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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 62.85 ± 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

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