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Summary

The objective of this study was to assess the potential of Hyaluronan Binding Assay (HBA) to predict freezability and fertilization capacity of dromedary camel spermatozoa, and to determine if conventional sperm quality parameters and precursor of A-Kinase Anchoring Protein 4 (proAKAP4) values correlate with HBA results.

Two semen samples were collected from each of six male camels. In experiment 1, the samples were assessed fresh (fresh), pre-freezing (PF) and post-thawing at 0 h (PT0H), and post-thawing at 1.5 h (PT1.5H). HBA slides coated with molecular layer of hyaluronan were used together with a proAKAP4 biomarker-test to validate semen quality of fresh samples and PT0H. Total motility (TM), Progressive motility (PM), motility-related kinematics, morphological abnormalities (MA), vitality (VIT), intact acrosome (IA) and mitochondrial membrane potential (MIT) were evaluated in fresh and PT0H and PT1.5H sperm to find possible correlations with HBA test results. In experiment 2, fertilizing ability of PT0H sperm was evaluated with a heterologous sperm penetration assay (SPA) using zona pellucida free goat oocytes resulting in values for number of oocytes (N), penetration rate (PEN), formation of male pro-nucleus (PN) and number of sperm penetrated per oocyte (SP/OC).

The results showed that dromedary camel spermatozoa bound to hyaluronan; there were no significant differences between males. However, there was no correlation between HBA in fresh sample and PT0H results. Furthermore, the proAKAP4 test results also did not differ between males and did not correlate with HBA results, with the exception of PT0H HBA and proAKAP4 results ($r = -0.62$; p-value 0.03). Of the conventional semen analysis parameters (TM, PM, VIT, IA, MIT, Volume, Viscosity, Concentration and MA) none were significantly different between males and only PM correlated with the HBA results in fresh sperm $r = 0.65$; (p-value 0.02). From LSMean values of computer-assisted semen analysis (CASA) kinematics of fresh spermatozoa, beat cross frequency (p-value 0.01), straightness (p-value 0.01) and curvilinear velocity (p-value 0.05) showed significant variation between males. From the PT0H samples, the lateral head deviation (p-value 0.01), average path velocity (p-value 0.04) and curvilinear velocity (p-value 0.02) varied between males, as did PT1.5H linearity (p-value 0.05). The kinematic straightness correlated with the HBA result for fresh semen ($r = 0.69$; p-value 0.01). The analysis for freezability was done using HBA results (fresh and PT0H) and post thaw values of different parameters (TM, PM, VIT, IA, MIT, PEN, PN and SP/OC); no significant correlations were found. In the SPA, dromedary camel sperm bound, penetrated, decondensed, and completed pro-nucleus formation in goat oocytes. Significant correlations were identified between fresh sperm HBA result and PEN (p-value 0.03) as well as with PN (p-value 0.03). The proAKAP4 test results from fresh sperm samples and PT0H results did not correlate with penetration assay results. There was an association between proAKAP4 and HBA at PT0H. In conclusion, the results suggested that the HBA score from fresh dromedary camel sperm may predict post-thaw IVF performance, but further investigation is needed.

Keywords: Dromedary camel sperm, freezability, semen analysis, sperm hyaluronan-binding assay.

Sammanfattning

Syftet med denna studie var att bedöma potentialen för Hyaluronan Binding Assay (HBA) att förutsäga frysbarhet och befruktningskapacitet hos dromedarspermier och att fastställa om konventionella spermiekvalitetsparametrar och prekursorn för A-Kinase Anchoring Protein 4 (proAKAP4) -värden korrelerar med HBA-resultaten.

Två spermaprov samlades från var och en av sex stycken dromedarer. I experiment 1 analyserades proverna som färska (fresh), innan frysning (PF), efter upptining vid 0 timmar (PT0H) och efter upptining vid 1.5 timme (PT1.5H). HBA-objektsglas med en hinna av hyaluron användes tillsammans med ett proAKAP4-biomarkertest för att validera spermiekvalitet i färska och PTH0-prov. Total motilitet (TM), progressiv motilitet (PM), motilitetsrelaterad kinematik, morfologiska abnormiteter (MA), vitalitet (VIT), intakt akrosom (IA) och mitokondriemembranspotential (MIT) undersöktes i färska spermier, i PT0H och i PT1,5H för att finna möjlig korrelation med HBAresultaten. I experiment 2 analyserades befruktningskapaciteten för PT0H-spermier med en heterolog spermiepenetrationsanalys (SPA) med getoocyter utan zona pellucida, vilket resulterade i värden för antal oocyter (N), penetrationshastighet (PEN), bildning av hanpronukleus (PN) och antal spermiepenetrationer per oocyt (SP/OC).

Resultaten visade att dromedarspermier binder till hyaluron; det fanns inga signifikanta skillnader mellan hanar. Det fanns det ingen korrelation mellan HBA i färska spermier och PT0H-resultaten. Vidare korrelerade inte proAKAP4-testresultaten med HBA-resultaten, med undantag av PT0H HBA- och proAKAP4-resultaten (r = - 0.62; p-värde 0.03). I de traditionella spermiekvalitetsparametrarna (TM, PM, VIT, IA, MIT, volym, viskositet, koncentration och MA) fanns inga signifikanta skillnader mellan hanarna och endast PM korrelerade med HBA-resultaten i färska spermier (r=0.65; p-värde 0.02). Från medelvärden av minstakvadratmetoden (LSMean) från en datorassisterad spermieanalys (CASA) visade följande värden signifikant variation i kinematiken i färsk sperma mellan hanar; korsfrekvens (p-värde 0.01), rakhet (p-värde 0.01) och kurvilinjär hastighet (p-värde 0.05). I proverna från PT0H varierade den laterala huvudavvikelsen (p-värde 0.01), medelvägshastighet (p-värde 0.04) och kurvilinjär hastighet (p-värde 0.02) mellan hanarna, så även PT1.5H linearitet (p-värde 0.05). Den kinematiska rakheten korrelerade med HBA-resultaten för färsk spermier (r=0.69, p-värde 0.01). Analysen för frysbarhet gjordes genom att använda HBA-resultaten (färska spermier och PT0H) och värden efter upptining för olika parametrar (TM, PM, VIT, IA, MIT, PEN, PN och SP/OC); inga signifikanta korrelationer kunde påvisas. I spermiepenetrationsanalysen kunde det konstateras att dromedarspermierna fäste, penetrerade, dekondenserade och fullbordade pronukleusformation i getoocyter. I färska spermier sågs signifikant korrelation mellan HBA-resultaten och PEN (p-värde 0.03) liksom med PN (p-värde 0.03). ProAKAP4-testresultaten från färska spermier och PT0H resultaten korrelerade inte med spermiepenetrationsanalysen. Det fanns ett samband mellan proAKAP4 och HBA vid PT0H. Sammanfattningsvis antydde resultaten att HBA-värden från färska dromedarspermier eventuellt kan förutse IVF-prestanda efter upptining, men vidare studier behövs.

Nyckelord: Dromedarsperma, frysbarhet, spermaanalys, spermie hyaluron bindning assay

Content

Abbreviations

AI	Artificial insemination
AKAP4	A-Kinase Anchoring Protein 4
ALH	Amplitude lateral head
BCF	Beat cross frequency
CASA	Computer-assisted semen analysis
COC	Cumulus oocyte complex
Conc	Concentration
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EN	Eosin-nigrosin stain
F1	Fraction 1
FCS	Foetal calf serum
FITC-PNA	Fluorescent isothiocyanate-conjugated peanut agglutinin
GB	Green buffer
HBA	Hyaluronan binding assay
HSP70	70 kilodalton heat shock protein family
HspA2	Extensive heat shock proteinA2
IA	Intact acrosome
ICSI	Intracytoplasmic sperm injection
LIN	Linearity
LSMean	Least-Squares Means
MA	Morphological abnormalities
PBS	Phosphate-buffered saline
PEN	Penetration rate
PI	Propidium iodide
PN	Male pro-nucleus
proAKAP4	Precursor of A–kinase anchor protein 4
R123	Rhodamine-123 dye
SEM	Standard error of mean
SY	Fluorescent probes SYBR-14
TCF	Tris-citrate-fructose

TM	Total motility
SLC	Single layer centrifugation
STR	Straightness
SPA	Sperm penetration assay
SP/OC	Number of sperm penetrated per oocyte
TCF	Tris-Citrate-Fructose Buffer
VAP	Average path velocity
VCL	Curvilinear velocity
Vis	Viscosity
VIT	Vitality
Vol	Volume
VSL	Straight line velocity

1. Introduction

Assisted reproduction technologies, including artificial insemination (AI), are important techniques in the improvement of production qualities and reproduction success of commercial camel production (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 superior males, animals would not have to be transported for mating, risk for the spread of infectious disease would be lower, reproductive lifespan would be extended and sperm samples could be transported around the world (Skidmore et al. 2018).

Dromedary camel semen characteristics have posed a great challenge for 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. 2017b).

Animal breeding soundness examination traditionally includes conventional semen analysis, it is a complex method that does not indicate the critical biochemical events that occur during the fertilization process (Suarez & 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 deployed at 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 have also reported HBA to have potential in predicting fertilization capacity in animal reproduction; Chun et al. (2005) found HBA score to associate with higher production rate on porcine embryos with normal chromosomal complement; Colleoni et al. (2011) reported some potential for assessing stallion fertility and Awan et al. (2021) found that the HBA test was capable of predicting the fertility of frozen-thawed Nili-Ravi buffalo bull spermatozoa (2021).

The HBA test 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). HA is a linear polysaccharide found in the extracellular matrix of cumulus oophorus covering the oocyte and has been observed to have critical role in the sperm selection during both *in vivo* (Redgrove et al. 2013) and *in vitro* fertilization (Worrilow et al. 2013). Formation of the binding sites for HA during sperm maturation is regulated by plasma membrane remodelling processes that eliminate the surplus cytoplasm and remodel the sperm plasma membrane. These spermiogenetic processes include development of the acrosome and tail growth (de Kretser et al. 1998; Huszar et al. 1997, 2000). Those spermatozoa binding to HA have completed the spermiogenetic process, having intact acrosomes, high vitality and transition of nuclear histone–to–protamine (Huszar et al. 2003; Nasr-Esfahani et al. 2008). They are also devoid of cytoplasmic retention, are of normal morphology, and have lower frequency of DNA fragmentation and chromosomal aneuploidies than spermatozoa that do not bind to HA (Huszar et al. 1997, Kovanci et al.2001).

Bound spermatozoa also express lower levels of apoptotic markers such as caspase 3 and cytoplasmic retention marker creatine phosphokinase (CK) (Cayli et al. 2004). Another important sperm maturity marker is testis-specific heat shock proteinA2 (HspA2) that belongs to the 70 kilodalton heat shock protein family (HSP70) (Ergur et al. 2002). It has been reported that HA-bound, more mature spermatozoa have higher expression of proteins from HSP70 family (Cayli et al. 2004). 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).

A novel study on Nili-Ravi buffalo spermatozoa reported that the HBA score was correlated with sperm freezability potential, which could be connected to the maturity level of the spermatozoa (Yogev et al. 2010). Thus, the HBA score might be associated with the expression level 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 (bulls; Peddinti et al. 2008, stallions; Blommaert et al. 2019, boars; Sergeant et al. 2019, rams; Riesco et al. 2020) and also identified in camel correlating with HBA results (Malo et al. 2021).

1.1. Purpose

The purpose of this study was to assess the potential of the HBA test to predict fertilization capacity and freezability of dromedary camel semen, and to determine if conventional sperm quality parameters and proAKAP4 values correlate with HBA results.

2. Literature review

2.1. The dromedary camel

The dromedary camel (*Camelus dromedaries*, figure 1*)* is the one-humped camel belonging to the suborder Tylopoda and family Camelidae. Together with the two-humped Bactrian camel (*Camelus bactrianus*), they are the predominant species of the genus *Camelus*, and are also known as Old World camelids (Schoch et al. 2020).

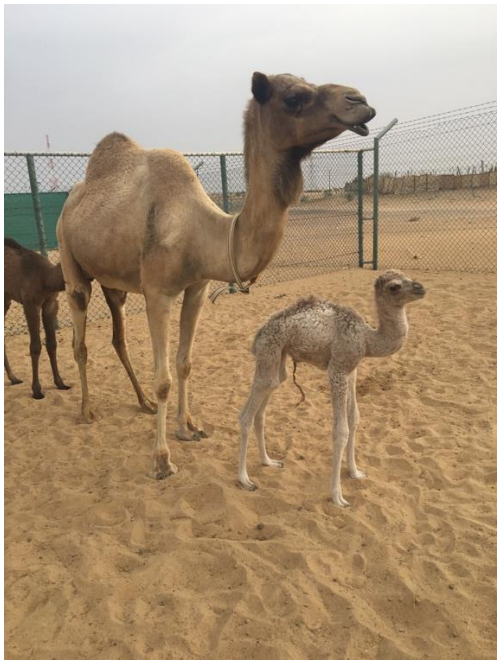

Figure 1. Dromedary camel with its newborn calf (on right) born from cryopreserved sperm at Camel Reproduction Center, Dubai, United Arabs Emirates (Picture by Anna Kotila-Ioannou 2019)

2.1.1. Semen characteristics

The physical and biological characteristics of camel semen vary greatly depending on the conditions of the semen collection, but are also affected by individual

animals and the collection frequency (Tibary et al. 2014; Al-Bulushi et al. 2018; Malo et al. 2019). Ejaculates are often of poor quality, varying even in different collections from the same individual, which poses a significant problem for developing reproductive processing and techniques (Bravo et al. 2000).

An important and challenging characteristic of camel semen is that it is extremely viscous. The degree of viscosity varies among males and is affected by the semen collection interval (Bravo et al. 2000). Viscosity is associated with seminal plasma, a secretion from the bulbourethral glands and the prostate (Tibary & Anouassi 1997), which is evenly distributed throughout the ejaculate (Skidmore et al. 2018). The seminal plasma traps spermatozoa into a fibrinous network, immobilizing them; this gel makes assessment and processing difficult unless it is possible to liquefy the ejaculate (Wani et al. 2011).

It was initially suspected that glycosaminoglycans (GAGs) secreted by the prostate or bulbourethral gland (Cowper's gland) were responsible for the seminal plasma viscosity (Mosaferi et al. 2005). However, GAG-degrading enzymes did not completely eliminate the semen viscosity from alpaca semen, suggesting that GAGs might not be the cause (Kershaw-Young et al. 2013). Enzymatic treatments with the proteases papain and proteinase K resulted in complete liquefication, indicating that proteins might be responsible for the viscosity. A possible candidate was a protein related to a large gel-forming mucin 5B protein secreted by glandular epithelial cells (Kershaw-Young & Maxwell 2012).

Camel ejaculates must be liquefied to be able to evaluate and process the semen. Enzymatic treatments can be effective, but they can also have deleterious effect on the spermatozoa. Monaco et al. (2016) found that papain-treated samples had elevated sperm head agglutination, Bravo et al. (2000) reported a decrease in the proportion of live spermatozoa and Ghoneim et al. (2010) observed a decrease in sperm vitality after enzyme treatment. Several studies reported spontaneous liquefication if semen was incubated at 30–37 °C for 15–30 min (Al-Qarawi et al. 2002; Wani et al. 2008), but the liquefication was only partial. Malo et al. (2016) suggested that semen dilution with Tris-Citrate-Fructose extender to 1:5–1:10 ratio, adjusted according to the viscosity level, combined with gentle pipetting for 30-60 min, liquefied the semen to the process-ready stage without compromising sperm motility and acrosome integrity.

Besides being extremely viscous, camel ejaculates are generally low in volume and have a low sperm concentration (Kershaw-Young & Maxwell 2012; Skidmore et al. 2018).

2.2. Artificial insemination

Figure 2. Semen collection with artificial vagina and teaser female. Dromedary camels mate in sternal recumbency (Picture by Anna Kotila-Ioannou 2019)

In recent decades, interest has grown in producing camels with improved genetic traits such as higher milk yield, meat quality, and better athletic abilities (Skidmore et al. 2013). However, the application of AI in camelids has proven to be challenging. Semen collection is difficult because of the camelids´ special mating behavior and its seasonality. Thus, camels only mate during the cooler season, mating takes place in sternal recumbency (figure 2), and ejaculation duration is long, varying from 5- to 20 minutes (Skidmore et al. 2013). Female camelids have induced ovulation that needs to be carefully timed for AI, and deep intrauterine insemination is recommended to improve pregnancy rates (Skidmore et al. 2013; Skidmore et al. 2018). Chilled semen should be inseminated within 72 hours of collection (Sieme et al. 1990); the option of using cryopreserved semen would place fewer constraints on AI. Despite relatively good post-thaw motilities that have been achieved with cryopreserved semen, fertilization success with this material has been poor (Anouassi et al. 1992; Skidmore et al. 2000; Morton et al. 2010). One of the major determinates of successful AI with frozen spermatozoa would be the adoption of a standardized protocol which optimizes sperm evaluation methods, diluent, packaging and the rates of freezing and thawing (Skidmore et al. 2013; Malo et al. 2018).

2.3. Cryopreservation

Cryopreservation involves controlled cooling, freezing and storage of spermatozoa. It is the best preservation technique for long-term storage of gametes (Skidmore et al 2013). Cells that are deep-frozen in liquid nitrogen (- 196°C) can be preserved almost indefinitely and transported around the world without time constraints (Skidmore et al. 2013). Cryopreserved semen enables selective breeding with short generation intervals, and the semen can be available without having the male present in the same location as the female (Holt & Penfold 2014).

The main impediments to cryopreservation of dromedary semen are that spermatozoa do not tolerate the freezing and thawing procedure well and the ejaculates are often poor in quality (Bravo et al. 2000; Morton et al. 2010; Crichton et al. 2015, Malo et al. 2017b). The viscous seminal plasma could contribute to low sperm cryosurvival (Malo et al. 2017b). It is possible that cryoprotectants and extender are not homogenously mixed because of the viscous seminal plasma, and are prevented from accessing the spermatozoa. Therefore, the combination of liquefaction efficiency of the seminal plasma and optimal cryoprotectant in the extender is extremely important for cryosurvival (Malo et al. 2018; Skidmore et al. 2018). Despite the improvements in freezing techniques and sperm processing, sperm freezability is still unpredictable (Yogev et al. 2010).

The poor quality of the semen can be addressed with sperm selection techniques. Malo et al. (2017a) used a silane-coated silica colloid in single layer centrifugation (SLC) to select live spermatozoa prior to freezing. The SLC treatment selected spermatozoa that showed improved cryosurvival and also improved in-vitro fertilization capacity (Malo et al. 2017b; Malo et al. 2018). However, it would be beneficial to be able to evaluate the potential freezability of the semen before cryopreservation to choose the spermatozoa that will tolerate preservation and retain their fertilizing capacity.

2.4. Prediction of freezability

Within-species variation of sperm freezing tolerance was demonstrated in a number of animals: mice (Songsasen & Leibo 1997), dogs (Yu et al. 2002), rhesus monkeys (Leibo et al. 2007), pigs (Thurston et al. 2002), and stallions (Ortega-Fergusola et al. 2009). Variability is significant even between the ejaculates from the same individuals (Waterhouse et al. 2006). The underlying mechanisms that cause such variations are unknown and conventional parameters for predicting sperm freezability have proven to be unreliable (Suarez & Pacey 2006; Casas et al. 2009).

Various reasons for variation in sperm freezability have been studied. Songsasen & Leibo (1997) demonstrated that mice from different genetic lines had differences in post-thaw sperm motility, suggesting a possible genetic basis for the variation in post-thaw sperm quality. Genetic markers, claimed to predict sperm freezability, have been reported in boars and goats (Thurston et al. 2002; Nikbin et al. 2014). Molecular markers such as sperm proteins, and seminal plasma proteins, including membrane channels and heat shock proteins, have been identified in boar semen (Rickard et al. 2015). It has also been reported that extensive heat shock protein (HspA2) expression is correlated with sperm maturation (Ergur et al. 2002).

The HBA test (CooperSurgical Fertility Solutions 2022) was introduced for assessing sperm quality by screening maturity, structurally normal morphology and high deoxyribonucleic acid (DNA) integrity of human spermatozoa. Yogev et al. (2010) assessed the prognostic value of the HBA test to predict freezability of human sperm. The results suggested that the predictive value of HBA for high freezability was significant (p-value 0.024), considering post-thaw motility $\geq 40\%$ to indicate good sperm cryosurvival. However, the study found that other conventional parameters such as motility, normal morphology and concentration had higher prognostic value than the HBA score. The authors concluded that sperm maturation may not be the most relevant parameter when it comes to sperm freezability (Yogev et al. 2010).

El Badry et al. (2015) studied camel sperm freezability from samples at different stages of maturity. Spermatozoa were obtained from the caudal epididymis, which is considered to be primarily storage for mature spermatozoa, and from caput and corpus regions of epididymis, where the spermatozoa are less functional and less morphologically mature (Glover & Nicander 1971). Caudal spermatozoa had significantly higher post-thaw vitality, total and progressive motility, mitochondrial activity, and non-fragmented DNA than caput and corpus epididymal spermatozoa. Caudal sperm also performed significantly better in in-vitro fertilization in trials of both fresh and frozen sperm samples, suggesting that mature spermatozoa have better freezability than immature spermatozoa.

2.5. Conventional semen evaluation

Sperm evaluation prior to AI or as part of a breeding soundness examination is done by macroscopic assessment of volume, colour, and viscosity of semen followed by microscopic evaluation, such as sperm concentration, motility, morphological features, viability, mitochondrial activity and acrosome integrity.

The volume of camel ejaculates varies between 2 and 10 mL (Skidmore et al. 2013) and is usually measured with a graduated collection tube. The colour of dromedary camel semen depends on the sperm concentration and proportion of seminal plasma but is generally milky white (Tibary et al. 2014; Skidmore et al. 2018). Viscosity is usually evaluated by measuring the strand (4-8 cm) that forms when sample is lifted from a glass slide using a pipette (thread test; Tibary & Anouassi 1997).

The normal range for sperm concentration is from 200 to 300 × 10^6/mL (Anouassi et al. 1992). Semen collection frequency affects sperm quality; once-a-week collection is recommended over a twice-a-week regimen, to maintain acceptable concentration and motility (Al-Bulushi et al. 2018). Sperm motility is one of the most important attributes of fertile spermatozoa. Motility assessment can be a highly subjective estimation with reduced predictive value if it is performed by visual microscopic examination (Lee et al. 1988). More precise assessment can be achieved with computer-assisted sperm analysis (CASA), a system that calculates several motility parameters of spermatozoa such as velocity and sperm head movement patterns. Thus, velocity average path (VAP; μm/s), velocity straight line (VSL; μm/s), velocity curvilinear (VCL; μm/s), amplitude of lateral head deviation (ALH, μM), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz) are evaluated. Although CASA evaluation has been used in a number of studies on camel sperm, no correlation to fertility has been found yet (Malo et al. 2016; Malo et al. 2017a & b; Malo et al. 2018).

Camel spermatozoa have the same anatomical features as other domestic mammals, and the same abnormalities can be found in morphological evaluation (Tibary & Anouassi 1997). Defects are located in head, midpiece and tail, and include proximal and distal cytoplasmic droplets (Tibary et al. 2014). An elevated proportion of spermatozoa with abnormal morphology has been reported to result in reduced fertility (Chandler et al. 1988). Cryoinjury can result in a decreased proportion of normal sperm, which could explain the low post-thaw fertility rate (Gravance et al. 1997). Damage to the acrosome is especially relevant, since it prevents fertilization (Saacke & White 1972).

Sperm vitality is measured by dye exclusion from spermatozoa having an intact membrane (Skidmore et al. 2013). Sperm mitochondria are one of the cell organelles most readily damaged during cryopreservation, resulting in loss of motility and fertility (Ball 2008; Pena et al. 2003; Gonzalez-Fernandez et al. 2012). Mitochondria play a major role in sperm energy production that is needed for flagellar movement and mitochondrial activity is therefore correlated with standard sperm parameters such as motility (Espinoza et al. 2009; Perry et al. 2011), and also for the changes occurring during capacitation. Evaluation of mitochondrial activity

is usually done with fluorescent probes that detect changes in mitochondrial membrane potential. Assays are typically done in situ due to the complex attachment of the mitochondria in the fibrous sheath. High and low fluorescence intensity indicate high and low mitochondrial membrane potential respectively (Perry et al. 2011; Moraes & Mayers 2018). It has been suggested that mitochondrial status might have a more prominent role in determining sperm freezability than its maturation status (Yogev et al. 2010). However, mitochondria are not the only energy source for flagellum.

Cryopreservation also affects acrosome integrity. Sperm acrosome integrity can be measured by for example Giemsa stain or the fluorescent isothiocyanate-conjugated peanut agglutinin (FITC-PNA) stain and the spermatozoa are classified as either having an intact acrosome or a non-intact acrosome based on the staining (Skidmore et al. 2018).

2.5.1. Sperm-Hyaluronan Binding Assay

The hyaluronan binding assay (HBA) is a commercial diagnostic test that is clinically used to assess human sperm maturity and function and to select spermatozoa for assisted reproduction (Huszar et al. 2006). Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan, which is a major component in cumulus oophorous that surround oocytes of most mammalian species and has a critical role in both *in vivo* and *in vitro* fertilization (Huszar 1999; Van Soom et al. 2002; Redgrove et al. 2013; Worrilow et al. 2013). The HBA test is based on the ability of mature spermatozoa to bind to HA via specific receptors in binding sites (Huszar et al. 2003). The synthesis of these sites is regulated by the plasma membrane remodeling processes during which the surplus cytoplasm is eliminated, and the sperm plasma membrane is remodeled (de Kretser et al. 1998; Huszar et al. 1997, 2000). Spermatozoa that have successfully completed the spermiogenetic process have an intact acrosome, high sperm vitality and transition of nuclear histone–to–protamine, devoid of cytoplasmic retention, normal morphology, and lower frequency of DNA fragmentation and chromosomal aneuploidies (Huszar et al. 1997, 2003; Kovanci et al.2001; Nasr-Esfahani et al. 2008). These properties are found in HA-bound spermatozoa.

Studies of human spermatozoa showed that the HBA assay can be an objective, standardized test to evaluate sperm functionality (Szucs et al. 2015). Spermatozoa binding to hyaluronan carry fewer chromosomal abnormalities (Yagci et al. 2010), are more mature on a developmental level (Huszar et al. 1990; Huszar et al. 2006; Huszar et al. 2007; Lazarevic et al. 2010) and demonstrate fewer morphological abnomalities (Szucs et al. 2015; Ye et al. 2006; Worrilow et al. 2004) than non HBA-binding spermatozoa. Human sperm selection through HBA resulted in

improved *in-vitro* fertilization rate (Breznik et al. 2013; Erberelli et al. 2017, Worrilow et al. 2013). The HBA score was found to predict *in-vivo* fertilization rates (Szucs et al. 2015), but other parameters of sperm quality might be better fertility indicators (Ye et al. 2006; Tarozzi et al. 2009; Lazarevic et al. 2010; Yildirim et al. 2015; Miller et al. 2019). In animal reproduction studies, the HBA score has been associated with production rate of porcine embryos with normal chromosomal complement (Chun et al. 2005). Some potential for assessing stallion sperm fertility was reported as the spermatozoa bound to HBA, but the test failed to predict the success of AI (Colleoni et al. 2011). A study on Nili-Ravi buffalo bull fertility reported high prediction value for HBA test (predicting 67.9% variability) from frozen-thawed sperm samples (Awan et al. 2021).

The predictive value of the HBA test for human sperm freezability was evaluated by Yogev et al. (2010). The result of the HBA with fresh spermatozoa had high predictive value for high freezability (motility $\geq$ 40%) but conventional parameters such as motility, concentration and normal morphology might be better indicators. The maturity of the spermatozoa may not be the main contributor to withstand the cryopreservation process and the mitochondrial organelle possibly plays an important role.

2.5.2. ProAKAP4

ProAKAP4 is precursor of A-Kinase Anchoring Protein 4 (AKAP4) protein, which when activated to AKAP4 integrates into the flagellum and allows protein kinase A (PKA) and protein kinase C (PKC) to regulate flagella movements. Therefore, proAKAP4 acts as reserve of sperm motility and has been found to correlate with total and progressive motility in stallion sperm (Blommaert et al. 2018). There is a strong association between proAKAP4 and ejaculate volume, viscosity and total motility in dromedary camel sperm (Malo et al. 2021). It is also considered as a functional and quality marker of spermatozoa (Sergeant et al. 2019). Commercialized enzyme linked immunosorbent assay (ELISA) kits use proAKAP4 as a biomarker to classify semen according to the concentration of the protein. These ELISA kits are available for several mammal species and have been reported to predict semen freezability in rams (Riesco et al. 2020) and seasonality of semen parameters in dromedary camels (Malo et al. 2021).

2.5.3. Sperm penetration assay

The SPA was described in 1976 by Yanagimachi et al. who observed that after removal of the zona pellucida from hamster ova, penetration by spermatozoa from other species was possible (Yanagimachi et al. 1976). Subsequently, SPA with zona-free hamster oocytes was used to assess sperm fertilizing ability in humans

(Corson et al. 1988), cattle (Eaglesome et al. 1990), mice (El-Sayed et al. 2010) and pigs (Oh et al. 2010). The SPA test measures the number of spermatozoa that are able to form a male pronucleus. To reach the pronucleus stage, the spermatozoa have to capacitate, fuse with the egg membrane, pass into the ovum followed by decondensation of the sperm head. The SPA test score is calculated from the ova that are penetrated or the average number of penetrations per ovum (Hwang & Lamb 2013). In cattle, the SPA protocol has been optimized for use with frozen-thawed sperm and it has led to accurate screenings to identify the most fertile bulls (Park et al. 2012). Crichton et al. (2016) found that camel spermatozoa penetrate and form a pro-nucleus in zona-free goat oocytes. In their study, heterologous zona-free goat oocytes were used to assess camel sperm function in vitro (Crichton et al. 2016). However, in vitro fertilization is the most meaningful method to evaluate sperm fertility *in-vitro*, since ultimately the spermatozoa have to be able to fertilize the oocyte (Skidmore et al. 2018).

The SPA is a time-consuming and expensive method and is not routinely used to evaluate semen. The relationship between the HBA and SPA results was studied for human spermatozoa by Lazarevic et al. (2010) to evaluate if HBA could replace the SPA in human andrology laboratories. However, there was no relationship between HBA and SPA results and it was concluded that HBA test cannot be used to predict the ability of the spermatozoa to penetrate oocytes. (Lazarevic et al. 2010)

2.6. Specific objectives

The primary objective of this study was to evaluate the ability of the HBA test to predict the fertilizing capacity and freezability of dromedary camel sperm. A secondary objective was to determine if conventional sperm quality parameters and proAKAP4 value correlate with the HBA result.

3. Materials and methods

3.1. Animals and semen collection

This study included ejaculates from six adult, dromedary camels from the Camel Reproduction Centre in Dubai. 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 (figure 3) (Skidmore et al. 2013). Collections were done during the rutting season between the end of February and the beginning of March. 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.

Figure 3. Bovine artificial vagina modified for dromedary camel semen collection (Picture by Anna Kotila-Ioannou 2019)

3.2. Media

Unless otherwise indicated, all chemicals were from Sigma-Aldrich Co. (St Louis, MO, USA).

Fresh 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 (0.22 m).

A two-step dilution with 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 fraction 2 (F2) consisted of F1 with the addition of 6% glycerol (see section 3.4. Cryopreservation and thawing procedures).

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 test for determination of the fraction of hyaluronan-binding sperm 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).

Cumulus oocyte complexes (COCs) used in a heterologous penetration assay 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. Oocyte maturation medium was modified North Caroline State University (NCSU)-23 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 0.8 mM cysteine (Petters & Wells, 1993). 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 sulphate (Crichton et al. 2016).

3.3. Semen preparation

The ejaculates were evaluated for volume (Vol; mL), colour, viscosity (Vis, scale 1-5), and diluted (1:2) with TCF at 37°C. Viscosity was measured by drawing semen up and down into 2 mL plastic pipettes and assessing the difficulty of pipetting. Score was based on scale of 1 to 5; 1 having no viscosity at all and 5 being unable to be pipetted at all. Viscosity was then eliminated by repeatedly gently pipetting the samples during incubation for 30 min in 37°C water bath (Malo et al. 2017b). Liquefied samples were evaluated for concentration (Makler counting chamber), TM, PM and kinematics (CASA), MA (Eosin-nigrosin), IA (Fitc-PNA), VIT (SYBR-PI), MIT (R123), proAKAP4 and HBA.

3.4. Cryopreservation and thawing procedures

Semen was prepared for cryopreservation and sample thawing according to the protocol developed by Malo et al. (2017b and 2018). Liquefied samples were centrifuged at 300 g 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 supplemented with 20% (v/v) egg yolk and 500 IU of catalase. Concentration was adjusted to $150x10^6$ spermatozoa/mL and cooled to 5ºC for 2 h in a water jacket. Second dilution (1:1) was done at 5º C, with F2 suspension; GB extender containing 20% egg yolk and 6% (v/v) glycerol, resulting in a final glycerol concentration of 3% and a final spermatozoa concentration of $75x10^6$. Semen was packaged in pre-cooled (0.5 mL) plastic straws and equilibrated in a cold cabinet for 30 min. Straws were frozen in the liquid nitrogen vapor 1 cm above the liquid nitrogen surface 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. Immediately after thawing, the following parameters were assessed: TM, PM and kinematics, VIT, IA, MIT, pro-AKAP4 and HBA. The *in vitro* fertilization parameters were measured PT0H. Post-thawing sperm TM, PM, kinematics, VIT, IA and MIT were assessed at PT1.5H.

3.5. Experimental design

In this experiment, two ejaculates per male were used. Samples were assessed fresh (fresh), pre-freeze (PF), and after thawing at 0 h (PT0H) and at 1.5 h (PT1.5H). The assessed parameters at each time points are shown below.

Table 1. Sample assessment times. Fresh, PF: pre-freeze, PT0H: post-thaw 0 h, PT1.5H: post-thaw 1.5 h and list of abbreviations.

	Fresh	PF	PT0H	PT1.5H
Concentration (Conc; M/mL), volume (Vol; mL)	✓	-	-	-
Viscosity (Vis; 1-5), colour, odour	✓	-	-	-
Total motility (TM; %) & progressive motility (PM, %)	✓	✓	✓	✓
Kinematics*	✓	✓	✓	✓
Vitality (VIT; %), intact acrosome (IA; %)	✓	-	✓	✓
Mitochondria (MIT; %) & morphological abnormality (MA; %)	✓	-	✓	✓
Hyaluronan Binding Assay (HBA; %)	✓	-	✓	-
Precursor of A-Kinase Anchoring Protein 4 (proAKAP4; %)	✓	-	✓	-
Sperm penetration assay (SPA)**	-	-	✓	-

* ALH: amplitude lateral head (µm)
BCF: beat cross frequency (Hz)
LIN: linearity (%)
STR: straightness (%)
VAP: average path velocity (µm/s)
VCL: curvilinear velocity (µm/s)
VSL: straight line velocity (µm/s)

** SPA: sperm penetration assay
N: number of oocytes
Pen: penetration rate (% of sperm)
PN: formation of male pro-nucleus (% of sperm)
SP/OC: number of sperm penetrated per oocyte

3.6. Sperm Assessment

3.6.1. Concentration

Diluted and liquefied fresh samples were further diluted 1:50 in saline solution with 4% paraformaldehyde. Concentration was measured from 10 µL of sample in a Makler counting chamber, counting 100 squares per sample by two people.

3.6.2. Motility

The TM, PM and kinematics were recorded with CASA (CEROS II®; Hamilton Thorne, MA, USA) connected to a Zeiss microscope (x10 objective, eyepiece, and a 37 °C temperature stage). A disposable chamber slide (Cytonix, Beltsville, MD, USA) was filled with 2µl of sample and five fields with minimum 400 sperms were captured. Sperm kinematics included VAP, VSL, VCL, ALH, STR, LIN and BCF. CASA settings are shown in appendix 1.

3.6.3. Morphological abnormalities

Semen samples were stained with eosin-nigrosin (EN) to assess sperm morphology (Agarwal et al. 2016). 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 100 x magnification (Olympus bx53). Abnormalities in the head such as miniature head, pyriform head, giant head and twin head were summarized in one class. Tail abnormalities such as coiled tail formed another class (figure 4, right). Droplets (regardless of the location) formed third class (figure 4, left). Two observers each evaluated 200 spermatozoa (total 400) and the proportion of abnormal spermatozoa was calculated from the combined score.

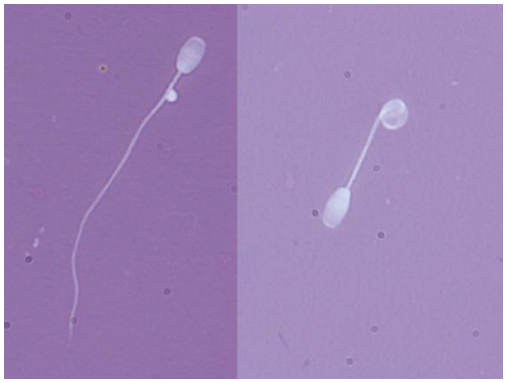

Figure 4. Eosin-nigrosin stained spermatozoa. Left: proximal droplet. Right: coiled tail (Picture by Anna Kotila-Ioannou 2019).

3.6.4. Vitality

Vitality was assessed by evaluating plasma membrane integrity. Live spermatozoa have an intact membrane whereas dead spermatozoa do not have an intact membrane (figure 5). 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% CO_2. After incubation, 2 µL of PI stain, 2 µL sperm immobilizing

Paraformaldehyde (4%) and 30 µL of TCF were added. Samples were observed by two people using fluorescence microscopy (Olympus bx53), each person classifying 200 spermatozoa (total 400 spermatozoa) as either live (SY-pos, green) or dead (PI-neg, red) (figure 5).

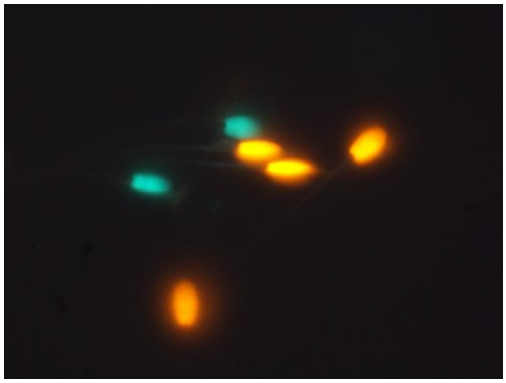

Figure 5. Green, intact membrane indicating live spermatozoa (SY-pos). Red, non-intact membrane, indicating dead spermatozoa (PI-pos) (Picture by Anna Kotila-Ioannou 2019).

3.6.5. Intact acrosome

The proportion of IA was evaluated with fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) with slight modifications to the method as described by Aboagla and Terada (2003) and Malo et al. (2019). 30 µL of sample were diluted with 140 µL of TCF. 6 µL PNA (2 µg/mL) dye and 1 µL of sperm immobilizing Paraformaldehyde (4%) was added. Two people classified 200 spermatozoa by fluorescence microscopy (Olympus bx53) either with intact acrosome (PNA-pos, green) or damaged acrosome (PNA-neg, no stain) (figure 6).

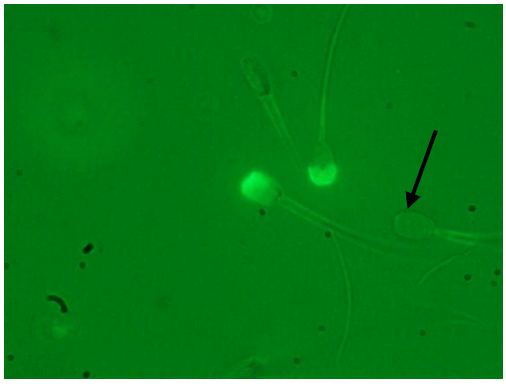

Figure 6. Bright green, intact acrosome (PNA-pos). Non-stained, damaged acrosome marked with black arrow (PNA-neg) (Picture by Anna Kotila-Ioannou 2019).

3.6.6. Mitochondrial Membrane Potential

Measurement of mitochondrial membrane potential was done using fluorescent Rhodamine-123 dye (R123) as described by Eskandani et al. (2016). R123 stains mitochondria in the intermediate piece in response to mitochondrial activation. High colour concentration indicates high activity and no colour low activity (figure 7). One hundred µL of semen sample was diluted with 900 µL of TCF and 5 µL of R123 (1 mg/mL) (final concentration 0.015 ng/mL). The sample was incubated for 10 min at room temperature in the dark and centrifuged at 300g/20min. 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.

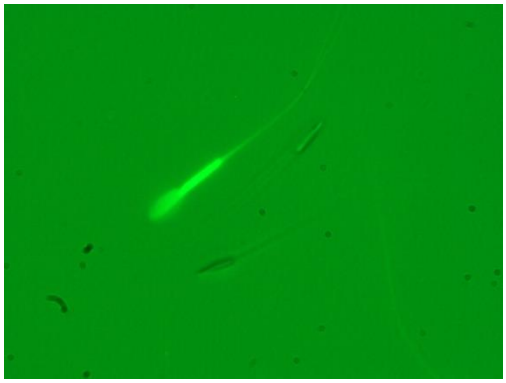

Figure 7. Bright green intermediate piece, high mitochondrial membrane potential (R123-pos) or dull green intermediate piece, low mitochondrial membrane potential (R123-neg) (Picture by Anna Kotila-Ioannou 2019)

3.6.7. Sperm-Hyaluronan Binding Assay

The HBA test was used according to the manufacturer´s instructions (Biocoat, Hosham, PA, USA). The HBA slide contained two test chambers, coated with a layer of hyaluronan. 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 and 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.

3.6.8. Precursor of A-kinase Anchoring Protein

The ProAKAP4 was used as a biochemical indicator to estimate sperm quality and sperm functionality (Delehette et al. 2018). The concentration of proAKAP4 was analyzed at the 4BioDx laboratory with ELISA assay Camel 4MID® Kit by following the manufacture´s protocol (Ref. 4VDX-19K11, 4BioDx, France). Fifty µL of thawed sample was mixed with 450 µL of the camel lysis buffer. One hundred µL of lysed semen sample was 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. The concentration of proAKAP4 was proportional to the colour intensity measured by spectrophotometry at a wavelength of 450 nm. The results were expressed in ng/10 million spermatozoa (ng/10 M spermatozoa).

3.6.9. Sperm Penetration Assay

The fertilizing ability of the frozen-thawed samples was evaluated using a SPA with in-vitro matured, zona-free goat oocytes. The method was described by Crichton et al. in 2016. Goat ovaries were obtained from the local slaughterhouse (Dubai, United Arab Emirates). The COCs were retrieved with a slicing technique by incising after rinsing the ovaries with phosphate buffered saline. These COCs were matured overnight in vitro and denuded the next day by removing the cumulus cells and zonae pellucidae and placed in fertilization media. 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 (30-45 per sample) were mounted on slides under vaseline-supported coverslips. The oocytes were fixed in a bath with 1 part acetic acid to 3 parts ethanol (v/v) was used for 72 hr. Fixed oocytes were stained with 1% (v/v) Lacmoid dye and viewed under ×100 microscope. Proportions of Pen, PN and SP/OC were calculated. Degenerated oocytes were excluded.

3.7. Statistical Analysis

Data from 48 samples were analyzed after checking using diagnostic plots. No apparent deviations from normality or homoscedasticity were detected. 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, treatment and time, and their interaction. Animal nested within time was set as a random factor. Degrees of freedom were determined according to Kenward and Roger (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 treatment.

Results are presented as Least square means (LSMeans) ± standard error of 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 0.05 to 0.10 were considered to show a trend towards significance.

4. Results

4.1. Experiment 1: HBA, proAKAP4 and conventional semen assessment

4.1.1. HBA binding results

Dromedary camel spermatozoa were able to bind to hyaluronan. The results for the HBA binding (%) are shown in appendix 2 and illustrated in figure 8. The binding of fresh sperm varied between 51.0 and 95.0 %. The frozen-thawed spermatozoa bound less, where the ratio varied between 3.0 and 24.0 %. No significant differences in binding between the males was demonstrated.

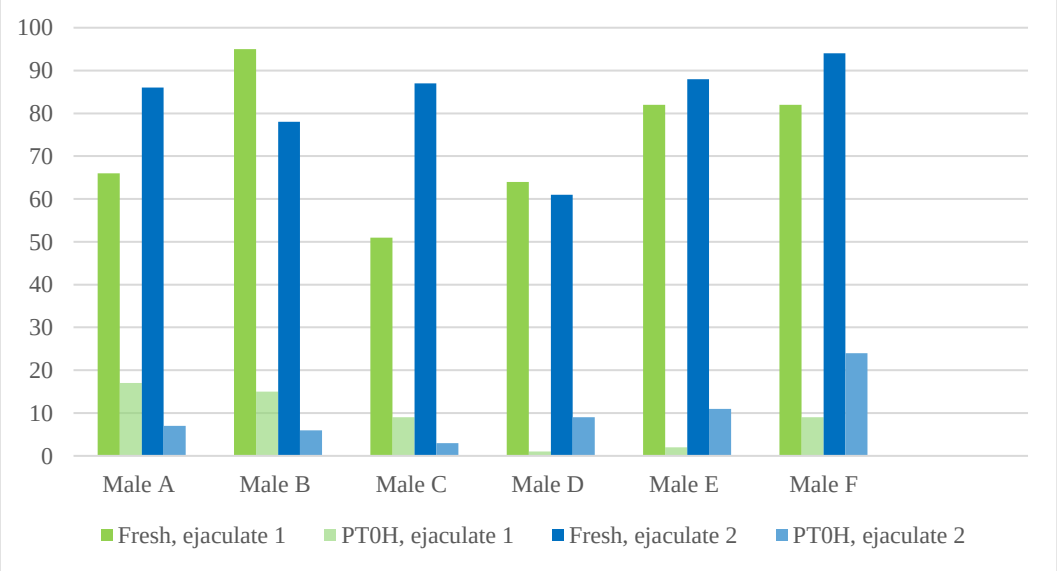

Figure 8. *Binding of spermatozoa in fresh semen and post thaw 0h (PT0H) to Hyaluronan Binding Assay (HBA; %) for two ejaculates from six males.*

4.1.2. ProAKAP4 results

The proAKAP4 results did not correspond well with the HBA results (table 2). This was especially noticeable for male B, where the proAKAP4 result in ejaculate 2 was high, unlike the HBA result. The LSMeans of HBA and proAKAP4 results are illustrated in figure 9.

Table 2. *Hyaluronan Binding Assay (HBA; %) and precursor of A-Kinase Anchoring Protein 4 (proAKAP; %) results for both ejaculates of all males from fresh (fresh) and after thawing at 0 h (PT0H) samples presented in LSMeans.*

HBA and proAKAP4 test results

	Ejaculate 1, fresh		Ejaculate 2, fresh	
Male	HBA	proAKAP4	HBA	proAKAP4
A	66.00	10.58	86.00	3.50
B	95.00	10.73	78.00	39.34
C	51.00	5.48	87.00	10.99
D	64.00	13.30	61.00	3.22
E	82.00	16.54	88.00	12.05
F	82.00	3.67	94.00	6.16

	Ejaculate 1, PT0H		Ejaculate 2, PT0H	
Male	HBA	proAKAP4	HBA	proAKAP4
A	17.00	8.25	7.00	20.35
B	15.00	4.42	6.00	18.38
C	9.00	14.74	3.00	14.69
D	10.00	16.47	9.00	13.90
E	20.00	6.54	11.00	10.33
F	9.00	11.81	24.00	12.68

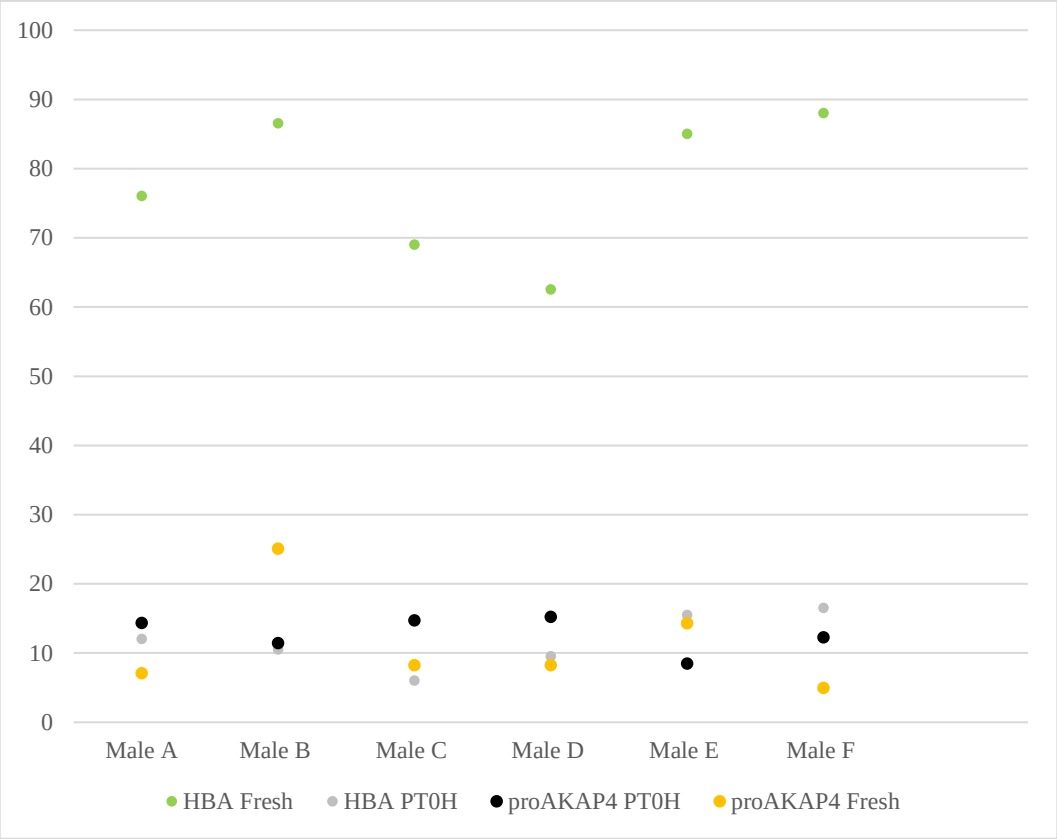

Figure 9. *LSMean values of Hyaluronan Binding Assay (HBA; %) and Pro A-Kinase Anchoring Protein 4 (proAKAP4; %) results in fresh (Fresh, n=12) and after thawing at 0 h samples (PT0H, n=12) from different males (n=6).*

4.1.3. Conventional semen analyses

The conventional semen analyses results (Vol, Vis, Conc, MA, TM, PM, VIT, IA & MIT), proAKAP4 test and CASA kinematics (ALH, BCF, LIN, STR, VAP, VCL, VSL) from fresh, PF, PT0H and PT1.5H samples are presented in tables 3, 4, 5, 6 and 7. Significant p-values ($p \leq 0.05$) are highlighted in red.

There were no differences between males for conventional analyses (table 3) and proAKAP4 results from fresh semen (table 4). Significant differences ($p \leq 0.05$) were found for LSMean values of CASA kinematics for BCF, STR and VCL (table 5).

Table 3. *Fresh semen volume (vol; mL) viscosity (Vis; 1-5), concentration (Conc; M/mL) and morphological abnormality (MA; %, n = 200) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from fresh semen for different males				
Male	Vol	Vis	Conc	MA
A	2.75	3.00	315.00	14.00
B	5.25	2.00	358.75	14.00
C	4.00	3.00	525.00	16.50
D	8.00	3.00	645.00	10.50
E	3.00	3.00	680.00	13.50
F	5.50	2.00	300.00	10.50
Mean	4.75	2.67	470.63	13.17
SEM	± 1.50	n/a	± 150.90	± 2.30
p-value	0.29	n/a	0.40	0.48

Table 4. *Fresh semen total motility (TM, %), progressive motility (PM, %), vitality (VIT, %), intact acrosome (IA, %), mitochondrial integrity (MIT, %) and proAKAP4 presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented*

LSMean values from fresh semen for different males						
Male	TM	PM	VIT	IA	MIT	proAKAP4
A	60.90	8.65	45.00	79.25	48.00	7.04
B	57.10	8.95	61.0	83.00	68.50	25.04
C	41.50	4.25	35.5	88.50	64.50	8.24
D	71.85	6.10	61.75	87.75	56.75	8.21
E	63.40	12.65	61.00	84.75	55.25	14.30
F	56.65	16.65	59.00	80.00	61.50	4.92
Mean	58.57	9.54	53.88	83.88	59.08	11.23
SEM	± 7.96	± 2.50	± 5.64	± 5.19	± 11.71	± 7.00
p-value	0.29	0.09	0.07	0.73	0.84	0.46

Table 5. *Fresh semen CASA kinematics; ALH (µM), BCF (Hz), LIN (%), STR (%), VAP (µm/s), VCL (µm/s) and VSL (µm/s) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from fresh semen for different males

Male	ALH	BCF	LIN	STR	VAP	VCL	VSL
A	10.89	18.49	29.34	64.29	90.14	201.05	57.41
B	10.08	18.97	30.51	65.74	83.30	183.91	54.94
C	11.14	20.90	27.61	59.05	96.35	209.04	56.31
D	11.48	21.73	25.60	59.09	101.81	238.06	59.76
E	10.17	20.59	28.52	67.82	81.82	195.46	55.18
F	9.17	18.19	35.46	71.18	80.86	165.18	57.56
Mean	10.49	19.81	29.51	64.53	89.05	198.78	56.86
SEM	± 0.54	± 0.51	± 1.75	± 1.44	± 6.64	± 11.94	± 5.50
p-value	0.15	0.01	0.07	0.01	0.27	0.05	0.99

There were no differences in pre-freeze values of TM, PM and CASA kinematics (ALH, BCF, LIN, STR, VAP, VCL and VSL) between males (table 5 & 6).

Table 6. *Pre-freeze (PF) semen values (n=12) for total motility (TM, %) and progressive motility (PM, %) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from pre-freeze samples for different males

Male	TM	PM
A	55.15	5.80
B	57.65	8.05
C	47.40	7.95
D	77.75	8.15
E	66.70	6.85
F	55.55	8.20
Mean	60.03	7.50
SEM	±9.83	± 2.63
p-value	0.42	0.98

Table 7. *Pre-freeze semen values for CASA kinematics; ALH (μM), BCF (Hz), LIN (%), STR (%), VAP (μm/s), VCL (μm/s) and VSL (μm/s) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from pre-freeze samples for different males							
Male	ALH	BCF	LIN	STR	VAP	VCL	VSL
A	9.97	19.49	28.52	58.97	91.81	194.24	53.91
B	10.05	18.02	30.37	62.63	94.81	199.06	58.42
C	9.98	20.08	29.22	56.61	98.98	201.58	55.96
D	10.42	20.35	28.46	55.02	100.33	201.84	55.49
E	9.64	20.84	26.91	58.85	82.95	184.91	48.74
F	10.03	18.30	29.38	62.55	89.37	194.12	55.26
Mean	10.02	19.51	28.81	59.10	93.04	195.96	54.63
SEM	± 0.61	± 0.90	± 1.93	± 2.07	± 6.15	± 11.07	± 3.88
p-value	0.97	0.29	0.86	0.18	0.45	0.88	0.65

There were no differences between the males in the TM, PM, VIT, IA, MIT, HBA score or proAKAP4 at PT0H (table 7). The LSMeans of CASA kinematics varied significantly ($p \leq 0.05$) between males in ALH, VAP and VCL values (table 8).

Table 8. *After thawing at 0 h values (PT0H) for total motility (TM, %), progressive motility (PM, %), viability (VIT, %), intact acrosome (IA, %), mitochondrial integrity (MIT, %), Sperm-Hyaluronan Binding Assay (HBA, %) and precursor of A–kinase anchor protein 4 (proAKAP4; %) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from post-thaw 0 h samples for different males							
Male	TM	PM	VIT	IA	MIT	HBA	proAKAP4
A	32.50	7.05	31.00	42.50	35.50	12.00	14.30
B	40.55	5.00	30.50	47.50	51.00	10.50	11.40
C	27.70	2.05	23.00	42.00	30.50	6.00	14.72
D	45.30	8.35	37.00	56.00	61.00	9.50	15.19
E	38.50	4.70	32.25	55.00	45.25	15.50	8.44
F	25.60	2.90	25.50	37.50	27.00	16.50	12.25
Mean	35.03	5.01	29.88	46.75	41.71	11.67	12.71
SEM	± 7.96	± 2.62	± 3.78	±7.29	± 7.49	± 5.00	± 3.4
p-value	0.48	0.57	0.26	0.47	0.10	0.67	0.72

Table 9. *After thawing at 0 h (PT0H) values for kinematics; ALH (µM), BCF (Hz), LIN (%), STR (%), VAP (µm/s), VCL (µm/s) and VSL (µm/s) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from post-thaw 0 h samples for different males

Male	ALH	BCF	LIN	STR	VAP	VCL	VSL
A	8.08	17.90	33.13	65.74	76.56	153.49	49.75
B	8.16	18.66	31.70	62.92	76.78	153.74	48.08
C	9.37	20.19	26.68	56.16	78.46	170.92	43.78
D	8.25	19.03	33.12	62.90	78.16	152.76	49.86
E	7.07	18.72	30.07	58.29	61.55	123.40	36.54
F	7.43	20.07	29.02	57.93	61.04	125.6	35.67
Mean	8.06	19.10	30.62	60.65	72.10	146.65	43.95
SEM	± 0.24	± 0.79	± 1.53	± 2.33	± 3.89	± 7.25	± 3.83
p-value	0.01	0.39	0.13	0.14	0.04	0.02	0.12

For the PT1.5H values of conventional analyses (TM, PM, VIT, IA, MIT) there were no significant differences among the males (table 9). However, LIN was significantly different ($p \leq 0.05$) between males (table 10). No statistical differences were detected for other PT1.5H values.

Table 10. *After thawing at 1.5 h values (PT1.5H) for total motility (TM, %), progressive motility (PM, %), viability (VIT, %), intact acrosome (IA, %), mitochondrial activity (MIT, %), Sperm-Hyaluronan Binding Assay (HBA,%) and precursor of A–kinase anchor protein 4 (proAKAP4; %). presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from post-thaw 1.5 h samples for different males

Male	TM	PM	VIT	IA	MIT
A	20.30	1.40	21.50	36.50	32.50
B	29.60	3.00	30.50	44.50	31.50
C	22.30	1.35	24.00	37.50	28.50
D	33.85	3.90	38.00	49.50	44.00
E	30.20	1.80	36.00	58.25	42.50
F	18.80	0.65	23.50	45.00	21.00
Mean	25.82	2.02	28.92	45.21	33.33
SEM	± 9.18	± 1.50	± 4.67	± 7.01	± 5.73
p-value	0.80	0.68	0.18	0.37	0.18

Table 11. *After thawing (PT1.5H) semen values for kinematics; ALH (µM), BCF (Hz), LIN (%), STR (%), VAP (µm/s), VCL (µm/s) and VSL (µm/s) presented in LSMeans for all the males in the study (sample n = 12). For variation between males, p-values are presented.*

LSMean values from post-thaw 1.5 h samples for different males

Male	ALH	BCF	LIN	STR	VAP	VCL	VSL
A	7.89	18.87	29.79	60.53	61.54	129.91	37.18
B	7.83	17.85	31.14	61.37	63.58	130.59	39.23
C	8.75	20.25	25.62	57.94	66.71	156.22	37.48
D	8.09	19.77	29.04	61.06	66.66	146.07	40.58
E	7.20	19.65	26.20	52.73	54.39	115.27	28.71
F	7.22	20.81	29.47	62.76	55.55	127.06	33.55
Mean	7.83	19.53	28.54	59.40	61.57	134.19	36.13
SEM	± 0.3	± 1.02	± 1.00	± 1.88	± 4.93	± 8.61	± 3.30
p-value	0.07	0.46	0.05	0.14	0.41	0.12	0.26

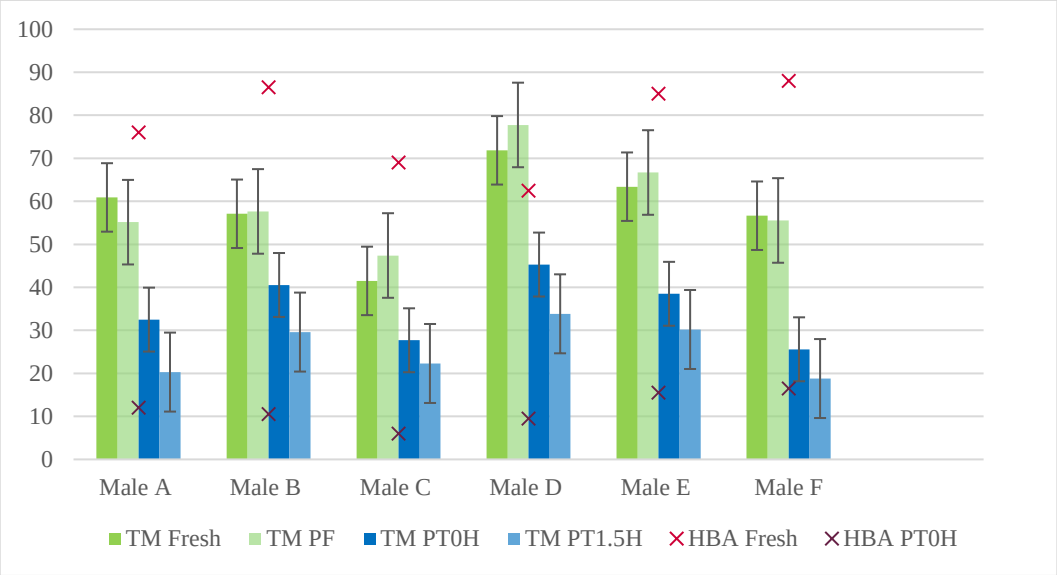

Figure 10. *LSMean (± SEM) values of total motility (TM, %) in different samples; fresh (Fresh), pre-freeze (PF), after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

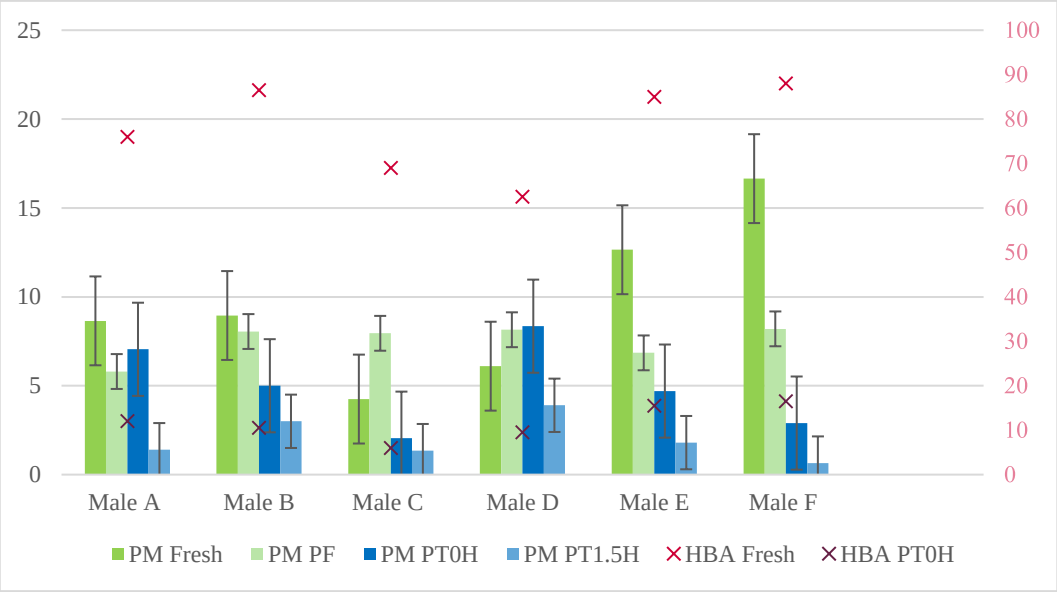

Figure 11. *LSMean (± SEM) values of progressive motility (PM; %) in different samples fresh (Fresh), pre-freeze (PF), after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

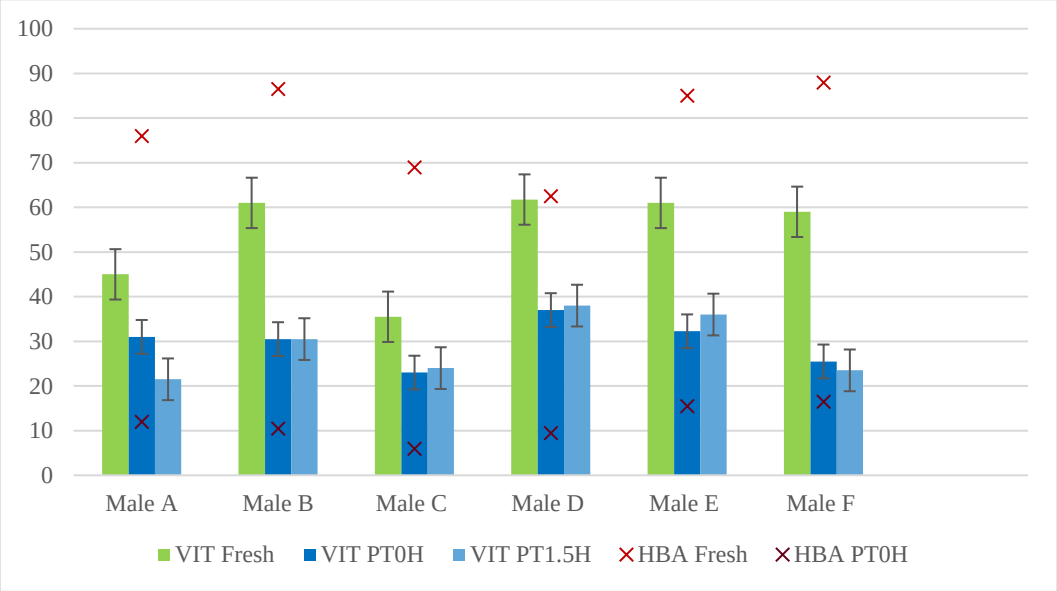

Figure 12. *LSMean (± SEM) values of vitality (VIT; %) in fresh (Fresh), after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) samples represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

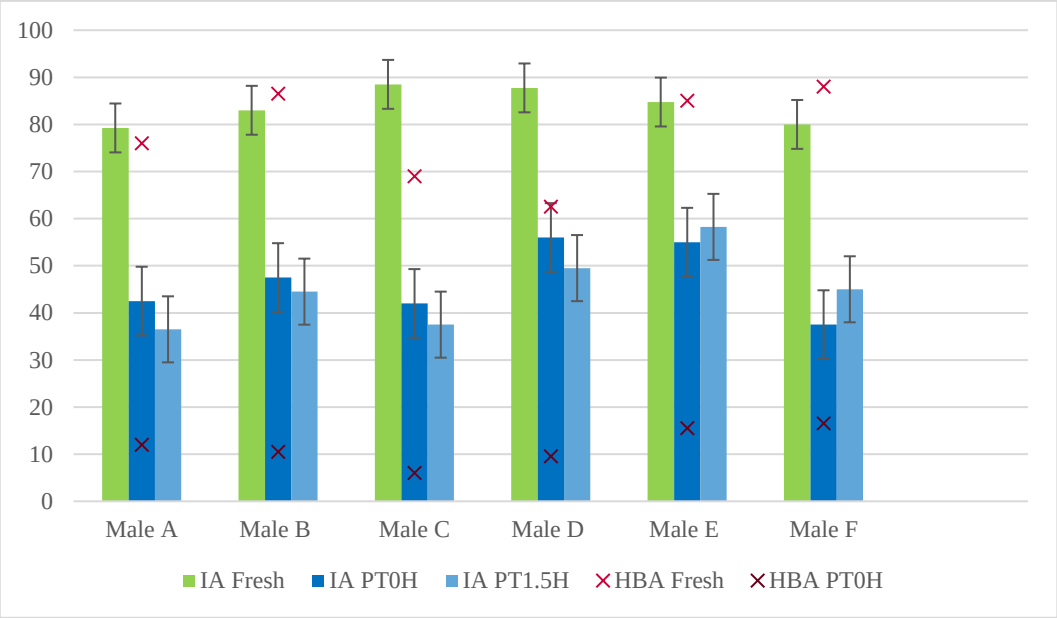

Figure 13. *LSMean (± SEM) values of intact acrosome (IA; %) in fresh (Fresh), after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) samples represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

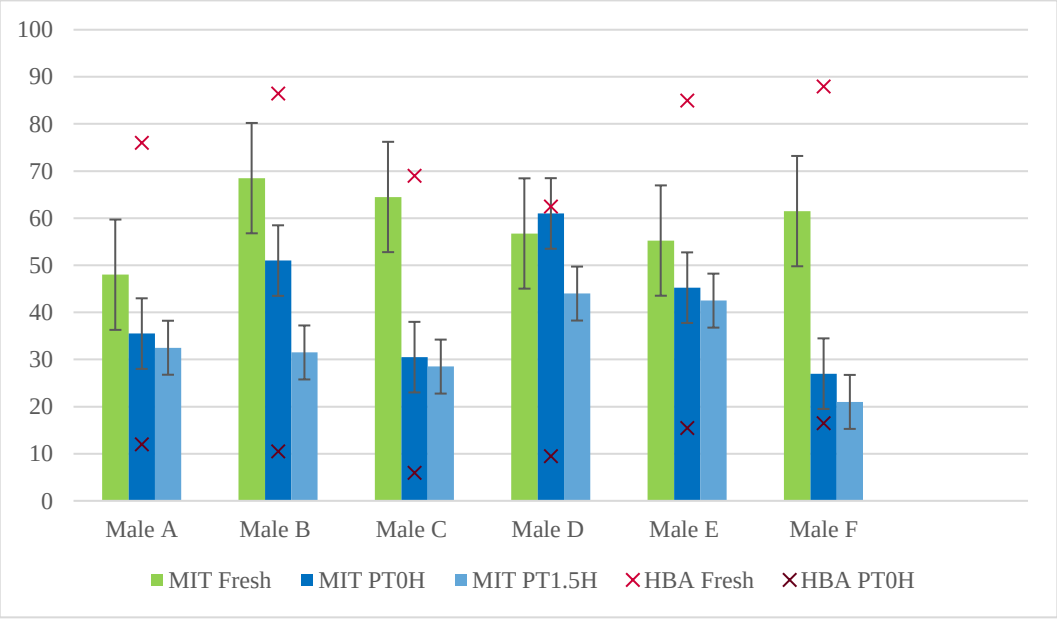

Figure 14. *LSMean (± SEM) values of mitochondrial activity (MIT; %) in fresh (Fresh), after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) samples represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

4.2. Experiment 2: Sperm penetration assay

The penetrating ability of camel spermatozoa was studied in the heterologous oocyte binding assay at PT0H. The camel spermatozoa bound, penetrated, decondensed, and completed pro-nucleus formation in goat oocytes (figure 15). The Pen varied from 0.25% to 78.37% (mean 46.07 ± 4.7%), the PN was 0% to 56.75% (mean 22.81 ± 5.3%) and the SP/OC varied from 1 to 2.41 (mean 1.70 ± 0.4).

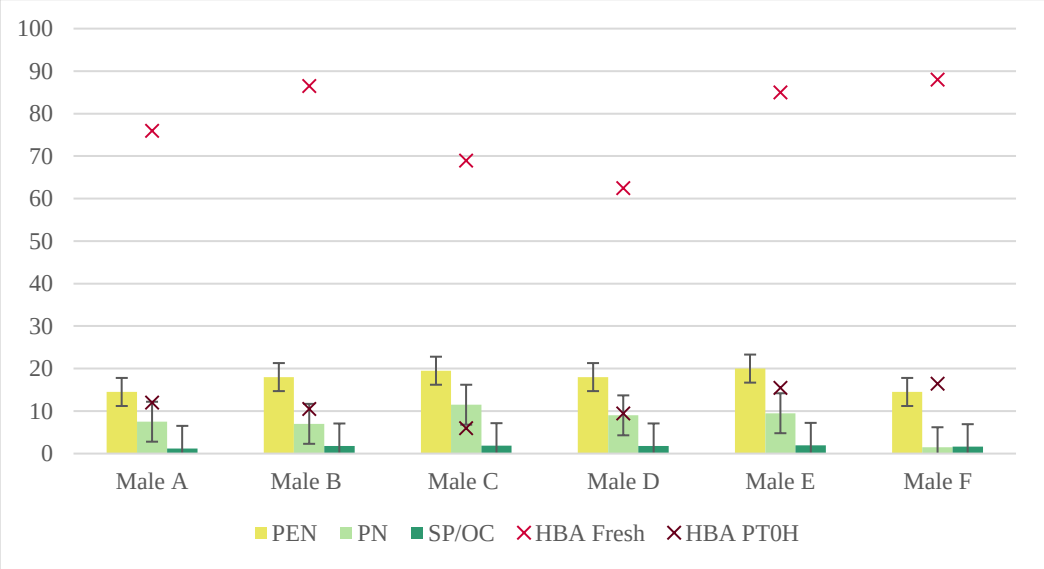

Figure 15. *LSMean (± SEM) values of sperm penetration assay (SPA) of after thawing at 0 h (PT0H) camel sperm; penetration rate (PEN; %), male pro-nucleus formation (PN; %) and sperm penetrated per oocyte (SP/OC) represented with bars. LSMean values of Hyaluronan Binding Assay (HBA; %) from fresh and PT0H samples shown with* X *symbol.*

4.3. Correlations

4.3.1. HBA correlations

No significant correlations ($p \leq 0.05$) were found between the HBA results from fresh samples and PT0H samples. The HBA PT0H and proAKAP4 PT0H samples were correlated (r = -0.62, p=0.03; table 12).

Table 12. *Correlations between Hyaluronan Binding Assay (HBA; %) results from fresh (Fresh) and after thawing at 0 h (PT0H) samples, and proA-Kinase Anchoring Protein 4 (proAKAP4; %) results from fresh and PT0H samples. Result presented in p-values (p) and correlations coefficients values (r). Significant p-value < 0.05 is highlighted in red.*

Parameters		proAKAP4, fresh	HBA, PT0H	proAKAP4, PT0H
HBA, fresh	p	0.81	0.45	0.42
	r	0.08	0.24	-0.26
proAKAP4, fresh	p		0.67	0.69
	r		-0.14	0.13
HBA, PT0H	p			0.03
	r			-0.62

The HBA and PM results from fresh samples were correlated (r = 0.13, p = 0.02; table 13). There were no significant correlations between HBA PT0H results and conventional semen parameters (TM, PM, VIT, AI, MIT, Vol, Vis, Con and MA) (table 13). The full tables with correlations are shown in appendix 3, 4 & 5.

Table 13. *Correlations between Hyaluronan Binding Assay (HBA; %) results and conventional semen parameters; total motility (TM; %), progressive motility (PM; %), vitality (VIT; %), intact acrosome (IA; %) and mitochondrial activity (VIT; %) for fresh (Fresh) and after thawing at 0 h (PT0H) samples. Correlation between HBA results and volume (Vol, mL), viscosity (Vis, 1-5), concentration (Con; M/mL) and morphological abnormalities (MA; %) on the bottom. Results presented in p-values (p) and correlation coefficient values (r).*

Parameters		TM	PM	VIT	IA	MIT
HBA, fresh	p	0.68	0.02	0.16	0.40	0.69
	r	0.13	0.65	0.44	-0.27	0.13
HBA, PT0H	p	0.82	0.98	0.15	0.27	0.75
	r	-0.07	-0.01	0.44	0.34	-0.10

Parameters		Vol	Vis	Con	MA
HBA, fresh	p	0.50	0.10	0.94	0.24
	r	-0.22	-0.49	-0.02	-0.37

Significant interaction (p = 0.01) was found between HBA ratio and STR (table 14) from freshspermatozoa.

Table 14. *Correlations between Hyaluronan Binding Assay (HBA; %) results and computer-assisted semen analysis (CASA kinematics; amplitude lateral head (ALH; µm), beat cross frequency (BCF; Hz), linearity (LIN; %), straightness (STR; %), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s) and straight line velocity (VSL; µm/s) for fresh (Fresh) and after thawing at 0 h (PT0H) samples. Results presented in p-values (p) and correlation coefficient values (r).*

Parameters		ALH	BCF	LIN	STR	VAP	VCL	VSL
HBA, fresh	p	0.53	0.10	0.09	0.01	0.41	0.34	0.46
	r	-0.20	-0.50	0.51	0.69	-0.26	-0.30	0.24
HBA, PT0H	p	0.09	0.96	0.86	0.65	0.15	0.12	0.28
	r	-0.50	-0.02	-0.06	-0.15	-0.44	-0.47	-0.34

4.3.2. Freezability study: correlations between HBA result and different post-thaw values

The correlations between HBA result and post-thaw (PT0H and PT1.5H) values of different attributes (TM, PM, VIT, AI, MIT, PEN, PN and SP/OC) were analysed to evaluate the ability of the HBA to predict freezability. The results are presented in tables 15 and 16. The IA value in PT1.5H (table 15) was found to be correlated with HBA PT0H results ($p < 0.05$). No other significant correlations were identified. The complete table of correlations is shown in appendix 6.

Table 15. *Correlations between fresh sample Hyaluronan Binding Assay (HBA; %) and both after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) results of total motility (TM; %), progressive motility (PM; %), vitality (VIT; %), intact acrosome (IA; %), mitochondrial activity (MIT; %), oocyte penetration rate (PEN; %), formation of male pro-nucleus (PN, %) and number of oocyte penetrating sperm (SP/OC) presented in p-values (p) and correlation coefficient values (r).*

Correlations of HBA result from fresh samples with different parameters at PT0H and PT1.5H

			PT0H	PT1.5H
HBA fresh	TM	p	0.38	0.44
		r	0.28	0.25
	PM	p	0.80	0.81
		r	0.08	-0.08
	VIT	p	0.85	0.64
		r	-0.06	0.15
	IA	p	0.81	0.39
		r	0.08	0.27
	MIT	p	0.77	0.20
		r	-0.09	-0.39
	PEN	p	0.08	n/a
		r	0.53	
	PN	p	0.34	n/a
		r	0.30	
	SP/OC	p	0.22	n/a
		r	0.39	

Table 16. *Correlations between after thawing at 0 h (PT0H) andHyaluronan Binding Assay (HBA; %) results and both after thawing at 0 h (PT0H) and after thawing at 1.5 h (PT1.5H) results of total motility (TM; %), progressive motility (PM; %), vitality (VIT; %), intact acrosome (IA; %), mitochondrial activity (MIT; %), oocyte penetration rate (PEN; %), formation of male pro-nucleus (PN; %), number of oocyte penetrating sperm (SP/OC)). Statistically significant p-value highlighted in red.*

Correlations of HBA result from PT0H samples with different parameters at PT0H and PT1.5H

			PT0H	PT1.5H
HBA, PT0H	TM	p	0.82	0.86
		r	-0.07	-0.06
	PM	p	0.98	0.56
		r	-0.01	-0.19
	VIT	p	0.15	0.96
		r	0.44	0.02
	IA	p	0.27	0.05
		r	0.34	0.57
	MIT	p	0.75	0.80
		r	-0.10	0.08
	PEN	p	0.37	n/a
		r	-0.29	
	PN	p	0.11	n/a
		r	-0.49	
	SP/OC	p	0.80	n/a
		r	0.08	

4.3.3. Correlations between HBA and sperm penetration assay results

The ability of the HBA test to predict in-vitro fertilization (IVF) performance was evaluated from correlation with the results of the penetration assay (figures 16, 17, 18).

The correlations between HBA results from fresh semen and penetration assay were significant for PEN (p-value = 0.03) and PN (p-value = 0.03). The correlation between SP/OC and HBA for fresh spermatozoa was not significant. The corresponding partial correlation coefficients with trendlines are illustrated in figures 16, 17 and 18.

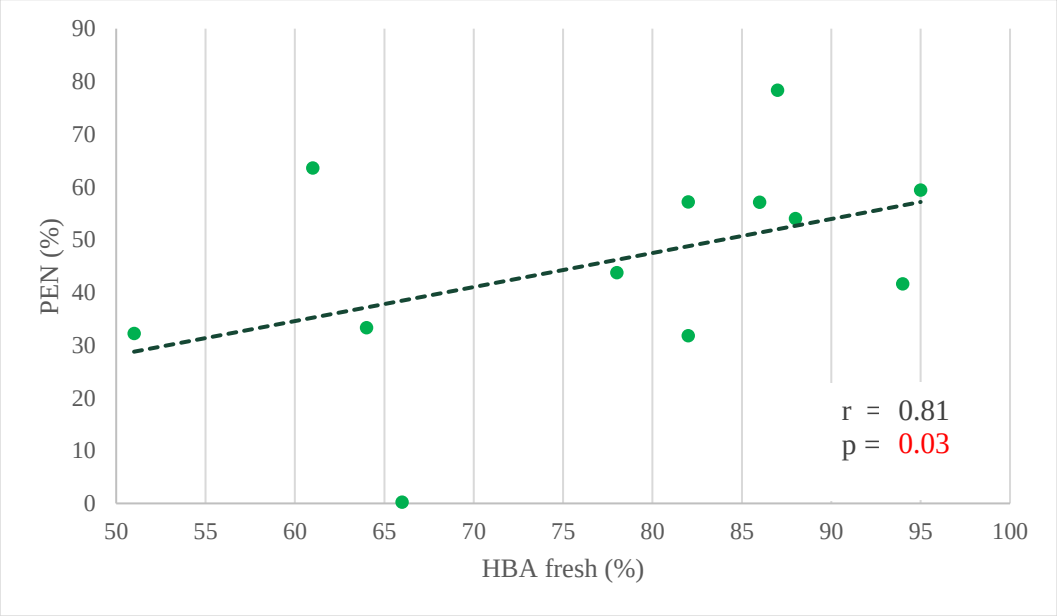

Figure 16. *Partial correlation coefficient between Hyaluronan Binding Assay (HBA; %) results from fresh semen and oocyte penetration rate values (PEN, %). Trendline illustrated with dashed line.*

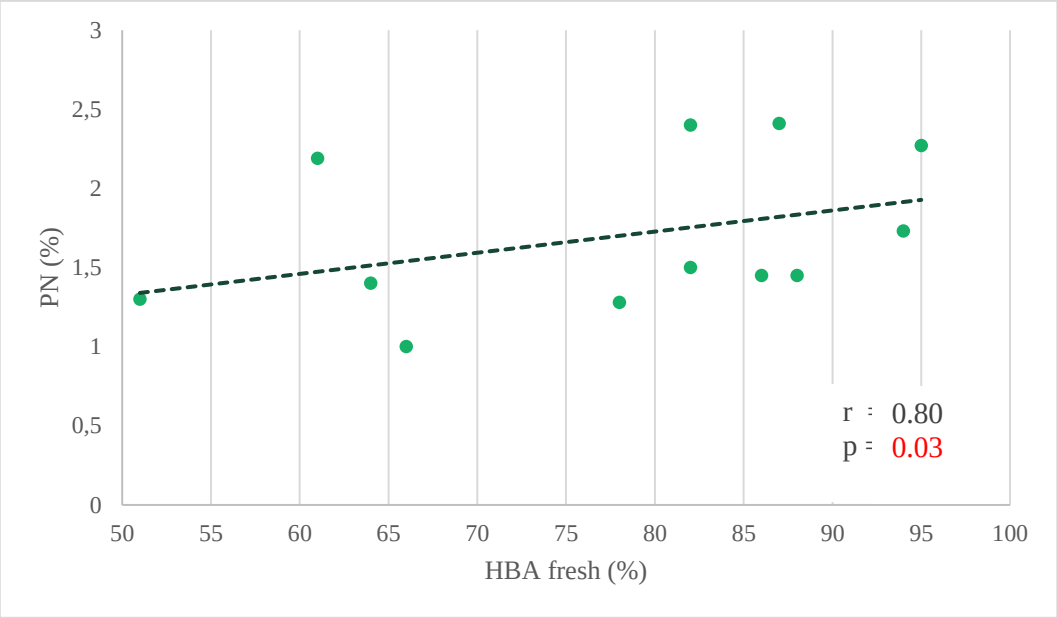

Figure 17. *Partial correlation coefficient (r) between Hyaluronan Binding Assay (HBA; %) results from fresh semen and formation of male pro-nucleus rate (PN, %). Trendline illustrated with dashed line.*

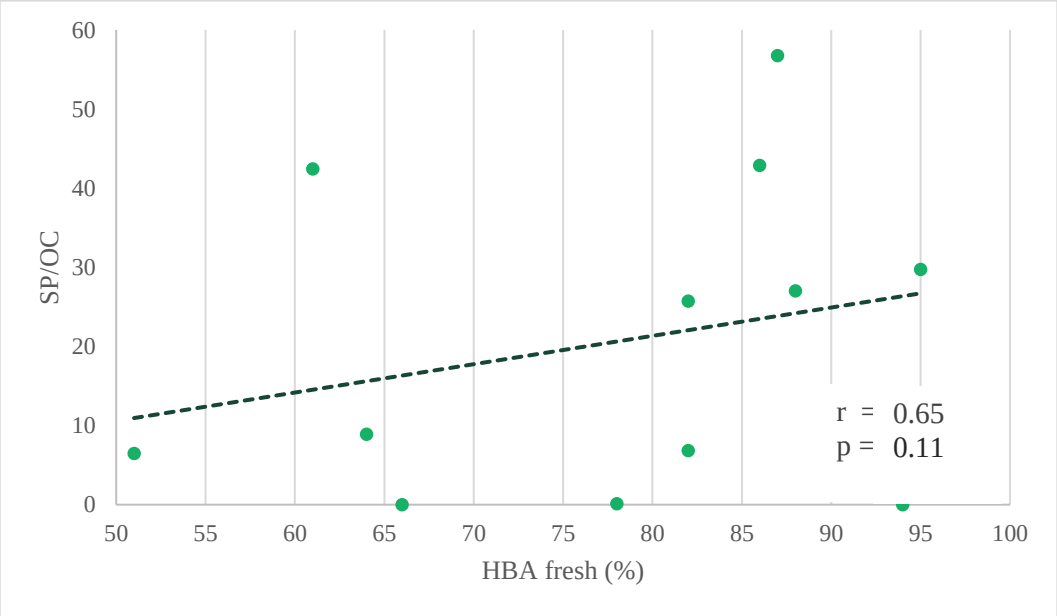

Figure 18. *Partial correlation coefficient (r) between Hyaluronan Binding Assay (HBA) results from fresh semen and number of oocyte penetrating sperm rates (SP/OC, %). Trendline illustrated with dashed line.*

The correlation between HBA from PT0H and SPA was not significant (figures 19, 20 and 21).

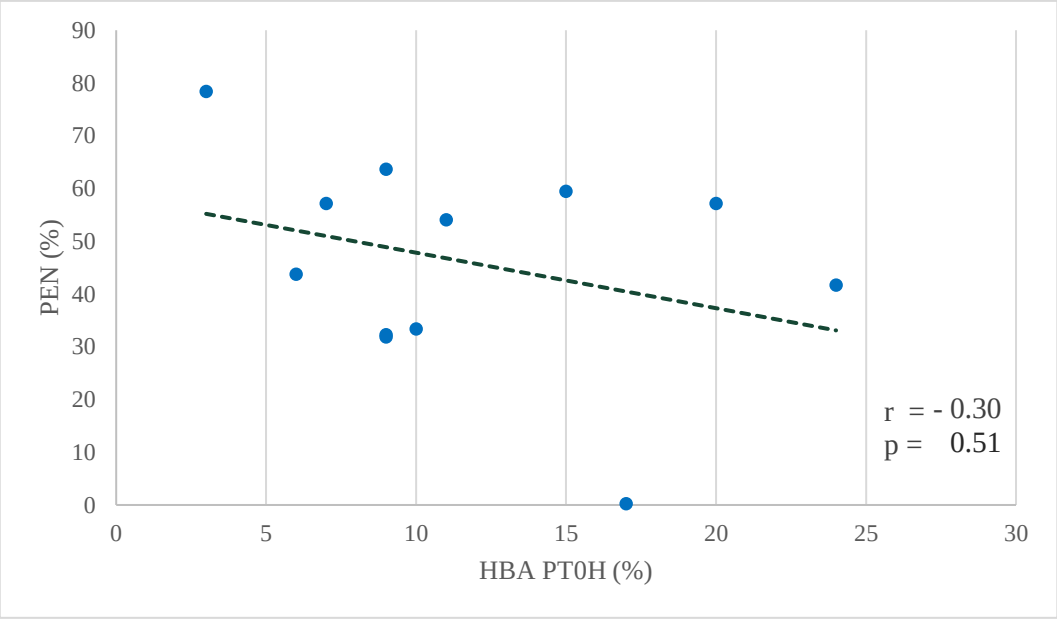

Figure 19. *Partial correlation coefficient (r) between Hyaluronan Binding Assay (HBA; %) results from after thawing at 0h (PT0H) sample and penetration rate (PEN, %). Trendline illustrated with dashed line.*

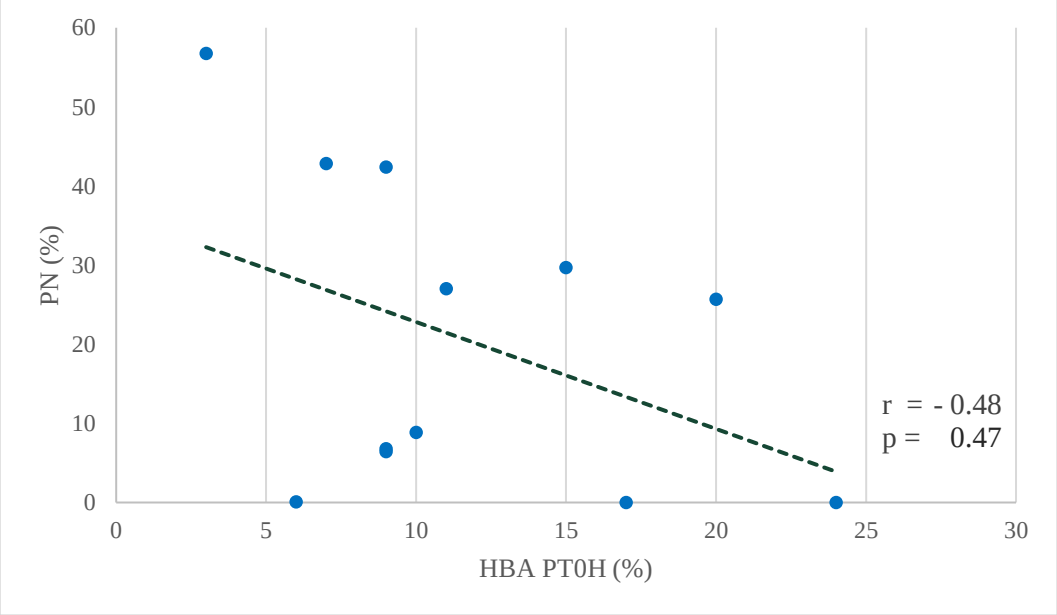

Figure 20. *Partial correlation coefficient (r) between Hyaluronan Binding Assay (HBA; %) results from after thawing at 0h (PT0H) sample and sperm penetration assay (SPA) values in percentages (PN, %). Trendline illustrated with dashed line.*

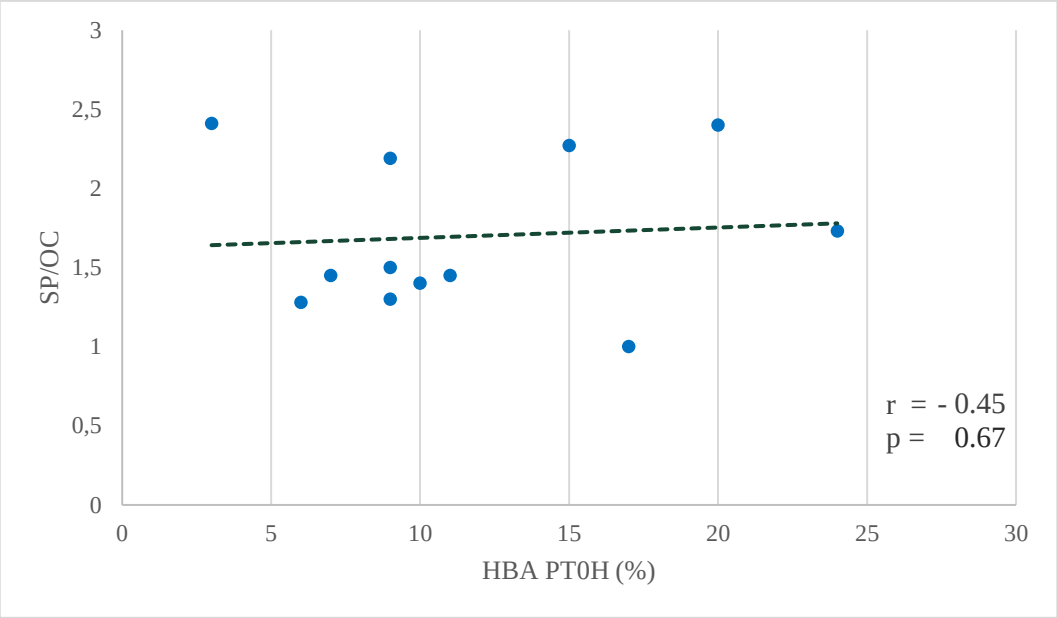

Figure 21. *Partial correlation coefficient (r) between Hyaluronan Binding Assay (HBA) results from fresh semen and number of sperm penetrated per oocyte (SP/OC). Trendline illustrated with dashed line.*

4.3.4. proAKAP4 correlations

There were no significant associations between proAKAP4 fresh or PT0H results and SPA parameters (PEN, PN and SP/OC).

Table 4. *Correlations between pro A–kinase anchor protein 4 (proAKAP4; %) results and sperm penetration assay parameters; penetration rate (PEN; %), male pro-nucleus formation (PN; %) and number of sperm penetrated per oocyte (SP/OC) for fresh (Fresh) and after thawing at 0 h (PT0H) samples. Results presented in p-values (p) and correlation coefficient values values (r).*

Parameters		PEN	PN	SP/OC
proAKAP4, fresh	p	0.47	0.27	0.32
	r	-0.33	-0.48	-0.45
proAKAP4, PT0H	p	0.59	0.98	0.42
	r	0.25	0.01	-0.36

5. Discussion

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

Our first finding was that dromedary camel spermatozoa bound to the HBA slides used in human and other species. The fresh sperm samples showed binding of 51 to 95 %, which is a higher proportion than reported with stallion spermatozoa (11 to 24%; Colleoni et al. 2011) or human spermatozoa (28 to 89 %; Hong et al. 2006). Frozen-thawed camel spermatozoa showed significantly less binding than fresh spermatozoa (3 to 24 %), and also less binding than frozen-thawed Nili-Ravi buffalo spermatozoa (27 to 57%; Awan et al. 2021). Low post-thaw binding was expected since dromedary camel spermatozoa do not tolerate freezing-thawing procedure well (Morton et al. 2010). Other factors potentially affecting the low binding rate could be interference from the semen extender that was used, sperm treatment and incubation time, as suggested in previous studies on stallion spermatozoa (Colleoni et al. 2011). It is not known whether receptors on camel spermatozoa function in the same way as on spermatozoa of other species and, therefore, results between species may not be comparable.

The HBA test 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 HBA test to be sensitive to select human spermatozoa with high PM and TM (Ye et al. 2006; Rashki et al. 2016). It has been suggested that only motile spermatozoa with normal morphology can bind to hyaluronan (Ye et al. 2006). The STR is related to the motility; in combination with VAP it is used to calculate the PM (Ringwelski 2020). However, this study did not find either VAP or TM to be significantly associated with HBA results. Since the HBA test detects mature spermatozoa (Biocoat 2022), a stronger correlation to some conventional parameters of sperm quality would have been expected, especially as higher VIT, TM and PM, MIT, and non-fragmented DNA have been

associated with greater sperm maturity (El Badry et al. 2015). Motility and morphology of human spermatozoa were associated with the HBA score (Ye et al. 2006; Rashki et al. 2016). A study with Nili-Ravi buffalo spermatozoa indicated a strong association between post-thaw HBA results and conventional parameters including PM, VIT 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, therefore, 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 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. In this study, the results of the SPA were indicative of sperm performance in HBA. Thus, HBA results from fresh samples and SPA results were significant for PEN and PN. The SP/OC showed no association with HBA score. These results are similar to those of previous studies on human spermatozoa where a high HBA score was associated with improved fertility *in-vitro* (Breznik et al. 2013; Worrilow et al. 2013) and *in-vivo* (Szucs et al. 2019; Awan et al. 2021). It should be noted that sperm-oocyte fusion in the goat oocyte penetration assay differs from the physiological situation, as the zona pellucida is absent. The assay also requires the spermatozoa to undergo spontaneous acrosome reaction when incubated for prolonged periods *in vitro*. Thus, it is possible that the results may not align with clinical IVF performance; further studies are needed to explore this possibility.

The associations between freezability, HBA results, conventional parameters of sperm quality, and sperm penetration assay results, were not significant. The results of the HBA and proAKAP4 test were not correlated for fresh sperm samples, but a correlation between them at PT0H was observed. Correlations between proAKAP4 test results and conventional parameters of sperm quality, sperm physical parameters or SPA results were not observed. The result is unexpected since a recent study reported that dromedary camel spermatozoa express proAKAP4, and the test results were strongly associated with vol, TM and visc (Malo et al. 2021).

In the present study, it was shown for the first time that HBA test might have potential to predict fertilization capacity of fresh dromedary camel sperm. Although the goat oocyte SPA is useful to assess camel sperm function *in vitro*, an *in vivo* study would be recommended to validate the results. Only six males and twelve

ejaculates were included in the study in total, and a future study with a larger number of animals should be conducted, preferably using semen from males of known good or bad fertility. A larger sample size might reveal correlations between other kinematics than PM and STR, as shown here. Also, the impact of the cryopreservation medium on sperm binding ability could be evaluated by repeating the assay after removing the cryoprotectant.

However, the results from this study are novel for the dromedary camel and are encouraging for the further investigation.

6. Conclusion

The HBA test was shown to have potential to predict the fertilizing capacity of fresh dromedary camel spermatozoa, although there was no association between HBA and proAKAP4. Of conventional parameters of sperm quality, only associations between HBA and PM and STR were identified. To establish the usefulness of the HBA test in dromedary camel sperm quality a further investigation with a larger sample size is required and an *in vivo* fertility association study would be recommended.

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Appendix 1

Appendix 1. *CASA settings*

Setup name: CAMEL PROGR		
Video capture	Frame capture speed (Hz)	60
	Frame count	30
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 (μm2)	101
	Head Size Min (μm2)	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
Morphology	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 Length	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

Appendix 2. *Binding % of fresh sperm ejaculates (fresh, n=12) and post-thaw 0 h (PT0H, n=12) sperm to Sperm-Hyaluronan Binding Assay (HBA; %) slides, as well as LSMean (± SEM) values for each male. For variation between males, p-values are presented.*

HBA binding of fresh and PT0H ejaculates for all samples		
Male, ejaculate	HBA, fresh	HBA, PT0H
A 1	66.0	17.0
A 2	86.0	7.0
LSMean (± SEM)	76.0 ± 10.0	12.0 ± 5.0
B 1	95.0	15.0
B 2	78.0	6.0
LSMean (± SEM)	87.0 ± 10.0	12.0 ± 5.0
C 1	51.0	9.0
C 2	0.87	3.0
LSMean (± SEM)	69.0 ± 10.0	6.0 ± 5.0
D 1	64.0	10.0
D 2	61.0	9.0
LSMean (± SEM)	63.0 ± 10.0	10.0 ± 5.0
E 1	82.0	20.0
E 2	88.0	11.0
LSMean (± SEM)	85.0 ± 10.0	16.0 ± 5.0
F 1	82.0	9.0
F 2	94.0	24.0
LSMean (± SEM)	88.0 ± 10.0	17.0 ± 5.0
p-value	0.39	0.46

Appendix 3. *Partial correlation statistics from fresh semen. Statistically significant p-values ($p \leq 0.05$) highlighted in red.*

	proAKAP4	ALH	BCF	LIN	STR	VAP	VCL	VSL	TM	PM	VIT	I	MIT	Conc	MA	Vol	Vis
HBA	0.8113	0.5277	0.0956	0.0899	0.0128	0.4096	0.3449	0.4475	0.6848	0.0210	0.1559	0.4041	0.6906	0.9414	0.2362	0.4960	0.1026
proAKAP4		0.2698	0.8762	0.5611	0.7831	0.0992	0.3155	0.0397	0.6714	0.4915	0.4868	0.5615	0.4250	0.3735	0.6620	0.7227	0.3840
ALH			0.1158	0.0608	0.0352	0.0002	<.0001	0.0674	0.4069	0.1607	0.5748	0.2782	0.9301	0.0953	0.9017	0.6205	0.0226
BCF				0.0009	0.0110	0.2325	0.0163	0.7041	0.9558	0.0389	0.7183	0.0852	0.8434	0.2189	0.8978	0.4431	0.0338
LIN					0.0011	0.3936	0.0162	0.3031	0.8011	0.0006	0.4194	0.0581	0.2642	0.6677	0.5737	0.7487	0.0118
STR						0.0762	0.0189	0.6371	0.6090	<.0001	0.1825	0.1794	0.6661	0.7770	0.4465	0.7252	0.0391
VAP							0.0005	0.0045	0.1791	0.4544	0.8904	0.6203	0.3143	0.0825	0.5527	0.1368	0.1152
VCL								0.1238	0.1882	0.1337	0.9867	0.1856	0.9571	0.1056	0.5009	0.3725	0.0161
VSL									0.0315	0.1626	0.3831	0.5844	0.1068	0.0736	0.1991	0.1362	0.8162
TM										0.1987	0.0064	0.3697	0.7554	0.3564	0.0116	0.1680	0.7604
PM											0.0753	0.0791	0.5094	0.7117	0.1638	0.7139	0.1156
VIT												0.6112	0.7885	0.6022	0.0087	0.3197	0.2287
IA													0.8101	0.7206	0.7211	0.4533	0.3999
MIT														0.2971	0.7213	0.1026	0.3262
Conc															0.7066	0.2627	0.1706
MA																0.3483	0.4952
Vol																	0.5440

Appendix 4. *Partial correlation statistics for pre-freeze (PF) results. Statistically significant p-values ($p \leq 0.05$) highlighted in red.*

	BCF	LIN	STR	VAP	VCL	VSL	TM	PM
ALH	0.31	0.26	0.34	0.12	<.0001	0.33	0.49	0.62
BCF		0.04	0.004	0.80	0.48	0.18	0.52	0.44
LIN			0.14	0.14	0.71	0.01	0.48	0.001
STR				0.23	0.41	0.46	0.41	0.59
VAP					0.01	0.002	0.11	0.04
VCL						0.06	0.57	0.80
VSL							0.23	0.01
TM								0.04

Appendix 5. *Partial correlations between precursor of A–kinase anchor protein 4 (proAKAP4; %) and sperm kinematics for after thawing at 0 h (PT0H) spermatozoa. Significant p-values ($p \leq 0.05$) highlighted in red.*

	proAKAP4	ALH	BCF	LIN	STR	VAP	VCL	VSL	TM	PM	VIT	IA	MIT
HBA	0.03	0.09	0.96	0.86	0.65	0.15	0.12	0.28	0.82	0.98	0.15	0.27	0.75
proAKAP4		0.29	0.55	0.93	0.72	0.65	0.47	0.76	0.69	0.75	0.10	0.05	0.83
ALH			0.83	0.42	0.87	0.002	<.0001	0.09	0.82	0.93	0.57	0.91	0.84
BCF				0.26	0.38	0.94	0.62	0.78	0.92	0.99	0.37	0.93	0.35
LIN					<.0001	0.27	0.93	0.01	0.10	0.001	0.03	0.35	0.34
STR						0.11	0.47	0.003	0.19	0.01	0.13	0.70	0.49
VAP							<.0001	<.0001	0.13	0.10	0.34	0.35	0.35
VCL								0.01	0.38	0.47	0.88	0.69	0.61
VSL									0.06	0.01	0.11	0.27	0.30
TM										0.005	0.02	0.004	0.01
PM											0.01	0.04	0.34
VIT												0.003	0.05
IA													0.07

Appendix 5. *Partial correlations between Hyaluronan Binding Assay (HBA; %), precursor of A–kinase anchor protein (4proAKAP4; %) results, and different parameters of sperm quality to predict freezability. Significant p-values ($p \leq 0.05$) highlighted in red.*

	proAKAP4 Fresh	HBA PT0H	proAKAP4 PT0H	TM PT0H	TM PT1.5H	PM PT0H	PM PT1.5H	VIT PT0H	VIT PT1.5H	IA PT0H	IA PT1.5H	MIT PT0H	MIT PT1.5H	PEN PT0H	PN PT0H	SP/OC PT0H
HBA Fresh	0.8113	0.4460	0.4200	0.3837	0.4368	0.8043	0.8061	0.8459	0.6431	0.8089	0.3928	0.7697	0.2043	0.0774	0.3447	0.2162
proAKAP4 Fresh		0.6700	0.6923	0.7293	0.3016	0.1523	0.5240	0.6067	0.7387	0.5125	0.7643	0.3044	0.5474	0.7877	0.5754	0.6759
HBA PT0H			0.0310	0.8210	0.8618	0.9840	0.5617	0.1527	0.9617	0.2725	0.0541	0.7456	0.8009	0.3650	0.1077	0.8007
proAKAP4 PT0H				0.6892	0.3928	0.7460	0.7004	0.1002	0.2964	0.0548	0.0069	0.8286	0.3873	0.8667	0.7546	0.2414
TM PT0H					0.0005	0.0049	0.0442	0.0173	0.0024	0.0035	0.1658	0.0070	0.0713	0.0547	0.0780	0.2064
TM PT1.5H						0.0011	0.0005	0.0488	0.0007	0.0051	0.0588	0.2788	0.0310	0.0195	0.0237	0.0348
PM PT0H							0.0020	0.0112	0.0473	0.0411	0.2285	0.3377	0.0353	0.2425	0.1304	0.4728
PM PT1.5H								0.0901	0.0123	0.0778	0.2122	0.5394	0.0219	0.1626	0.0906	0.1236
VIT PT0H									0.0281	0.0003	0.0130	0.0544	0.0126	0.7643	0.8552	0.4835
VIT PT1.5H										0.0009	0.0200	0.0715	0.0164	0.0298	0.0656	0.0113
IA PT0H											0.0045	0.0702	0.0094	0.1626	0.2629	0.0499
IA PT1.5H												0.5199	0.1169	0.3029	0.7125	0.0793
MIT PT0H													0.1532	0.7368	0.8550	0.9746
MIT PT1.5H														0.8441	0.5632	0.6094
PEN PT0H															<.0001	0.0012
PN PT0H																0.0165