DOI: 10.54083/ResBio/7.1.2025/11-15 *Res. Bio.*, 2025, 7(1):11-15

Research Biotica

e-ISSN: 2582-6743
January-March, 2025

Article ID: RB210 Research Article

Leakage of Sperm Membrane Protein, Cholesterol, ALT and AST during Cryopreservation of Swamp Buffalo Bull Semen

D.J. Talukdar[1*], K. Ahmed[2], Papori Talukdar[2] and Pankhi Priya Bora[1]

[1]*College of Veterinary Sciences & A.H., Central Agricultural University, Aizawl, Mizoram (796 015), India*
[2]*College of Veterinary Science, Assam Veterinary and Fishery University, Khanapara, Assam (781 022), India*

Corresponding Author
D.J. Talukdar
✉: dibya26@gmail.com

Conflict of interests: The author has declared that no conflict of interest exists.

How to cite this article?

Talukdar, D.J., Ahmed, K., Talukdar, P., *et al*., 2025. Leakage of Sperm Membrane Protein, Cholesterol, ALT and AST during Cryopreservation of Swamp Buffalo Bull Semen. *Research Biotica* 7(1), 11-15. DOI: 10.54083/ResBio/7.1.2025/11-15.

Abstract

A total of 28 ejaculates collected from four Swamp buffalo bulls aged five to eight years maintained at the Network Project on Swamp Buffalo, College of Veterinary Science, Khanapara, Guwahati, Assam were used to study the leakage of sperm membrane protein, cholesterol, ALT and AST during cryopreservation of Swamp buffalo bull semen in Tris-egg yolk-citrate-glycerol extender. The mean levels of sperm membrane protein (SMP) in fresh, after equilibration and after freezing were 5.13±0.12, 3.95±0.10 and 3.83±0.11 mg/ 10^9 spermatozoa, respectively. The mean levels of cholesterol in fresh, after equilibration and after freezing were 21.95±0.44, 18.27±0.40 and 14.74±0.60 µg/ 10^8 spermatozoa, respectively. The mean levels of sperm membrane proteins and cholesterol were significantly (p<0.01) lower after equilibration and freezing. The mean AST activity was 12.63±1.14, 28.60±1.71 and 57.20±4.52 units/ 10^8 spermatozoa while the mean ALT activity was 0.89±0.11, 1.73±0.21 and 4.00±0.20 units/ 10^8 spermatozoa in fresh, after equilibration and freezing, respectively. The mean activity of AST and ALT was significantly (p<0.01) higher after equilibration and freezing. From the present study, it can be concluded that cryopreservation induces leakage of sperm membrane protein, cholesterol, ALT and AST of the swamp buffalo bull semen, which is an indicator of cryodamage.

Keywords: ALT, AST, Bull semen, Cholesterol, Sperm membrane protein, Swamp buffalo

Introduction

Preservation of semen samples under liquid nitrogen leads to considerable changes in the morphological and functional attributes, which ultimately leads to poorer fertility of preserved semen as compared to fresh semen (Ibrahim, 2024). Cryodamage of buffalo spermatozoa is more severe because of its distinctive physiology, especially the presence of more polyunsaturated phospholipids in the sperm membrane, making them more susceptible to freezing stress than bull spermatozoa, leading to significant loss of viability, membrane integrity and DNA integrity (Said *et al*., 2024). Cryopreservation increases ROS production, damaging lipids, proteins and DNA leading to poor fertility of preserved buffalo spermatozoa (Talukdar *et al.,* 2015a; Arunkumar *et al*., 2022). Numerous mechanisms have been ascribed to the reduced fertility of preserved semen; among them are capacitation-like changes in frozen-thawed spermatozoa (Talukdar *et al.*, 2015a). Studying cryocapacitation in swamp buffalo spermatozoa is vital because the freeze-thaw process induces premature capacitation-like changes. These alterations cause membrane destabilization, calcium influx and an untimely acrosome reaction, ultimately shortening sperm lifespan in the female tract and leading to significantly lower post-thaw fertility compared to fresh semen. Moreover, preserved spermatozoa have a transformed membrane state, which is functionally similar to capacitation (Watson, 1995; Talukdar *et al.,* 2017). Cryo-capacitation is one of the major factors associated with reduced and poor survivability of preserved spermatozoa in the female genital tract (Talukdar *et al.*, 2015b). There is leakage of sperm membrane proteins, cholesterol and other biomolecules which reduces the fertility of preserved semen (Chavda *et al*., 2022). Cholesterol efflux leads to changes in

Article History
RECEIVED on 07th August 2024 RECEIVED in revised form 15th February 2025 ACCEPTED in final form 01st March 2025

membrane structure, increased bi-layer penetrability and fluidity that give rise to the capacitated state of the sperm cells (Talukdar, 2014). There are also changes in sperm membrane proteins, which might be due to sub-lethal damage occurring during preservation leading to loss of sperm surface proteins (Kadirvel *et al.*, 2011). Although cryopreservation-induced biochemical alterations have been investigated in cattle and Murrah buffalo, limited information is available regarding leakage of sperm membrane protein, cholesterol and transaminases in swamp buffalo semen during cryopreservation. Moreover, by understanding how cryocapacitation affects the depletion of membrane proteins and cholesterol allows researchers to design improved extenders. Supplementing with protective agents, such as cholesterol-loaded cyclodextrins (CLC), antioxidants, or novel extenders, can stabilize membranes and preserve fertilization capacity (Talukdar *et al.*, 2017). Improving the cryosurvival of buffalo semen is the foundation of successful AI programs. Swamp buffaloes are essential agricultural assets (for draft power and meat/milk production) in Southeast Asia and parts of India, so optimizing their reproductive efficiency directly impacts livestock breeding (Talukdar *et al.*, 2015b). That is why the present study was undertaken to assess the changes in swamp buffalo sperm cells during preservation under liquid nitrogen with respect to transaminases activities, sperm membrane protein and cholesterol content.

Materials and Methods

A total of 28 semen samples were collected using the artificial vagina method from each of the four swamp buffalo bulls aged five to eight years maintained at the Network Project on Swamp Buffalo, College of Veterinary Science, AAU, Khanapara, Guwahati, Assam (Latitude: 26.1240° N, Longitude: 91.8178° E). Immediately after collection, the ejaculates were evaluated for volume, concentration and initial motility. Semen samples having volume 1.0 ml or more, mass activity 3+ or more and initial sperm motility of 70% or more were utilized for the present study. The experimental plan of study was duly approved by the Institution Animal Ethics Committee of College of Veterinary Science, AAU, Khanapara, Assam (Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/12-13/164).The semen sample was evaluated for sperm membrane protein levels (Cheema *et al.*, 2011), cholesterol levels (Srivastava *et al.*, 2013b) and ALT and AST activity was estimated using a commercially available kit method. The ejaculates found suitable were extended (1:10) with Tris extender [Tris (hydroxymethyl) amino methane: 2.422 g; citric acid monohydrate: 1.36 g; fructose: 1.0 g; penicillin G sodium: 1000 IU ml^{-1}; streptomycin sulphate: 100 mg ml^{-1}; double distilled water up to 100 ml] with 20% egg yolk and 6.4% glycerol to yield approximately 60 million motile sperm ml^{-1} (Foote, 1970). French mini straws (0.25 ml) of four different colours were used to fill the extended semen. A particular colour of the straw was used for a particular animal (bull). The straws were filled by suction at room temperature. The straws were cooled gradually to 5 °C @ 1 °C per 3 minutes with the help of crushed ice cubes. One hour before the end of 4 hours equilibration period, the straws were taken out of the water and wiped dry using pre-cooled (5 °C) towels. After drying, the straws were arranged horizontally in a freezing rack and then transferred into an insulated freezing container (thermocol box) so that the straws were 4 cm above the level of liquid nitrogen. Immediately after 10 minutes of freezing in liquid nitrogen vapour, the straws were collected in a goblet full of liquid nitrogen and transferred to a liquid nitrogen container for storage. After 24 hours of storage in liquid nitrogen, the frozen semen was thawed in warm water at 37 °C for 30 seconds and evaluation was done after that.

The data obtained from present study subjected to statistical analysis and significance of difference was calculated by using Duncan's Multiple Range Test (SPSS software version 20.0) and presented as mean ± standard error (SE).

Results and Discussion

The mean levels of sperm membrane protein (SMP) in fresh, after equilibration and after freezing were 5.13±0.12, 3.95±0.10 and 3.83±0.11 mg/ 10^9 spermatozoa, respectively (Table 1; Figure 1). As per report, in the course of capacitation, sperm surface proteins are modified, added or removed and an array of proteins have been shown to undergo tyrosine phosphorylation in different species (Cheema *et al.*, 2011; Srivastava *et al.*, 2013a) and in fertilization, these mammalian sperm membrane proteins are also involved in the penetration of cumulus matrix, recognition of zona pellucida and fusion with the oocyte plasma membrane (Bansal, 2010; Talukdar *et al.*, 2016). In present study, the mean level of sperm membrane protein differed significantly (p<0.01) between the stages of evaluation, *i.e.*, at fresh, after equilibration and after freezing. The mean level of sperm membrane protein was significantly (p<0.01) decreased after equilibration and freezing compared with fresh. The mean level of sperm membrane protein recorded after equilibration and freezing in the present study was higher than that reported by Dhanju *et al.* (2001). The alteration in the sperm membrane proteins might be due to sub-lethal damage that occurred during cryopreservation, leading to loss of sperm surface proteins (Lessard *et al.*, 2000; Abdelnour *et al.*, 2024), segregation of membrane proteins (De Leeuw *et al.*, 1990), inactivation of membrane-bound enzymes and decreased lateral protein diffusion within the membrane (Arunkumar *et al*., 2022).

The mean levels of cholesterol in fresh, after equilibration and after freezing were 21.95±0.44, 18.27±0.40 and 14.74±0.60 µg/ 10^8 spermatozoa, respectively (Table 1; Figure 1). During capacitation, the sperm enzymes get inactivated which ultimately cause efflux of the cholesterol and influx of Ca^{2+} through the plasma membrane and outer acrosomal membrane and thus, resulting into acrosomal reaction (Srivastava *et al.*, 2013b). Various reports suggested an active participation of the sperm plasma membrane in the process of capacitation, mainly through the loss of cholesterol (Talukdar *et al.*, 2015). The cholesterol efflux during *in vitro* capacitation increases the disorder of phospholipid packing and results in increased bilayer permeability (Kadirvel *et al.*, 2009; Talukdar *et al.*, 2016). Similar to physiological and *in vitro* capacitation, a significant reduction of cholesterol content after cryopreservation was observed in the present study. The mean level of cholesterol differed significantly (p<0.01) between the stages of evaluation, *i.e.*, at fresh, after

equilibration and freezing. The mean level of cholesterol was significantly (p<0.01) decreased after equilibration and freezing than fresh. The mean level of cholesterol recorded after equilibration and freezing in the present study was higher than the values reported by Kadirvel *et al.* (2009) and Srivastava *et al.* (2013b). Membrane cholesterol has stabilizing effect on spermatozoa membrane; hence, any change in its content is expected to induce reorganization or destabilization of the membrane as reported by Srivastava *et al*. (2013a). The alteration in the cholesterol level might be due to damage to their membrane during freezing (Chavda *et al*., 2022). Cholesterol efflux leads to changes in membrane design, increased bilayer permeability and fluidity that give rise to the capacitated state of the sperm cells (Talukdar *et al.*, 2015b).

Table 1: Changes in sperm membrane protein, cholesterol, AST and ALT levels during cryopreservation of swamp buffalo semen (n = 28)

Parameters ↓ \ Stage →	Fresh	Equilibrated	Frozen-thawed	F-value
SMP (mg/ 10^9 sperm)	5.13^{a}±0.12	3.95^{b}±0.10	3.83^{c}±0.11	37.88^{**}
Cholesterol (µg/ 10^8 sperm)	21.95^{a}±0.44	18.27^{b}±0.40	14.74^{c}±0.60	53.57^{**}
AST (unit/ 10^8 spermatozoa)	12.63^{a}±1.14	28.60^{b}±1.71	57.20^{c}±4.52	61.95^{**}
ALT (unit/ 10^8 spermatozoa)	0.89^{a}±0.11	1.73^{b}±0.21	4.00^{c}±0.20	75.42^{**}

**Means with different superscripts within a row differ significantly (p<0.01)

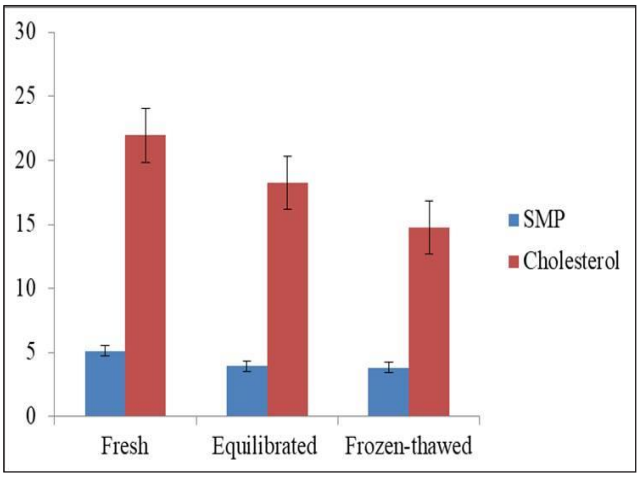

Figure 1: Sperm membrane protein (SMP) (mg/ 10^9 sperm), cholesterol (µg/ 10^8 sperm) level of fresh, equilibrated and frozen-thawed swamp buffalo spermatozoa

The mean AST activity was 12.63±1.14, 28.60±1.71 and 57.20±4.52 units/ 10^8 spermatozoa in fresh, after equilibration and after freezing, respectively; while the mean ALT activity was 0.89±0.11, 1.73±0.21 and 4.00±0.20 units/ 10^8 spermatozoa in fresh, after equilibration and after freezing, respectively (Table 1; Figure 2). The mean activity of AST and ALT differed significantly (p<0.01) between the stages of evaluation, *i.e.*, at fresh, after equilibration and after freezing. The mean activity of AST and ALT was significantly (p<0.01) higher after equilibration and freezing than in fresh samples. The mean activity of AST and ALT in the present study after equilibration was lower than the values reported by Nath *et al.* (1996) and higher than the values reported by Nagendrakumar and Kathiresan (2009). The present values of AST and ALT after freezing were lower than the values reported by Nath *et al.* (1996) and higher than the values reported by Pratap *et al.* (1999) and El-Harairy *et al.* (2011). The activity of transaminases (AST and ALT) is a good indicator of semen quality. Good-quality semen was characterized by lower AST and ALT activities, as reported by Taha *et al.* (2000). This may be due to temperature shock and sperm cell injury associated with freezing (Singh *et al.*, 1991), changes in mitochondrial sheath with loss of protein from the mid-piece (Graham *et al.*, 1974) and an increase in cell membrane permeability with or without rupture (Talukdar *et al.*, 2015b).

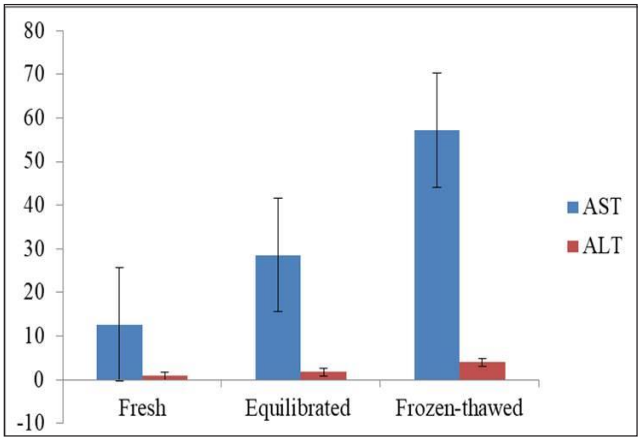

Figure 2: Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) level of fresh, equilibrated and frozen-thawed swamp buffalo spermatozoa (unit/ 10^8 spermatozoa)

Conclusion

In the present study, it was observed that the mean levels of sperm membrane protein (SMP), cholesterol were significantly reduced (p<0.01) while preserving the Swamp buffalo bull semen in Tris-egg yolk-citrate-glycerol extender. The mean activity of AST and ALT were significantly (p<0.01) increased after equilibration and freezing of Swamp buffalo spermatozoa. It can be concluded that there was a breakage of sperm plasma membrane, loss of membrane selective permeability, change in membrane fluidity, leakage and aggregation of phospholipids, proteins and enzymes.

Future research on swamp buffalo semen aims to mitigate cryocapacitation, premature capacitation-like changes that reduce post-thaw viability. Key directions include: (i) evaluating antioxidants (*e.g.*, melatonin) to neutralize oxidative stress; (ii) developing novel extenders and cryoprotectants; (iii) assessing biomarkers of cryodamage (*e.g.*, specific proteins or enzyme leakage); and (iv) correlating biochemical changes with actual conception rates. Exploring these mechanisms will help preserve membrane stability and boost artificial insemination success rates in swamp buffalo breeding programs.

Ethical Statement

The authors declare that no generative artificial intelligence tools were used in drafting or preparing this manuscript. The manuscript was written, interpreted and revised solely by the authors, who take full responsibility for its content.

References

Abdelnour, S.A., Khalil, W.A.,. Khalifa, N.E, Khalil, F.M.A., Hassan, M.A.E., 2024. L-Proline: A promising tool for boosting cryotolerance and fertilizing ability of cryopreserved sperm in animals. *Animal Reproduction Science* 263, 107429. DOI: https://doi.org/10.1016/j.anireprosci.2024.107429.

Arunkumar, R., Kumaresan, A., Sinha, M.K., Elango, K., King, J.P.E.S., Nag, P., Karuthadurai, T., Baithalu, R.K., Mohanty, T.K., Kumar, R., Datta, T.K., 2022. The cryopreservation process induces alterations in proteins associated with bull sperm quality: The equilibration process could be a probable critical control point. *Frontiers in Endocrinology* 13, 1064956. DOI: https://doi.org/10.3389/fendo.2022.1064956.

Chavda, B.P., Vala, K.B., Solanki, G.B., Prajapati, S.G., Singh, V.K., 2022. Caffeine supplementation in semen extender protects buffalo spermatozoa from cryodamage. *Indian Journal of Veterinary Sciences and Biotechnology* 18(4), 1-5. DOI: https://doi.org/10.48165/ijvsbt.18.4.01.

Cheema, R.S., Bansal, A.K., Bilaspuri, G.S., Gandotra, V.K., 2011.Correlation between the proteins and protein profile(s) of different regions of epididymis and their contents in goat buck. *Animal Science Papers and Reports* 29(1), 75-84.

De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology* 27(2), 171-183. DOI: https://doi.org/10.1016/0011-2240(90)90009-S.

Dhanju, C.K., Cheema, R.S., Kaur, S.P., 2001. Effect of freezing on proteins and protein profiles of sperm membrane extracts and seminal plasma of buffalo bulls. *Asian-Australasian Journal of Animal Sciences* 14(12), 1678-1682. DOI: https://doi.org/10.5713/ajas.2001.1678.

El-Harairy, M.A., Eid, L.N., Zeidan, A.E.B., El-Salaam, A.M.A., El-Kishk, M.A.M., 2011. Quality and fertility of the frozen-thawed bull semen as affected by the different cryoprotectants and glutathione levels. *Journal of American Science* 7(5), 791-801.

Foote, R.H., 1970. Fertility of bull semen at high extension rates in tris-buffered extenders. *Journal Dairy Science* 53(10), 1475-1477. DOI: https://doi.org/10.3168/jds.S0022-0302(70)86417-7.

Graham, E.F., Crabo, B.G., Schmel, M.K.L., 1974. Utilization of enzyme assay in developing techniques for freezing semen. In: *Proceedings of VIIIth International Zootechnical Symposium*, Milan, Italy. pp. 95-111. [c.f. Animal Breeding Abstract 42: 411].

Ibrahim, M.A., 2024. Bull sperm cryopreservation: An overview on the current status and future perspectives. *The German Journal of Veterinary Research* 4(1), 9-22. DOI: https://doi.org/10.51585/gjvr.2024.1.0071.

Kadirvel, G., Kumar, S., Kumaresan, A., Kathiravan, P., 2009. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Animal Reproduction Science* 116(3-4), 244-253. DOI: https://doi.org/10.1016/j.anireprosci.2009.02.003.

Kadirvel, G., Kathiravan, P., Kumar, S., 2011. Protein tyrosine phosphorylation and zona binding ability of *in vitro* capacitated and cryopreserved buffalo spermatozoa. *Theriogenology* 75(9), 1630-1639. DOI: https://doi.org/10.1016/j.theriogenology.2011.01.003.

Nagendrakumar, S.B., Kathiresan, D., 2009. Effect of type of freezing on the functional characteristics of frozen thawed bull spermatozoa. *Indian Journal of Animal Reproduction* 30(2), 13-18.

Lessard, C., Parent, S., Leclerc, P., Bailey, J.L., Sullivan, R., 2000. Cryopreservation alters the level of the bull sperm surface protein P25b. *Journal of Andrology* 21(5), 700-707. DOI: https://doi.org/10.1002/j.1939-4640.2000.tb02138.x.

Nath, K.C., Ahmed, K., Dutta, G., Barthakur, T., Borgohain, B.N., 1996. Semen quality and release of certain enzymes during the course of freezing bull spermatozoa. *Indian Journal of Animal Reproduction* 17(2), 130-131.

Pratap, N., Reddy, V.N.V., Sharma, P.A., Honnappa, T.G., Devraj, M., Krishnaswamy, A., Arrora, V.K., 1999. Estimation of transaminases (AST and ALT) in cryopreserved Murrah buffalo semen. *Indian Journal of Animal Reproduction* 20(2), 159-160.

Said, S., Maulana, T., Iskandar, H., Kaiin, E.M., Khaerunnisa, I., Putra, W.P.B., Hasan, F., Arifiantini, R.I., 2024. Sperm protein profile and their correlation with frozen semen quality of indigenous Indonesian buffalo bulls. *Journal of Advanced Veterinary and Animal Research* 11(4), 846-855. DOI: http://doi.org/10.5455/javar.2024.k836.

Singh, J., Pangaonkar, G.R., Biswas, R.K., Shrivastava, A.K., 1991. Studies on leakage of transaminases from buffalo spermatozoa in relation to deep freezing in certain extenders. *Indian Journal of Animal Reproduction* 12(2), 135-140.

Srivastava, N., Jerome, A., Srivastava, S.K., Ghosh, S.K., Amit, K., 2013a. Bovine seminal PDC-109 protein: An overview of biochemical and functional properties. *Animal Reproduction Science* 138(1-2), 1-13. DOI: https://doi.org/10.1016/j.anireprosci.2013.02.008.

Srivastava, N., Srivastava, S.K., Ghosh, S.K., Amit, K., Perumal,

P., Jerome, A., 2013b. Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa. *Asian Pacific Journal of Reproduction* 2(2), 126-131. DOI: https://doi.org/10.1016/S2305-0500(13)60132-3.

Taha, T.A., Abdel-Gawad, E.I., Ayoub, M.A., 2000. Monthly variations in some reproductive parameters of Barki and Awassi rams throughout 1 year under subtropical conditions 2. Biochemical and enzymatic properties of seminal plasma. *Animal Science* 71(2), 325-332. DOI: https://doi.org/10.1017/S135772980005517X.

Talukdar, D.J., 2014. Morphological and functional characterization of *in vitro* capacitated and frozen thawed swamp buffalo spermatozoa. *Ph.D. Thesis*, Assam Agricultural University, Guwahati, India.

Talukdar, D.J., Ahmed, K., Talukdar, P., 2015a. Cryocapacitation and fertility of cryopreserved semen. *International Journal of Livestock Research* 5(6), 11-18.

Talukdar, D.J., Ahmed, K., Deori, S., Das, G.C., 2015b. Heparin-induced *in vitro* capacitation changes of swamp buffalo spermatozoa. *Turkish Journal of Veterinary & Animal Sciences* 39(5), 629-633. DOI: https://doi.org/10.3906/vet-1501-16.

Talukdar, D.J., Ahmed, K., Deka, B.C., Sinha, S., Deori, S., Das G.C., 2016. Cryo-capacitation changes during cryopreservation of swamp buffalo spermatozoa. *The Indian Journal of Animal Sciences* 86(4), 397-400. DOI: https://doi.org/10.56093/ijans.v86i4.57723.

Talukdar, D.J., Ahmed, K., Sinha, S., Deori, S., Das, G.C., Talukdar, P., 2017. Cryopreservation induces capacitation-like changes of the swamp buffalo spermatozoa. *Buffalo Bulletin* 36(1), 221-230.

Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development* 7(4), 871-891. DOI: https://doi.org/10.1071/RD9950871.