\times$ mL^{-1} (spz/mL) and kept at 37 °C. The percentages of viable spermatozoa and morphological abnormalities (head, midpiece and tail) were evaluated on eosin/nigrosin (E/N) stained smears at $200 \times$ and $1,000 \times$ magnification, respectively, counting a minimum of 200 spermatozoa (Lorton, 2014).

Sperm dilution and freezing

Samples with a progressive motility ≥75% and a minimum concentration of 200×10^6 spz/mL ($n = 3$ samples) were diluted to a final concentration of 100×10^6 spz/mL and a final composition of Tris 247 mM, citric acid 87 mM, glucose 66 mM, cephalexin (0.1%) (modified from Batista et al., 2011), and clarified egg yolk 20%. The clarified egg yolk was prepared according to Fernández-Santos et al. (2009). After 1.5 h of equilibration at 4 °C, samples were further diluted to reach the final concentration of 4% glycerol and 50×10^6 spz/mL. After an additional 1.5 h at 4 °C, samples were loaded into pre-cooled 0.5-mL straws, placed for 15 min 4 cm above liquid nitrogen (LN) level, and then plunged into LN.

Thawing and post-thaw evaluation

Thawing was performed at 46 °C for 20 s (Crichton et al., 2015); for each sample, three straws were evaluated, after 10-min incubation at 37 °C, for: viability, intact plasma membranes (IPMs), intact acrosomes (IAs), motility and progressive motility.

The sperm viability was evaluated on E/N stained smears (Lorton, 2014). IPMs were evaluated through hypo-osmotic swelling test (HOS test) on a minimum of 200 spermatozoa (Skidmore et al., 2013). IAs were evaluated through a FITCH-PNA/DAPI fluorescence staining (Desantis et al., 2010; Rateb et al., 2019). Fluorescent images (jpg) were acquired at $20 \times$ magnification using a 488-nm filter photomicroscope Eclipse Ni-U (Nikon, Japan) equipped with a digital camera (DS-U3, Nikon, Japan). Acrosomes with uniform green fluorescence were considered intact, whereas non-uniform/non-stained acrosomes were considered damaged and lost acrosomes, respectively (Fig. 1) (Morton et al., 2010).

Motility parameters were evaluated at 37 °C, through a Computer-Assisted Sperm Analyser (CASA) (Ivos12, Hamilton Thorne, USA). Since at the time of the experiment reference settings for DC sperm progressive motility were not available, the CASA system was set using the cut-off values reported in Table 1 (Experiment 1). Total and progressive motility, percentages of rapid, medium, slow and
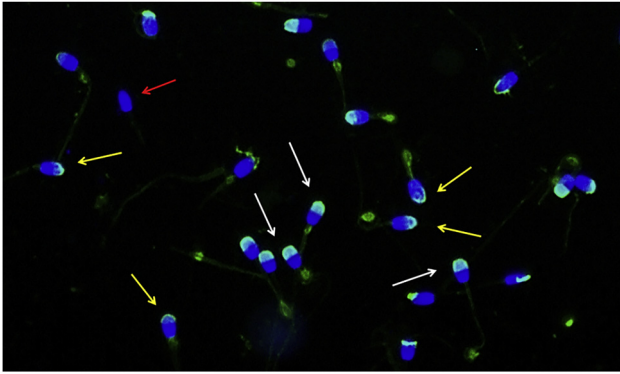
Fig. 1. Fluorescence staining (FITC-PNA/DAPI) of frozen-thawed epididymal spermatozoa.
* The red arrow indicates a lost acrosome, whereas yellow and white arrows indicate damaged and intact acrosomes, respectively

Table 1. Setting parameters for CASA evaluation of dromedary camel epididymal spermatozoa

	Experiment 1	Experiment 2[a]
Sample acquisition rate	60 images/s	60 images/s
Acquired fields	7	7[b]
Motile sperms	VAP > 20 μm/s	VAP > 20 μm/s
Progressively motile sperms	VAP > 50 μm/s and STR > 75%	VAP > 40vμm/s and STR > 70%
Slow spermatozoa	20 μm/s < VAP <3 0 μm/s	20 μm/s < VAP < 30 μm/s

[a]Setting according Malo et al. (2019a).
[b]Modified from 5 to 7 fields.

static spermatozoa were evaluated in addition to 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 above-mentioned semen parameters were evaluated again, after 3-h incubation at 37 °C (Thermal Stress Test; ThS Test) (López-Urueña et al., 2015).

Experiment 2

The SP used in Experiment 2 was obtained through semen collections (with an artificial vagina) from two adult dromedary bulls of proven fertility. The selected ejaculates ($n = 2$) (non-contaminated, volume ≥4 mL, sperm concentration >100 × 10^6 spz/mL and total motility >80%) were allowed to spontaneously liquefy at 4 °C, pipetted to reduce residual viscosity, and centrifuged twice at 1,000×g for 30 min. The two SP samples were checked for the absence of spermatozoa, then mixed with each other in equal volume and stored in aliquots at −80 °C until use.

Testes and epididymides ($n = 18$) were collected following the castrations of 9 adult males (6–15 years of age), and kept at 4 °C for 24 h, until RF.

Experiment 2A

The recovered ES samples (experimental units $n = 15$) were evaluated for volume, concentration, and motility score (0–5; 0: no motility; 1: motility between 0 and 20%, 2: motility between 20 and 40%; 3: motility between 40 and 60%; 4: motility between 60 and 80%; 5: motility between 80 and 100%) (Monaco et al., 2015). Eosin/nigrosin stained smears were also prepared for the evaluation of sperm morphology to be carried out at a later time. Samples with a motility score ≥3 ($n = 13$) were split into two portions (control and 15% SP) and processed to reach the composition of Tris 268.28 mM/L, citric acid 79.7 mM/L, lactose 152.64 mM/L, glucose 27.75 mM/L, 20% clarified egg yolk, glycerol 4% (modified from El-Bahrawy, 2017), and the final concentration of 50 × 10^6 spz/mL. Pre-freezing viability, total and progressive motility were evaluated by two operators, as described in Experiment 1, and samples showing pre-freezing progressive motility ≥40% ($n = 13$) were frozen with the same described technique.

Two straws for each sample and treatment were thawed, kept in Eppendorf tubes at 37 °C for 10 min, and then evaluated for viability (E/N) and total and progressive motility.

Experiment 2B

Twenty paired frozen samples were thawed, then evaluated for total and progressive motility, viability, and acrosome

Table 2. Experiment 1: Parameters of dromedary camel epididymal spermatozoa samples ($n = 8$) collected through retrograde flushing technique

Parameter	Unit	Mean	St. Dev.	Median	Q1	Q3
Volume	mL	1.52	0.43	1.48	1.06	2.00
Sperm concentration	×10^6 spz/mL	332.5	199.48	360	163	418
Total recovered sperms	×10^6 spz	508.13	319.14	540	163	830
Total motility	%	74.63	10.54	76.00	62.50	85.00
Progressive motility	%	65.63	15.22	70.00	50.00	78.75
Viable spermatozoa	%	84.13	8.85	83.00	77.75	92.25
Abnormal sperm cells	%	65.75	10.03	65.75	57.38	74.75
Abnormal tails[a]	%	63.12	10.43	60.75	50.63	67.75
Abnormal midpieces	%	1.38	1.13	1.00	0.63	2.25
Abnormal heads	%	1.25	1.07	1.00	0.25	2.25

[a]Proximal and distal cytoplasmic droplets included.

Table 3. Experiment 1: Pre-freezing parameters of dromedary camel epididymal spermatozoa semen samples ($n = 3$) diluted in a Tris-citrate-glucose extender (20% clarified egg yolk and 4% glycerol) and equilibrated for 3 hours at 4 °C

Parameter	Unit	Mean	St. Dev.	Median	Q1	Q3
Viability	%	95.2	3.68	96.5	91	98
Motility	%	85.7	4.01	86	81.5	89.5
Progressive motility	%	78.2	5.20	76.5	74	84
Intact plasma membranes	%	86.2	8.43	90	76.5	92

integrity either after 10 min (T0) or after 60 min (T1) of incubation at 37 °C. The same parameters were also evaluated through E/N stained smears as well as by CASA, according to Malo et al. (2019a).

Statistical analysis

In Experiment 1, post-recovery, pre-freezing and post-thaw epididymal semen parameters were analysed through descriptive statistics; the results were expressed as mean, standard deviation, quartile 1 (Q1) and quartile 3 (Q3).

The parameters of ES samples collected in Experiment 2 were analysed through descriptive statistics. In Experiment 2A, pre-freezing and post-thaw semen parameters of control and treated (15% SP) samples were analysed using the non-parametric Wilcoxon test. In Experiment 2B, post-thaw data recorded at 10 min (T0) and 60 min (T1) after thawing were tested for normality using the Anderson test. Since the distribution was non-normal, the data were log-transformed and then analysed using a 2-way ANOVA Generalized Linear Model procedure with treatments (Control and SP),

time (T0 and T1) and their interaction as fix factors. Since the interaction was never significant, the original data (not normally distributed) were analysed using the U-Mann test to identify possible differences due to the treatment (Control and SP) and time (T0 and T1). The statistical significance was always set at $P < 0.05$. All statistical analyses were performed using Genstat 19th edition.

RESULTS

Experiment 1

Table 2 summarises the semen parameters of the eight collected samples. A mean volume of 1.52 mL of sperm-buffer solution with an average concentration of 332×10^6 spz/mL was collected; the mean number of total recovered spermatozoa was $508 \pm 319 \times 10^6$. Within the eight samples, six showed total and progressive motility above 75 and 65%, respectively. Descriptive statistics of pre-freezing and post-thaw parameters of selected samples ($n = 3$) are reported in Tables 3 and 4, respectively.

Experiment 2

Descriptive parameters of the 15 samples collected in Experiment 2 are reported in Table 5. Overall, a mean volume of 1.57 mL of sperm-buffer solution with an average of 480.15×10^6 spz/mL was collected. The mean number of total recovered spermatozoa was $708 \pm 205 \times 10^6$. Eleven samples showed a motility score ≥ 4 and, after equilibration, 13 samples were selected for freezing.

In experiment 2A, SP samples were not statistically different from control pre-freezing or post-thaw; a reduction of sperm viability was noticed after thawing but it was not

Table 4. Experiment 1: Post-thaw and post thermal stress test (3 h at 37 °C) parameters of dromedary camel epididymal spermatozoa ($n = 3$) collected through retrograde flushing and frozen with a Tris-citrate-glucose extender (20% clarified egg yolk and 4% glycerol). Motility and kinematic parameters were evaluated through computer assisted sperm analyser

Parameter	Unit	Post-thaw					Post thermal stress (180 min)				
		Mean	St. Dev.	Median	Q1	Q3	Mean	St. Dev.	Median	Q1	Q3
Viability	%	83.54	3.37	84.30	81.00	85.95	64.37	14.21	71.20	51.75	73.95
IPM	%	69.38	9.56	68.10	61.50	77.05	56.06	7.69	55.25	51.33	62.40
Intact acrosome	%	37.39	9.56	35.87	27.16	47.66	24.33	7.89	27.40	17.04	32.38
Motility	%	75.33	3.91	77.00	71.50	78.50	41.33	18.03	39.00	26.00	57.50
Progressive motility	%	17.22	4.89	16.00	13.50	20.50	6.89	4.54	6.00	3.50	10.00
Rapid	%	60.22	6.26	61.00	55.00	65.00	28.44	17.29	29.00	12.00	41.50
Medium	%	14.89	4.43	14.00	10.50	18.00	12.89	3.10	12.00	10.50	15.50
Slow	%	5.56	2.46	5.00	3.50	7.00	3.00	1.00	3.00	2.00	3.50
VAP	µm/s	79.14	9.04	78.30	70.85	85.55	67.08	12.99	71.80	54.30	77.15
VSL	µm/s	45.42	7.60	43.20	40.15	49.25	36.78	7.82	39.40	28.30	42.95
VCL	µm/s	129.26	19.30	127.80	113.65	148.50	122.61	23.18	124.50	110.25	140.10
ALH	µm/s	4.64	0.53	4.60	4.20	5.10	4.86	0.95	5.20	4.35	5.55
BCF	HZ	14.12	3.88	14.40	10.60	16.35	17.01	4.93	15.00	13.05	22.60
STR	%	54.22	4.47	54.00	51.50	57.50	56.56	12.72	52.00	51.00	55.50
LIN	%	35.89	5.33	36.00	31.50	40.50	30.56	3.81	32.00	27.00	33.00

IPM: Intact plasma membranes; VAP: average path velocity; VSL: straight line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity.

Table 5. Experiment 2: Sperm parameters of dromedary camel epididymal semen samples ($n = 15$) collected through retrograde flushing

Parameter	Unit	Mean	St. Dev.	Median	Q1	Q3
Volume	mL	1.57	0.33	1.5	1.3	1.85
Sperm concentration	$\times 10^6$ spz/mL	480.15	206.88	419	351.5	568
Total recovered sperms	$\times 10^6$ spz	708.57	205.18	668.8	605.95	840.75
Sperm motility (0–5)	Score (0–5)	3.92	0.49	4	4	4
Abnormal sperm cells	%	52.31	10.44	55	41.5	60.5
Abnormal tails[a]	%	49.77	10.66	51	38	59
Abnormal midpieces	%	2.54	1.85	3	0.5	4
Abnormal heads	%	1.31	1.03	1	0.5	2

[a]Proximal and distal cytoplasmic droplets included.

significantly different from control (Graphs 1 and 2). In experiment 2B, as expected, the incubation time had a significant negative effect on many semen parameters with reduction of values from T0 to T1 except for straightness ($P < 0.01$): viability ($P < 0.05$), intact acrosome ($P < 0.01$), motility ($P < 0.01$), progressive motility ($P < 0.01$), rapid spz ($P < 0.01$), medium spz ($P < 0.01$), static spz ($P < 0.01$), VAP ($P < 0.01$) (Graphs 3–9). In contrast, the supplementation of 15% SP plasma to the freezing extender had a positive effect on progressive motility ($P < 0.05$) and sperm kinematic parameters: VAP ($P < 0.05$), VSL ($P < 0.001$), VCL ($P < 0.001$), ALH ($P < 0.05$), STR ($P < 0.05$), LIN ($P < 0.05$) (Graphs 10–16).

DISCUSSION

In this paper the parameters of DC ES collected by the use of the RF technique are described in detail. The freezability of ES and the effect of SP supplementation on frozen-thawed semen parameters were also evaluated, for the first time. Eight out of ten (Experiment 1) and fifteen out of eighteen (Experiment 2) tails of epididymides were successfully flushed and 19 of the 23 obtained samples were considered to be of good/excellent quality (motility >70%). DC males reach puberty at the age of 3 years but the maximum

potential fertility is reached at the age of 7 years (El-Wishy, 1988). The higher amount of total spermatozoa collected in Experiment 2 (Tables 2 and 5) could be ascribed to the higher age of bulls in Experiment 2, but this hypothesis should be confirmed by further studies.

Waheed et al. (2011) obtained a mean total amount of 360×10^6 spz, with an average motility of 36%, by mincing DC epididymal tails. Reference data about DC ES parameters are lacking in the literature; therefore, it is difficult to compare the results of the present study with those of other studies where ES were collected (Moawad et al., 2011; Wani and Hong, 2018).

Comparing RF with cutting, Turri et al. (2012) observed higher total motility and viability in bull epididymal sperm samples obtained through RF as compared to mincing. Santiago-Moreno et al. (2009) observed, in *Capra pyrenaica*, substantial benefits of the RF technique over cutting because of the larger number of recovered sperm cells and the reduced amount of post-thaw acrosome damage and morphological abnormalities. Hori et al. (2015) concluded that RF could be used as a standard technique in dogs and that cutting may be used when RF is unsuccessful. Evaluating the results of the present work, RF could be considered a satisfactory and easy-to-perform technique for recovering DC ES even though a true comparison should be made with the other techniques, for defining the gold standard procedure.

To the best of our knowledge, this is the first evaluation, by CASA, of the post-thaw motility and kinematic parameters of frozen-thawed DC ES samples. The post-thaw motility data observed in Experiment 1 (Table 4) are similar to those observed on frozen-thawed DC ejaculated spermatozoa, selected by single-layer centrifugation by Malo et al. (2019a), even though we used more restrictive parameters for the identification of progressively motile spermatozoa (Table 1).

In Experiment 2 semen samples showed acceptable pre-freezing motility, albeit the post-thaw values were unsatisfactory (Graphs 4 and 11). The exact reason for these findings remain unknown but it could be hypothesised that the 24-h storage time of epididymides may have affected the freezability of sperm. Alternatively, the diluent used in Experiment 2 did not protect the ES efficiently from cryoinjury. Turri et al. (2014) observed that buck epididymis could be stored at 5 °C up to 48 h before the post-thaw effect
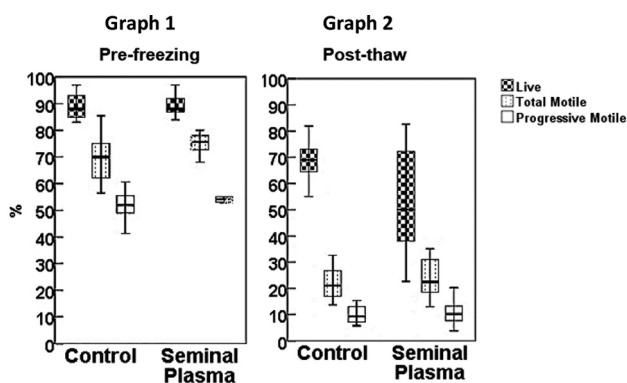
Graphs 1 and 2. Experiment 2A: Pre-freezing and post-thaw semen parameters of dromedary camel epididymal semen samples ($n = 13$) diluted with a Tris-citrate-lactose extender (20% clarified egg yolk and 4% glycerol) with and without the supplementation of 15% seminal plasma
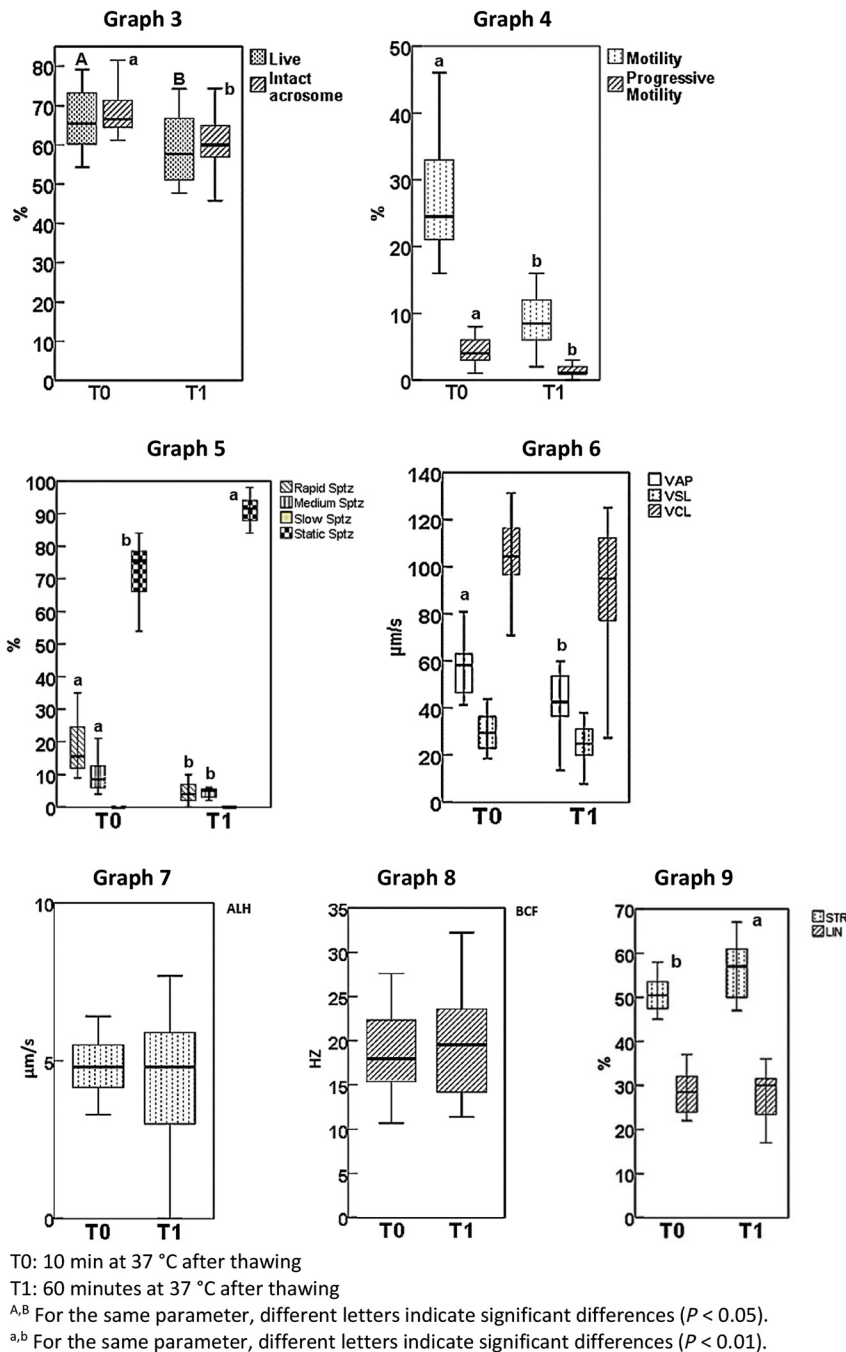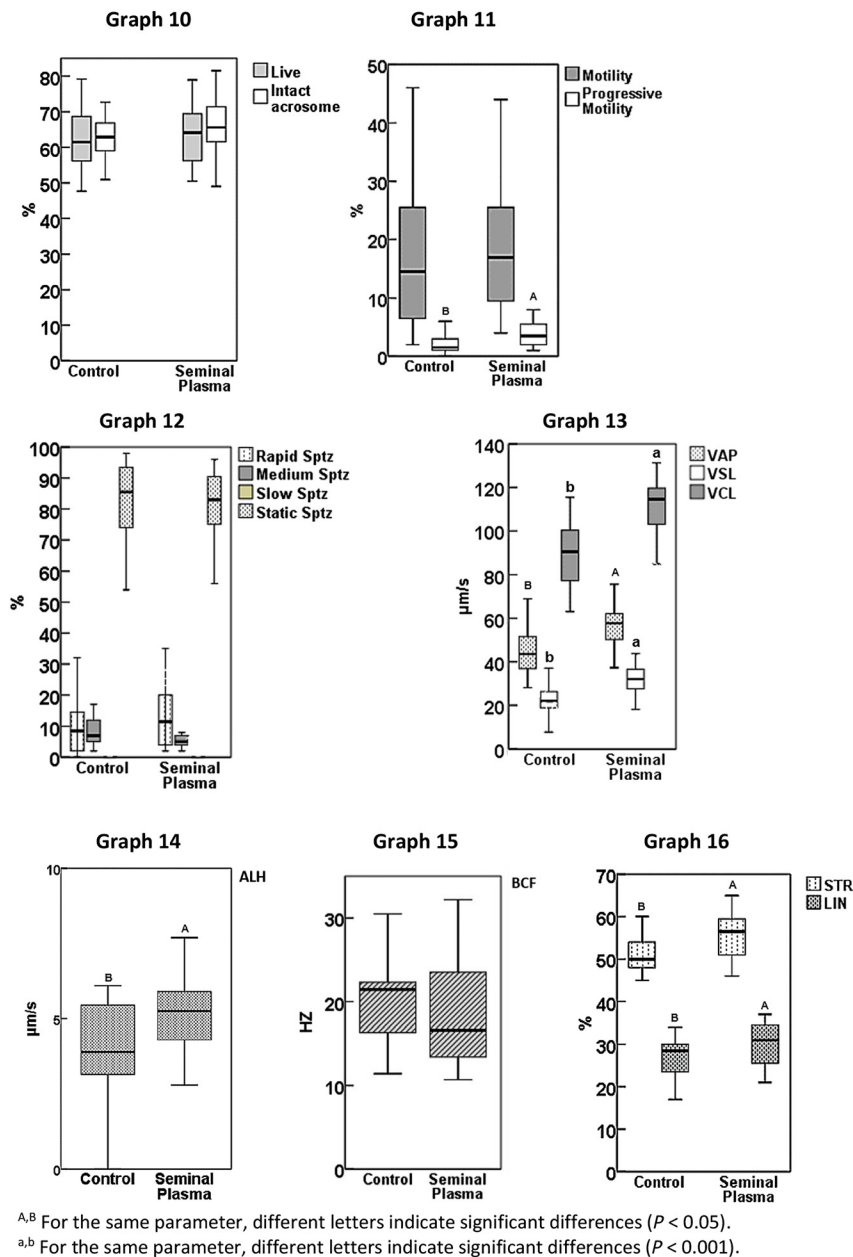
Graphs 3–9. Experiment 2B: Effect of time (T0, T1) on post-thaw semen and kinematic parameters of dromedary camel epididymal semen samples ($n = 20$) diluted with a Tris-citrate-lactose extender (20% clarified egg yolk and 4% glycerol) extender. Motility and kinematic parameters were evaluated through computer assisted sperm analyser

of storage could manifest itself. Martins et al. (2009) found that post-thaw motility of bull spermatozoa was significantly reduced after a 72-h storage time of epididymides as compared with 0, 24 and 48 h. In contrast, Monteiro et al. (2013) observed a significant decrease of post-thaw progressive motility in stallion spermatozoa collected from the epididymis after 12-h storage at 5 °C.

In previous studies, with subjective evaluation of ES post-thaw motility, Abdoon et al. (2013) observed higher post-thaw total motility ($47 \pm 5\%$) by using Ovixcell®. By using Shotor diluent, El-Badry et al. (2015) obtained total and progressive motility values of $47.4 \pm 1.3\%$ and $32.8 \pm 1.3\%$, respectively. More recently, post-thaw CASA evaluation of ejaculated spermatozoa revealed total and progressive motility values of $38.6 \pm 0.8\%$ and $10 \pm 0.7\%$ for spermatozoa diluted with Triladyl® (Swelum et al., 2019), and Malo et al. (2019b) observed total motility values ranging from 33% to 46% and progressive motility from 8 to 18.5% for spermatozoa frozen with Green Buffer®. In the light of the above-mentioned studies and according to the present

Graph 10 · **Graph 11** · **Graph 12** · **Graph 13** · **Graph 14** · **Graph 15** · **Graph 16**

A,B For the same parameter, different letters indicate significant differences ($P < 0.05$).
a,b For the same parameter, different letters indicate significant differences ($P < 0.001$).

Graphs 10–16. Experiment 2B. Post-thaw semen and kinematic parameters of dromedary camel epididymal semen samples ($n = 20$) diluted with a Tris-citrate-lactose extender (20% clarified egg yolk and 4% glycerol) with and without 15% seminal plasma

results, the effects of the epididymal storage time and of the freezing diluents on post-thaw ES motility parameters require further investigations.

This is the first study that evaluated the effect of SP supplementation on the post-thaw parameters of DC ES. The 15% SP supplementation was chosen considering the work of Kershaw-Young and Maxwell (2011) who found that 10 and 25% SP had a protective effect on alpaca ES maintained at 37 °C for 3 h, as compared with control (0% SP), 50 and 100% SP dilution. Contrary to expectations (reduced sperm motility due to a residual micro-viscosity was also hypothesised as a side effect), the CASA analysis revealed that SP had a significant effect on some sperm

kinematic parameters (Graphs 10–16). Fumuso et al. (2018) observed that llama ES incubated at 37 °C for 3 h had higher progressive motility when 10 or 50% SP was added as compared to 100% SP supplementation. In the same species, Fumuso et al. (2019) observed that the pre-freezing supplementation of 10% SP leads to higher than 50% post-thaw motility (subjective evaluation). However, significant differences were not detected in either experiment comparing 10 and 50% SP supplementation with control samples.

In stallion semen preservation protocols, addition of 5–20% SP is recommended since beneficial effects on sperm motion characteristics for up to 96 h of storage have been observed (Neuhauser et al., 2015). Subjective motility

evaluations showed that the pre-freezing exposure of stallion ES to SP could improve the pre-freezing but not the post-thaw motility values (Tiplady et al., 2002). It has also been observed that the kinematic parameters of stallion sperm are positively influenced by the post-thaw supplementation of SP and that the sperm kinematic values improve until the limit of 50% SP is added to the extender (Neuhauser et al., 2015, 2018). According to the present results, it seems that there is a positive effect of SP on post-thaw motility of DC ES as observed also in the equine species.

In conclusion, RF is a valuable procedure for the collection of DC ES and the obtained samples could be used for evaluating the DC semen processing procedure. The supplementation of 15% SP seems to have beneficial effects on post-thaw motility and sperm kinematic parameters of DC ES.

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