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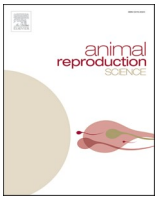

Use of a sperm-Hyaluronan binding assay for evaluation of sperm quality in dromedary camels

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ABSTRACT

The objective of this study was to assess the ability of camel spermatozoa to bind in the Hyaluronan Binding Assay (HBA), to determine if conventional sperm quality parameters, in vitro fertilization capacity, and precursor of A-Kinase Anchoring Protein 4 (proAKAP4) values correlate with HBA results. The potential to predict post-thaw fertilization performance from HBA for fresh dromedary camel sperm was also evaluated. Semen samples were collected and assessed both fresh and post thawing, at 0 h and 1.5 h. Conventional semen analysis, HBA, and a proAKAP4 biomarker-test were used to validate sperm quality. A heterologous sperm penetration assay using zona pellucida-free goat oocytes was used to assess in vitro sperm fertilizing capacity. The results showed that dromedary camel spermatozoa bound to hyaluronan with no correlation between results from fresh samples and after thawing. Furthermore, the proAKAP4 test results showed a negative correlation with HBA at 0 h after thawing ($r = -0.62$; $P = 0.03$). In the conventional analysis, only progressive motility ($r = 0.65$; $P = 0.02$) and straightness correlated with HBA for fresh semen ($r = 0.69$; $P = 0.01$). In the sperm penetration assay, a moderate but non-significant correlation was identified between fresh sperm HBA and penetration ($r = 0.52$; $P = 0.07$). In conclusion, results suggested that HBA can be used to assess camel sperm properties, but further investigation is needed to understand its correlation with other sperm quality parameters. The HBA score from fresh dromedary camel sperm was unable to predict post-thaw fertilization performance.

1. Introduction

Assisted reproductive technologies in camels, including artificial insemination (AI), provide many advantages to commercial animal breeding to produce genetically superior animals and increase reproductive success (Skidmore et al., 2013). Accurate sperm quality assessment and optimized cryopreservation techniques, combined with AI, would provide many advantages for dromedary camel breeding. Several females could be inseminated with a single ejaculate from a superior male, animals would not have to be transported for mating, the risk of transmitting infectious disease would be lower, reproductive lifespan would be extended and sperm

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samples could be transported around the world (Skidmore et al., 2018).

Dromedary camel semen characteristics have posed a great challenge to the development of cryopreservation techniques. Viscous seminal plasma makes it difficult to evaluate and process camel ejaculates (Wani et al., 2011), and dromedary camel sperm quality varies significantly between individuals and under different climate and housing conditions (Fatnassi et al., 2017; Malo et al., 2021). Camel spermatozoa also tolerate freeze-thawing poorly; cryopreservation affects sperm quality and fertilization potential considerably (Bravo et al., 2000; Morton et al., 2010; Crichton et al., 2015; Malo et al., 2017).

The hyaluronan binding assay (HBA) is based on the ability of spermatozoa that are mature, functional, and capable of fertilization to bind to hyaluronic acid (HA) (Huszar et al., 2003), which is a linear polysaccharide found in the extracellular matrix of the cumulus oophorus covering the oocyte. It plays a critical role in sperm selection during both in vivo (Redgrove et al., 2013) and in vitro fertilization (Worrilow et al., 2013). Plasma membrane remodeling during epididymal transit is accompanied by the formation of HA binding sites. Thus, spermatozoa acquire these binding sites as they become mature. Spermatozoa capable of binding to HA are considered to have intact acrosomes, high membrane Integrity, and low protamine deficiency (De Kretser et al., 1998; Huszar et al., 1997; Huszar et al., 2000). They are also devoid of cytoplasmic retention, are of normal morphology, and have a lower frequency of DNA fragmentation and chromosomal aneuploidies than spermatozoa that do not bind to HA (De Kretser et al., 1998; Huszar et al., 1997; Huszar et al., 2000; Nasr-Esfahani et al., 2008; Kovanci et al., 2001). Bound spermatozoa also express lower levels of apoptotic markers such as caspase 3 and the cytoplasmic retention marker, creatine phosphokinase (CK) (Cayli et al., 2004).

Conventional semen analysis is confined to an indirect evaluation of sperm function. Quality parameters that are relatively easy to assess, such as sperm motility evaluation, morphology and acrosome status do not necessarily indicate the critical biochemical events that occur during the fertilization process (Suarez and Pacey, 2006). It would be beneficial to have an accurate, simple technique to predict dromedary camel sperm freezability and fertilization capacity. The HBA test has been employed in human andrology laboratories to predict in vivo fertility of sperm samples and also for sperm selection for intracytoplasmic sperm injection (ICSI) (Esterhuizen et al., 2015). Novel studies also reported that HBA may have potential in predicting fertilization capacity in animal reproduction; Chun et al. (2005) found that the HBA score was associated with a higher production of porcine embryos with a normal chromosomal complement; Colleoni et al. (2011) reported some potential for assessing stallion sperm fertility with HBA, and Awan et al. (2021) found that the HBA was capable of predicting the fertility of frozen-thawed Nili-Ravi buffalo bull spermatozoa.

Another important sperm maturity marker is testis-specific heat shock protein A2 (HspA2), which belongs to the 70 kilodalton heat shock protein family (HSP70) (Ergür et al., 2002). It was reported that HA-bound spermatozoa showed higher expression of proteins from the HSP70 family. Specifically, HspA2 is known as a down-regulator of apoptosis that likely alleviates the adverse effects of several types of stress in the cells and provides sperm protection during cryopreservation (Neuer et al., 2000; Holt et al., 2015; Yogev et al., 2010). A novel study on Nili-Ravi buffalo spermatozoa reported that the HBA score was correlated with sperm freezability potential, which could be associated with the maturity level of the spermatozoa (Awan et al., 2021). Thus, the HBA score might be associated with the expression of HspA2 and the tolerance of spermatozoa to cryopreservation.

ProAKAP4 is a profusely expressed protein of the sperm fibrous sheath that is well-conserved in mammals and reptiles (Delehedde et al., 2018; Nixon et al., 2019; Sergeant et al., 2019; Greither et al., 2020). ProAKAP4 has emerged as a novel marker of fertility in many species, such as bulls (Peddinti et al., 2008), stallions (Blommaert, 2018), rams (Riesco et al., 2020) and was also identified in camel (Malo et al., 2021).

The objective of this study was to determine whether the HBA, conventional sperm quality parameters and proAKAP4 values are related in dromedary camel spermatozoa. A further aim was to determine if the results in fresh semen reflect post-thaw sperm quality.

2. Material and methods

2.1. Experimental design

Six males (two ejaculates per male) were used. Samples were assessed just after collection (fresh), after thawing at 0 h (PT0H), and 1.5 h (PT1.5 H). Liquefied samples and samples PT0H were evaluated for HBA slides and proAKAP4 biomarker-test as described below. Total motility (TM, %), Progressive motility (PM, %), motility-related kinematics, viability (VI,%), intact acrosome (IA, %), and mitochondrial membrane potential (MIT, %) were evaluated in fresh and post-thaw samples (PT0H and PT1.5 H) to find possible correlations with HBA test results. Volume (Vol, mL), viscosity (Vis, scale 1–5), concentration (Conc; million sperm/mL), and morphological abnormalities (MA, %) were also assessed in fresh samples. The fertilizing ability of PT0H sperm samples was evaluated with a heterologous sperm penetration assay (SPA) using zona pellucida free goat oocytes, resulting in values for a number of oocytes (N), penetration rate (PEN), formation of male pro-nucleus (PN) and number of sperm penetrated per oocyte (SP/OC) to determine correlations between fresh HBA and fertilizing ability after thawing.

2.2. Animals and semen collection

Six dromedary camels from the Camel Reproduction Centre in Dubai were used in this study. The guidelines of the Animal Care and Use Committee of the Camel Reproduction Center (UAE) were followed in all the procedures. Semen was collected using a modified bovine artificial vagina (Skidmore et al., 2013). Collections were made between the end of February and the beginning of March, during the rutting season. Collections from the same animals were made three days apart. After the collection, samples were immediately transported to the onsite laboratory and placed in a 37°C water bath.

2.3. Media

Unless otherwise indicated, all chemicals were from Sigma-Aldrich Co. (St Louis, MO, USA). Ejaculates were diluted in Tris-Citrate-Fructose Buffer (TCF, pH = 6.9; 340 mOsm) composed of 300 mM Tris, 94.7 mM citric acid, and 27.8 mM fructose (Evans and Maxwell, 1987). Bovine serum albumin (0.05 %) and ethylenediaminetetraacetic acid (EDTA) (10 mM) were added with 4 % (v:v) egg-yolk and the solution was filter-sterilized (Merck Millex™-GS Sterile Syringe Filter Unit, MCE, 0.22 μm).

A two-step dilution with a freezing extender was performed before cryopreservation. Fraction 1 (F1) comprised Green Buffer (GB) freezing extender (IMV: L'Aigle, France) supplemented with 20 % (v:v) egg-yolk and 500 IU of catalase, and fraction 2 (F2) consisted of F1 with the addition of 6 % glycerol (Malo et al., 2018).

2.4. Cryopreservation and thawing procedures

The ejaculates were diluted 1:5 in TCF. Viscosity was eliminated by repeatedly gently pipetting the samples during incubation for 30 min in 37°C water bath [9]. Liquefied samples were evaluated for TM, PM and kinematics, IA, VI, MIT, proAKAP4, and HBA.

Semen was prepared for cryopreservation and thawed according to the protocol developed by Malo et al. (2017, 2018, 2019). Liquefied samples were centrifuged at 300 xg for 20 min to remove supernatant containing TCF and seminal plasma. The supernatant was carefully removed with a Pasteur pipette and discarded. The pellet with spermatozoa was re-suspended with F1 suspension. Concentration was adjusted to 150×10^6 spermatozoa/mL and cooled to 5ºC for 2 h in a water jacket. The second dilution (1:1) was done at 5° C, with F2 suspension, resulting in a final glycerol concentration of 3 % and a final sperm concentration of 75×10^6. Sperm samples were packaged in pre-cooled (0.5 mL) plastic straws and equilibrated in a cold cabinet for 30 min. Straws were frozen in vapor 1 cm above the surface of liquid nitrogen for 15 min and were then plunged into liquid nitrogen (-196ºC) for storage.

For post-thaw studies, two straws from each sample were thawed by placing them in a circulating water bath at 60ºC for 10 seconds.

2.5. Sperm assessment

2.5.1. Concentration

Diluted and liquefied fresh samples were further diluted 1:50 (v:v) in saline solution with 4 % paraformaldehyde. Concentration was measured from 10 μL of sample in a Makler counting chamber.

2.5.2. Motility

Sperm motility assessment was performed by Computer Assisted Sperm Analysis (CASA) as described previously [36] using the CEROS II (Hamilton Thorne; MA; USA) at 10X on the heated stage of a phase contrast microscope (AX10 Zeiss, Gottingen, Germany). A disposable chamber slide (Cytonix, Beltsville, MD, USA) was filled with 2 μl of sample, and five fields with a minimum of 400 spermatozoa were captured. Sperm kinematics included average path velocity (VAP; μm/s), straight-line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz). The settings of the CASA were as follows: frame capture speed 60 Hz, Elongation Max 65 %, and Head size Max 101 μm^2. Motility was classified as follows: progressive motility (PM): STR 70 % and VAP 40 μm/s; slow VAP 20 μm/s, VSL 30 μm/s; static VAP 4 μm/s VSL 1 μm/s.

2.5.3. Morphological abnormalities

Semen samples were stained with eosin-nigrosine (EN) to assess sperm morphology [39]. One drop of semen was combined with one drop of EN and smeared on a glass slide. Slides were placed on a warm plate to dry and were viewed at 100x oil-immersion objective (Olympus bx53). Abnormalities in the head (microhead, macrohead, pyriform, giant, twin), tail abnormalities (coiled, bent, thick), and cytoplasmatic droplets were counted. In total. 400 spermatozoa were evaluated per sample.

2.5.4. Viability

Viability was assessed by evaluating plasma membrane integrity. Live spermatozoa have an intact membrane whereas dead spermatozoa do not have an intact membrane. The fluorescent probes SYBR-14 (SY) and propidium iodide (PI) were used according to the manufacturer's instructions (L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands). A working solution was prepared with 1.5 μL of SY and 49 μL of TCF; 6 μL of working solution combined with 30 μL of sample were incubated for 10 min at 38°C and 5 % CO2. After incubation, 2 μL of PI stain, 2 μL spermatozoa immobilizing paraformaldehyde (4 %), and 30 μL of TCF were added. The stained samples were evaluated by two people using fluorescence microscopy (Olympus bx53), each person classifying 200 spermatozoa (total 400 spermatozoa) as either live (SY-positive, green) or dead (PI-positive, red).

2.5.5. Intact acrosome

The proportion of spermatozoa with an intact acrosome (IA) was evaluated with fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) with slight modifications to the method described by Aboagla and Terada (38) and Malo et al. (39). Thirty μL of sample were diluted with 140 μL of TCF. Six μL PNA (2 μg/mL) dye and 1 μL of spermatozoa immobilizing paraformaldehyde (4 %) were added. Two people each classified 200 spermatozoa by fluorescence microscopy (Olympus bx53) as having either an intact acrosome (PNA-negative, no stain) or a damaged acrosome (PNA-positive, green).

2.5.6. Mitochondrial membrane potential

Measurement of mitochondrial membrane potential was done using fluorescent Rhodamine-123 dye (R123) as described by Eskandari and Momeni (2016). R123 stains mitochondria in the intermediate piece in response to mitochondrial activation. Fluorescence indicates high activity. One hundred μL of each sperm sample was diluted with 900 μL of TCF and 5 μL of R123 (1 mg/mL) (final concentration 5 μg/mL). The sample was incubated for 10 min at room temperature in the dark and centrifuged at 300 xg for 20 min. The supernatant was removed by pipetting and discarded. The pellet was re-suspended with 150 μL of TCF, and 2 μL of paraformaldehyde (4 %) was added to immobilize the spermatozoa. Two people analyzed the sample by fluorescence microscopy (Olympus bx53), each evaluating 200 spermatozoa, and the proportion of spermatozoa with active mitochondria were calculated.

2.5.7. Sperm-hyaluronan binding assay

The HBA® Sperm-Hyaluronan Binding Assay was manufactured by Biocoat, Inc. (Horsham, PA, USA) and purchased from Medical Device & QA Services Ltd. (Hale, United Kingdom). The HBA® Sperm-Hyaluronan Binding Assay is a ready-to-use microscope glass slide for the determination of the fraction of hyaluronan-binding spermatozoa in a semen (Biocoat, Inc. 2022). Each test slide has two identical chambers coated with a layer of hyaluronan, covalently attached to the chamber surface (Biocoat, Inc. 2022). The HBA test was carried out according to the manufacturer's instructions. For each trial, one chamber was loaded with 8 μL of freshly mixed semen sample. A Cell-Vu gridded (Cell-vu®, Millennium Sciences, Inc. Miami Beach, FL, USA) cover slip was placed on top, avoiding bubble formation. The slide was placed on a warm plate covered with a cardboard box to avoid exposure to light during the 10 min incubation time. Samples were examined under light microscope with 400x magnification by two people, both assessing 100–170 spermatozoa as being either bound or unbound. Bound spermatozoa showed vigorous flagellar activity without any PM. The mean values were used to achieve the proportion of hyaluronan-bound sperm.

2.5.8. Precursor of A-kinase anchoring protein

The ProAKAP4 was used as a biochemical indicator to estimate sperm quality and sperm functionality [28]. The concentration of proAKAP4 was analyzed at the 4BioDx laboratory with the ELISA assay Camel 4MID® Kit following the manufacturer's protocol (Ref. 4VDX-19K11, 4BioDx, France). Fifty μL of the thawed sample was mixed with 450 μL of the camel lysis buffer. One hundred μL of lysed sperm sample were loaded in each well of the 96-well Camel 4MID® Kit plate. The plate was placed on an orbital shaker at 300 rpm and incubated at ambient temperature (20–22 ºC). The concentration of proAKAP4 was proportional to the color intensity measured by spectrophotometry at a wavelength of 450 nm. The results were expressed in ng/10 million spermatozoa (ng/10 M spermatozoa).

2.5.9. Sperm penetration assay

The fertilizing ability of the frozen-thawed samples was evaluated using a sperm penetration assay (SPA) with in-vitro matured, zona-free goat oocytes. The method was described by Crichton et al. (2016) Goat ovaries were obtained from the local slaughterhouse (Dubai, United Arab Emirates). The Cumulus oocyte complexes (COCs) were retrieved using a slicing technique by incising after rinsing the ovaries with phosphate-buffered saline. The COCs were washed in phosphate-buffered saline (PBS) composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, and 1.46 mM CaCl2·2H2O, 0.34 mM sodium pyruvate, 5.4 mM glucose and 70 mg/mL kanamycin. The oocyte maturation medium was a modified North Caroline State University (NCSU)-23 medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum (FCS) and 0.8 mM cysteine. Fertilization medium was M199 with Earle's salts supplemented with 6 % FCS (v:v), 0.91 mM sodium pyruvate, 3.05 mM d-glucose, 2.92 mM calcium lactate, 50 IU kanamycin, and 30 μg/mL streptomycin sulfate.

These COCs were matured in drops (30 oocytes per drop) in vitro overnight and denuded the next day by removing the cumulus cells and zona pellucida, before placing them in fertilization media. The oocytes were pooled and split between the different samples (thawed sperm samples from 12 batches; 2 ejaculates/ 6 males) (approx. 30–45 oocytes per sample). Thawed semen samples were centrifuged and re-suspended with fertilization medium to a concentration of 1×10^6 motile sperm/mL. Denuded oocytes were incubated (39°C, 5 % CO_2 in humidified air) with spermatozoa for 18–24 hr. Oocytes were mounted on slides under vaseline-supported coverslips. The oocytes were fixed in a bath with 1 vol acetic acid to 3 volumes ethanol (v/v) for 72 hr. Fixed oocytes were stained with 1 % (v/v) Lacmoid dye and viewed under ×100magnification. Proportions of Pen, PN, and SP/OC were calculated. Degenerated oocytes were excluded.

2.6. Statistical analysis

Data from 36 samples [6 males, 3 evaluation times (Fresh, PT 0 H, PT 1.5 H), 2 ejaculates per male] were analyzed. No apparent deviations from normality or homoscedasticity were detected. The assumptions were checked using diagnostic plots. This was done by creating scatterplots with the residuals against the dependent variables. A mixed model approach as implemented in the mixed procedure of the SAS system (SAS Institute Inc. 2017, Version 9.4.) was used. The fixed part of the models included animal, sample, and time, and their interaction. Animal nested within time was set as a random factor. Degrees of freedom were determined according to Gravance et al. (1997). Scheffe correction was applied. The MANOVA option was used in the multivariate GLM model to obtain partial correlations for the studied variables. The model included the fixed effects of animal and sample. Results are presented as Least square means (LSMeans) ± standard error of the mean (SEM). The alpha value for this experiment was chosen to be 5 % (the experiment is confident in 95 %) and *P* values were compared based on the selected alpha value. The *P* values and F values were taken into consideration, with $P < 0.05$ values being considered as statistically significant (based on selected alpha level). However, *P* values in a

range of 0.05–0.10 were considered to show a trend towards significance (Ntallaris et al., 2023).

3. Results

3.1. HBA binding results and proAKAP4

Dromedary camel spermatozoa were able to bind to hyaluronan. The results for the HBA binding (%) with fresh sperm are shown in Table 1, and for frozen thawed sperm in Table 2. The binding of fresh sperm varied between 63.0 % and 88.0 %. The frozen-thawed spermatozoa boundless, where the ratio varied between 6.0 % and 17.0 %. No significant differences in binding between the males were observed.

3.2. Conventional semen analyses

The conventional semen analysis results (TM, PM, VI, IA & MIT) and CASA kinematics (ALH, BCF, LIN, STR, VAP, VCL, VSL) from fresh semen samples are presented in Table 1. Other parameters measured in fresh samples (Vol, Vis, Conc, and MA) are shown in the Supplementary Table 1. There were no differences between males for conventional analyses and proAKAP4 results from fresh semen. Significant differences ($P \leq 0.05$) among males were found for some CASA kinematics (BCF, STR, and VCL).

There were no differences between the males in the TM, PM, VI, IA, MIT, HBA score or proAKAP4 at PT0H (Table 2). The CASA kinematics ALH, VAP and VCL varied significantly ($P \leq 0.05$) among males. For the conventional analyses (TM, PM, VI, IA, MIT) at PT1.5 H, there were no significant differences among the males (Table 3). However, LIN was significantly different ($P \leq 0.05$) between males.

3.3. Sperm penetration assay

The penetrating ability of camel spermatozoa was studied using the heterologous oocyte binding assay at PT0H. The camel spermatozoa bound, penetrated, decondensed, and completed pro-nucleus formation in goat oocytes. The PEN varied from 36.76 % to 55.60 % (48.13 ± 4.62), the PN varied from 3.41 % to 31.60 % (21.33 ± 5.45), and the SP/OC varied from 1.23 to 1.93 (1.70 ± 0.14).

3.4. Correlations

All corrections are shown in Supplementary Tables 2 and 3 in Supplementary material.

3.4.1. HBA correlations

No significant correlation was found between the HBA results and proAKAP4 for fresh samples. In fresh samples, significant correlations were found between the HBA and PM (r = 0.65, $P = 0.02$) and STR (r = 0.69, $P = 0.01$). A moderate but non-significant correlation was found between HBA and BCF (r = −0.50, $P = 0.09$), LIN (r = 0.51, $P = 0.08$), and viscosity (r = −0.49, $P = 0.10$). The HBA PT0H and proAKAP4 PT0H samples were negatively correlated (r = −0.62, P =0.03). The IA value in PT1.5 H was found to be correlated with HBA PT0H results (r = −0.56, P =0.05).

Table 1
Values (Means ±SEM) for dromedary camel sperm hyaluronan binding assay, precursor of A-kinase Anchoring Protein total and progressive motility, kinematics, viability, acrosome integrity and mitochondria activity in fresh samples (n=12).

	A	B	C	D	E	F	*P*
HBA	76 ± 0.10	87 ± 0.08	69 ± 0.18	63 ± 0.10	85 ± 0.03	88 ± 0.06	0.520
ProAKAP4	7.04 ± 3.54	25.04 ± 14.30	8.24 ± 2.75	15.19 ± 1.28	14.3 ± 2.24	4.92 ± 1.24	0.460
TM	71.85 ± 7.96	63.40 ± 7.96	57.10 ± 7.96	60.90 ± 7.96	41.50 ± 7.96	56.65 ± 7.96	0.290
PM	6.10 ± 2.50	12.65 ± 2.50	8.95 ± 2.50	8.65 ± 2.50	4.25 ± 2.50	16.65 ± 2.50	0.091
ALH	11.48 ± 0,54	10.17 ± 0.54	10.08 ± 0.54	10.89 ± 0.54	11.14 ± 0.54	9.17 ± 0.54	0.153
BCF	21.73 ± 0.51[a]	20.59 ± 0.51	18.97 ± 0.51	18.49 ± 0.51	20.90 ± 0.51	18.19 ± 0.51[b]	**0.012**
LIN	25.60 ± 1.75	28.52 ± 1.75	30.51 ± 1.75	29.34 ± 1.75	27.61 ± 1.75	35.46 ± 1.75	0.073
STR	59.09 ± 1.44[a, c]	67.82 ± 1.44	65.74 ± 1.44	64.29 ± 1.44	59.05 ± 1.44[c]	71.18 ± 1.44[b]	**0.013**
VAP	101.81 ± 6.64	81.82 ± 6.64	83.30 ± 6.64	90.14 ± 6.64	96.35 ± 6.64	80.86 ± 6.64	0.273
VCL	238.06 ± 11.94	195.46 ± 11.94	183.91 ± 11.94	201.05 ± 11.94	209.04 ± 11.94	165.18 ± 11.94	**0.053**
VSL	59.76 ± 5.50	55.18 ± 5.50	54.94 ± 5.50	57.41 ± 5.50	56.31 ± 5.50	57.56 ± 5.50	0.991
VI	61.75 ± 5.64	61.00 ± 5.64	61.00 ± 5.64	45.00 ± 5.64	35.50 ± 5.64	59.00 ± 5.64	0.074
IA	87.75 ± 5.19	84.75 ± 5.19	83.00 ± 5.19	79.25 ± 5.19	88.50 ± 5.19	80.00 ± 5.19	0.732
MIT	56.75 ± 11.71	55.25 ± 11.71	68.50 ± 11.71	48.00 ± 11.71	64.50 ± 11.71	61.50 ± 11.71	0.846

Different letters across rows indicate differences (P < 0.05). Hyaluronan binding assay (HBA, %), Precursor of A-kinase Anchoring Protein (ProAKAP4; ng/10 M spermatozoa), Total motility (TM, %), Progressive motility (PM, %), Average path velocity (VAP; μm/s), straight line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), beat cross frequency (BCF, Hz), viability (VI,%) and acrosome and mitochondrial integrity (IA and MIT, %).

Table 2
Values (Means ±SEM) for hyaluronan binding assay, precursor of A-kinase Anchoring Protein, dromedary camel sperm total and progressive motility, kinematics, viability, acrosome integrity and mitochondria activity at 0 H after thawing (n=12).

	A	B	C	D	E	F	*P*
HBA	12 ± 0.05	11 ± 0.04	6 ± 0.03	10 ± 0.01	16 ± 0.04	17 ± 0.07	0.670
ProAKAP4	14.3 ± 6.05	11.4 ± 6.98	14.72 ± 0.02	15.19 ± 1.28	8.44 ± 1.89	12.25 ± 0.43	0.720
TM	45.40 ± 7.62	38.50 ±7.62	40.55 ± 7.62	32.50 ± 7.62	27.70 ± 7.62	25.60 ± 7.62	0.479
PM	8.38 ± 12.65	4.70 ± 2.62	5.00 ± 2.62	7.05 ± 2.62	2.05 ± 2.62	2.90 ± 2.62	*0.568*
ALH	8.25 ± 0.24	7.07 ± 0.24[c]	8.15 ± 0.24	8.07 ± 0.24	9.37 ± 0.24[a]	7.43 ± 0.24[b,c]	**0.005**
BCF	19.02 ± 0.79	18.72 ± 0.79	18.65 ± 0.79	17.90 ±0.79	20.19 ± 0.79	20.07 ± 0.79	0.389
LIN	33.10 ± 1.53	30.07 ± 1.53	31.70 ± 1.53	33.12 ±1.53	26.68 ± 1.53	29.01 ±1.53	*0.127*
STR	62.89 ± 2.33	58.28 ± 2.33	62.91 ±2.33	65.74 ± 2.33	56.15 ± 2.33	57.92 ± 2.33	0.143
VAP	78.15 ± 3.89	61.55 ± 3.89	76.77 ± 3.89	76.56 ± 3.89	78.46 ± 3.89	61.03 ± 3.89	**0.043**
VCL	152.76 ± 7.25	123.40 ± 7.25[a]	152.74 ± 7.25	153.49 ± 7.25	170.90 ± 7.25[b]	125.60 ± 7.25	**0.020**
VSL	49.86 ± 3.83	36.54 ± 3.83	48.08 ± 3.83	49.74 ± 3.83	43.78 ± 3.83	35.66 ± 3.89	0.116
VI	37.00 ± 3.78	32.25 ± 3.78	30.50 ± 3.78	31.00 ± 3.78	23.00 ± 3.78	25.50 ± 3.78	*0.258*
IA	56.00 ± 7.29	55.00 ± 7.29	47.50 ± 7.29	42.50 ± 7.29	42.00 ± 7.29	37.50 ± 7.29	0.465
MIT	61.00 ± 7.49	45.25 ±7.49	51.00 ± 7.49	35.50 ± 7.49	30.50 ± 7.49	27.00 ± 7.49	0.104

Different letters among columns indicate differences ($P < 0.05$). Average path velocity (VAP; μm/s), straight line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head displacement (ALH, μM), straightness (STR, %), linearity (LIN, %), beat cross frequency (BCF, Hz), viability (VI,%) and acrosome and mitochondrial integrity (IA and MIT, %).

Table 3
Values (Means ±SEM) for dromedary camel sperm total and progressive motility, kinematics, viability, acrosome integrity and mitochondria activity at 1.5 H after thawing (n=12).

	A	B	C	D	E	F	*P*
TM	33.85 ± 9.18	30.20 ± 9.18	29.60 ± 9.18	20.30 ± 9.18	22.30 ± 9.18	18.80 ± 9.18	0.800
PM	3.9 ± 1.5	1.8 ± 1.5	3.00 ± 1.5	1.40 ± 1.50	1.35 ± 1.50	0.65 ± 1.50	0.675
ALH	8.08 ±0.30	7.2 ± 0.3	7.83 ± 0.30	7.88 ± 0.30	8.75 ± 0.30	7.22 ± 0.30	0.072
BCF	19.76 ± 1.02	19.65 ± 1.02	17.85 ± 1.02	18.86 ± 1.02	20.25 ± 1.02	20.81 ± 1.02	0464
LIN	29.04 ± 1.00	26.20 ±1.00	31.13 ± 1.00[a]	29,34 ±1,75	25.61 ± 1.00[b]	29.47 ± 1.00	**0.045**
STR	61.05 ± 1.88	52.72 ± 1.88	61.36 ± 1.88	60.53 ± 1.88	57.93 ± 1.88	62.75 ± 1.88	0070
VAP	66.66 ± 4.93	54.39 ± 4.93	63.58 ± 4.93	62.54 ± 4.93	66.71 ± 4.93	55.54 ± 4.93	0.410
VCL	146.07 ± 8.61	115.27 ± 8.61	130.59 ± 8.61	129.91 ± 8.61	156.22 ± 8.61	127.00 ± 8.61	0.115
VSL	40.58 ± 3.30	28.76 ± 3.30	39.22 ± 3.22	37.18 ± 3.30	37.48 ± 3.30	33.54 ± 3.30	0.263
VI	38.00 ± 4.67	36.00 ± 4.67	30.50 ± 4.67	21.50 ± 4.67	24.00 ± 4.67	23.50 ± 4.67	0.177
IA	49.50 ± 7.01	58.25 ± 7.01	44.50 ± 7.01	36.50 ± 7.01	37.50 ± 7.01	45.00 ± 7.01	0.367
MIT	44.00 ± 5.73	42.50 ± 5.73	31.50 ±5.73	32.50 ± 5.73	28.50 ± 5.73	21.00 ± 5.73	0.179

Different letters among columns indicate differences ($P < 0.05$). Average path velocity (VAP; μm/s), straight line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head displacement (ALH, μM), straightness (STR, %), linearity (LIN, %), beat cross frequency (BCF, Hz), viability (VI,%) and acrosome and mitochondrial integrity (IA and MIT, %).

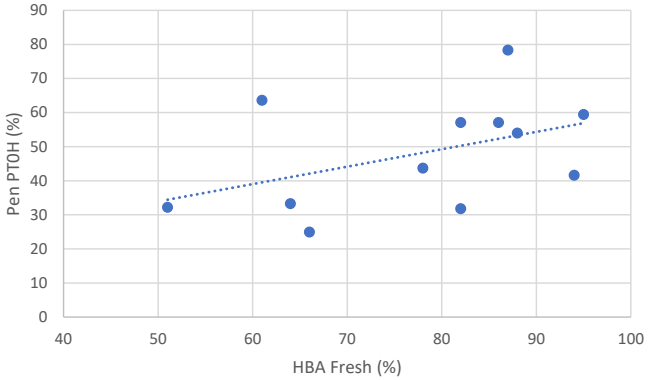

Fig. 1. Moderate but non-significant correlation between Hyaluronan Binding Assay (HBA; %) results for fresh dromedary camel semen and oocyte penetration rate values (Pen, %) in the Sperm Penetration Assay (SPA) with zona-free goat oocytes (n=12) in post-thawed samples. The trendline is illustrated by a dashed line ($r = 0.52$; $P = 0.07$).

3.4.2. Freezability study: correlations between HBA result in fresh samples and different post-thaw values

The correlations between HBA results in fresh samples and post-thaw (PT0H and PT1.5 H) values of different attributes (TM, PM, VI, AI, MIT, PEN, PN, and SP/OC) were analyzed to evaluate the ability of the HBA to predict freezability. There were no significant correlations between HBA PT0H results and conventional semen parameters (TM, PM, VI, AI, MIT, Vol, Vis, Con and MA). Only a moderate but not significant correlation was found between HBA results from fresh semen and Pen ($r = 0.52$; $P = 0.07$) (Fig. 1).

3.4.3. Other correlations

ProAKAP4 showed a negative correlation with VSL ($r = -0.59$, $P = 0.03$) in fresh samples. Interestingly, viscosity showed correlations with several kinematics: ALH ($r = 0.64$, $P = 0.02$), BCF ($r = 0.61$, $P = 0.03$), LIN ($r = -0.69$, $P = 0.01$), STR ($r = -0.60$, $P = 0.03$) and VCL ($r = 0.67$, $P = 0.01$). Morphological abnormalities showed negative correlations with TM ($r = -0.69$, $P = 0.01$) and VI ($r = -0.71$, $P = 0.008$), although VI showed a positive correlation with TM ($r = 0.73$, $P = 0.006$). Some other correlations among kinematics can be observed in the tables in Supplementary material.

4. Discussion

A fast and accurate test that assesses sperm quality and predicts the fertilizing capacity and freezability of camel sperm would increase the efficiency and success of assisted reproduction in this species. In this study, the prognostic value of HBA was assessed for the first time for dromedary camel sperm; possible associations between the HBA result, conventional parameters of sperm quality, the proAKAP4 test, and in vitro fertilization ability were studied.

Our first finding was that dromedary camel spermatozoa bound to the HBA slides used for human sperm and other species. The fresh sperm samples showed binding of 63–88 %, which is a higher proportion than reported with stallion spermatozoa (11–24 %) (Colleoni et al., 2011) or human spermatozoa (28–89 %) (Ye et al., 2006). The reason for the higher HBA binding of dromedary camel sperm compared to the rates reported for other species is not known. It is possible that epididymal passage in the dromedary camel is longer than in some other species, such as the stallion or human, thus allowing more time for plasma membrane re-modelling and the formation of hyaluronan binding sites. This suggestion is speculation since there is a paucity of information on epididymal transit time in dromedary camels. In any species epididymal transit time may be influenced by extraneous factors such as season and frequency of ejaculation, which are not necessarily reported in studies on HBA. We can also speculate that dromedary camel sperm may be more mature when ejaculated than the sperm of other species due to the ejaculate characteristics. Dromedary camel semen is characterised by a low volume and low sperm concentration, as well as the viscous seminal plasma. These factors contribute to fewer sperm being available for potential fertilization when the ejaculate is deposited in the female, possibly necessitating a degree of membrane maturity that facilitates binding to the zona pellucida to ensure reproductive efficiency. Since the camel is an induced ovulator, the time from ejaculation to potential fertilization is well-defined, which might also necessitate a more advanced state of maturity in the sperm leaving the epididymis than in species that are spontaneous ovulators where the time spent in the sperm reservoirs in the female can be prolonged. The observation that thawed dromedary camel epididymal sperm achieved better results in IVF than thawed ejaculated sperm suggests that seminal plasma may have an inhibiting effect on sperm binding (Scholkamy et al., 2016), although it should be noted that the epididymal and ejaculated sperm samples came from different males. Frozen-thawed camel spermatozoa showed significantly less binding than fresh spermatozoa (6–17 %), and also less binding than frozen-thawed Nili-Ravi buffalo spermatozoa (27–57 %) (Awan et al., 2021). Low post-thaw binding was expected, since dromedary camel spermatozoa do not tolerate the freezing-thawing procedure well (Morton et al., 2010). Apart from the lack of an optimised protocol, the viscous seminal plasma of the camel (as previously mentioned) hinders the distribution of cryoprotectant within the sample and its penetration into the sperm. Other factors potentially affecting the low binding rate could be interference from the semen extender, sperm sample, and incubation time, as suggested in previous studies on stallion spermatozoa (Colleoni et al., 2011).

During cryopreservation, the sperm membrane becomes less flexible due to lipid phase changes and redistribution of cholesterol (Yeste, 2016), thus distorting membrane channels and receptors. It is, therefore, possible that the hyaluronan receptor in the membrane of thawed dromedary camel sperm is physically unable to bind to hyaluronan, despite the ability of the same receptor to bind in fresh sperm. This possibility could explain why fresh dromedary camel sperm bound well in the HBA whereas thawed dromedary camel sperm from the same individuals did not. Another source of cryodamage could be due to the size of the sperm head, which is relatively small in the dromedary camel compared to other species (O`Brien et al., 2022) Alternatively, the presence of a higher proportion of egg yolk in the cryopreservation medium than in the Tris buffer medium for fresh sperm could have interfered with binding in the HBA for cryopreserved sperm. Further work is needed to optimise the cryopreservation of dromedary camel sperm, but once improved protocols are established it would be interesting to try the HBA again with thawed sperm. Similar studies with other membrane receptors, such as aquaporin 3, also did not reveal any association with freezability in dromedary camel sperm (O`Brien et al., 2022).

The HBA did not show any important correlation with conventional parameters of sperm quality other than PM and the CASA kinematic STR for fresh spermatozoa. Other authors have reported the HBA to be able to select human spermatozoa with high PM and TM (Rashki Ghaleno et al., 2016). It was suggested that only motile spermatozoa with normal morphology can bind to hyaluronan (Rashki Ghaleno et al., 2016). Our study, however, did not find either VAP or TM to be significantly associated with HBA results. Since the HBA detects mature spermatozoa, a stronger correlation to some conventional parameters of sperm quality would have been expected, especially as higher V, TM and PM, MIT, and non-fragmented DNA have been associated with sperm maturity (El-Badry et al., 2015). Motility and morphology of human spermatozoa were associated with the HBA score (Ye et al., 2006; Rashki Ghaleno et al., 2016). A study with Nili-Ravi buffalo spermatozoa indicated a strong association between post-thaw HBA results and

conventional parameters including PM, VI, and IA (Awan et al., 2021). On the other hand, a study on human spermatozoa found no relationship between HBA score, fertility, and conventional semen parameters (Esterhuizen et al., 2015). However, in the latter study, fertility was assessed with ICSI, for which sperm motility, morphology and zona binding with IA would not have been essential for fertilization to occur. It is also not known if the HBA score correlates with other conventional parameters for camel spermatozoa not included in this study.

In this study, the relationship between HBA result and fertilizing capacity was established from the SPA trial using goat oocytes. The SPA method was previously reported to demonstrate the ability to assess camel sperm function in-vitro (Crichton et al., 2016) but has not been used to predict the correlation with in vivo fertility due to lack of optimised protocols for artificial inseminaiton in camels (Skidmore et al., 2018). The HBA results from fresh samples and SPA results after thawing showed a moderate but not significant correlation for Pen. The PN and SP/OC showed no association with HBA score in fresh samples. These results are pioneering in camels and in other species as the correlation of HBA in fresh samples and in vitro fertilization ability of thawed sperm (as a predictor of freezability) has not been reported before in any species. Only previous studies on human spermatozoa have shown that a high HBA score in fresh samples was associated with improved fertility of the fresh sample in vitro (Worrilow et al., 2013; Breznik et al., 2013) and in vivo (Szucs et al., 2019). It should be noted that sperm-oocyte fusion in the goat oocyte penetration assay differs from the physiological situation with homologous material, as the zona pellucida is removed to allow heterologous sperm penetration. The assay also requires the spermatozoa to undergo spontaneous acrosome reaction when incubated for prolonged periods in vitro. It is, thus, possible that the results may not align with clinical IVF performance; further studies are needed to explore this possibility.

Correlations between proAKAP4 test results and conventional parameters of sperm quality, sperm physical parameters, or SPA results were observed only for VSL. The result was unexpected since a recent study reported that dromedary camel spermatozoa express proAKAP4, and the test results were strongly associated with vol, TM, and viscosity (Malo et al., 2021). It is not known why the results of the present study differ from the previous one from the same research group, since the methods and some of the observers were the same in both cases. The males were different, indicating possible male-male variation.

To establish the usefulness of the HBA test in evaluating dromedary camel sperm quality, further investigations with a larger sample size are required and an in vivo fertility study would be recommended. While the heterologous SPA used in this study provides an indication of sperm functionality, it does not provide a true representation of events in vivo or even in a homologous zona binding assay, since the zona is removed from the oocytes to allow penetration in the SPA. It does, however, provide an indication that some sperm function is retained in these sperm samples (Crichton et la.2016). Availability of camel oocytes is scarce because camel meat is not consumed in United Arab Emirates so discarded ovaries cannot be obtained in slaughter houses, thus limiting the possibility of performing an in vitro fertilization trial, and camel artificial insemination is, at present, in its infancy (Skidmore et al., 2018), requiring optimization of protocols before it can be considered reliable as a means of assessing sperm function. The SPA, therefore, although not ideal, is the best option for sperm function assessment available for our work at the present time.

5. Conclusions

In the present study, it was shown for the first time that the HBA can be used with dromedary camel spermatozoa. The assessment of HBA in fresh samples, however, did not show the potential to predict the fertilizing capacity of dromedary camel sperm after thawing. In the conventional analyses, only progressive motility ($r = 0.65$; $P = 0.02$) and straightness correlated with the HBA result for fresh semen ($r = 0.69$; $P = 0.01$).

CRediT authorship contribution statement

J.A. Skidmore: Resources, Funding acquisition. **Clara Malo:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **A Kotila-Ioannou:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **JM Morrell:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **T Ntallaris:** Formal analysis. **M Gargallo:** Writing – original draft, Data curation.

Declaration of Competing Interest

No conflict of interest to declare.

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