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Full Length Article

The influence of caffeine supplementation and concerted utilization of enzymatic and mechanical semen liquefaction on freezability of dromedary camel spermatozoa

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ABSTRACT

Assisted reproductive technologies have been reported to improve reproductive efficiency and genetic potential in camelids. Two experiments were carried out to determine efficiency of centrifugation in the presence of a mucolytic agent for liquefaction of dromedary semen. In the first experiment, three groups, namely: I. Tris lactose (TL, control), II. Tris lactose supplemented with amylase (TL_A) and III. Tris lactose supplemented with amylase followed by seminal plasma removal via centrifugation (TL_A$_{-Cent.}$) and re-suspension into enzyme free TL media. After equilibration, control group recorded 6 2.85 ± 4.28% motility and 1.3 ± 0.13 viscosity score, while TL_A group values were 72.88 ± 3.30% and 0. 83 ± 0.07%, respectively. TL_A$_{-Cent.}$ group showed significant viscosity reduction (0.33 ± 0.05) and motility decline 47.85 ± 3.04% with increment in abnormalities and detached acrosome. The second experiment investigated the effect of caffeine addition to tolerate enzymatic and mechanical stress. Using 4 mM caffeine in amylase-treated semen (TL_AC) improved post-thaw motility 50.0 ± 1.29% and recovery rate 77.8 ± 3.83% compared to the control (40.17 ± 2.79% and 62.55 ± 8.39%), respectively. Caffeine supplemented centrifuged samples (TL_AC$_{-Cent.}$) showed superiority ($P < .05$) in post-thaw motility and recovery rate (38.33 ± 6.41%, 62.76 ± 8.10%) compared to centrifuged samples TL_A$_{-Cent.}$ without caffeine addition (25.00 ± 2.88% and 40.47 ± 3.48%), respectively. Sperm kinetics showed that TL_A exhibited high ($p < .05$) values for mostly all sperm kinetics. Caffeine treatments showed superiority in velocity curved line (VCL, μm/s) 94.24 ± 8.44 for TL_AC and 104.25 ± 8.72 for the TL_AC$_{-Cent.}$ group compared to 86.8 ± 5. 54 for TL_A, and 85.73 ± 5.99 for the TL_A$_{-Cent.}$ groups. In conclusion, preforming a combined enzymatic-mechanical protocol in the presence of an antioxidant may be crucial for refinement of camel semen cryopreservation.

1. Introduction

High variations of camelids semen physical characteristics; namely volume and sperm concentration, are always observed [1,2]. This leads to inaccuracy of processed semen concentration, and does not allow precise adjustment for semen doses. Most of semen processing techniques depend on various dilution rates ranging from 1:1 to 1:4 [3–5]. However the final doses concentration adjustment is adversely affected by both raw semen concentration variation and seminal plasma viscosity. Moreover, there is no controversy regarding the negative effect of camel seminal plasma viscosity, which is considered the most defeating challenge that hampers the adequate evaluation of raw semen characteristics and further processing steps till cryopreservation. Semen viscosity elimination attempts usually depend on conventional methods, either mechanically [6–8] or enzymatically [9–12] with controversial results.

The removal of seminal plasma prior to cryopreservation of semen was recently studied in different species [13,14]. Semen centrifugation is a reliable approved technique in stallions' semen processing, to overcome the negative effects of seminal plasma during cryopreservation [15], and to adjust dose concentration [16]. Semen centrifugation in camelids was used as a mechanical treatment for seminal plasma viscosity elimination with limited success [7,8].

Caffeine is known as a heterocyclic compound, which has an influence in promoting hyper-activation in human and bull [17,18]. The alkaloid caffeine and its catabolic products theobromine and xanthine exhibit both antioxidant and prooxidant properties that have been regarded responsible for enhancing sperm motility and improving fertilization [19].

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Frozen equine semen supplemented with caffeine increased post-thaw motility and potential longevity [20]. Also, caffeine citrate was a good additive for preserving seminal characteristics in bucks semen [21], with reported effectiveness of caffeine addition in increasing sperm motility in rabbits [22]. More recently, the positive effect of caffeine addition on turkey spermatozoa motility was reported [23].

Caffeine in camel semen processing for IVM and IVF *in vitro* trials, showed improvement in semen characteristics during processing [24–26]. Furthermore, the addition of caffeine improved motility of individual camel spermatozoon and decreased acrosomal damage in semen used for female *in vivo* trials [27,28].

Therefore, the current investigation aimed to reduce the seminal plasma viscosity and to provide a manageable insemination dose through a modified protocol for camel semen processing for cooling and freezing, in addition to investigating the role of antioxidant addition in improving sperm freezability through combining both enzymatic and mechanical treatments in one modified protocol.

2. Materials and methods

2.1. Location of the study, animals and semen collection

This investigation was performed in Tharb camel hospital (Assisted Reproductive Technologies Department), Qatar. Semen ejaculates were collected from 5 healthy bulls, three times a week during the breeding season (December 2016–February 2017). The bulls aged 9–15 years and had an average body weight 622 ± 40. 12 kg. Animals were fed pelleted concentrate feed mixture (crude protein 14%) supplemented with barley, dried dates and dry Berseem hay (*ad lib.*), and were allowed to drink twice a day.

Semen collection was performed at 6:30 am using a female restrained in sternal recumbency position. A 42 cm bovine artificial vagina (AV), adjusted at 40–45°C and lubricated from the inside with sperm-friendly vaseline (Mini-tube Vaseline, 1000 g, REF.: 11905/0100), was utilized. Semen was retrieved from the AV by evacuation of water and holding the AV in a standing position to allow slopping of viscous semen downwards to the collecting graduated tube [10].

2.2. Physical characteristics assessment

Sperm motility assessment was performed on a 37°C warm stage phase-contrast microscope (Carl ZEISS, AX10_Lab. A1, Germany). Five fields at 400× magnification were analyzed. Freshly prepared eosin-nigrosin was used for sperm livability (live and dead sperm, %). Primary abnormalities [abnormal heads (large, small, tapering, pyriform, vacuolated, double head)], secondary abnormalities [abnormal mid-piece, (distended or irregular, abnormally thick/thin), abnormal tails (short, multiple, broken, coiled, absent, bent, presence of cytoplasmic droplets)] and acrosome integrity were assessed using a phase-contrast microscope (Carl ZEISS, AX10_Lab. A1, Germany) at 1000× magnification by adding 50 μL of semen to 200 μL of a 0.02% glutaraldehyde solution, and the percent of any abnormal sperm or with intact/damaged acrosome were recorded [29]. Seminal plasma viscosity was assessed visually (on a scale of 1–3) as (3 = highly viscous; 0 = complete liquefaction) through a thread test technique used for assessment of semen viscosity [30].

2.3. Extenders preparation

2.3.1. Tris lactose diluent preparation (TL)

Tris-lactose egg yolk extender composed of Tris buffer (3.025%), Lactose (5.5%), Citric acid (1.67%), Glucose (1%), and supplemented with 20% fresh egg-yolk, to reach a final pH of 7.9 and 0.359 osmol/kg osmotic pressure. The extender was subjected to 15 min centrifugation at 23,000×*g*, followed by filter paper filtration to get rid of any solid particles. Thereafter, the extender was divided into two portions A & B. Portion A represented the cooling extender (free of glycerol) which was used for initial dilution with a ratio 1:1, while portion B was supplemented with 6% glycerol to be added after 2 h of equilibration at 5°C with a ratio 1:1 to make the diluted semen with a final glycerol level of 3% and a total equilibration period of 4 h at 5°C with a final dilution rate 1:3 [10]. Unless stated otherwise, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3.2. Amylase-supplemented diluent (TL_A)

A concentration of 2.5 μL/mL α-amylase enzyme [TERMAMYL SUPRA®, Novozymes (Novo Nordisk), Denmark] extracted from *Bacillus licheniformis* was used in this experiment for seminal plasma viscosity elimination [10,31]. Tris lactose extender was supplemented with amylase (TL_A), the amylase was added to the extender just before semen dilution.

2.3.3. Caffeine-supplemented diluent (TL_AC)

For preparing the Tris lactose amylase extender supplemented with caffeine (TL_AC), a (4 mM) concentration of caffeine [Sigma-Aldrich, China, Pcode: 1001176428] was used in this experiment. This concentration was based on a preliminary study along with concentrations cited in [25,26].

2.4. Experimental design

2.4.1. Experiment 1

The experiment aimed to assess the effect of seminal plasma removal after 2 h of the enzymatic treatment with α-amylase, and re-suspension of rich sperm fraction pellet in α-amylase-free Tris-Lactose extender. In general, ejaculates with low volume (<3 mL) and raw motility <50%, as well as azoospermic or contaminated ejaculates were excluded. For this experiment, semen ejaculates (n = 3 ejaculates per male) were used in the treatments after being assessed for raw physical parameters. The semen ejaculate was split, and each part was either diluted 1:1 with Tris lactose (TL) and served as control or was diluted 1:1 with Tris lactose supplemented with α-amylase (TL_A). The latter portion was divided into two equal parts [TL_A and TL_A$_{-Cent.}$]. The three groups were maintained at 5°C for 2 h. For the first two groups, portion B supplemented with 6% glycerol was added after 2 h of equilibration at 5°C with a ratio 1:1 to make the diluted semen with a final glycerol level of 3% and a total equilibration period of 4 h at 5°C with a final dilution rate 1:3. The third portion (TL_A$_{-Cent.}$) was subjected to low speed centrifugation (700×*g* for 10 min) under cooling at 5°C (LABOCON, IMPC_10R, USA, cooling centrifuge). Immediately after centrifuging the supernatant was aspirated. An objective evaluation of non-recovered sperm in the discarded supernatant was performed immediately after centrifugation has ceased. Only 15% of the supernatant were retained along with the soft sperm rich fraction pellet. The soft sperm rich fraction pellet was re-suspended in Tris lactose extender free of amylase with 3% glycerol level and was kept again under 5°C for the remaining of the 4 h equilibration period. Sperm physical characteristics (motility, livability, acrosomal integrity, primary and secondary abnormalities) were assessed after initial dilution as well as at 2 and 4 h of equilibration.

2.4.2. Experiment 2

With the fact of low sperm/seminal plasma ratio which is common in dromedary camels, raw ejaculate may encourage the removal of the seminal plasma as an elective technique for sperm processing in this species especially for low concentrated raw

semen samples. In this experiment, 3 ejaculates per male were collected. After routine examination of ejaculates, the ejaculates were immediately diluted to a final dilution rate of 1:3 with TL, TL_A, TL_AC extenders supplemented with 3% glycerol. Initial motility (M_I) was assessed immediately after dilution and before placing all treatments into the cooling cabinet at 4°C for 4 h. Twenty minutes before packing, each of TL_A and TL_AC groups were split into two portions, one portion of each treatment was concentrated by low speed centrifugation (700×*g* for 10 min) under cooling at 5°C (LABOCON, IMPC_10R, USA), and 50% of the supernatant was excluded, the centrifuged samples were represented in the results as TL_A$_{-Cent.}$ and TL_AC$_{-Cent}$. The methodology was modified in respect to Alvarenga et al. [16]. Prior to cryopreservation, the treatments were checked for pre-freezing motility (M_{PF}) just before packing in 0.5 mL French straws using a Mini-tube automatic filling and sealing machine (model MPP Uno). The treatments were frozen using computer controlled cryo-freezer (Mini-tube type: Ice Cube 14S) through a rapid freezing curve that starts at −80°C then within 5 min dropped down to −120°C for 15 min. Later, preserved semen doses were examined using slow thawing techniques (40°C for 40 s) for assessment of post thaw motility (M_{PT}), acrosomal integrity, primary and secondary abnormalities and recovery rate % (RR) that showed recovered motility of the frozen spermatozoa post thawing compared to the pre-freezing motility. Sperm dynamics, including distance curved line (DCL, μm), distance average path (DAP, μm), distance straight line (DSL, μm), sperm velocity curved line speed (VCL, μm/s), velocity average path (VAP μm/s), velocity straight line (VSL, μm/s), sperm linearity movement (LIN = VSL/VCL), sperm straightness movement (STR = VSL/VAP), sperm balanced movement, and Wobble (WOB = VAP/VCL) were analyzed using a computer assisted sperm analysis (CASA) system [Sperm Vision Lite, a registered trademark of the Mini-tube, USA]. Prior to conducting the evaluation, the device was calibrated for camel normal sperm bio-metrics.

2.5. Statistical analysis

The obtained data were statistically analyzed using one-way analysis of variance with the software program [32]. ANOVA procedure of SAS was used. Mean differences were tested by Duncan's Multiple Range tests when significant *P* value was obtained at 95% confidence [33].

3. Results

3.1. Experiment 1

Two hours after semen collection and addition of diluents, an increase ($P < .05$) in sperm motility was observed in amylase supplemented group (TL_A, 73.34 ± 3.4%) compared to the control group (TL, 63.34 ± 4.41%). The results showed that the motility dropped significantly after re-suspended in the extender reaching 47.85 ± 3.04% versus 73.33 ± 3.04% prior to centrifuging (Table 1).

The results in (Table 1) clarified that, sperm livability for TL extender was 87.34 ± 0.92%, compared to amylase supplemented extender (TL_A) 81.57 ± 1.68%, with a significant decline reaching 70.25 ± 3.71% after centrifuging and re-suspension of the soft semen rich fraction pellet for the (TL_A$_{-Cent.}$) treatment. The (TL_A$_{-Cent.}$) group recorded higher ($P < .05$) values for primary abnormalities (7.16 ± 0.27%) compared to the TL_A group (5.68 ± 0.35%) and the control (5.00 ± 0.29%). The results also revealed that the control group (TL) showed lower ($P < .05$) values for secondary abnormalities (7.41 ± 0.45%), compared to TL_A and TL_A$_{-Cent.}$ groups being 9.83 ± 0.53% and 9.71 ± 0.43%, respectively. Likewise, the same trend was observed for acrosomal integrity percentage 8.00 ± 0.76%, 9.57 ± 0.60% and 11.14 ± 0.65% for TL, TL_A and TL_A$_{-Cent.}$ groups, respectively (Table 1).

On a scale of 1–3, after 4 h equilibration at 5°C, the (TL_A) treatment showed reduced viscosity (0.83 ± 0.07) in comparison to the control group (1.3 ± 0.13). Despite of the declined motility for the centrifuged samples (TL_A$_{-Cent.}$) after re-suspension in enzyme free Tris lactose (TL), the viscosity was almost totally eliminated reaching (0.33 ± 0.05). Through visual eye observation the TL_A$_{-Cent.}$ group showed a high sperm recovery rate exceeding 85% after examining the aspirated supernatant showing very low concentration of highly motile sperm.

3.2. Experiment 2

Five treatments were evaluated in this experiment, in order to determine the potential of caffeine addition to the freezing media to tolerate the enzymatic and the mechanical stress during semen processing prior to cryopreservation.

Post thaw motility illustrated in (Fig. 1) showed an increment in (TL_A) and (TL_AC) groups compared to the control being 49.00 ± 7.31%, 50.00 ± 1.29% and 40.17 ± 2.79%, respectively. In the meantime, both centrifuged groups (TL_A$_{-Cent.}$ and TL_AC$_{-Cent.}$) showed a lower post thaw motility (25.00 ± 2.88% and 38.33 ± 6.41%, respectively) compared to (TL, TL_A and TL_AC) groups, with superiority ($P < .05$) for the caffeine supplemented treatment (TL_AC$_{-Cent.}$) versus the (TL_A_Cent.) treatment (Fig. 1).

TL_AC$_{-Cent.}$ treatment also showed an acceptable recovery rate (62.76 ± 8.10%), comparable to the control group (62.55 ± 8.35%), with a significant superiority ($P < .05$) upon the TL_A$_{-Cent.}$ (40.47 ± 3.48%). Both TL_A and TL_AC recovery rates none-significantly surpassed all other treatments reaching 76.85 ± 6.94% and 77.8 ± 3.83%, respectively.

Results in (Fig. 2) showed no differences within the treatments for primary abnormalities. A significant improvement for the caffeine supplemented groups was reported for secondary abnormalities being 5.00 ± 0.25% for TL_AC and 5.33 ± 0.80% for

Table 1
Physical properties (Means ± SE) of diluted cooled semen (5°C) after α-amylase supplementation and centrifugation.

	Motility (%)			Livability (%)			Primary abnormalities (%)			Secondary Abnormalities (%)			Detached acrosome (%)		
	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
TL	64.34^{Aa} ± 5.48	63.34^{Ab} ± 4.41	62.85^{Ab} ± 4.28	91.00^{Aa} ± 1.31	87.67^{Ba} ± 0.91	87.34^{Ba} ± 0.92	4.25^{Ac} ± 0.39	5.00^{Ab} ± 0.21	5.00^{Ab} ± 0.29	5.80^{Bb} ± 0.38	5.91^{Bb} ± 0.24	7.41^{Ab} ± 0.45	6.25^{Bab} ± 0.44	7.8^{ABa} ± 0.38	8.00^{Ab} ± 0.76
TL_A	67.14^{Aa} ± 3.66	73.34^{Aa} ± 3.04	72.88^{Aa} ± 3.30	91.43^{Aa} ± 0.76	88.29^{Aa} ± 1.05	81.57^{Ba} ± 1.68	5.08^{Ab} ± 0.33	5.08^{Ab} ± 0.39	5.68^{Ab} ± 0.35	8.00^{Aa} ± 0.67	8.00^{Aa} ± 0.71	9.83^{Aa} ± 0.53	6.50^{Ba} ± 0.56	8.16^{ABa} ± 0.58	9.57^{Bb} ± 0.60
TL_A$_{-Cent}$	65.00^{Aa} ± 3.89	73.33^{Aa} ± 3.04	47.85^{Bb} ± 3.04	87.50^{Aa} ± 1.82	84.00^{Aa} ± 2.32	70.25^{Bb} ± 3.71	6.00^{Ba} ± 0.15	6.15^{Ba} ± 0.27	7.16^{Aa} ± 0.27	7.94^{Ba} ± 0.79	7.94^{Ba} ± 0.61	9.71^{Aa} ± 0.43	5.85^{Cb} ± 0.47	9.14^{Ba} ± 0.63	11.14^{Aa} ± 0.65

$^{A, B, C}$Means with different superscripts in the same raw are significantly different at $P < .05$.
$^{a, b, c}$Means with different superscripts in the same columns are significantly different at $P < .05$.

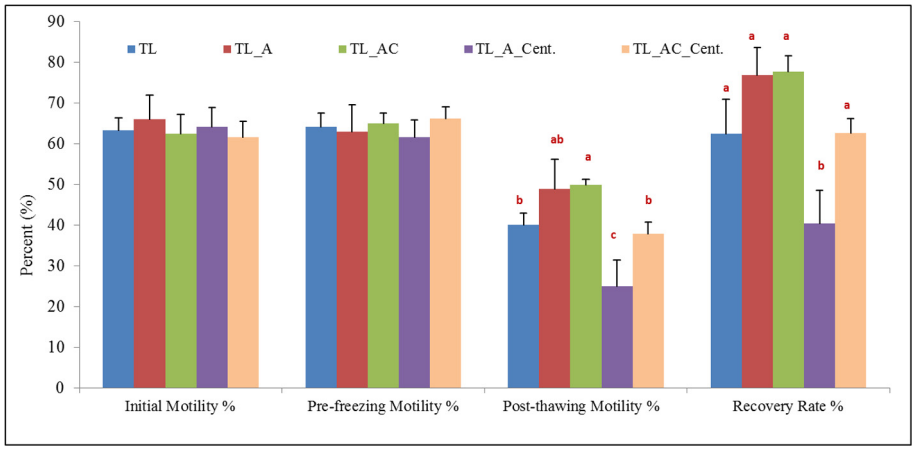

Fig. 1. Effect of caffeine supplementation on sperm post-thaw motility and sperm recovery after cryopreservation.

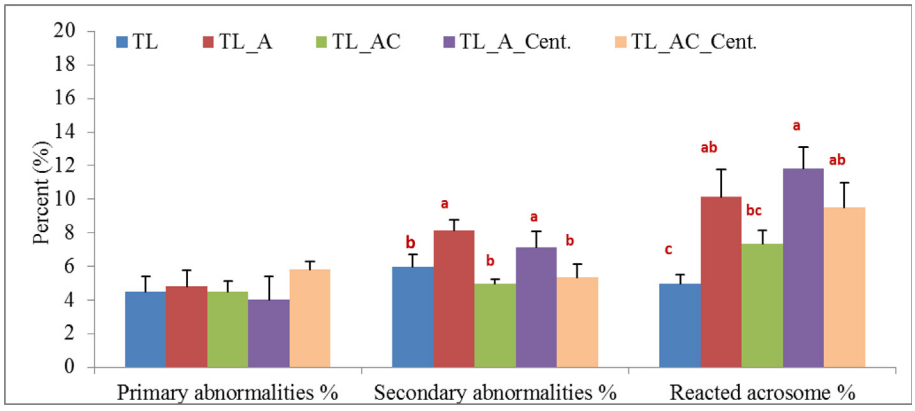

Fig. 2. Effect of caffeine supplementation on sperm post-thaw physical properties.

TL_AC$_{_Cent.}$ compared to TL and TL_A$_{_Cent.}$ groups that showed secondary abnormalities' percentage of 8.16 ± 0.61 and 7.16 ± 0.9 1, respectively.

The data revealed that, acrosomal integrity (Fig. 2) showed the same aforementioned trend observed for secondary abnormalities, with reduced values of reacting acrosome in TL_AC and TL_AC$_{_Cent}$ groups (7.33 ± 0.81% and 9.50 ± 1.3% respectively), in comparison to TL_A (10.16 ± 1.61%) and TL_A$_{_Cent.}$ (11.83 ± 1.49%), with a significant superiority for control being as low as 5.00 ± 0.52%.

Post-thaw evaluation of sperm kinetics of the frozen thawed samples exhibited high values ($P < .05$) for the TL_A treatment (DCL, DAP, VAL, VSL, LIN, STR and WOB). Nevertheless, caffeine supplemented treatments showed superiority in velocity curved line (VCL, μm/s) 94.24 ± 8.44 for TL_AC and 104.25 ± 8.72 for the TL_AC$_{_Cent.}$, compared to 86.8 ± 5.54 for TL_A and 85.73 ± 5.99 for the TL_A$_{_Cent.}$ treatments as shown in (Figs. 3A, 3B, 3C). The TL_AC treatment also showed a significant increase in (DSL, μm), despite the fact that caffeine supplementation did not affect positively all sperm kinetics. All treatments supplemented with amylase surpassed the control group in almost all sperm kinetics.

4. Discussion

Semen processing steps may differ in species that have problematic semen handling and unique freezing steps. In equine, commercial semen processing protocols depend on removing most of the seminal plasma through centrifugation as prolonged exposure to seminal plasma was deleterious during equine semen freezing [34]. However, in camelids, the problem of the seminal plasma mainly refers to its high viscous nature that causes trapping of sperm cells and, hence, hinders sperm motility [5].

Through visual observations, the results showed sperm cells recovery more than 85%. The supernatant was almost free of spermatozoa; except for few highly motile sperms. Immediate swim up for highly motile sperms during aspiration soon after centrifugation was recorded in equine [15]. This agreed with the suggestion that, as the sperm pellet is very soft due to the enzymatic treatment, the highly motile sperm immediately swim up soon after centrifugation step ceases. In dromedary camels, using ultra-speed centrifugation for raw ejaculated semen immediately after collection showed good post-thaw motility with very low concentration of cryopreserved doses [7]. As most of the rich sperm fraction was trapped in the supernatant leading to at least 50% loss of sperm cells in the discarded viscous supernatant. Morton et al. [8] recovered only 62.7% without affecting either sperm motility or acrosome integrity after centrifugation with difficulty in removing the supernatant as the semen was still viscous and did not liquefy. This resulted in a significant loss of sperm cells. In the current investigation, results of sperm recovery seem to be surpassing former trials, as centrifuging herein was for diluted samples, not raw samples, in addition to the presence of a mucolytic agent prior to centrifuging. The mucolytic agent had a partial liquefaction effect and softened the viscous nature of the raw semen allowing

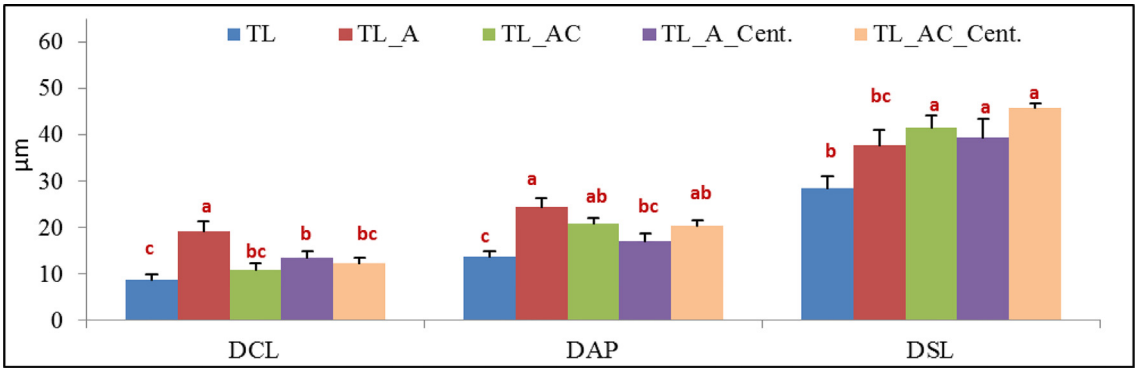

Fig. 3A. Effect of caffeine supplementation on post-thaw sperm dynamics [Distance curved line (DCL, μm), Distance average path (DAP, μm), Distance straight line (DSL, μm)].

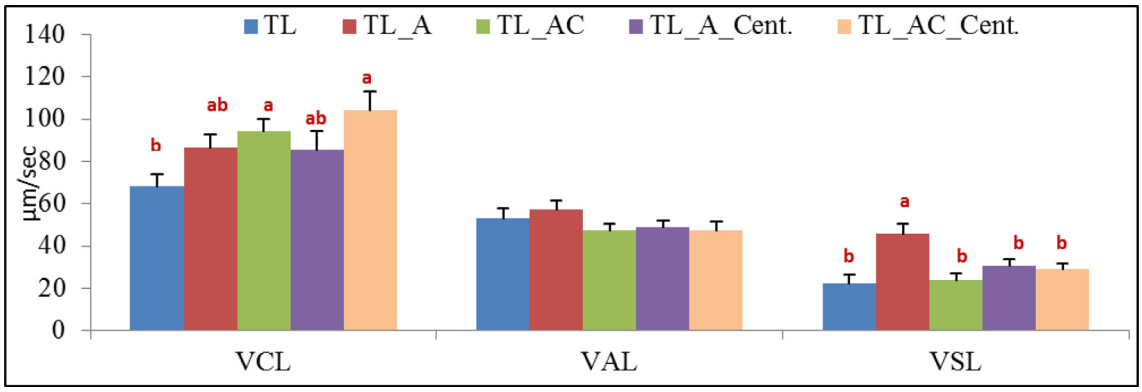

Fig. 3B. Effect of caffeine supplementation on post-thaw sperm dynamics [Velocity curved line (VCL, μm/s), Velocity average path (VAP μm/s), Velocity straight line (VSL, μm/s)].

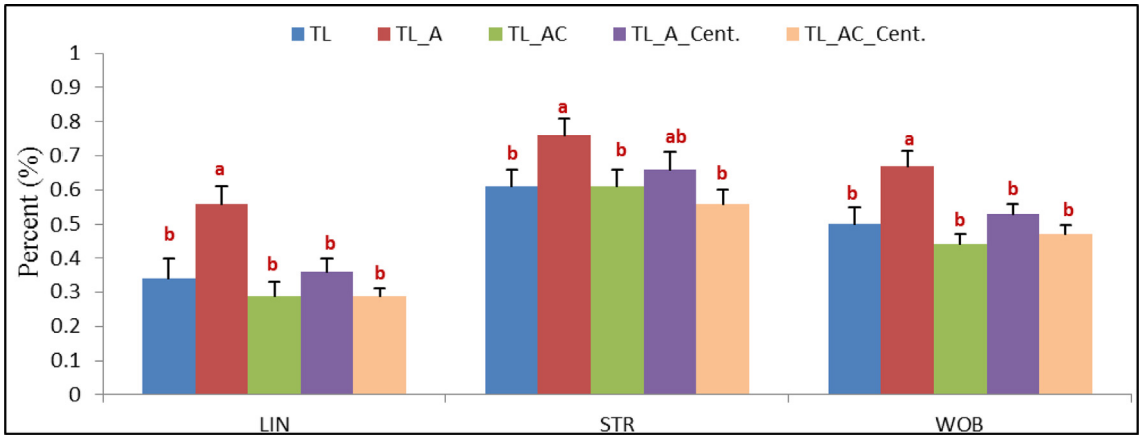

Fig. 3C. Effect of caffeine supplementation on post-thaw sperm dynamics [Linearity movement (LIN = VSL/VCL), Straightness movement (STR = VSL/VAP), Balanced movement, Wobble (WOB = VAP/VCL).

liberation of more sperm cells. This would explain the higher recovery of sperm cells after centrifugation.

The results also revealed that centrifugation severely impaired motility percentage with an increase in abnormalities and detached acrosome compared to the non-centrifuged treatments or the control. These findings were not in accordance with previous results noted that the raw sperm quality of boar semen was not affected significantly by centrifugation during processing [35], or even post-thaw motility of centrifuged semen prior to cryopreservation in dog semen processing [36]. The decline in motility percentage and increased acrosomal reaction after re-suspension of the sperm cells (soft sperm pellet) in Tris lactose extender may refer to centrifuging conditions used in the experiment (700×g for 10 min at 5°C) compared to (600 g for 7 min) those recommended by [8]. Short time centrifugation with high force was not harmful to porcine processed semen [37], another reason for motility reduction may be altered to inadequate adjustment of the Tris lactose re-suspension media components, especially for the adjustment of the osmotic pressure after disposing most of the seminal plasma. Recently, in bovine, seminal plasma removal reduced rate of *in vitro* produced embryos and did not improve the quality of thawed semen [13,14]. This may be referred to the role of seminal

plasma components, especially proteins being essential for the sperm cells during capacitation [38].

Contrary to the results of this study, Morton et al. [8] demonstrated that centrifugation was not effective in removing the viscous seminal plasma. They recommended enzymatic methods which are more effective in reducing semen viscosity, although their detrimental effects on sperm structure and viability due to type, exposure time and concentration of the used enzyme. Herein, the idea was to combine enzymatic and mechanical treatments in one modified protocol to benefit from viscosity elimination via enzymatic treatment and reduce the exposure time and concentration of the used enzyme after re-suspension in enzyme free media.

Due to the individual variation between males, as well as variations between collected semen specimens, it was assumed that setting various centrifugation protocols (with different g-force and/or time) to match with each ejaculate characteristics is not effective during application of several semen specimens [8,39]. In this regard, it is suggested to have more adapted, combined protocol based on enzymatic action, even with proteolytic enzymes having more reliable results about viscosity elimination despite their damaging effect on sperm cells (unpublished data) and sperm cell agglutination [11], followed by harvesting of sperm cell through more adaptable centrifuged scheme and re-suspension of semen pellet in an enzyme free freezing media.

Nowadays, semen processing and cryopreservation are considered routine procedures, optimizing and improving the final cryopreserved semen is an aim. Oxidative stress is a major factor affecting semen quality after processing steps.

The results of this investigation revealed that caffeine addition, improved physical semen characteristics post-thaw, namely abnormalities and acrosomal integrity percentage. In rabbits, no acrosomal damage was observed after supplementation with 5–10 mM of caffeine [40]. In Kankrej bulls, an increase in acrosomal integrity, viability and motility of spermatozoa with a decrease in abnormal sperm in EDTA and caffeine supplemented diluents was reported [41]. In boars, diluent supplemented with caffeine showed no acrosomal membrane damage [42]. Similarly, in Murrah buffalo, caffeine significantly reduced abnormalities [43]. Most recent in *in vitro* trials on camel semen, epididymal camel spermatozoa diluent supplemented with antioxidants had a positive effect in maintaining the function and morphology as well as membrane integrity of sperm [44].

The results of this study were contrary to those reported by Špaleková et al. [45] who observed no effect on the acrosome integrity in frozen ram semen. This could be attributed to the dose of caffeine or the cryopreservation steps, including equilibration period and time of exposure to the caffeine addition. As reported in the current study, no significant difference in post-thaw motility between amylase treated group (TL_A) and when supplemented with caffeine (TL_AC). In contrary to what had been reported in camels, that the addition of 10 mM caffeine to camel semen extender significantly increased motile spermatozoa and decreased percentage of acrosomal damage [27], but this effect could be related to higher dose of caffeine used in his trial.

Based on the present data, sperm kinetics showed significant superiority in caffeine supplemented group, particularly in distance straight line (DSL) and velocity curved line (VCL). Nevertheless, both amylase and caffeine treatments either solely or in conjugation, significantly surpassed all sperm kinetics of the non-treated control group (TL). Imoedemhe et al. [46] recorded enhanced velocity and LIN in fresh human sperm due to caffeine addition, while an improvement in VSL, VAP, LIN, and STR patterns in fresh boar semen was also recorded [47]. In Garut ram, a concentration of 4 mM of caffeine was the optimum dosage to reach the highest motility and velocity of sperm [48]. While in frozen, thawed boar semen, caffeine enhanced progressive motility, STR and LIN [42]. Also, adding caffeine to the thawing media, led to an observed improvement in kinetic features of spermatozoa correlated with caffeine addition in boars [49]. The stimulatory effect of caffeine on kinetic activity and spermatozoa respiration was suggested to be due to the fact that it converts the inactive form of glycogen phosphorylase to the active form through breaking down of glycogen into simple sugar, which might be utilized during long time preservation of semen [50]. In rams, Budai et al. [51] also reported that, antioxidants addition during semen cryopreservation could improve post-thaw motility and survival rates, as antioxidants act as a free radical scavenger protecting and defending the sperm against reactive oxygen species (ROS). As a result of excessive ROS production that consequently leads to lipid peroxidation, decreased motility and viability and increased sperm morphological defects [52]. The caffeine enhances utilization of fructose [53], and stimulates both respiration and sperm metabolism [45].

5. Conclusions

A modified dual combined enzymatic-mechanical protocol for viscosity reduction with care to optimize semen handling and processing centrifugation conditions accompanied with a suitable enzymatic treatment in the presence of an antioxidant may be crucial for the refinement of camel semen processing. That, subsequently, may improve the efficiency of semen cryopreserved doses in camelids.

Competing interests

No financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper.

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