EFFECT OF SUPPLEMENTATION OF CAFFEINE ON SPERM MOTILITY AND LIVEABILITY IN MARWARI STALLIONS

SOURABH KANT[1], YASH PAL[2], RA LEGHA[2], TARU SHARMA[1], TR TALLURI*[2]

[1]ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh- -243122, India

[2]Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, Rajasthan-334001, India

Submitted : 02-07-2021 Accepted : 06-08-2021

ABSTRACT

Six ejaculates from each three adult 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 during pre-freeze stage and cryopreserved by following standard protocol. The sperm kinetic parameters, membrane functionality and integrity were evaluated with computer-assisted sperm analysis, a hypo-osmotic swelling test and epifluorescent microscopy, respectively. The present study revealed that, adding caffeine to stallion semen extender at 0.1, 0.5 and 2 mM levels had positive impact on seminal parameters compared to the control semen. Sperm kinetics (VAP, VSL and STR) and motility were increased in the caffeine treated groups in comparison to the control group. Among these, 2mM caffeine supplemented group the post-thaw sperm motility and other functional attributes have significantly improved compared to the other treatment and control groups.

***Key words*:** Caffeine; Jack sperm; cryopreservation; kinetic parameters; acrosome integrity

INTRODUCTION

The population of the equines are drastically declining for the past decade and there is an urgent need to conserve and propagate this valuable indigenous equine germplasm (Talluri et al., 2016). One of the best strategies to conserve the germplasm of any breed is through cryopreservation of gametes. Artificial Insemination (AI) with cryopreserved semen is more common in the equine breeding industry (Rota et al., 2012).

Unfortunately, pregnancy rates after frozen semen AI are lower compared to fresh or cooled semen in equines. Since cryopreservation of equine semen is one of the difficult activities wherein it causes membrane damage or apoptosis. The successful use of cryopreserved sperm largely depends on cryosurvival rates, species and individuals of the same species (Vidament et al., 2009). In fact, only 20% of fertile stallions produce sperms that survive the freezing and thawing processes (Tischner, 1979). Cryopreservation is known to degrade potential fertility of spermatozoa by causing death of about 50 per cent of the cells and altered characteristics of many of the damages generally are consequences of mechanical and osmotic phenomenon, oxidative stress, increased membrane permeability, lipid peroxidation and subsequent membrane damage during cooling, freezing and thawing.

Caffeine is a heterocyclic compound that promote hyper activation of bull spermatozoa (Ho and Suarez, 2001b and Marques and Suarez, 2004) for improved fertilization (Ho and Suarez, 2001a; Pavlok *et al*., 2001; Marquez and Suarez, 2004). by increasing the cAMP levels within the cell, *viz*., bulls (Barakat et al.2015) and boars (Yamaguchi et al. 2013). In view of the above, present study was carried out to evaluate the effect of supplementation of caffeine on stallion sperm motility and liveability.

MATERIALS AND METHODS

Six healthy Marwari breed stallions, ranging between 4 and 10 years of age, maintained at Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, under uniform conditions of feeding and management were used for the study. The present study has been carried out with the approval of the Institute's Animal Ethics Committee of ICAR-National Research Centre on Equines, Hisar, India. The semen from stallions was collected (6 collections from each stallion, a total of 18 collections), twice per week using artificial vagina (Colorado model) equipped with a disposable liner as per the protocol (Soni et al., 2017). Semen collection, evaluation and processing for freezing were done as previously described (Talluri et al. 2012). Immediately after semen collection, seminal parameters

Corresponding Author: raotalluri79@gmail.com; [1]Sourabh Kant: MVSc Scholar (kantsourabh951@gmail.com); [2]Yash Pal, Director , ICAR-National Research Centre on Equines, Hisar (yashpal1888@gmail.com); [2]RA Legha: Principal Scientist; ralegha@gmail.com; [2]TR Talluri: Senior Scientist, Equine Production Campus, [2]ICAR-National Research Centre on Equines, Bikaner, Rajasthan (raotalluri79@gmail.com) and Taru Sharma: [1]ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, 243122, India (gts553@gmail.com).

like appearance, volume, colour, consistency, pH were recorded by visual observation. For calculating total gel volume, the gel free semen volume was deducted from fresh semen collected before sieving. The other semen parameters that were evaluated were total and progressive sperm motility, sperm concentration, liveability and Hypo-osmotic swelling test (HOST) to determine plasma membrane integrity.

After performing macroscopic and microscopic evaluation of fresh semen, the semen samples were processed, extended and frozen according to the described methods (Talluri et al., 2012). Semen samples having progressive motility >50% were processed further for cryopreservation. Gel free semen was mixed with modified Citrate-EDTA primary extender in the ratio of 1:1 and centrifuged at 650 g for 3 min. The supernatant was discarded and sperm pellet was extended with Glucose-EDTA-lactose secondary extender having cryoprotectant agent (5% Di-methyl Formamide) and 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 150-200 X 10^6 sperms/ml. The diluted semen was kept in semen cooling cabinet at 4ºC for 2 h as equilibration period. Semen samples were again assessed for pre-freeze seminal characteristics. Equilibrated semen was manually filled in 0.5 ml straws using vacuum pump. The straws were sealed using PVC powder and were cooled for 30 min at 4ºC. The straws were spread over freezing racks, 4 cm above liquid nitrogen (LN2) in a traditional styrofoam box for exposure to LN2 vapours for 10-12 min and then plunged directly into LN2. Later, the semen straws were transferred to LN2 cryocans. The microscopic evaluation of frozen thawed sperm was done at least 24 h after storage. The straws were thawed in 37ºC water bath for 30 sec. Each frozen thawed semen sample was evaluated for determining the post-thaw motility, live and dead percentage and plasma membrane integrity through hypo-osmotic swelling (HOS) test as per the standard procedures.

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 37ÚC. The sperm morphology and live/dead status was determined according to methods described previously (eosin/nigrosine) (Kumar et al. 2020). Evaluation of the plasma membrane/ functional integrity of sperm membrane were determined by HOS test as described by Kumar et al. (2019). The number of spermatozoa (%) with tail coiling (HOS +ve) was recorded for each sample. The experiment was repeated five times to obtain a consistent result.

Fresh and post-thaw sperm quality parameters were analysed in duplicate. The statistical analysis was performed using SPSS (SPSS, V12.0, SPSS Inc., Chicago, IL, USA). Before the analyses, normal distribution of data was confirmed. As repeated collection of semen was done on same stallion/jack at different time intervals, a repeated measure ANOVA was done to partition the variability attributable to differences between treatments and individual variation among stallion/jack/ subjects in treatment groups. Statistical analyses were performed using the SPSS 20.0 statistical software package. One- way ANOVA was used to test statistical differences between different treatment groups. Pair wise comparisons (or post hoc test) were performed using the T-method (Tukey's honestly significant difference method).

RESULTS AND DISCUSSION

The physio-morphological attributes of fresh, semen of Marwari horses are presented in Table 1. Semen volume, gel volume and gel free semen volume were significantly (P<0.05) different between Marwari horses. A significant improvement in pre-freeze progressive semen motility was observed with 1.0 mM (67.2±2.65%) and 2.0 mM (68.6±2.93%) caffeine concentration in comparison to control group (53.6±2.31%) in Marwari horse. However, there was no significant effect of media containing 0.1mM, 0.5mM caffeine on the pre freezing progressive motility. At post thaw stage also, the average progressive motility (mean ±SE) of Marwari horses was observed to be significantly different (P<0.05) at 0.5mM (50.27±2.19), 1mM (51.38±1.44) and 2mM (51.11±0.95) concentration of caffeine in comparison to control (40.66±1.61). However, there was no significant effect of media containing 0.1mM caffeine on the post thawing progressive motility. Caffeine at higher concentration (5.0mM) caused a reduction of bull and ram sperm motility (Bird *et al*, 1989; El-Gaafary, 1987). The improvement in progressive motility as seen in the present study might be due to addition of caffeine which stimulates sperm metabolism, enhances fructose utilization, improves respiration and causes an increase in cAMP levels (Milani *et al.*, 2010).

The average live sperm count (mean±SE) of Marwari horses was observed to be significantly different (P<0.05) at 1mM (70.22±1.53) and 2mM (73.11±1.73) concentration of caffeine in comparison to control (61.05±1.79). There was no significant difference in the live and dead spermatozoal count in the 0.1mM and 0.5 mM treatment groups to that of control. The similar trend was also observed at post thaw stage also. In contrast to our results, Sipek *et al.* (2014) observed that addition of caffeine adversely affected sperm viability in boar whereas, Makler *et al.* (1980) and Yamaguchi *et al.* (1994)

could not find any effect of caffeine on viability of sperm in human and boar.

There was no significant effect of media containing 0.1mM, caffeine on the pre-freezing and post thaw HOS reacted sperm. HOS reacted sperm (mean±SE) was observed to be significantly different (P<0.05) at 0.5mM (62.72±1.15), 1mM (63.61±1.75) and 2 mM (67.27±1.17) concentration of caffeine in comparison to control (56.16±1.42). At post thaw stage, there was significant difference between the control groups and 0.5mM, 1mM and 2mM caffeine groups. These findings were supported by Shukla and Misra (2014) which showed improvement in post thaw motility at 1.0 and 3.0mM caffeine concentration in dog (Milani *et al.,*2010). Total and progressive motility of the sperm was increased after 24h of incubation in presence of 1 or 4 mM of caffeine (Spalekova *et al*., 2011).

CONCLUSION

The present study was conducted to evaluate the effect of supplementation of caffeine at various concentration to the semen extender in Marwari breed stallions and concluded that the caffeine supplementation has induced beneficial effect on post thaw seminal parameters of equine semen and there is need to explore the effects of caffeine at concentrations more than 2.0 mM.

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 Incharge, Equine Production Campus, Bikaner, Rajasthan for his kind cooperation.

REFERENCES

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.

Bird, J. M., Carey, S. and Houghton, J. A. 1989. Motility and acrosomal changes in

ionophoretreated bovine spermatozoa and their relationship with in vitro penetration of zona-free hamster oocytes. Theriogenology. *32:* 227-242

El-Gaafary, M. N. 1987. The characteristics of semen from Welsh Mountain and Cambridge rams. PhD Thesis, UCNW, Baangor, 258 pp

Ho, H. C. and Suarez, S. S. 2001b. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction. Biol. Reprod. *122:* 519–526

Kumar P, Mehta JS, Ravi SK, Dedar RK, Purohit GN, Legha RA,Tripathi BN, Talluri TR. Cholesterol Loaded Cyclodextrin Supplementation Enhances the Cholesterol-to-Phospholipid Ratio and Diminishes Oxidative Stress in Jack Spermatozoa During Cryopreservation. Journal of Equine Veterinary Science. 2020 Nov;94:103237. DOI: 10.1016/j.jevs.2020.103237

Makler, A., E. Makler, J. Itzkovitz and J.M. Brandes. 1980. Factors affecting sperm motility. IV. Incubation of human semen with caffeine, kallikrein and other metabolically active compounds. Fertil. Steril. *33*: 624-630

Marquez, B. and Suarez, S. S. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperaktivation. Biol. Reprod. *70*: 1626- 1633.

Milani. A. and Fontbonne, C. A. 2010. Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology. 74*(1):* 153–164

Pavlok, A., Kubelka, M. and Peknicova, J. 2001. The effect of various capacitation active compounds and capacitation time on the in vitro fertility and protein tyrosine phosphorylation profiles of bovine spermatozoa. Zygote. *9*: 25-38.

Prashant Kumar, Ashok Kumar, J S Mehta, G N Purohit, S K Ravi, Yash Pal, R A Legha,B N Tripathi and TR Talluri. Impact of supplementation of semen extender with antioxidants on quality of cooled or cryopreserved Marwari stallion spermatozoa. *Indian Journal of Animal Sciences* 90 (10): 00–00, October 2020/Article.

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 Bullettin* 33: 32–36.

Sípek, J., Kopecká, V., Kunetková, M. and Pøinosilová, P. 2014. Effects of caffeine,

equilibration time and thawing temperatures on the quality of boar insemination

doses. Res. pig breed., *8(2):*16-19

Soni Y, Talluri T R, Kumar A, Ravi S K, Mehta J S, 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.

Spalekova, E., Makarevich, A. V. and Pivko, J. 2011. Effect of caffeine on parameters of ram sperm motility. Slovak. J. Anim. Sci., *44(2):*78-83

Thirumala Rao Talluri, Arangasamy A, S K Ravi, Yash Pal, A. K. Gupta and R.K. Singh. (2012). Characteristics of fresh and frozen semen of Zanskari stallions. *Indian Vet J.* 89 (7): 62-64

Thirumala Rao Talluri, S.K. Ravi, J. Singh, R.A. Legha, Yash Pal, A.K. Gupta, R.K. Singh and B.N. Tripathi. Feb 2016. Some reproductive parameters of Zanskari Mares reared in an otganized farm under tropical climate. *Indian Journal of Animal Sciences.* 86 (2): 163–167,

Tischner M. Evaluation of deep-frozen semen in stallions. *J Reprod Fert.* 1979. pp. 53–59.

Vidament M., Vincent P., Martin F.-X., Magistrini M., Blesbois E. Differences in ability of jennies and mares to conceive with cooled and frozen semen containing glycerol or not Animal Reproduction Science, 112 (2009), pp. 22-35

Wang X, Sharma RK, Sikka SC, Thomas AJ, Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 2003;80:531– 535.

Watson, P.F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and assessment of their post-thawing function. Reprod Fertil Dev 7, 871-891.

Table 1. Quantitative and qualitative seminal parameters in the fresh semen of Marwari stallions

Sr No	Seminal attribute	Mean±SE
	Total semen volume	49.44±4.12
	Gel free semen Volume	16.11±1.54
	Colour	Creamy white to milk white
	Consistency	Thin to thick
	pH	7.49±0.03
	Initial Motility	84.72±2.04
	Progressive sperm motility	73.88±1.37
	Sperm concentration	277.55±7.92
	Live sperm (%)	78.24±1.05
	HOST (%)	69.88±1.08
	Sperm abnormalities	12.11±0.63

Table 2. Seminal parameters of pre-freeze semen treated with different concentrations of Caffeine

Stage of cryopreservation ⇨ / Seminal attributes ⇩		Pre- freeze	Post- thaw
Progressive motility (%)	Control	53.61±2.31	40.66±1.61^{a}
	0.1mM	60.55±2.38^{ab}	46.11±1.74^{ab}
	0.5mM	63.61±2.41^{b}	50.27±2.19^{b}
	1mM	67.22±2.65^{b}	51.38±1.44^{b}
	2mM	68.61±2.93^{b}	51.11±0.95^{b}
Live sperm count (%)	Control	61.05±1.79^{a}	48.94±2.09^{a}
	0.1mM	64.77±1.53^{ab}	54.11±2.28^{ab}
	0.5mM	66.88±1.90^{abc}	58.05±2.18^{bc}
	1mM	70.22±1.53^{bc}	60.61±1.71^{bc}
	2mM	73.11±1.73^{c}	62.27±1.28^{c}
HOST (%)	Control	56.16±1.42^{a}	34.50±1.16^{a}
	0.1mM	60.16±1.54^{ab}	37.16±1.27^{ab}
	0.5mM	62.72±1.15^{bc}	39.38±1.22^{bc}
	1mM	63.61±1.75^{bc}	39.94±1.75^{bc}
	2mM	67.27±1.17^{c}	42.55±1.00^{c}