The Pharma Innovation Journal 2022; 11(7): 1519-1522

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(7): 1519-1522

www.thepharmajournal.com
Received: 13-04-2022
Accepted: 24-06-2022

Anklesh Katara
Assistant Professor, Department of Veterinary Gynaecology and Obstetrics, M.B. Veterinary College, Dungarpur, Rajasthan, India

Mitesh Gaur
Assistant Professor, Department of Veterinary Gynaecology and Obstetrics, CVAS Navania, Udaipur, Rajasthan, India

Rohit Juneja
Veterinary officer, Veterinary Hospital Pundlota, Nagaur, Department of Animal Husbandry, Jaipur, Rajasthan, India

Ishwar mal Harizan
Assistant Professor, Department of Veterinary Clinical Complex Medicine, M.B. Veterinary College, Dungarpur, Rajasthan, India

Corresponding Author:
Rohit Juneja
Veterinary officer, Veterinary Hospital Pundlota, Nagaur, Department of Animal Husbandry, Jaipur, Rajasthan, India

Effect of various concentration of caffeine on pre-freeze and post thaw quality of Surti buffalo (*Bubalus bubalis*) bull semen

Anklesh Katara, Mitesh Gaur, Rohit Juneja and Ishwar mal Harizan

Abstract
The present study evaluated the beneficial effects of incorporation of caffeine as an additive in Tris fructose egg yolk citrate extender in Surti buffalo bull semen. Extended semen was divided in 4 equal parts and caffeine was added at 0.5 mM (Treatment-1), 1.0 mM (Treatment-2), 1.5 mM (Treatment-3) with one control group having no additives at all. Pre-freeze semen parameter individual progressive motility were found to be significantly higher ($P< 0.05$) in semen samples treated with caffeine (Treatment-1 and Treatment-2) in comparison to control. Similarly, effects of caffeine as an additive in semen were also recorded in semen thawed after 0hr and 48hr of cryopreservation. The post thaw (at 0hr) individual sperm progressive motility percentage were significantly higher ($P< 0.05$) in Treatment-1 and Treatment-2 compared to control. At 48hr also post thaw individual progressive motility were significantly lower ($P< 0.05$) in 0.5 mM caffeine (Treatment-1) and 1.0 mM caffeine (Treatment-2) compared to control, but the 0.5 mM caffeine (Treatment-1), had the highest pre-freeze and post thaw (0hr and 48hr) individual progressive motility percentage compared to other three groups. It was concluded that caffeine at 0.5 mM of concentration showed significant ($P< 0.05$) improvement in pre-freeze and post thaw semen quality in comparison to concentrations of 1 mM or 1.5 mM; and is sufficient to induce a beneficial effect on cryopreservation of Surti buffalo semen.

Keywords: Various concentration, caffeine, pre-freeze, Surti buffalo, *Bubalus bubalis*

Introduction
Frozen semen technology has been the pillar behind artificial insemination and one of the major strengths of assisted reproduction. The discovery of liquid nitrogen and different extenders has made it relatively easy for the long-term preservation of bovine frozen semen.

The dairy industry has witnessed an increased demand for semen from superior sires. India has the largest breeding infrastructure in the world (64 frozen semen bull stations and more than 1 lac A.I. centres). Presently, total annual semen production is around 100 million frozen semen straws. However, the result of A.I. is much below the desired level. As such, A.I. as a tool for livestock development is hardly applicable to 30% to 35% of bovine population. (NDDB 2019) [23].

Cryopreservation of semen is the most viable biotechnology for faster and increased genetic improvement in many species allowing for improved herd performance and productivity. (Bilodeau *et al*. 2000) [4] (Kim *et al*. 2010) [18] Freezing and thawing induce the detrimental effects on sperm ultrastructural, biochemical and functional damage (Watson 2000) [36], resulting in a reduction of motility, membrane integrity and fertilizing ability (Nichi *et al*. 2006) [24]. In bovine semen, enzymatic (catalase, superoxide/dismutase, glutathione peroxidase/reductase) and non-enzymatic (vitamin C and E, glutathione, cysteine) antioxidants are present to protect the spermatozoa from the reactive oxygen species molecules. However, the levels of inherent antioxidants are inadequate to protect the sperm integrity against oxidative stress during cryopreservation (Nichi *et al.* 2006) [24]. Egg yolk at different concentration is commonly added to freezing extenders as a source of lipoproteins to provide protection against cold shock. Nitric oxide is an important mediator of sperm function (Ortega *et al*. 2009) [25] and since egg yolk is a nitric oxide scavenger, the level of egg yolk must be considered.

Caffeine is a xanthine alkaloid compound that acts as a stimulant in humans. Caffeine is sometimes called guaranine when found in guarana, mateine when found in mate, and theine when found in tea. It is found in the leaves and beans of the coffee plant, in tea, yerba mate, and guarana berries, and in small quantities in cocoa, the kola nut and the yaupon holly.

Caffeine at concentration of 5 mM may cause decrease in fertilization capacity of human and bull sperm, but in rabbits higher concentration (10 mM) may cause increased motility (Ibrahim *et al.* 2015) [16]. Caffeine was reported to induce an increase in intracellular calcium and an immediate hyperactivation of sperm (Colas *et al.* 2010) [8]. The essential role of calcium on sperm capacitation has been proved in several mammalian species (Yanagimachi 1994) [37] and an increase in intracellular Ca2+ concentration has been shown in hyper-activated flagella (Ho, Suarez 2001) [15] and a stimulating effect on bull semen in a lower concentration of caffeine (Bird *et al.* 1989) [5]. Caffeine may have a direct effect on cellular metabolism, and such effect depends on the concentration of calcium ions. Additionally, cattle IVF have been improved by the application of heparin alone or through its synergistic effects when used with caffeine (Park *et al.* 1989) [26].

Materials and Methods

8 Surti buffalo bulls of the age group between 6.5–7.5 years and weighing between 445-520 kg, reared at Network Project on Buffalo Bull (Surti) Improvement at Livestock Research Station, Vallabhnagar, Udaipur which is situated in campus of College of Veterinary Sciences, Navania, Vallabhnagar Udaipur, in the state of Rajasthan.

All the buffalo bull were fed green fodder, hay and compounded concentrate mixture as per farm schedule. These animals were maintained under identical nutritional hygienic and management conditions. A total of 8 ejaculates, collected in a sterilized AV, were used to evaluate the effect of stimulant caffeine on semen cryopreservation. After collection, the semen samples were immediately transferred to a water bath maintained at 35 ºC and kept under laminar air flow to avoid exposure to direct sunlight and bacterial contamination. Semen samples were evaluated for progressive sperm motility (Tomar 1997) [34], immediately after collection. Soon after the neat semen evaluation, sperm concentration was evaluated by haemocytometer method. Each ejaculate was diluted in tris fructose egg yolk citrate extender. The ejaculates after dilution were split into four parts and treated as follows:

1. Part 1: Diluted in TCFY (Control)
2. Part 2: Diluted in TCFY + 0.5 mM caffeine (T_1)
3. Part 3: Diluted in TCFY + 1.0 mM caffeine (T_2)
4. Part 4: Diluted in TCFY + 1.5 mM caffeine (T_3)

Diluted semen was put in French medium straws and sealed with PVA powder. These straws were kept for equilibration at 4ºC for 4 h. Subsequently the semen samples were frozen using the horizontal liquid nitrogen vapour freezing technique (Verma *et al.* 1975) [35]. Frozen semen samples were thawed at 37ºC for 1 minute, immediately (0 h) and 48 h after freezing and examined for progressive sperm motility.

All the chemicals used in the experiment were from Sigma-Aldrich, USA.

Statistical Analysis

The results were subjected to one way analysis of variance and the paired t-test (Snedecor and Cochran 1994) [31].

Results and Discussion

The results of the trial conducted to evaluate the effect of 0.5 mM (T1), 1.0 mM (T2) and 1.5 mM (T3) concentrations of caffeine are summarized in Table 1. As evident from the table, significant ($P< 0.01$) beneficial effect of 0.5 mM caffeine on the progressive sperm motility. The difference in the effect of 1.0 and 3.0 mM caffeine was also significant ($P< 0.01$). Supporting reports of better effect of 2.0 mM caffeine on cryopreservation of buffalo (Fattouh *et al.* 1985) [12] (Fattouh, Abdou 1991) [11] and bovine (Garbers *et al.* 1971) [13] semen as compared to 4.0 or 6.0 mM has also been recorded in the past. There was enhanced fertilizing ability when semen was diluted with 2.0 mM caffeine as compared to that diluted with 5.0 or 10.0 mM caffeine (Aitken *et al.* 1983) [1].

These reports and the significant beneficial effect of 1 mM caffeine as compared to the control and other three concentrations, recorded in the present study, suggests that even lower concentrations of caffeine may be effective in protecting sperm from cryoinjuries.

Table 1: Effect of Caffeine supplementation on percent progressive motility of Surti buffalo bull spermatozoa at pre-freeze and post-thaw stage (Mean ± SE, n=32).

Stage of semen freezing	Control	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3(T3)
Pre-freeze	70.37±0.65^{c}	73.50±0.5^{a}	73.12±0.63^{ab}	71.37±0.90^{bc}
Post-thaw (0hr)	64.43±0.43^{b}	66.37±0.41^{a}	65.62±0.52^{a}	63.93±0.77^{b}
Post-thaw (48hr)	63.06±0.22^{b}	67.00±0.54^{a}	66.93±0.60^{a}	63.81±0.65^{b}

Values are presented as mean ± standard error of mean of thirty two replicates. Different superscripts within a row indicate significant difference ($P< 0.05$). Control, T1, T2 and T3 contained 0.0, 0.5, 1.0 and 1.5 mM concentration of caffeine.

Table 2: Analysis of variance showing the influence of additives at pre-freeze, 0hr and 48hr of preservation on individual sperm progressive motility percentage.

Source of Variation	D.F.	Individual Progressive Motility					
		Pre freeze		0hr		48hr	
		M.S.	F.	M.S.	F.	M.S.	F.
Treatment	3	17.36	4.56*	9.844	3.984*	34.00	15.01**
Error	28	3.8		2.47		2.26	

*$P\leq 0.05$, **$P\leq 0.01$

The mean value of individual sperm progressive motility of Surti buffalo bull semen was 74.00±0.35% in present study. Earlier study reported mean value of individual sperm motility of Surti buffalo bull as 80.76±0.39% (Chaudhary *et al.* 2017) [6] and 75.00±0.69% (Dhami *et al.* 2016) [9]. The average individual progressive motility (%) for 3 Murrah buffalo bulls was reported as 78.42±0.70 (Shukla, Misra 2014) [29]. Bhakat *et al.* (2015) [3] reported the overall least squares mean values of Murrah buffalo and crossbred bulls individual progressive sperm motility (%) as 60.64±0.02 & 46.73±0.03% respectively. Also, Bhakat *et al.* (2011) [2] reported the average individual progressive motility in Murrah buffalo as 66.63±0.44% (with a range of 61.58 to 71.93%). Koonjaenak, Rodriguez (2007) [19] reported the average individual sperm progressive motility in swamp buffalo to be greater than 65%. Sperm progressive motility is an important criterion of semen quality (Lasley 1951) [20] and is an important determinant of success rate of the fertilization and ability of spermatozoa to withstand the stress of cryopreservation process. Semen samples must contain good numbers of forward moving spermatozoa for optimum fertility. Usually, the progressive motility of a good semen

sample should range between > 50 to 90 percent.

In the present study, effect of various concentrations of caffeine (0.5 mM, 1.0 mM and 1.5 mM) on the pre-freezing semen attributes in Surti buffalo bull were studied after the equilibration period of 4ºC for 4 hours.

A significant improvement in pre-freezing sperm progressive motility of sperm was observed with 0.5 mM (73.50±0.50%) and 1.0 mM (73.12±0.6%) caffeine concentration in comparison to control (70.37±0.65%) in Surti buffalo bulls. In earlier study, Shukla, Misra (2014) [29] reported higher pre-freezing individual progressive motility of sperm with 1.0 mM (78.05±0.82%) and 3.0 mM (77.78±0.72%) caffeine concentration in comparison to control (76.67±0.90%) in Murrah buffalo bulls. Singh, Raina (2000)[30] reported significantly better pre-freezing individual sperm progressive motility with 4.0 mM (68.00±1.70%) caffeine in comparison to control (61.46±2.10%) in Murrah buffalo bulls.

Patel, Siddiquee (2012) [27] also reported increased pre-freezing individual progressive motility of sperm with 0.5% caffeine (77.95±0.58%) concentration in comparison to control (74.73±0.58%) in Kankrej bulls. Our results were supported by Kant (2016) [17] who showed significant improvement in pre-freezing individual progressive motility of sperm at different concentration of caffeine *viz*. 1.0 mM (67.2±2.65%) and 2.0 mM (68.6±2.93%), in comparison to control (53.6±2.31%) in Marwari horse.

The improvement in individual progressive motility of sperm as seen in the study may be due to addition of caffeine which stimulates sperm metabolism, enhances fructose utilization, improves respiration and causes an increase in cAMP levels (Milani, Fontbonne 2010) [22]. EL-Gaafary *et al.* (1990) [10] reported that addition of different levels of caffeine in either EYC and Tris diluted bull semen (10, 20, 40 and 80 mM/100 ml of diluents) increased the percentage of motile spermatozoa as compared to control. Whereas, Cohen *et al.* (1977) [7] reported that in ram, high concentrations of caffeine caused adverse effects on the sperm motility. Bird *et al.* (1989) [5] also postulated that caffeine at higher concentration (5.0mM) may cause a reduction of bull sperm motility.

Haesungcharern, Chulavatnatol (1973) [14] reported that cAMP levels increase energy production by accelerating the glycolysis and tricarboxylic acid cycle and their utilization by the motile apparatus of the spermatozoa. However more extensive studies may clarify the mechanism by which caffeine enhances motility of spermatozoa at lower concentrations whereas reduces the same at higher concentrations.

Surti buffalo bull, a significant ($P< 0.05$) improvement in the post thaw motility was observed with caffeine concentration of 0.5 mM (66.37±0.41%) and 1.0 mM (65.62 ±0.52%) in comparison to control (64.43±0.43%) and 1.5 mM (63.93±0.77%). These findings were supported by Shukla, Misra (2014) [29] who showed improvement in post thaw motility at 1.0 mM (70.00±0.99%), 3.0 mM (65.83±0.88%) and 5.0 mM (63.05±0.67%) caffeine concentration in buffalo and Milani, Fontbonne (2010) [22] also reported improvement in post thaw motility at 2.5 mM (26.9±5.00%), 5.0 mM (25.6±5.80%) and 7.5 mM (24.6±5.80%) caffeine concentration in dog. Patel, Siddiquee (2012) [27] also reported higher post thaw motility with 0.5% caffeine (60.17±1.14%) concentration in comparison to control (56.83±0.34%) in Kankrej bulls. Singh, Raina (2000) reported increase in post thaw motility with 4.0 mM (50.50±4.04%) caffeine in comparison to control (44.50±3.70%) in buffalo bulls. Our results were also supported by Kant (2016)[17] who showed improvement in post thaw motility at different concentration of caffeine in comparison to control in Marwari horses and exotic donkeys. Further, Spalekova *et al.* (2011) [32] reported that in ram progressive sperm motility of the sperm was increased after 24 hr of incubation in presence of 4.0 mM (60%) of caffeine in comparison to control (40%). Špaleková *et al.* (2014) [33] studied the effect of caffeine on motility and viability indices of coolied-stored ram spermatozoa and reported that caffeine significantly ($P< 0.05$) increased sperm motility and progressive movement. The improvement in post thaw motility as seen in the present study may also be due to the stimulatory effect of caffeine upon sperm metabolism, fructose utilization, respiration and an increase in cAMP levels (Makler *et al.* 1980) [21] (Yanagimachi 1994) [37] (Milani, Fontbonne 2010) [22].

In Surti buffalo bull, a significant ($P< 0.05$) improvement in the post thaw motility was observed with caffeine concentration of 0.5 mM (67.0±0.54%) and 1.0 mM (66.93±0.60%) in comparison to control (63.063±0.22%) and 1.5 mM (63.81±0.65%). These findings were supported by Shukla, Misra (2014) [29] who showed improvement in post thaw motility at 1.0 mM (70.28±1.05%), 3.0 mM (66.11±0.89%) and 5.0 mM (62.78±1.02) caffeine concentration in buffalo. Spalekova *et al.* (2011) [32] also reported that in ram progressive motility of the sperm was increased after 48 hr of incubation in presence of 4.0 mM (50%) caffeine in comparison to control (31%). Špaleková *et al.* (2014) [33] studied the effect of caffeine on motility and viability indices of coolied-stored ram spermatozoa and reported that caffeine significantly ($P< 0.05$) increased sperm motility and progressive movement and maintained this value for 72 hours. Fattouh, Abdou (1991) [11] reported that addition of caffeine (2.0, 4.0 and 6.0 mM) in buffalo semen diluted (1:2) with lactose diluent before freezing, resulted in a significant increase in the post-thaw motility of spermatozoa over the 3 hours incubation period.

Caffeine at 0.5 mM of concentration showed significant ($P< 0.05$) improvement in pre-freeze and post thaw semen quality in comparison to concentrations of 1 mM or 1.5 mM; and is sufficient to induce a beneficial effect on cryopreservation of Surti buffalo semen.

Caffeine has been used for stimulating the kinetic activity and respiration of bovine spermatozoa. It was later found to stimulate the progressive motility of both the fresh and preserved sperm of various species like bull, ram, stallion, boar, donkey. Caffeine improved the sperm fertilizing ability and it had no apparent teratogenic effects on mammals (Schilon *et al.* 1978) [28].

Caffeine was reported to induce an increase in intracellular calcium and an immediate hyperactivation of sperm (Colas *et al.* 2010) [8]. The essential role of calcium on sperm capacitation has been proved in several mammalian species (Yanagimachi 1994) [37].

References

1. Aitken RJ, Best F, Richardson DW, Schats R, Simm G. Influence of caffeine on movement characteristics, fertilizing capacity and ability to penetrate cervical mucus of human spermatozoa. Journal of Reproduction Fertility. 1983;67:19-27.
2. Bhakat M, Mohanty TK, Raina VS, Gupta AK, Khan

HM. Frozen Semen Production Performance of Murrah Buffalo Bulls Buffalo. Bulletin. 2011;30:2.
3. Bhakat M, Mohanty TK, Singh S, Gupta AK, Chakravarty AK, Singh P. Influence of Semen Collector on Semen Characteristics of Murrah Buffalo and Crossbred Bulls. Advances in Animal and Veterinary Sciences. 2015;3(4):253-258.
4. Bilodeau JF, Blanchette S, Gagnon C, Sirad MA. Levels of antioxidant defences are decreased in bovine spermatozoa after a cycle of freezing and thawing. Molecular Reproduction and Development. 2000;55:282-288.
5. Bird JM, Carey S, Houghton JA. Motility and acrosomal changes in ionophore treated bovine spermatozoa and their relationship with *in vitro* penetration of zona-free hamster oocytes. Theriogenology. 1989;32:227-242.
6. Chaudhary PJ, Dhami AJ, Chaudhari DV, Hadiya KK, Patel JA. comparative Study of Gir Cattle and Surti Buffalo Bulls Semen under Middle Gujarat Climate. Indian Journal of Veterinary Sciences & Biotechnology. 2017;13:56-61.
7. Cohen MS, Colin MJ, Golimbu M, Hotchkiss RS. Effects of Prostaglandins on Sperm Motility. Fertility and sterility. 1977;28:78-85.
8. Colas C, Cebrian-Perez JA, Muinoblanco T. Caffeine induces ram sperm hyperactivation independent of camp-dependent protein kinase. International Journal of Andrology. 2010;33:187-197.
9. Dhami AJ, Chaudhari DV, Varghese O. Quality traits of fresh, refrigerated and cryopreserved buffalo bull semen and their interrelationships. Indian Journal of Veterinary Sciences & Biotechnology. 2016;12:57-62.
10. El-Gaafary MN, Daader AH, Ziedan A. Effect of caffeine on bull semen quality and sperm penetration into cervical mucus. Animal Reproduction Science. 1990;23:13-19.
11. Fattouh El-S M, Abdou MSS. Effect of caffeine on the post-thaw motility of buffalo spermatozoa. Theriogenology. 1991;36:149-154.
12. Fattouh El-SM, Seida AA, Nasr MT, Abou-Ahmed MM. Effect of Caffeine on the motility of ejaculated and epididymal buffalo spermatozoa. Veterinary Medicine Journal Egyptian. 1985;33:261-271.
13. Garbers Dl, First Nl, Sullivan JJ, Lardy HA. Stimulation and maintenance of ejaculated bovine spermatozoa respiration and motility by caffeine. Biology of Reproduction. 1971;5:336-339.
14. Haesungcharern A, Chulavatnatol M. Stimulation of human spermatozoal motility by caffeine. Fertility and Sterility. 1973;24:9.
15. Ho HC, Suarez SS. An inositol 1,4,5-trisphosphate receptor-gated intracellular ca2+ store is involved in regulating sperm hyperactivated motility. Biology of Reproduction. 2001;65:1606-1615.
16. Ibrahim AH, Mohamed AD, Fatma AM, Galewan, Mohamed AD. Effect of various concentrations of caffeine, pentoxifylline, and kallikrein on hyperactivation of frozen bovine semen. Biomed Research International, 2015;7.
17. Kant S. Effect of caffeine as an additive in semen extender to improve frozen thawed semen quality of Marwari horses and exotic donkeys. M.V.SC Thesis, Deemed University, ICAR-Indian Veterinary Research Institute Izatnagar (UP), 2016.
18. Kim SH, Yu DH, Kim YJ. Effects of Cryopreservation on Phosphatidylserine Translocation, Intracellular Hydrogen Peroxide, and DNA Integrity in Canine Sperm. Theriogenology. 2010;73:282-292.
19. Koonjaenak S, Rodriguez-Martinez H. Assessment of semen quality in Swamp Buffalo AI Bulls in Thailand, Italian Journal animal Science. 2007;6:701-704.
20. Lasley JF. Spermatozoan motility as a measure of semen quality. Journal of Animal Science. 1951;10:211.
21. Makler A, Makler E, Itzkovitz J, Brandes JM. Factors affecting sperm motility. Fertility and Sterility. 1980;33:624-630.
22. Milani A, Fontbonne CA. Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology. 2010;74(1):153-164.
23. NDDB. National Dairy Development Board, annual report 2018-19. 2019 www.nddb.coop.
24. Nichi M, Bols PEJ, Zuge RM, Barnabe VH, Goovaerts IGF, Barnabe RC, *et al*. A review on goat sperm cryopreservation. Small Ruminant Research. 2006;63:215-225.
25. Ortega FC, Gonzalez FL, Macias Garcia B, Salazar SC, Morillo RA, Rodriguez MH. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biology of Reproduction. 2009;81:1106-1111.
26. Park CK, Ohgoda, Niwa K. Penetration of bovine follicular oocytes by frozen-thawed spermatozoa in the presence of caffeine and heparin. Journal of Reproduction and Fertility. 1989;86(2):577-582.
27. Patel BR, Siddiquee GM. Effect of semen diluent additives on spermatozoal viability of Kankrej bull semen following cryopreservation. Wayamba Journal of Animal Science. 2012;(3):77-80.
28. Schilon M, Paz G, Homonnai ZT, Schoenbaun M. The effect of Caffeine citrate on guinea pig epididymal spermatozoa: Motility and fertilizing capacity. International Journal of Andrology. 1978;1:416-423.
29. Shukla MK, Misra AK. Caffeine as a semen additive to improve murrah buffalo (Bubalus bubalis) semen cryopreservation. Buffalo Bulletin. 2014;33(1):32-36.
30. Singh P, Raina VS. Effect of caffeine, camp and cattle seminal plasma on freezability of buffalo bull semen. Asian-Australasian Journal of Animal Sciences. 2000;13:901-905.
31. Snedecor GW, WG Cochran. Statistical Methods. The Iowa State Univ Press, USA, 1994.
32. Spalekova E, Makarevich A, Pivko J. Effect of caffeine on parameters of ram sperm motility. Slovak Journal of Animal Science. 2011;44(2):78-83.
33. Špaleková E, Makarevich AV, Kubovičová E, Ostró A, Chrenek, P. Effect of caffeine on functions of cooling-stored ram sperm in vitro. Journal Acta Veterinaria Brno. 2014;83:19-25.
34. Tomar NS. Artificial Insemination and Reproduction of Cattle and Buffaloes, 4th ed. Saroj Prakshan, Allahabad, 1997.
35. Verma MC, Saxena VB, Tripathi SS, Singh R. A note on deep freezing of Murrah and Jersey bull semen. Indian Journal of Animal Science. 1975;45:970-971.
36. Watson PF. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science. 2000;60-1:481-492.
37. Yanagimachi R. Mammalian Fertilization, Raven Press, New York. 1994;189-317.