Indian Journal of Animal Sciences **91** (3): 182–187, March 2021/Article
https://doi.org/10.56093/ijans.v91i3.114141

Supplementation of caffeine to extender improves post-thaw seminal attributes of Poitou donkey semen

SOURABH KANT[1], YASH PAL[2], R A LEGHA[3], S K RAVI[3], T R TALLURI[3]✉ and TARU SHARMA[1]

ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh 243 122 India
and
ICAR-National Research Centre on Equines, Bikaner, Rajasthan 334 001 India

Received: 30 March 2021; Accepted: 12 July 2021

ABSTRACT

The current study was conducted with an objective of elucidating the effect of addition of various levels of caffeine to jack semen on seminal attributes at pre- and post-thaw stage. Six ejaculates from each three adult jack stallions were collected via artificial vagina using estrus jenny as dummy. Caffeine was added to semen extender at 0.1, 0.5 and 2 mM at pre-freeze stage and the semen was cryopreserved according to standard protocol. Sperm motility, viability and other functional attributes were assessed at pre-freeze and post-thaw stages. The present study revealed that, adding caffeine to jack semen extender at 0.1 mM, 0.5 mM or 2 mM levels had positive impact on seminal parameters compared to the control semen. Sperm kinetics (VAP, VSL and STR) and motility increased in the caffeine treated groups in comparison to the control group. The caffeine at 2.0 mM concentration resulted in better post-thaw sperm motility, viability, and acrosome integrity as compared to 1 or 0.5 mM of caffeine and control. Caffeine supplementation also enhanced post-thaw sperm kinematic parameters, which implies its potential as an alternative antioxidant supplement. It was concluded that, 2 mM caffeine supplementation to donkey semen significantly improved post-thaw sperm motility and other functional attributes as compared to the control. However, the potential benefits of caffeine on the fertility rates needs to be investigated.

Keywords: Acrosome integrity, Caffeine, Cryopreservation, Jack sperm, Kinetic parameters

The domestic donkey (*Equus asinus*) is an important livestock species vastly used across the world as pack animal and also used for skin and meat purpose (Canisso *et al*. 2011). With the rapid mechanization and industrialization, their number has greatly declined in industrialized countries (Contri *et al*. 2010). Considering the declining donkey population and multiple use of the animals as mentioned before, the use of assisted reproductive biotechnologies has become relevant for conservation and preservation of the domestic donkey species population.

The studies on cryopreservation of horse stallion sperm have been reported since many years (Avanzi *et al*. 2015), however, the studies involving donkey stallion semen are quite small (Ortiz *et al*. 2015). Despite the excellent semen quality, fertility rates of frozen jack semen are still low (0%–36%) (Vidament *et al.* 2009) in comparison to mares (33%–53%) (Canisso *et al*. 2011). Moreover, the fertility rate of frozen semen is far lower than that of fresh or chilled semen used in breeding programmes (Watson 2000). Sperm cryopreservation is also associated with depletion of ATP and loss of chromatin integrity (Aitken and Fisher 1994). These conditions seriously affect the motility, viability and fertilizing potential of sperm leading to significant loss of viable spermatozoa thus affecting the fertility rates in AI programmes (Rota *et al*. 2012).

In domestic livestock, post-thaw semen motility parameters have been improved by supplementation of various cyclic adenosine monophosphate (cAMP) enhancers. Methylxanthines, like caffeine was found to improve motility and conception rates by increasing the cAMP levels within the cell, viz. bulls (Barakat *et al*. 2015) and boars (Yamaguchi *et al*. 2013). In bovine species, caffeine has been used in the hyperactivation (Tartaglione *et al*. 2004.). Caffeine has a direct effect on cellular metabolism, and such effect depends on the concentration of caffeine, Ca^{2++} ions and the immediate hyperactive activation of incubated sperm (Colas *et al*. 2010). We hypothesized that the addition of caffeine to the jack spermatozoa at pre-freeze stage, in various concentrations during processing would result in improvement of sperm motility and quality.

MATERIALS AND METHODS

Jacks and semen collection: Three healthy jack stallions

Present address: [1]ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh. [2]ICAR-National Research Centre on Equines, Hisar, Haryana. [3]Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, Rajasthan. ✉Corresponding author e-mail: raotalluri79@gmail.com

of Poitou breed (*Martina franca*) aged between 4 to 10 years, and weighing between 355–380 kg body weight and were maintained under uniform conditions of feeding and management. These jacks were previously trained for donating the semen and routinely used for AI programs. All the jacks were provided with *ad lib.* water and no special diet was provided. Semen ejaculates were collected twice a week during breeding season (Year 2016, March-June) in the morning hours (6.0–7.30 AM) using a pre-lubricated artificial vagina (AV), containing warm water (42°C to 45°C) and in the presence of a jenny in estrus. Initially, the jacks' semen was collected for four days continuously to empty the sperm reservoir and to stabilize the sperm parameters. Eighteen semen ejaculates from three jacks (six ejaculates from each three stallions) were collected for the current study (n=3; r=6). Reaction time was measured by using a stop watch and counting the time between the entry of a jack into the breeding facility and mounting on a jenny. The collected semen ejaculate was shifted immediately to the laboratory for further evaluation and processing. Unless otherwise mentioned, all chemicals utilized in this study were procured from Sigma-Aldrich (St. Louis, MO). All the analyses were performed in the laboratory under standard operative procedures.

Semen processing and cryopreservation: The semen was collected directly into a clean dry graduated bottle attached to the latex cone of the AV. The tubes containing semen were marked and placed in a water bath at 37°C immediately after collection. Total ejaculate volume of the semen was recorded as visible from graduated collection bottle, filtered through sterilized gauze and gel free semen volume was noted. Gel free semen was washed and extended with primary extender (Citrate- EDTA extender) in equal ratio, and divided in to five equal aliquots. These tubes were centrifuged at 650 g for 10 min to obtain sperm rich pellet fraction. The supernatant containing seminal plasma was discarded and obtained sperm pellet was extended further with secondary semen extender (Glucose- Lactose-EDTA, with Dimethyl formamide (DMF) @ 5% as cryoprotectant) having different concentrations of caffeine, i.e. 0 (control), 0.1, 0.5, 1.0 and 2.0 mM, respectively to obtain a final concentration of 250×10^6/ml.

French medium straws of 0.5 ml capacity were filled with extended semen by automatic straw filling and sealing machine (IMV, France) and kept at 4°C for 2 h equilibration in cooling cabinet. After equilibration and before freezing (Pre-freeze stage of cryopreservation) once again all the samples were analyzed for the seminal attributes. After equilibration, the straws filled with diluted semen were laid horizontally onto a wired net and lowered into a styrofoam box containing liquid nitrogen two inches above for 10–12 min before plunging in to liquid nitrogen (LN_2). After 24 h of its storage, straws from each group were thawed at 37°C for 30 sec for post-thaw semen evaluation.

Semen evaluation

Assessment of motility and livability: The total and progressive motility parameters were evaluated using a Computer Assisted Semen Analyzer (CASA) (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA) equipped with a thermostage (MiniTherm®, Hamilton Thorne Inc. Beverly, MA, USA) maintained at 37°C. For determining the total and progressive motility of the spermatozoa, 5 µl diluted aliquots of semen sample (50 µl of semen sample dissolved in 1 ml of 2.94% Sodium citrate diluting fluid to make a 1 : 20 dilution) was loaded in disposable chambers with a 20 µm chamber depth (Leja® Standard Count 8 Chamber Slide, 20 µm, Leja® Products BV, Netherlands). We recorded the sperm motility attributes, straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s) and average path velocity (VAP, µm/s) and other parameters like linearity LIN%, beat cross frequency (BCF) (Hz) and amplitude of lateral head displacement (ALH). The CASA analysis was set up at 75 Hz (frame per second), 45 frames, minimum contrast of 80, and minimum cell size of four pixels. Cells were considered progressively motile with at least 50 µm/second average path velocity and 75% straightness. A minimum of 10 microscopic fields were analysed for each assessment, which included at least 200 cells. Concentration of the spermatozoa was estimated using a hemocytometer chamber.

Stained sperm smear was prepared in duplicate, by using eosin-nigrosine staining and 200 sperm per slide were evaluated. One drop (~5 µL) of semen was placed together with one drop (~5 µL) of dye on a slide, and a smear was made after 1 min incubation on a stage warmer at 37°C. The spermatozoa were classified as viable and were not stained by eosin, while the non-viable showed the pink-red stained nuclei. From each one, a sample of 200 sperm cells were counted under common optical microscope.

Assessment of plasma membrane functional integrity: The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoa plasma membranes. The HOS test was performed by incubating 30 µl of semen with 300 µl of a 150 mOsmol/kg fructose-base hypo-osmotic solution at 37°C for 60 min. After incubation, 20 µl of the mixture was spread on a warm slide with a cover slip, subsequently, the sperms were analysed by counting 200 sperm cells from each sample in phase contrast microscope (Nikon Intech Co. Ltd., Japan) (400×). The number of swollen spermatozoa out of 200 was counted; swelling was characterised by a coiled tail, indicating that the plasma membrane is intact.

Sperm acrosome integrity: Giemsa stain was used to assess the acrosome integrity of jack spermatozoa as per the protocols described previously by Soni *et al.* (2019). Briefly, diluted semen drop was kept on clean grease free slide and thin smear was prepared. After air drying, the smear slide was fixed in methanol for 15 min and then after washing, the fixed slide was kept in working solution of Giemsa for 90 min. Excess stain was removed by washing under gentle stream of water. It was dried in air and examined under the bright field 100×oil immersion

objective. Around 300 spermatozoa were assessed in different fields of a slide and the same were expressed in percentage.

Assessment of DNA integrity: For detecting the DNA intactness of spermatozoa, the method described by Soni *et al*. (2019) was adopted. Briefly, a small aliquot (20 µl) of sperm suspension was smeared on a glass slide. The smear was air dried and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3 : 1). Once rinsed and air dried, the slides were stained for 5 min with freshly prepared Acridine orange (AO) stain. After washing and drying, the slides were examined using a fluorescent microscope (Nikon, Japan; excitation of 450–490 nm). Two hundred sperm per sample were counted under the epifluorescence microscope (480/550 nm). Sperm heads with intact chromatin showed green fluorescence and those with denatured (non-intact DNA) chromatin had orange-red or yellow fluorescence. The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of spermatozoa with red, orange, or yellow fluorescence to all spermatozoa counted per sample.

Statistical analysis: All results were expressed as the mean±standard error (SE), and were considered significant at P<0.05. Data pertaining to fresh, equilibration and post thaw stages of cryopreservation was analysed by one-way ANOVA using Tukey' s test for normality for all. The factorial model included the effect of various concentrations of caffeine as independent variables and percent post thawed progressive motility, live sperm count, acrosome intact sperm, hypo-osmotic swelling positive sperm and DNA integrity as dependent variables. Data was subjected to ANOVA, using the Post-hoc procedure from Statistical software package, version 20 (SPSS 20, SPSS Inc., Chicago, IL, US). For fresh, unextended semen, the paired t-test model was used in order to see the variability between individual jacks as well as between the control and treatment groups.

RESULTS AND DISCUSSION

The mean±SE values observed for fresh seminal parameters are listed in Table 1. Furthermore, the process of cryopreservation had a detrimental impact on the movement of spermatozoa because it affects not only the percentage of spermatozoa that retains the ability to move (total motility) and to move rectilinearly (progressive motility) but also reduced the qualitative parameters of the donkey spermatozoa significantly (Table 2 and Figs 1, 2). As reported in the present study (Fig. 1), the sperm velocity (VCL, VSL, VAP, % LIN, % STR) in the post-thawed semen samples were significantly lesser than in the fresh and cooled semen (Fig. 2). Similarly, a reduction in ALH (µm), WOB (%) and BCF (Hz) were recorded.

Table 1. Seminal characteristics of fresh semen in Poitou donkeys *(n=18 collections)

Seminal attribute	Poitou donkey
Reaction time (min)	8.45±3.17*
Total semen volume (ml)	65.00±4.04**
Gel volume (ml)	16.11±1.24
Gel free semen volume (ml)	49.44±3.54
Color	Creamy to milky white
Consistency	Thin to thick
pH	7.37±0.04
Sperm concentration(×10^6)	268.05±10.15**
Progressive motility (%)	80.55±1.50*
VAP (µm/s)	132.4±13.2
VSL (µm/s)	133.9±10.4
ALH (µm)	8.6±2.4
BCF (Hz)	37.2±2.5
STR (%)	84.7±2.4
LIN (%)	58.4±4.8
Live sperm count (%)	80.11±1.00*
Acrosomal integrity (%)	72.16±1.34
HOST (%)	68.27±1.13**
DNA intactness (%)	72.72±1.53
Sperm abnormalities (%)	11.11±0.63*

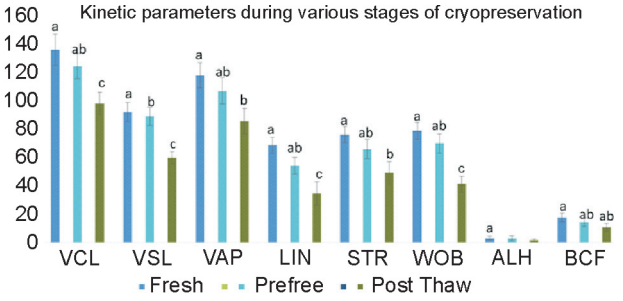

Fig. 1. Kinematic properties of jack spermatozoa at various stages of cryopreservation. *Mean values in the semen attributes with different superscripts (a, b, c and d) differ significantly (P≤0.05). VCL (µm/s), Curvilinear velocity; VSL (µm/s), Straight line velocity; VAP (µm/s), Average path velocity; LIN (%), Linearity; STR, Straightness; WOB, wobble effect; ALH (µm), Amplitude of linear head; BCF (Hz), Beat cross frequency.

Table 2. Mean±SE of seminal characteristics of untreated Poitou donkey semen at fresh, pre-freeze and post-thaw stage

Seminal attribute	Fresh	Pre-freeze	Post-thaw
Progressive motility (%)	80.55±1.50^{a}	65.0±1.93^{ab} (19.30)	35.27±1.17^{b} (56.2)
Live sperm count (%)	80.11±1.00^{a}	67.50±1.59^{ab} (15.7)	38.27±1.18^{b} (52.2)
Acrosomal integrity (%)	72.16±1.34^{a}	61.94±1.41^{ab} (14.16)	39.16±1.22^{b} (45.73)
HOST (%)	68.27±1.13^{a}	55.16±1.05^{ab} (19.20)	31.77±0.70^{b} (53.46)
DNA intactness (%)	72.72±1.53^{a}	58.83±1.30^{ab} (19.10)	40.33±1.26^{b} (44.54)

Note: Values in parenthesis indicate the percentage of difference from fresh semen.

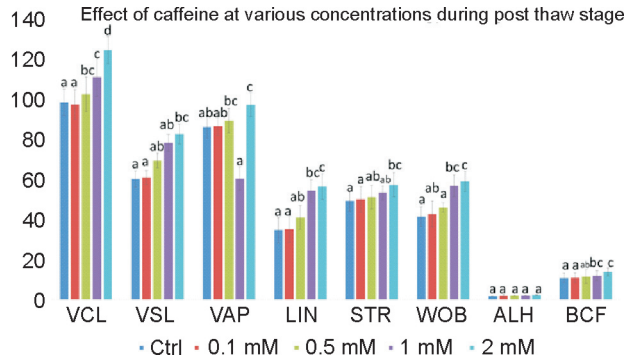

Fig. 2. Effects of different caffeine concentrations on sperm kinematic properties during post thaw stage. *Mean values in the semen attributes with different superscripts (a, b, c and d) differ significantly (P≤0.05). VCL (µm/s), Curvilinear velocity; VSL (µm/s), Straight line velocity; VAP (µm/s), Average path velocity; LIN (%), Linearity; STR, Straightness; WOB, wobble effect; ALH (µm), Amplitude of linear head; BCF (Hz), Beat cross frequency.

Progressive motility: The various parameters defining the kinetics of the spermatozoa (VSL, VAP, VCL, etc.,) were recorded and compared throughout at different stages of cryopreservation. In addition, the sperm kinematics showed a progressive decline (P<0.05) during the cryopreservation process (Fig. 2). There is significant effect of process of cryopreservation as directly observed from the declining results of kinetic parameters from fresh stage to the post-thaw stage. Greater variability and significant difference in the kinetic properties were noticed between the control and treatment groups (Fig. 2). The average progressive motility of Poitou donkeys was observed to be significant (P≤0.05) at 1 mM and 2 mM concentration of caffeine in pre-freeze semen and at 0.5 mM, 1 mM and 2 mM concentration of caffeine in post thaw semen respectively in comparison to control group (Table 3). Greater sperm kinematics were recorded in the group treated with 2 mM concentration of caffeine (Fig. 2). In addition, there was no individual variation found between individual jacks at pre- and post-freezing progressive motility between the treated and control groups.

Sperm viability: The similar staining patterns that were reported for equine spermatozoa were observed in the frozen-thawed jack semen samples. The percentage of live sperm count of donkeys was recorded to be significant (P≤0.05) at 1 mM and 2 mM concentration of caffeine in pre-freeze semen and the viability was found to be significant in the treatment groups of caffeine in comparison to control in post-thaw semen (Table 3). In addition, there was no individual variation found between jacks at pre- and post-freezing stages of cryopreservation for this parameter.

Acrosome integrity: The average acrosome integrity of Poitou donkeys was observed to be significantly higher (P<0.05) in treatment groups of caffeine in comparison to control in pre-freeze as well as post-thaw semen. However, there was no significant (P<0.05) effect of media containing 0.1 mM caffeine on the pre-freezing acrosomal integrity (Table 3). Between the treatment groups of caffeine, in 2 mM caffeine, there was individual variation found between the jacks in case of acrosomal integrity.

DNA intactness: The DNA intactness percentage of donkey semen at pre-freeze stage was found to be significant (P<0.05) in all the caffeine treated (0.1 mM, 0.5 mM, 1 mM and 2 mM) groups in comparison to control group, whereas during post- thaw stage, except for 0.1 mM caffeine group, all other groups showed significant increase in the DNA integrity of donkey semen. In addition, there was an individual variation found between the jacks in case of DNA intactness both at pre-freeze and post- thaw stages of cryopreservation.

Plasma membrane functionality (HOST) of spermatozoa: The average percentage of HOS reacted sperm of jacks was found to be significant (P<0.05) in the treatment groups of caffeine in comparison to control group at both pre- and post -freeze stage of cryopreservation.

Cryopreservation of semen is an important biotechnological tool, which is useful for the conservation of biodiversity of donkey species (Kumar *et al*. 2018). However, the results obtained in donkey semen

Table 3. Effect of different concentrations of caffeine on the qualitative seminal parameters of Poitou donkey semen at various stages of cryopreservation

Stage of cryopreservation	Seminal attribute	Control	0.1 mM	0.5 Mm	1.0 mM	2.0 mM
Pre-freeze	Progressive motility (%)	65.0±1.93^{a}	66.66±1.80^{ab}	68.33±2.25^{ab}	73.33±1.93^{bc}	76.11±1.34^{c}
	Live sperm count (%)	67.50±1.59^{a}	70.44±1.42^{ab}	72.38±1.74^{ab}	76.11±1.55^{bc}	78.72±1.16^{c}
	Acrosomal integrity (%)	61.94±1.41^{a}	64.94±1.03^{ab}	68.27±0.76^{bc}	70.94±0.90^{cd}	73.83±0.85^{d}
	HOST (%)	55.16±1.05^{a}	58.72±1.04 ^{ab}	61.55±1.03^{bc}	63.55±0.88^{c}	67.33±0.67^{d}
	DNA intactness (%)	58.83±1.30^{a}	64.00±1.41^{b}	65.61±1.14^{bc}	69.11±0.89^{cd}	72.66±0.95^{d}
Post-thaw	Progressive motility (%)	35.27±1.17^{a}	39.16±1.29^{ab}	42.22±1.72^{bc}	45.27±1.10^{cd}	47.77±1.08^{d}
	Live sperm count (%)	38.27±1.18^{a}	42.61±1.13^{ab}	46.16±1.52^{bc}	50.38±1.20^{c}	56.22±1.10^{d}
	Acrosomal integrity (%)	39.16±1.22^{a}	43.11±1.38^{ab}	46.83±1.60^{b}	53.33±1.37^{c}	57.27±0.97^{c}
	HOST (%)	31.77±0.70^{a}	34.11±0.61^{b}	36.83±0.53^{c}	38.33±0.54^{c}	41.11±0.52^{d}
	DNA intactness (%)	40.33±1.26^{a}	45.33±1.51^{ab}	48.44±1.53^{b}	53.61±1.19^{c}	58.11±0.85^{c}

*Mean values in the same columns with different superscripts (a, b, c and d) differ significantly (P≤0.05).

cryopreservation were found highly variable (Legha *et al*. 2013, Prashant Kumar *et al*. 2019). Most of the procedures used for cryopreservation of donkey semen have been adopted from those reported in horse stallions. Unfortunately, cryopreservation of donkey semen is not as easy as cattle and other livestock species, mainly due to wide variations in membrane composition and modifications during the freezing process and a lesser resistance of their sperm membrane to osmotic imbalances during temperature variations (Morris *et al*. 2007). An appropriate additive/antioxidant in freezing extender should, therefore be added to enhance the quality of frozen-thawed jack spermatozoa. The additives may vary in efficacy based on species and concentration and whether fresh, cooled or frozen semen is used.

The fresh seminal characteristics recorded in the present study are in agreement with the data on this breed reported earlier in the literature (Morris *et al*. 2007). In the current study, as observed from the results, it was found that cryopreservation had detrimental effect on motility and other seminal quality parameters. The present study results are in corroboration with the kinetic parameters reported for jack semen previously (Prashant *et al*. 2019). A significant decrease in total and progressive motility was recorded in all jack stallions, supporting the general agreement that the semen of the donkey was less efficiently frozen and freezing process can contribute to a decline in sperm motility, loss of membrane integrity and acrosome structure and DNA integrity (Karger *et al.* 2017).

In general, the results of the present study demonstrated that the addition of caffeine to the extender improved the kinetic and motility patterns in the semen samples supplemented with caffeine. This stimulatory effect of caffeine on sperm kinetic activity may be explained by the fact that caffeine converts glycogen phosphorylase from its inactive form to the active form by breaking down of glycogen into simple sugar, which might be utilized during cryopreservation. Motility enhancers like caffeine imposed positive effects in fresh and frozen-thawed semen of several species, including humans (Carrel and Aston 2013), bulls (Zhang *et al.* 2014) and stallions (Stephens *et al.* 2013).

In the current study, it was found that sperm motility increased significantly at a higher concentration of caffeine (2 mM), but was not affected at lower caffeine concentrations (0.1 or 0.5 mM). Stephens *et al*. (2013) demonstrated a non-significant increase in motility parameters after the addition of caffeine at concentrations of 1, 2 and 3.5 mmol/L to thawed equine semen. The addition of 5 mM of caffeine also significantly enhanced the motility of bovine semen (Barakat *et al*. 2015). Caffeine also improved the qualitative parameters of spermatozoa in boars (Yamaguchi *et al*. 2013) and laboratory mice (Nabavi *et al* 2013). The effect of caffeine on sperm characteristics may be species-specific; as the sperm, motility was adversely affected with high concentration of caffeine in ram and human at 5 mM (El-Shahat *et al*. 2016).

In this study, it was hypothesized that the additional ATP generated by the presence of caffeine would increase not only the activity of spermatozoa but also the life span of the sperm cells, and there was indeed an increase in the livability of caffeine-treated spermatozoa compared to that of untreated control for both parameters. Non-significant differences noticed for various seminal parameters at 0.1 and 0.5 mM of caffeine may be because concentration of caffeine used may have been inadequate to elicit desired results.

The acrosome integrity and DNA intactness of the donkey spermatozoa were found to be significantly increased with the addition of caffeine to the semen extender. Detainment of acrosome cap and its role in fertilization is vital and hence assessment of acrosome integrity of spermatozoa is an important qualitative parameter to assess. Acrosomal integrity was enhanced after supplementation with 5, 10 mM of caffeine in rabbits (El-Gaafary *et al.* 1994). Moreover, acrosomal integrity, viability, motility and hypo-osmotic swelling test increased in semen specimens treated by caffeine with a decrease in abnormal sperm in bovine bulls and buffalo bulls (Shukla and Misra 2014). Furthermore, supplementation of 1 mM of caffeine to ram semen reduced acrosomal membrane damage. The caffeine exhibits both antioxidant and pro-oxidant properties that have been regarded responsible for enhancing sperm qualitative parameters. In agreement to the previous studies conducted on horse stallions (Blottner *et al.* 2001) and jack stallions (Rota *et al.* 2008) semen preservation, individual variability between the jacks was recorded in the majority of semen parameters in this study.

Improvement in the majority of spermatozoa kinematic and qualitative parameters *in-vitro* might be expected to improve pregnancy and conception rates after insemination with frozen-thawed donkey semen; however, *in-vivo* fertility trials are required to assess the effect of addition of caffeine on the fertility of cryopreserved donkey semen.

The present study demonstrated that the supplementation of the freezing extender with various increasing concentrations of caffeine (0.1, 0.5, 1 and 2 mM) improved post-thaw donkey sperm quality in terms of motility variables, acrosome integrity, and DNA integrity compared to the control samples. The findings of the present study reveals that caffeine has hyperactivation efficacy at 2 mM concentration compared to other concentrations. This would allow a more efficient use of cryopreserved donkey semen for artificial insemination and the conservation of endangering species by creating the semen reserves. Further experiments should be conducted to evaluate *in-vivo* fertility rates of frozen-thawed donkey spermatozoa supplemented with caffeine.

ACKNOWLEDGEMENTS

The authors would like to thank the Director, ICAR-NRC on Equines, Hisar for providing excellent research facilitates for carrying out the present research study and also thank In-charge, Equine Production Campus, Bikaner, Rajasthan for his kind cooperation.

REFERENCES

Aitken J and Fisher H. 1994. Reactive oxygen species generation and human spermatozoa – the balance of benefit and risk. *Bioessays* **16**: 259–67.

Avanzi B R, Ramos R S, Araujo G H M, Fioratti E G, Trinca L A and Dell'Aqua Jr J A. 2015. Fixed-time insemination with frozen semen in mares: is it suitable for poorly fertile stallions? *Theriogenology* **83**: 1389–93.

Barakat I A H, Danfour M A, Galewan F A M and Dkhil M A. 2015. Effect of various concentrations of caffeine, pentoxyfylline, and kallikrein on hyperactivation of frozen bovine semen. *Biomedical Research International*: 948575.

Blottner S, Warnke C, Tuchscherer A, Heinen V and Torner H. 2001. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. *Animal Reproduction Science* **65**: 75–88.

Canisso I F, Carvalho G R, Morel M D, Ker P G, Rodrigues A L and Silva E C. 2011. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. *Equine Veterinary Journal* **43**: 179e83.

Carrel D T and Aston K I. 2013. *Spermatogenesis: Methods and Protocols*. New York, USA, Humana Press.

Colas C, Cebrian-Perez J A and Muino-Blanco T. 2010. Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. *International Journal of Andrology* **33**: e187e97.

Contri A, De Amicis I, Veronesi M C, Faustini M, Robbe D and Carluccio A. 2010. Efficiency of different extenders on cooled semen collected during long and short day length seasons in Martina Franca donkey. *Animal Reproduction Science* **120**: 136–41.

El-Gaafary M N. 1994. Quality and fertility of cooled rabbit semen supplemented with cyclic-AMP stimulators. *Animal Reproduction Science* **34**: 3070–313.

El-Shahat K H, Taysser M I, Badr M R and Zaki K A. Effect of heparin, caffeine and calcium ionophore A23187 on *in vitro* induction of the acrosome reaction of fresh ram spermatozoa. *Asian Pacific Journal of Reproduction* **5**:148–55.

Karger S, Geiser B, Grau M, Heuwieser W and Arlt S P. 2017. Short communication: Progressive motility of frozen-thawed canine semen is highest five minutes after thawing. *Reproduction Domestic Animals* **52**: 350–52.

Kumar R, Ravi S K, Dholpuria S, Purohit G N, Pushp M K, Solanki S, Ganguly S and Pratap C. 2018. Study on fresh semen characteristics in donkeys. *Journal of Entomology and Zoological Studies* **61**: 227e9.

Legha R A, Yash Pal, Ravi S K and Dedar R K. 2013. Physical and biochemical properties of Indian jack semen. *Animal Science Reporter* **7**(4): 153–60.

Miró J, Vilés K, García W, Jordana J and Yeste M. 2013. Effect of donkey seminal plasma on sperm movement and sperm polymorphonuclear neutrophils attachment *in vitro*. *Animal Reproduction Science* **140**: 164–72.

Mirshokraei P, Hassanpour H, Mehdizadeh A and Akhavan Taheri M. 2011. Pentoxifylline induces capacitation and acrosome reaction and improves quality of motility in canine ejaculated spermatozoa. *Research in Veterinary Science* **91**: 281e4.

Morris G J, Faszer K, Green J E, Draper D, Grout B W and Fonseca F. 2007. Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. *Theriogenology* **68**: 804–12.

Nabavi N, Todehdehghan F and Shiravi A. 2013. Effect of caffeine on motility and vitality of sperm and *in vitro* fertilization of outbreed mouse in T6 and M16 media. *Iranian Journal of Reprodution and Medicine* **11**: 741–6.

Naz R K and Rajesh P B. 2004. Role of tyrosine phosphorylation in sperm capacitation/ acrosome reaction. *Reproduction Biology Endocrinology* **2**: 75.

Ortiz I, Dorado J, Morrell J M, Crespo F, Gosálvez J and Gálvez M J. 2015. Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: do they have the same effect regardless of the quality of the sample? *Theriogenology* **84**: 294–300.

Prashant Kumar, Rabindra Kumar, Jitendra Singh Mehta, Ashok Kumar Chaudhary, Sanjay Kumar Ravi, Sharat Chandra Mehta, Mohd. Matin Ansari, Ram Avtar Legha, Bupendra Nath Tripathi and Thirumala Rao Talluri. 2019. Ameliorative effect of ascorbic acid and glutathione in combating the cryoinjuries during cryopreservation of exotic Jack semen. *Journal of Equine Veterinary Science* **81**: 102796.

Rota A, Magelli C, Panzani D and Camillo F. 2008. Effect of extender, centrifugation and removal of seminal plasma on cooled preserved Amiata donkey spermatozoa. *Theriogenology* **69**: 176–85.

Rota A, Panzani D, Sabatini C and Camillo F. 2012. Donkey jack (*Equus asinus*) semen cryopreservation: studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. *Theriogenology* **78**: 1846–54.

Shukla M K and Misra A K. 2014. Caffeine as a semen additive to improve Murrah buffalo (*Bubalus bubalis*) semen cryopreservation. *Buffalo Bulletin* **33**: 32–36.

Soni Y, Talluri T R, Kumar A, Ravi S K, Mehta J S and Tripathi B N. 2019. Effects of different concentration and combinations of cryoprotectants on sperm quality, functional integrity in three Indian horse breeds. *Cryobiology* **86**: 52e7.

Stephens T D, Brooks R M, Carrington J L, Cheng L, Carrington A C and Porr C A. 2013. Effects of pentoxifylline, caffeine, and taurine on post-thaw motility and longevity of equine frozen semen. *Journal of Equine Veterinary Science* **33**: 615e21.

Tartaglione C M and Ritta M N. 2004. Prognostic value of spermatological parameters as predictors of *in vitro* fertility of frozen-thawed bull semen. *Theriogenology* **62**: 1245e52.

Watson P F. 2000. The causes of reduced fertility with cryopreservation semen. *Animal Reproduction Science* **60**: 481–92.

Yamaguchi S, Suzuki C, Noguchi M, Kasa S, Mori M, Isozaki Y, Ueda S, Funahashi H, Kikuchi K, Nagai T and Yoshioka K. 2013. Effects of caffeine on sperm characteristics after thawing and inflammatory response in the uterus after artificial insemination with frozen-thawed boar semen. *Theriogenology* **79**: 87e93.

Zhang J, Guo H, Su J, Zhao L, Li Y, Sun W, Han H, Hu S, Zhao G, Li Y, Dai Y and Li X. 2014. A comparison of the effects of pentoxifylline on quality of fresh and frozen-thawed bull spermatozoa. *Agriculture Biotechnology* **3**: 20–29.