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Ramifications of protease-based liquefaction of camel semen on physical, kinematic and surface glyco-pattern of cryopreserved spermatozoa

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ABSTRACT

The efficiency of incorporating different proteases in the diluent for reducing camel semen viscosity, and subsequent ramifications on morpho-functional and glycan surface properties of cryopreserved spermatozoa were investigated. Ejaculates (n = 48) were collected from three adult camels, *Camelus dromedarius*, during the breeding season (January - March). A portion of each raw ejaculate was evaluated for sperm physical and morphological traits, whereas the other portion was divided into three aliquots assigned for the following liquefaction treatments: control (untreated), 0.1 mg/mL papain or 5 U/mL bromelain. All samples were diluted with Tris-lactose diluent containing the anti-enzyme E-64 to neutralize both proteases before being processed for cryopreservation. Post-thaw physical and kinematic properties of spermatozoa were analyzed using a computer-assisted sperm analysis (CASA) system. The sperm surface glycocalyx pattern was evaluated with a panel of 14 fluorescent lectins. Although bromelain was more effective in elimination of semen viscosity, there was a negative correlation between bromelain supplementation and values for the variables: normal sperm, intact acrosome and intact sperm cell membrane. Bromelain supplementation, compared to papain-treated and control samples, was positively correlated with secondary sperm abnormalities, increased straight-line velocity (VSL, μm/s) and straightness (%) of spermatozoa. Results from the glycan analysis indicated that both proteases did not affect the N-linked glycan content of the entire sperm surface, whereas the treatment with proteases induced little change in N-acetylgalactosamine and fucose terminating glycans in the tail region of the sperm. Functional studies are needed to evaluate the sperm fertility rates of bromelain- and papain-treated semen for application in camel assisted reproductive technologies.

1. Introduction

The highly viscous nature of *camelidae* semen is considered the major impediment for application of assisted reproductive

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techniques aiming to improve the reproductive efficiency in *camelidae* species. Although this semen feature is essential for maintaining sperm viability and fertilization capacity within the female reproductive tract following natural mating (Deen et al., 2005; Vaughan and Tibary, 2006), it impedes qualitative sperm assessment (Deen, 2008; El-Bahrawy, 2010; Shekher et al., 2012) and disrupts homogenous mixing of semen with nutrients and cryoprotective compounds. There have, therefore, been efforts in recent years to develop semen liquefaction procedures.

Several techniques were used to reduce semen viscosity and to improve *camelidae* sperm motility. For example, mechanical liquefaction of semen by stirring (Niasari-Naslaji et al., 2007), needling and pipetting (Morton et al., 2008), centrifugation (El-Bahrawy, 2010) and treatment with ultrasonic waves (Rateb, 2016) have been conducted in semen processing protocols with different success rates. Supplementing semen diluent with different enzymes that function via different pathways (i.e., long-chain carbohydrate cleavage; El-Bahrawy, 2010), or amino acid chain cleavage (Shekher et al., 2012; Kershaw-Young et al., 2013, 2017; Monaco et al., 2016) were also investigated for their capacity to reduce semen viscosity. Most recently, both mechanical and enzymatic techniques were successfully used in concert to reduce dromedary semen viscosity (El-Bahrawy, 2017). Nontheless, the effects emerging when utilizing such reagents on sperm characteristics, particularly following cryopreservation, were not consistent among the studies.

According to some authors, semen viscosity was attributed to presence of a highly condensed network of muco-polysaccharides (Mosaferi et al., 2005; El-Bahrawy, 2010; Skidmore et al., 2013), while in other studies proteins in the seminal plasma were thought to be the primary factors contributing to semen viscosity (Kershaw-Young et al., 2013; Mal et al., 2016; Monaco et al., 2016). With this latter consideration, different proteases were used to reduce semen viscosity but the effects on spermatozoa after processing differed markedly among the studies.

Both bromelain and papain have peptidase–like activity (Rawlings and Barrett, 1994) that induces cleavage of piptide bonds in amino acid chains (Kershaw-Young et al., 2013). Results from a study of Pattinson et al. (1990) indicated that incubating normal human spermatozoa with papain led to impairment of oocyte penetration in the zona-free hamster egg penetration test, which may reflect alterations of constituents in the sperm acrosomal region. Nonetheless, the negative effects of such proteases on sperm structure and function are not completely understood (Kershaw-Young et al., 2017).

The sperm glycocalyx is a dense carbohydrate layer that, along with proteins and lipids, coats the sperm membrane surface (Schroter et al., 1999). Typically, sperm surface glycocalyx undergoes a marked rearrangement during the process of sperm formation, maturation, capacitation and acrosome reaction. The glycocalyx, therefore, affects sperm maturation and motility (Tollner et al., 2012; Tecle and Gagneux, 2015). Furthermore, subtle changes in the glycocalyx has been reported to markedly affect sperm fertilization potential (Purohit et al., 2008; Xin et al., 2016). In addition, cryopreservation of spermatozoa has been reported to affect sperm glycan characteristics in birds (Long, 2006; Pelaez et al., 2011), sheep (Pini et al., 2017) and humans (Wu et al., 2017; Xin et al., 2018). These studies, besides focusing on the glycosylation pattern of mammalian sperm glycocalyx, have been conducted using different lectins. Lectins, which are proteins/glycoproteins isolated from a wide variety of plant and animal sources, are particularly well suited for having actions on specific glycoconjugates because of the specificity and capacity of these compounds to have actions on sugar isomers as well as at branch, and linkage sites, as well as at the site where there are terminal modifications of complex glycans (Sharon and Lis, 2004).

There have been no studies for which there are reports on the effects of protease treatment on frozen-thawed camel sperm motility and glycocalyx. The present study was conducted to qualitatively evaluate the effectiveness of incorporating two proteases (i.e., bromelain and papain) in the semen dilution medium to reduce camel semen viscosity. Subsequent ramifications on physical and kinematic properties of cryopreserved spermatozoa, as well as the effects of treatments on the surface glycosylation pattern were investigated.

2. Materials and methods

2.1. Animals and management

The study was conducted at the Artificial Insemination Laboratory, Mariout Research Station (Latitude 31° 00′ N; Longitude 29° 47′ E), Alexandria, Egypt, as well at the Department of Emergency and Organ Transplantation, University of Bari Aldo Moro, Italy. Three adult dromedary bulls, *Camelus dromedarius*, aged 15–22 years, with an average body weight of 650 ± 50.0 kg and body condition score 2.5 ± 0.5 (Faye et al., 2011) were used during the breeding season (January-March). The males were housed individually in shaded pens throughout the period of the study. The males were fed a concentrate mixture composed of 50% corn, 47% barley, 2% minerals, and 1% salt at the rate of 4 kg/animal/day. Egyptian clover, *Trifolium alexandrinum*, hay was provided ad libitum and fresh water was available once daily at midday. Prior to conducting the study, all camels were clinically examined to assure they were free of diseases or reproductive disorders. All procedures were conducted conforming to the EU Directive for protection of experimental animals (2010/63/EU).

2.2. Preparation of sperm cryopreservation medium

Unless otherwise stated, all reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). A glycerolized (3% glycerol, v/v) tris-lactose egg yolk extender was prepared for dilution of semen using procedures that were previously described (El-Bahrawy et al., 2017). The medium was prepared 24 h prior to each collection session and was stored at 4 °C until used.

2.3. Semen collection

A total of 48 ejaculates were collected from the three camels, 16 ejaculates each, during the breeding season (January - March) by using an artificial vagina (AV) and a female "teaser" animal. Semen was collected at 07.00 h twice-weekly using the procedures described by Bahrawy et al. (2012). Disposable polyethylene sheaths were used to inlayer the AV, and double-wall collection tubes were used to maintain the ejaculates at 37 °C during the collection sessions. Immediately after collection each ejaculate was transported to the laboratory, which was directly adjacent to the collection area, in a portable water bath adjusted at 37 °C, for physical and morphological assessment.

2.4. Experimental design

Only ejaculates where sperm had an initial total motility (oscillatory and/or progressive) exceeding 90% were processed. Mean values of physical and morphometric traits of raw semen throughout the period of the study were: 5.8 ± 0.7 mL, 4.2 ± 0.1, 90.8 ± 2.4%, 88.3 ± 2.3%, 94.0 ± 0.4% and 85.7 ± 2.4% for ejaculate volume, motility score (5 = highly motile, 0 = immotile; Crichton et al., 2015), viability, normal sperm, intact acrosome and intact sperm cell membrane, respectively.

The other portion of raw semen was divided into three aliquots assigned for the following liquefaction treatments: control (untreated), 0.1 mg/mL papain (Sigma-Aldrich, USA) or 5 U/mL bromelain (Sigma-Aldrich, USA). Treated samples were placed in a water bath (37 °C) for 5 min, and were mechanically mixed with the liquefaction enzymes using a pipette once every 30 s. Afterwards, each sample was diluted (1:1) with non-glycerolized Tris-lactose. The medium of treated samples was supplemented with 10 µM N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64; Sigma-Aldrich, USA) to neutralize the proteolytic enzymes (Kershaw-Young and Maxwell, 2012). All diluted semen groups were then equilibrated for 90 min at 4 °C, thereafter another portion of chilled glycerolized medium (6%, v/v) was added to each sample to have a final concentration of 3% glycerol in the medium. All specimens were equilibrated for another 90 min at 4 °C before being packed in 0.5 mL French straws using a Minitüb filling and sealing machine (Model 133, Minitüb, Germany). Prior to packing, the sperm count was determined for control and liquefied chilled samples using a hemocytometer (Neubauer, improved double, Superior Ltd, Germany) and sperm concentration was adjusted at 50×10^6 sperm/straw. Cryopreservation was conducted by placing the straws in a Minitüb biological freezer exposing the cells to nitrogen vapor (−80 °C) for 10 min. Immediately after, the straws were plunged into liquid nitrogen (−196 °C). The frozen straws were stored in liquid nitrogen until further assessment.

2.5. Computer-assisted sperm analysis (CASA)

The frozen straws (5 per treatment) were thawed using a programmable thawing device (mini-tube, Germany) adjusted to 38 °C for 40 s. After thawing, each sample was assessed for sperm physical and kinematic properties using a computer-assisted semen analysis (CASA) system (Mira-9000, Mira Lab, Egypt) following the world health organization strict criteria for sperm analyses (WHO, 2010). Prior to assessment, the system was calibrated for normal camel sperm motility and morphometric properties. The reference limits for sperm motility and morphology were set according to WHO (2010) cut-off values. A 10 µl drop of semen was loaded onto the CASA's slide for dynamic sperm analyses and covered with a 10 x 10 mm cover slip. At least 200 sperm from ten random bright fields were evaluated by spermolyzer software (Spermolyzer, Mira Lab, Egypt) for motility variables (i.e., total motility (%), progressive motility (%), non-progressive motility (%), proportion of immotile sperm (%) and viability (%) at 400 x magnification). Additionally, sperm kinematics in terms of straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), wobble movement coefficient (WOB, %), linearity (LIN, %) and straightness (STR, %), were also determined.

Sperm viability (%) was evaluated by mixing and smearing 20 µl of semen and 10 µl of freshly-prepared eosin-nigrosin stain on a warm stage. Sperm abnormalities (%) and acrosome integrity (%) were analyzed after staining fixed semen smears using Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt). Preparation and staining of smears were conducted according to the manufacturer's instructions, and stained smears were evaluated using CASA at 1000x magnification. The hypo-osmotic swelling (HOS) test was used to evaluate integrity of sperm plasma membranes as previously described (Mosaferi et al., 2005). At least 200 sperm were evaluated using a phase contrast microscope (Leica, Germany) at 40x magnification.

2.6. Determining effects on sperm glycocalyx pattern

After thawing, spermatozoa of control and treated samples were prepared as previously described (Accogli et al., 2017). Briefly, spermatozoa were fixed in 4% (v/v) buffered paraformaldehyde, pH 7.4, for 45 min at room temperature (RT) and then pelleted using centrifugation at 800 x *g* for 5 min. After supernatant removal, sperm were washed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.4), smeared on poly-L-lysine coated glass slides and air-dry fixed. Dried slides were then incubated at room temperature for 1 h in a darkened area with appropriate dilutions of 14 fluorescent biotinylated lectins (Table 1) diluted in 0.05 M Tris–HCl-buffered saline (TBS) (pH 7.4). All lectins were obtained from Vector Laboratories (Burlingame, CA, USA). After incubation, the slides were rinsed three times in the same buffer and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, USA). The controls (negative staining) included: i. substitution of the substrate medium with buffer lacking lectin; ii. incubation with each lectin in the presence of its hapten sugar (0.2 Min Tris buffer). All samples placed on slides were assessed using a fluorescent photomicroscope (Eclipse Ni-U, Nikon, Japan) equipped with a digital camera (DS-U3, Nikon, Japan). Exposition conditions were

Table 1
List of 14 fluorescent lectins, the sugar specificities and inhibitory sugars utilized to visualize glycan structural content of cryopreserved camel spermatozoa after semen liquefaction using proteolytic enzymes.

Lectin abbreviation	Concentration (μg/mL)	Lectin source	Sugar specificity	Inhibitory sugar
SNA	15	*Sambucus nigra*	Neu5Acα2,6 Gal/GalNAc	NeuNAc
RCA_{120}	20	*Ricinus communis*	Terminal Galβl,4GlcNAc	Galactose
Con A	15	*Canavalia ensiformis*	Terminal/internal αMan > αGlc	Mannose
PHA-E	20	*Phaseolus vulgaris E*	bisected complex GlcNAc βl-2Man	Mannose
PHA-L	20	*Phaseolus vulgaris E*	GlcNAcβl,2Man	Mannose
sWGA	15	*Triticum vulgaris*	Terminal/internal βGlcNAc	GlcNAc
GSA I-B_4	20	*Griffonia simplicifolia*	Terminal αGal	Galactose
HPA	20	*Helix pomatia*	GalNAcαl,3GalNAc	GalNAc
DBA	25	*Dolichos biflorus*	Terminal GalNAcαl,3(LFucαl,2)Galβl,3/4GlcNAcβl	GalNAc
SBA	20	*Glycine max*	Terminal α/βGalNAc	GalNAc
PNA	25	*Arachis hypogaea*	Terminal Galβl,3GalNAc	Galactose
GSA II	20	*Griffonia simplicifolia*	Terminal D-GlcNAc	GlcNAc
LTA	25	*Lotus tetragonolobus*	Terminal αL-Fuc	Fucose
UEA I	20	*Ulex europaeus*	Terminal L-Fucαl,2Galβl,4GlcNAcβ	Fucose

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid; s, succinylated.

maintained for all images, and the images were analyzed using the NIS Elements BR software (Ver. 4.20; Nikon, JP).

2.7. Statistical procedure

The data were assessed using the Shapiro-Wilk's test and were found to have a normal distribution. Levene's test was used to determine homogeneity of variance and data were ascertained to be homogenous. A one-way analysis of variance (ANOVA) was used to compare post-thaw physical, morphological and kinematic properties of spermatozoa among all semen groups. The threshold of significance was set at 5% and the differences between means were detected using the Tukey's *post-hoc* test. The correlations between liquefaction treatments and post-thaw semen characteristics were determined using Spearman's correlation coefficient. The data were analyzed using IBM-SPSS statistics for windows (IBM Corp., 2013). The results are expressed as means ± standard error of mean (SEM).

3. Results

3.1. Effects on post-thaw sperm variables

Reduction in semen viscosity by using either of the proteolytic enzymes, prior to cryopreservation, affected ($P < 0.05$) the values for all sperm physical and morphological variables (Table 2). Treatment with both proteases improved post-thaw sperm motility compared to the control samples (Table 2). Likewise, there were similar trends for the percentage of progressive motility (Table 2). There was no difference in post-thaw sperm viability (live sperm, %) among the three semen groups. Treatments with both bromelain and papain resulted in the least ($P < 0.05$) percentages of normal sperm, intact acrosomes and intact sperm cell membranes (Table 2). The major form of observed abnormalities were of the secondary type, and comprised presence of distal/translocated

Table 2
Post-thaw computer-assisted evaluation of cryopreserved dromedary camel sperm characteristics after semen liquefaction using different proteolytic enzymes (mean ± SEM).

Variable	Liquefaction treatment		
	Control	Bromelain	Papain
Total motility (%)*	56.9 ± 3.9 ^{b}	71.5 ± 2.8 ^{a}	61.9 ± 1.4 ^{ab}
Progressive motility (%)**	22.1 ± 4.1 ^{b}	35.3 ± 2.7 ^{a}	25.6 ± 2.3 ^{ab}
Sperm vitality (%)	70.0 ± 2.9	83.3 ± 1.7	75.0 ± 1.9
Normal sperm (%)	54.7 ± 0.9 ^{a}	21.0 ± 0.6 ^{b}	20.7 ± 1.2 ^{b}
Primary abnormalities (%)	2.0 ± 0.5	3.7 ± 0.3	3.0 ± 0.6
Secondary abnormalities (%)	43.3 ± 1.3 ^{b}	75.3 ± 3.5 ^{a}	76.3 ± 2.1 ^{a}
Acrosome integrity (%)	52.3 ± 1.2 ^{a}	13.3 ± 0.9 ^{b}	7.7 ± 0.4 ^{c}
Intact sperm cell membrane (%)	31.3 ± 1.9 ^{a}	7.7 ± 0.7 ^{b}	6.7 ± 0.9 ^{b}

$^{a-c}$Means with different superscripts in the same row are significantly different at $P < 0.05$.
* Motility was evaluated based on total sperm movement; i.e. oscillatory and progressive movements.
** Rapid (class-A) and slow (class-B) progressive movement were considered (WHO, 2010).

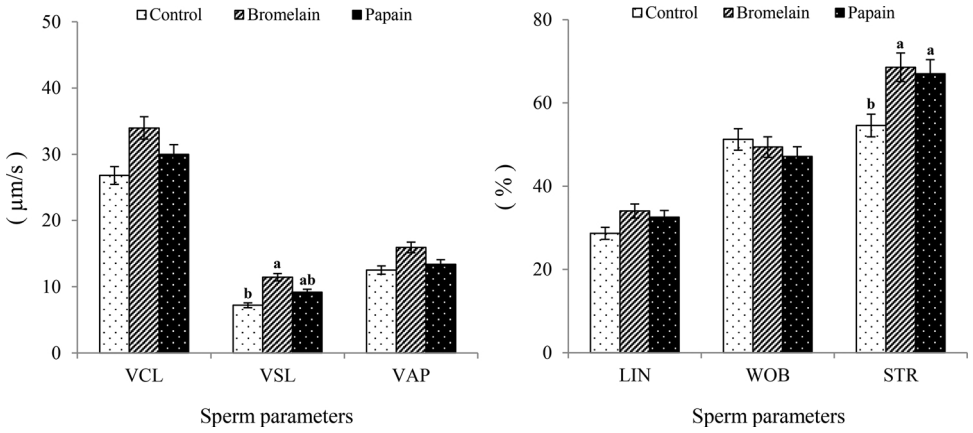

Fig. 1. CASA-derived kinematics of post-thaw dromedary camel spermatozoa after liquefaction of semen by different proteolytic enzymes (mean ± SEM). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity (VSL/VCL); WOB, wobble movement coefficient (VAP/VCL); STR, straightness (VSL/VAP).

cytoplasmic droplets with tail openings and/or defective tails (bent, folded or coiled tails) (Table 2). Furthermore, treatment of samples with bromelain or papain resulted in the least ($P < 0.05$) proportion of spermatozoa with intact acrosomes as compared to control samples (Table 2).

In addition, there was a negative correlation ($P < 0.01$) between the values when there was bromelain supplementation for percentages of normal sperm, intact acrosomes and intact sperm cell membranes (r = -0.77, -0.95 and -0.80, respectively), while there was a positive correlation ($P < 0.01$) for secondary sperm abnormalities ($r = 0.90$).

3.2. Effects on post-thaw sperm kinematics

The results for CASA-derived motility and velocity patterns indicated that both liquefaction treatments only affected ($P < 0.05$) straight-line velocity (VSL, µm/s) and straightness (%) of spermatozoa (Fig. 1). In this respect, both bromelain- and papain-treated samples had a greater ($P < 0.05$) VSL compared to control samples, with no significant difference between both protease-treated samples, with values 11.4 ± 0.5, 9.2 ± 0.4 and 7.2 ± 0.4 µm/s, respectively. There was also a similar trend for sperm straightness with corresponding values of 68.6 ± 3.4, 67.0 ± 3.3 and 54.6 ± 3.1% ($P < 0.05$), respectively (Fig. 1).

3.3. Effects on post-thaw sperm glycan structures

The lectin-binding pattern of the glycocalyx for control, bromelain and papain-treated semen is summarized in Table 3. The comparison of the lectin reactivity revealed four glycocalyx lectin-binding patterns, which are subsequently described in this

Table 3
Lectin staining pattern of the glycocalyx from control, bromelain- and papain-treated camel spermatozoa.

Lectin	Head									Tail		
	Acrosomal cap			Equatorial region			Post-acrosomal cap					
	C	B	P	C	B	P	C	B	P	C	B	P
SNA	–	–		–	–	–	–	–	–	+	+	+
RCA_{120}	±	±	±	–	+*	+*	–	–	–	+	+	+
Con A	+	+	+	+	+	+	±	±	±	+	+	+
PHA-E	±	±	±	–	–	–	–	–	–	+	+	+
PHA-L	–	–	–	–	–	–	–	–	–	+	+	+
sWGA	+	+	+	+		+				±	±	±
GSA I-B_4	+*	+*	–	–	–	–	–	–	–	±	±	±
HPA	+	+	+	–	–	–	–	–	–	+	±	±
DBA	–	–	–	–	–	–	–	–	–	+	+	+
SBA	–	–	–	–	–	–	–	–	–	+	+	+
PNA	+	+	+	–	–	–	–	–	–	±	±	±
GSA II	–	–	–	–	–	–	–	–	–	±	±	–
LTA	–	–	–	–	–	–	–	–	–	–	+	–
UEA I	–	–	–	–	–	–	–	–	–	±	±	±

C, control; B, bromelain; P, papain; s, succinyl; *, few reactive cells; -, negative reaction; ± , faintly visible reaction; +, intense positive reaction.

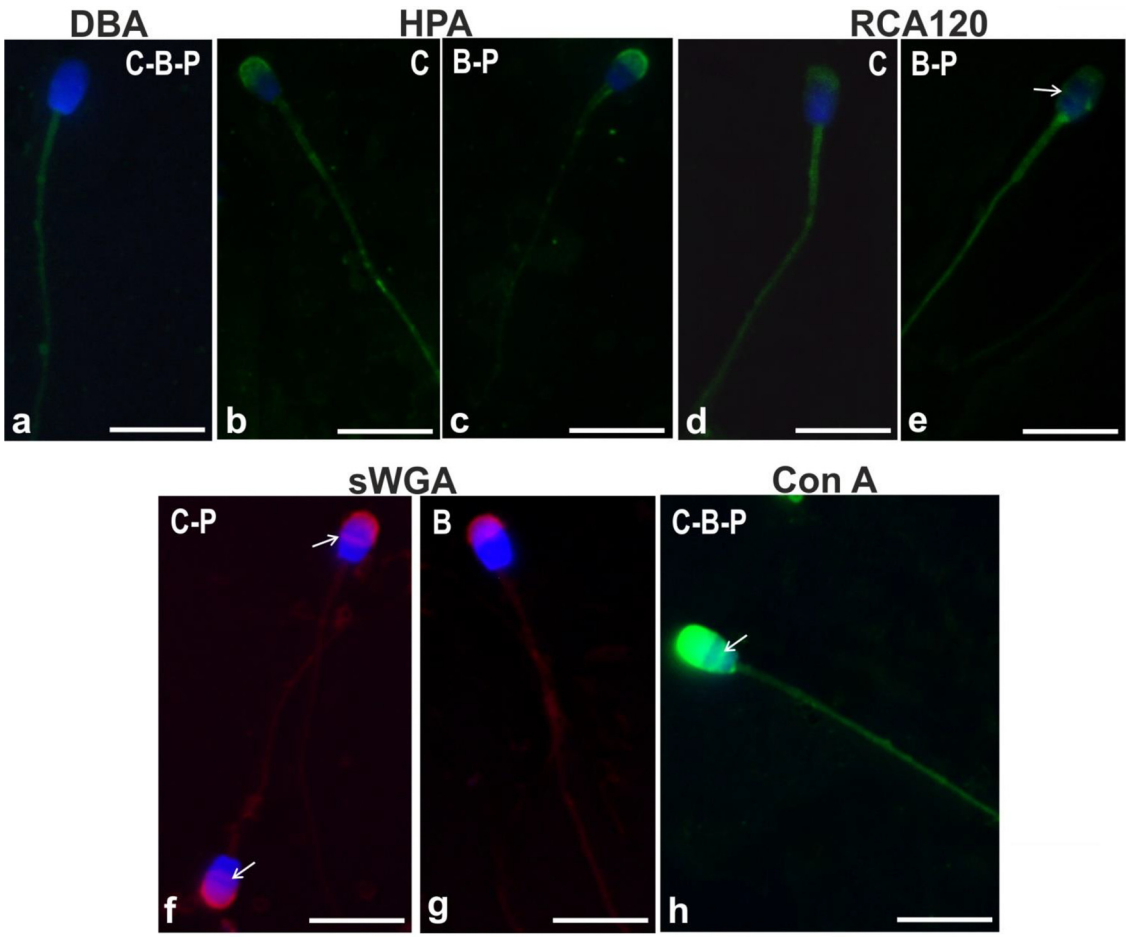

Fig. 2. Representative illustration of lectin-binding pattern of ejaculated dromedary sperm; a) DBA staining of dromedary sperm; b) HPA staining of the control spermatozoa; c) HPA staining of papain-treated spermatozoa; Note the lesser reactivity of the tail compared to b. d) RCA_{120} reactivity of control spermatozoa; e) RCA_{120} affinity of enzyme-treated spermatozoa; Note the binding sites in the equatorial zone compared to d; f) succinyl WGA binding to the control and papain-treated spermatozoa; g) succinyl WGA affinity for bromelain-treated sperm; Note the absence of the equatorial zone reactivity compared to f; h) Con A staining pattern of papain-treated spermatozoa; Note the faint reactivity in the post-equatorial region; B, bromelain; C, control; P, papain; s, succinyl; Arrow, equatorial region; Bar: 10 μm.

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The fluorescent biotinylated lectins, SNA, PHA-L, DBA, SBA, UEA I, GSA II and LTA, bound to the tail of the sperm (Fig. 2a). The fluorescent biotinylated lectins, SNA, PHA L, DBA, DBA and UEA I, reacted with the tail of all sperm cells evaluated, whereas with the fluorescent biotinylated lectin, GSA II, there was no staining in the tail of papain-treated spermatozoa and for the fluorescent biotinylated, LTA, there were binding sites in the tail of bromelain-treated sperm. For the fluorescent biotinylated lectins, PHA-E, HPA, PNA, and GSA I-B4, there were binding sites in the cap and tail of all the sperm samples. Bromelain and papain treatment did not affect the fluorescent biotinylated lectin, PHA-E and PNA staining intensity, in the different parts of the spermatozoa, whereas there was a reduced fluorescent biotinylated lectin, HPA, reactivity at the sperm tail (Fig. 2b, c). Furthermore, papain-treatment inhibited the fluorescent biotinylated lectin staining of GSA I-B4 in the sperm head.

The fluorescent biotinylated lectin, RCA120 and succinyl WGA bound to the acrosomal cap and tail of all sperm samples. Furthermore, in the equatorial region of the sperm head there was binding of the fluorescent biotinylated lectin, RCA120, after bromelain and papain treatments (Fig. 2d), and lack of fluorescent biotinylated lectin, succinyl WGA reactivity, after treatment with bromelain (Fig. 2e,f). Lastly, Con-A reacted with the entire surface of normal spermatozoa (Fig. 2g).

4. Discussion

In the present study, the effect of liquefying camel semen using bromelain and papain prior to cryo-storage on the physical, kinematic and glycosylation pattern of spermatozoa was investigated. The results from assessment of the sperm motility pattern indicated that supplementing cryopreservation medium with bromelain was more effective in reducing semen viscosity as compared to papain treatment. The results of post-thaw sperm kinematics also indicated similar effects as a result of bromelain treatment. This

was clearly evident because bromelain-treated semen had 1.3, 1.6 and 1.6 times higher total motility, progressive motility and straight-line velocity than sperm of control samples, respectively. This finding is consistent with that form previous studies with camels (Monaco et al., 2016; El-Bahrawy, 2017). Results of the present study also showed that liquefying semen with either of the powerful proteases, even after using an E-64 anti-enzyme, resulted in an increase in sperm secondary abnormalities and reduced post-thaw sperm acrosome and membrane integrities. Similar results were reported by El-Bahrawy (2017). This finding, however, is not consistent with results of Kershaw-Young et al. (2017) in alpacas. From this study, it was reported that supplementing semen diluent with papain enhanced sperm motility and viability without affecting acrosomal or DNA integrities post thaw when the anti-enzyme E-64 was used to terminate papain activity.

Camel semen viscosity was attributed either to presence of mucopolysaccharides and/or proteins in the seminal plasma. Such viscosity inhibits spermatozoa motility (Zeidan and Abbas, 2003; Deen et al., 2005). Reducing semen viscosity allows for spermatozoa motility to be enhanced as a result of increased total and progressive motility. Because the anti-enzyme E-64 was used in the present research, it is suggested that the increased percentages of abnormalities and altered acrosome, as well as damaged sperm cell membranes could be either due to spermatozoa undergoing the freezing/thawing cycle (Bagchi et al., 2008), or the amount of E-64 used was not sufficient to effectively inhibit the effects of both proteases.

In the meantime, even subtle rearrangements in the sperm surface glycocalyx have been reported to affect sperm fertilization capacity (Xin et al., 2016). Also, cryopreservation of spermatozoa has detrimental effects on sperm glycan structures (Wu et al., 2017). In camelides, these effects are speculated to be greater when cryopreserved spermatozoa are subjected to enzymatic liquefaction of semen using proteases.

In the present study, the results of lectin histochemistry showed that the acrosomal cap of control spermatozoa contained binding sites for RCA120, Con A, PHA-E, succinyl WGA, GSA I-B4, HPA, and PNA. This indicates presence of both N- and O-linked glycans. N-glycans were highly mannosilated and were bisected types (Con A, PHA-E) containing N-acetylglucosamine (succinyl WGA) and terminating with N-acetyllactosamine (RCA120). Sperm O-glycans contained the core 1 disaccharide (Galβ1,3GalNAc; termed T antigen; PNA) and a terminal αGalNAc (HPA). There was no change in the staining intensity of these lectins in the acrosomal cap of both bromelain- and papain treated sperm. There were also a few spermatozoa containing a terminal α-galactose (GSA I-B4) in the acrosomal cap of control and bromelain-treated spermatozoa. Papain-treated sperm did not have this sugar in the acrosomal cap. This finding indicates that bromelain treatment could preserve the acrosomal cap glycocalyx of dromedary spermatozoa to a greater extent than papain treatment. There is Con A affinity in the acrosomal region of the sperm of several mammalian species such as the rabbit (Nicolson et al., 1977), boar (Jiménez et al., 2003), dog (Kawakami et al., 2004), stallion (Desantis et al., 2010), buffalo bull (Accogli et al., 2017), and ram (Pini et al., 2017). This conserved N-linked glycan family could have considerable physiological importance. Con A reactivity has been associated with the presence of pro/acrosin which in boars has N-glycosylation sites (Töpfer-Petersen et al., 1990). There is an importance of mannose in binding of sperm to the human zona pellucida (Cheng et al., 1995). In the current study, Con A binding sites were detected in the equatorial segment of the head of all sperm samples and the two enzymatic treatments did not affect the staining intensity. Interestingly, the equatorial segment of the sperm head is the place where fusion with the oocyte membrane is initiated in mammals (Yanagimachi, 1988; Fujihara et al., 2012). This finding elucidates that an N-glycan that contains large amounts of mannose could be important for dromedary spermatozoa fertilization capacity. The presence of O-linked oligosaccharides results in there being HPA and PNA reactivity in the acrosomal region of sperm. Interestingly, PNA binding has been considered a feature of acrosome intactness (Cheng et al., 1996). The O-glycans are considered important in sperm–zona binding during the process of fertilization (Bleil and Wassarman, 1988).

The tail of control sperm reacted with all the lectins for which assessments occurred in the present study, except with LTA, indicating there was an absence of glycans containing α1,3-linked fucose. The treatment with enzymes to reduce dromedary semen viscosity led to some change in the glycosylation pattern of this part of the sperm cell. The α1,3-linked fucose was also not detected in the papain-treated sperm in which there was not detection of terminal N-acetylglucosamine residues (GSA II). Furthermore, the papain incubation resulted in a reduction of HPA-reactive glycans terminating with αN-acetylgalactosamine. There was a similar reduction in bromelain-treated sperm.

In the present study, treatment with both of the endopeptidases effectively reduced dromedary semen viscosity due to actions on seminal plasma proteins. It, however, appears as though the treatment of semen with these endopeptidases induced alterations in the structure of sperm cells due to cleavage of sperm cytoskeletal proteins (e.g. hydrophobic and lipophilic peptides) rather than inducing modifications in the sperm surface glycocalyx pattern. The changes in sperm glycan structure in the present study are attributed to the proteolytic actions rather than the deglycosylation activity of both enzymes. In this regard, the treatments with the two proteases resulted in very few changes in the glycosylation pattern of camel sperm glycocalyx. Specifically, the treatments with bromelaine and papain did not affect the distribution and intensity of staining signal for lectins in the entire sperm surface that typically bind to the core of N-linked sugars such as Con-A, PHA-E, and PHA-L. The presence of N-linked glycans along the entire sperm surface has also been detected using Con-A in fresh sperm of several mammalian species (see Desantis et al., 2010 and Accogli et al., 2017 for references).

The extensive presence of N-glycans contributes greatly to the physicochemical properties of the glycocalyx due to the extraordinary flexibility (high degree of rotation and movement possible about each glycosidic bond) and microheterogeneity (variation in chain length, branching and monosaccharide composition) of n-glycans (Schröter et al., 1999). It has been suggested that oligomannosidic chains could be recognition signals for the elimination of sperm that do not have the capacity for fertilization during passage through the female reproductive tract (Nardone et al., 1985). The presence of RCA_{120} binding sites on the equatorial region of enzyme-treated sperm, as well as the change in HPA and GSA II affinity detected on the tail of these sperm could depend on the physical damage of cell surface produced by crystal formation or osmotic changes that occur during the freezing process (Talaei et al.,

2010).

5. Conclusions

On the basis of the present results, it can be inferred that bromelain- and papain-based liquefaction of camel semen improved post-thaw motility and velocity patterns of spermatozoa while markedly affecting sperm morphometric characteristics. Meanwhile, treatments with both proteases resulted in very few changes in the glycosylation pattern of camel sperm glycocalyx. Further functional studies are needed to evaluate the sperm fertilization capacity of bromelain- and papain-treated semen for application of assisted reproductive technologies (ARTs) in production of dromedary camels.

Authorship contributions

GL, RS, KB, DS, DM conceived and designed the study. RS, MK, DM, DS, GA acquired the data. RS, MK, DS, DM, IA, AK, GA analyzed and interpreted the data. GL, RS, KB, DS drafted the manuscript. All authors read and accepted the final manuscript.

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