RESEARCH ARTICLE

Effect of Supplementation of Antioxidant Sericin in Semen Extender on Post-Thaw Sperm Quality, Oxidative Status and Fertility of Jaffarabadi Buffalo Bull Semen

Hardipsinh P. Vijyeta[1]*, Fulabhai S. Kavani[2], Arshi A. Vagh[3], Gajendrasingh B. Solanki[1], Karsan B. Vala[2], Vivek K. Singh[4], Arjan J. Dham[5]

Abstract

The present investigation was undertaken on semen of six Jaffarabadi buffalo bulls, aged 6-8 years (n=6x6=36 ejaculates) with an objective of determining the influence of different concentrations of Sericin, *viz.,* 0.0% control (T0), 0.25 % (T1), 0.50 % (T2), 0.75 % (T3), 1.0 % (T4) in AndroMed® extender on the quality of sperm parameters, oxidative status and fertility of cryopreserved semen. The semen samples were evaluated at pre-freeze (on dilution) and post-thaw stages. The pre-freeze sperm motility, viability, plasma membrane integrity, acrosome integrity and total antioxidant capacity of semen were significantly ($p<0.05$) higher with lower sperm abnormalities in control and 0.25% or 0.50% sericin supplemented extender over others, while in post-thawed semen the values of all these parameters were significantly ($p<0.01$) higher with lower sperm abnormalities in 0.25% followed by 0.50% Sericin groups over control and 0.75% Sericin, while 1.0% was found to be detrimental by suppressing all sperm parameters even below the control values. Lipid peroxidation (MDA production) status however did not vary much between treatment, although it was lower ($p<0.05$) with 0.25% and 0.50% Sericin at post-thaw stage. The first service conception rates of frozen-thawed semen with inclusion of 0.25 % and 0.50 % Sericin in extender were significantly higher (44.66±1.43 % and 42.66±1.76 %) as compared to those of 0 %, 0.75%, and 1% concentrations (38.66±1.33, 29.66±1.89 and 28.66±2.13 %, respectively). Thus the study concluded that since supplementation of Sericin @ 0.25 or 0.50% (w/v) concentration in AndroMed® extender significantly improved post-thawed sperm quality parameters, oxidative status and fertility of cryoprserved Jaffarabadi buffalo semen over control and higher levels, it may be added routinely in the extender during cryopreservation of buffalo semen for better outcome.

Key words: Cryopreservation, Fertility, Jaffarabadi buffalo semen, Oxidative markers, Sericin, Sperm quality.

Ind J Vet Sci and Biotech (2024): 10.48165/ijvsbt.20.2.16

Introduction

The Jaffarabadi buffalo, a cornerstone of the rural economy in the Saurashtra region of India, plays a crucial role in agricultural activities and resource provision for local communities. However, its reproductive efficiency and conception rate, especially with artificial insemination, face challenges due to limitations in bull semen cryopreservation techniques (Gordon, 1996). Artificial insemination (AI) is instrumental in improving buffalo genetics but requires long-term sperm storage. Unfortunately, lower deep-freeze-resistance of buffalo sperm compared to cattle sperm restricts its practicality (Andrabi *et al.,* 2008; Andrabi, 2009). Buffalo AI employing frozen-thawed sperm often results in disappointingly low conception rates (30%) compared to cattle (Anzar *et al.,* 2003). Cryopreservation processes harm buffalo sperm, affecting motility, viability, plasma membrane, and acrosome integrity (Kadirvel *et al.,* 2009).

Sericin, derived from silkworms, is a hydrophilic silk protein rich in amino acids, particularly serine and glycine, with a molecular weight of 10-400 kDa. It boasts antioxidant properties that inhibit tyrosinase and lipid peroxidation. Sericin finds applications in cell culture, cryopreservation,

[1]Cattle Breeding Farm, Kamdhenu University, Junagadh-362001, Gujarat, India

[2]Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Science & AH, Kamdhenu University, Junagadh-362001, Gujarat, India

[3]Department of Veterinary Medicine, College of Veterinary Science & AH, Kamdhenu University, Junagadh-362001, Gujarat, India

[4]Department of Veterinary Physiology & Biochemistry, College of Veterinary Science & AH, Kamdhenu University, Junagadh-362001, Gujarat, India

[5]Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Science & AH, Kamdhenu University, Anand-388001, Gujarat, India

Corresponding Author: Dr. Hardipsinh P. Vijyeta, Assistant Research Scientist, Cattle Breeding Farm, Kamdhenu University, Junagadh-362001, Gujarat, India. e-mail: hpvijyeta@gmail.com

How to cite this article: Vijyeta, H. P., Kavani, F. S., Vagh, A. A., Solanki, G. B., Vala, K. B., Singh, V. K., & Dham, A. J. (2024). Effect of Supplementation of Antioxidant Sericin in Semen Extender on Post-Thaw Sperm Quality, Oxidative Status and Fertility of Jaffarabadi Buffalo Bull Semen. Ind J Vet Sci and Biotech. 20(2), 79-84.

Source of support: Nil

Conflict of interest: None

Submitted 18/11/2023 **Accepted** 20/12/2023 **Published** 10/03/2024

and embryo quality enhancement. In the context of buffalo semen cryopreservation, Sericin has demonstrated its ability to enhance motility, viability, membrane integrity with reduced lipid peroxidation and cryocapacitation in bovine semen (Chaturvedi *et al.,* 2021, 2023). Its antioxidant property contributes to improved sperm quality and fertility (Kato *et al.,* 1998; Cao and Zhang, 2017; Patel *et al.,* 2019; Dhami *et al.,* 2020; Chaturvedi *et al.,* 2023). The primary objective of the current study was to investigate the impact of varying concentrations of Sericin supplementation to the Andromed extender during cryopreservation on the functional capacity and fertility of Jaffarabadi buffalo bull semen.

Materials and Methods

For this study, six sexually mature Jaffarabadi buffalo bulls, 6 to 8 years old, were selected from the semen station of Cattle Breeding Farm, Kamdhenu University, Junagadh, India. These bulls were chosen for their prime health, freedom from ailments, robust libido, and excellent clinical condition. They were subjected to uniform management practices and received a carefully tailored diet according to Government of India's MSP guidelines, including 0.8 kg of concentrate, 1.6 kg of dry fodder, and 4.16 kg of green fodder per 100 kg of body weight, supplemented with a daily mineral mixture. The bulls had unrestricted access to clean water and a daily routine that included exercise, bathing, and grooming. Semen collection from these bulls was carried out twice weekly with meticulous attention to temperature control and hygiene of artificial vaginas. The ejaculates were assessed for routine semen quality and those (6x6=36) having initial motility >75% were selected for the current study.

The ejaculates were divided into five equal fractions, each subsequently diluted to a final concentration of 80 million sperm/mL using the AndroMed® extender supplemented with varying concentrations of Sericin, *i.e.,* 0.0% control (T0), 0.25% (T1), 0.50% (T2), 0.75% (T3), and 1.0% (T4). The extended semen was carefully sealed in 0.50 mL French straws by an automatic machine, and evaluated for percent individual sperm motility, viability, abnormality, plasma membrane integrity (HOST test, Jeyendran *et al.,* 1984), and acrosome integrity (Kutty *et al.,* 1996) using standard procedures. The straws of extended semen were gradually cooled down to 4°C and equilibrated for a period of 4 h. The straws were then subjected to freezing in LN2 vapour at a temperature of -140°C for 7-8 min, after which they were promptly transferred to the goblets filled with liquid nitrogen at -196°C. The post-thaw semen analysis was conducted the next day, after thawing the straws in a water bath at 37°C for 30 seconds, and assessing again all above five sperm quality parameters in all five aliquots.

Moreover, the extent of peroxidative membrane damage was quantitatively determined through the measurement of lipid peroxidation (LPO) and total antioxidant capacity (TAC) in seminal plasma at both pre-freeze (on dilution) and post-thaw stages, using standard procedures and kits procured from HiMedia Lab Pvt. Ltd., Mumbai. The semen ejaculates cryopreserved using Sericin having post-thaw motility of > 50 % were used to inseminate the Jaffarabadi buffaloes at the University Farm, Junagadh, as well as in the field by AI workers. In all 1500 Jaffarabadi buffaloes (50/bull/treatment, *i.e.,* 300/treatment) were inseminated with frozen semen containing different concentration of Sericin. Pregnancy was confirmed by per-rectal palpation after 60 days of AI in non-returned buffaloes and the first service conception rates were calculated. This extensive experiment was aimed to determine the influence of different Sericin concentrations on the quality and fertility of cryopreserved semen, and judge the best one.

The data obtained for various sperm parameters and oxidative markers were analyzed by using one-way ANOVA. Duncan's post-hoc test was used to determine significant differences between the treatment means at $p<0.05$. Conception rates were compared by Chi-square test (Snedecor and Cochran, 1994).

Results And Discussion

The results of pre-freeze (on dilution) and post-thaw semen quality parameters, *viz.,* individual sperm motility, live sperm, abnormal sperm, plasma membrane integrity (HOST), acrosome integrity, lipid peroxidation (MDA production) and total antioxidant capacity observed in semen cryopreserved with different concentrations of sericin in Andromed extender are furnished in Table 1, and Figures 1 to 6.

Sperm Quality Parameters

From the results, it was evident that the pre-freeze sperm motility was highest in Control group (T0) followed by T1 and T2, all were at par and significantly higher than T3. Post-thaw motility in T1 and T2 however displayed significantly higher ($p<0.05$) values in comparison to the T0, T3, and T4 groups (Table 1, Fig. 1). Sperm viability at the pre-freeze stage was significantly ($p<0.05$) higher in the control group (T0) compared to T1, T2, T3, and T4 treatments, and all treatments differed significantly ($p<0.05$) from one another with reduced values at higher concentration of Sericin. However, at the post-thaw stage, both T1 and T2 groups (Fig. 2) displayed notably higher viability percentages followed by T3 in comparison to the T0 and T4 groups ($p<0.05$). The sperm abnormalities depicted inverse trend, and was lowest in T1 and T2 groups at both pre-freeze and post-thaw stage. With respect to plasma membrane and acrosome integrity, both T0 and T1 groups demonstrated significantly higher HOST reactive sperm and acrosome integrity percentages followed by T2 group at the pre-freeze stage compared to T3 and T4 groups ($p<0.05$). Conversely, after thawing, T1 and T2 groups displayed markedly higher HOST reactive sperm percentages followed by T0 as compared to T3 and T4 groups ($p<0.05$, (Fig. 3), while the acrosome integrity percentage was significantly

Table 1: Effect of Sericin on physico-morphological sperm parameters and antioxidant markers (MDA, µM/mL and TAC, µM/mL) in seminal plasma of Jaffarabadi bull (n=6) semen before and after cryopreservation

Parameters	Pre-freeze stage					Post-thaw stage				
	Control(T0)	T1	T2	T3	T4	Control(T0)	T1	T2	T3	T4
Individual motility (%)	85.55 ±0.56^{c}	84.72 ±0.60^{bc}	84.77 ±0.51^{bc}	82.27 ±0.41^{a}	83.88 ±0.49^{b}	54.44 ±0.56^{b}	56.94 ±0.54^{c}	56.52 ±0.56^{c}	50.00 ±0.20^{a}	49.16 ±0.37^{a}
Live sperm (%)	86.02 ±0.26^{e}	84.83 ±0.27^{d}	83.52 ±0.34^{c}	79.94 ±0.24^{b}	77.83 ±0.32^{a}	52.11 ±0.31^{a}	64.66 ±0.41^{c}	63.94 ±0.28^{c}	54.75 ±0.82^{b}	51.44 ±0.67^{a}
Abnormal sperm (%)	5.55 ±0.26^{a}	5.41 ±0.29^{a}	5.41 ±0.23^{a}	6.80 ±0.22^{b}	7.52 ±0.20^{c}	14.44 ±0.30^{c}	10.22 ±0.25^{a}	11.16 ±0.25^{b}	15.55 ±0.18^{d}	16.38 ±0.32^{e}
Plasma membrane integrity (HOST) (%)	76.86 ±0.25^{d}	75.86 ±0.28^{d}	73.38 ±0.25^{c}	69.88 ±0.48^{b}	68.22 ±0.70^{a}	43.44 ±0.32^{c}	53.05 ±0.50^{d}	51.88 ±0.48^{d}	42.13 ±0.40^{b}	37.72 ±0.46^{a}
Acrosome integrity (%)	85.30 ±0.35^{d}	84.63 ±0.37^{d}	82.83 ±0.31^{c}	78.55 ±0.82^{b}	76.47 ±0.62^{a}	51.25 ±0.26^{c}	57.91 ±0.29^{e}	55.36 ±0.39^{d}	43.27 ±0.40^{b}	40.86 ±0.32^{a}
MDA (µM/mL)	7.71 ±0.05	7.58 ±0.07	7.65 ±0.07	7.78 ±0.10	7.82 ±0.12	8.41 ±0.04^{b}	8.01 ±0.06^{a}	8.07 ±0.07^{a}	8.42 ±0.12^{b}	8.81 ±0.12^{c}
TAC (µM/mL)	317.39 ±15.73^{b}	373.36 ±7.97^{c}	348.09 ±9.54^{bc}	280.32 ±13.15^{a}	259.80 ±9.86^{a}	302.25 ±7.27^{c}	350.30 ±22.26^{d}	333.33 ±18.00^{cd}	260.36 ±7.85^{b}	218.18 ±11.95^{a}

Means with different superscripts within the raw differ significantly between treatment at p<0.05 level.

higher in T1 group followed by T2 and T0 in comparison to T3 and T4 groups (p<0.05, Fig. 4). Specifically, treatment groups T1 and T2 consistently exhibited superior sperm quality parameters both before freezing and after thawing compared to the control group (T0) and other treatments (T3 and T4) (Table 1). In a recent study, sericin (5 mg/mL) in TFYG extender was reported to be effective in reducing cryodamage, cryo-capacitation with improved post-thaw sperm quality and conception rates by ameliorating ROS mediated oxidative damage in Gir cattle and Murrah buffalo semen (Chaturvedi *et al.,* 2021, 2023), and similar were the observations of Kumar *et al.* (2015), Demra *et al.* (2017) and Patel *et al.* (2019) on sperm quality parameters and oxidative stress.

Oxidative Markers

The study examined malondialdehyde (MDA) and TAC levels in Jaffarabadi bull semen before and after cryopreservation in various treatment groups. During the pre-freeze stage, all groups had statistically similar seminal plasma MDA levels. However, after thawing, MDA levels in T1 and T2 groups were found to be significantly lower (p<0.05) compared to T0, T3, and T4 groups, suggesting reduced oxidative damage with 0.25% and 0.5% sericin (Fig. 5). However, the mean total antioxidant activity in Jaffarabadi bull semen before and after cryopreservation varied across treatment groups. During both pre-freeze and post-thaw stage, T1 and T2 group exhibited significantly higher total antioxidant activity compared to T0, T3, and T4 groups (p<0.05). The levels of TAC with T3 and T4 treatments were significantly (p<0.05) lower than even control group at both pre-freeze and post-thaw stage, with inverse trend of MDA (Fig. 5, 6). These observations indicated improved antioxidant defense mechanism with T1 and T2 treatments. These findings underscore the potential benefits of specific sericin concentrations (0.25% and 0.5%) in enhancing sperm quality and protecting against oxidative stress during cryopreservation, while higher sericin concentrations (0.75% and 1%) were less effective or rather detrimental or toxic to sperm (Table 1) as has been reported by Demra *et al.* (2017) and Patel *et al.* (2019) with Murrah bull semen.

Buffalo bull sperms are known to be rich in polyunsaturated fatty acids in its plasma membrane (Kadirvel *et al.*, 2009) making sperms highly susceptible to lipid peroxidation (LPO). Cryopreservation process induces apoptosis like changes in cryopreserved buffalo semen along with reduction in basic semen quality parameters like motility and plasmalemma integrity (Khan *et al.,* 2009). The damage to the sperms seems to be caused by the liberation of free radicals during cryopreservation which subsequently effects phospholipid composition of sperm membrane (Demra *et al.,* 2017).

First Service Conception Rate

The overall mean first service conception rates obtained in Jaffarabadi buffaloes inseminated using cryopreserved semen with different concentrations of Sericin (0, 0.25, 0.50,

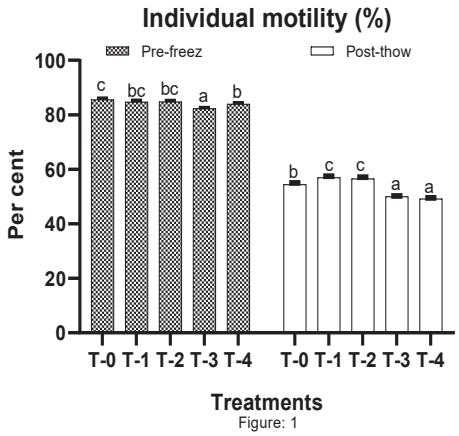

Figure: 1

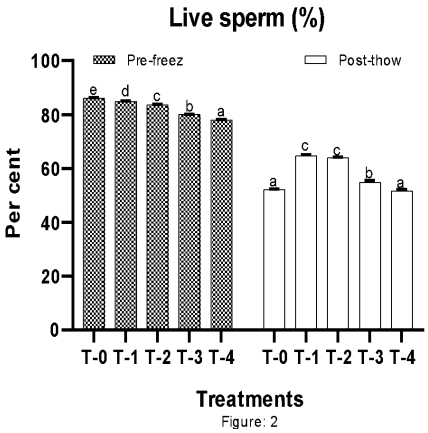

Figure: 2

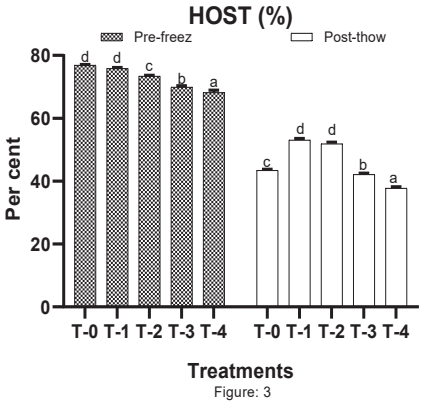

Figure: 3

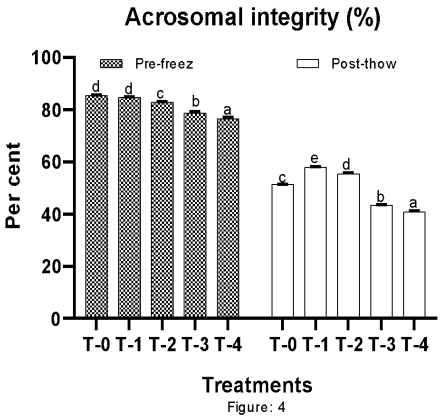

Figure: 4

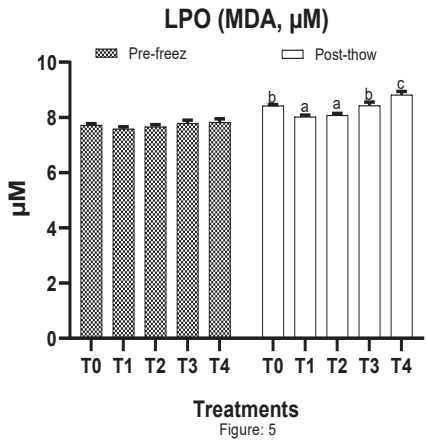

Figure: 5

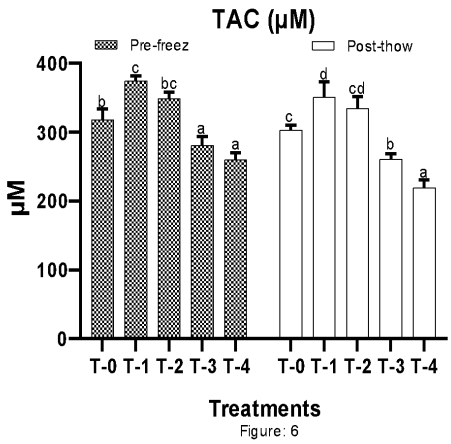

Figure: 6

Fig. 1 to 6: Sperm quality and oxidative parameters of fresh and frozen-thawed semen of Jaffarabadi buffalo bull semen with different levels of antioxidant Sericin in Andromed extender.

0.75, 1.00% w/v) in Andromed extender were 38.66±1.33 % in T0; 44.66±1.43 % in T1; 42.66±1.76 % in T2; 29.67±1.89 % in T3 and 28.00±2.13 % in T4 group. It was significantly ($p<0.05$) higher in T1 and T2 groups as compared to that of the T0, and treatments T3 and T4 in fact suppressed the first service conception rates (Table 2), and supported the observations on sperm quality and oxidative status recorded with different levels of Sericin. The effect of sericin supplementation on fertility outcomes has been studied by a few workers over control with beneficial results (Dhami *et al.,* 2020; Chasturvedi *et al.,* 2023), but the results with varying levels of sericin could not be found in the literature. Akhter *et al.* (2010) found similar fertility rates between Bioxcell and tris-citric egg yolk extender in Nili-Ravi buffalo bulls, while Crespilho *et al.* (2012) recorded significantly higher fertility rate with lecithin based than the egg yolk based extender.

Table 2: First service conception rate of Jaffarabadi buffalo bull semen cryopreseved in Andromed extender with different concentration of sericin (Mean ± SE)

Sericin Treatment	No. AI	No. of buffaloes pregnant	First service conception rate (%)
T0 (Control)	300	116	38.66 ± 1.33^{b}
T1 (0.25%)	300	134	44.66 ± 1.43^{c}
T2 (0.50%)	300	128	42.66 ± 1.76^{bc}
T3 (0.75%)	300	89	29.67 ± 1.89^{a}
T4 (1.00%)	300	84	28.00 ± 2.13^{a}

Means with different superscripts within column differ significantly at P<0.05 level.

Dash *et al.* (2008) reported that sericin decreased lactate dehydrogenase, catalase, and thiobarbituric acid reactive substance in H_2O_2-treated cells. Sericin has been reported as a novel cryopreservation agent of mammalian and insect cell lines (Sasaki *et al.,* 2005). Sericin also possesses the biological activity of preventing cell death and promoting cellular growth (Masakazu *et al.,* 2003). It also serves as a protectant against various stresses such as cryoprotectants, ethanol, surfactants, heating and cooling stresses (Sasaki *et al.,* 2005). Our findings were consistent with the observations of Terada *et al.* (2002), who reported that 1% sericin had harmful effects on the various mammalian cell lines.

The extender supplemented with 0.25 and 0.5% sericin, resulted in the higher sperm motility, plasma membrane integrity, with lower lipid peroxidation and higher antioxidant enzyme activity including fertility. These findings strongly concurred with the observations of Kumar *et al.,* 2015) and Chaturvedi *et al.* (2023) in Murrah buffalo and Gir bull semen. Likewise, Patel *et al.* (2019) reported sericin @ 0.50 and/or 0.25% as an optimal effective dose to reduce cryodamage and improve post-thaw sperm motility in both cattle and buffalo semen. Sericin supplementation to the extender reduced the oxidative stress and enhanced inherent antioxidant enzyme activity protecting lipid peroxidation and sperm membrane integrity. On the basis of above findings, it can be hypothesized that sericin protects the sperm cells during dilution, cooling and freezing-thawing against cryoprotectant toxicity through unknown mechanism. This study also revealed that sericin acts as potent antioxidant and free radical scavenger that reduces lipid peroxidation and protects sperm from free radicals and oxidative damage.

Conclusion

The findings of the present study revealed that the sericin supplementation in semen extender improves post-thaw motility, viability, plasma membrane integrity, acrosome integrity and antioxidant status of buffalo cryopreserved semen. The post-thawed sperm quality results were the best with supplementation of 0.25-0.5% sericin in semen extender by reducing the oxidative stress damage.

Acknowledgement

Authors are grateful to the Research Scientist and Head of CBF, Junagadh and the Kamdhenu University authorities for their cooperation and providing all infrastructural facilities including buffalo bull semen and funds needed for this work.

References

Akhter, S., Ansari, M.S., Rakha, B.A., Andrabi, S.M.H., Anwar, M., & Ullah, N. (2010). Effect of fructose addition in skim milk extender on the quality of liquid Nili-Ravi buffalo (*Bubalus bubalis*) semen. *Pakistan Journal of Zoology, 42*(3), 227-231.

Andrabi, S.M.H. (2009). Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reproduction in Domestic Animals, 44*(3), 552-569.

Andrabi, S.M.H., Akhter, S., Ansari, M.S., Ullah, N., & Qayyum, M. (2008). Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Reproduction in Domestic Animals, 43*(3), 272-278.

Anzar, M., Farooq, U., Mirza, M. A., Shahab, M., & Ahmad, N. (2003). Factors affecting the efficiency of artificial insemination in cattle and buffalo in Punjab, Pakistan. *Pakistan Veterinary Journal, 23*(3), 106-113.

Cao, T.T., & Zhang, Y.Q. (2017). The potential of silk sericin protein as a serum substitute or an additive in cell culture and cryopreservation. *Amino Acids*, *49,* 1029-1039.

Chaturvedi, D., Dhami, A.J., Chaudhari, D.V. & Patel, A.C. (2023). Evaluation of cryoprotective, cryocapacitation inhibitory and fertility enhancing potential of RU-486, sericin and taurine in cattle and buffalo semen. *Indian Journal of Animal Research,* online published, DOI: 10.18805/IJAR.B-4913.

Chaturvedi, D., Dhami, A. J., Chaudhari, D. V., & Pathan, M. M. (2021). Effect of mifepristone, sericin and taurine in tris extender on oxidative markers and quality of fresh and frozen-thawed bovine spermatozoa. *International Journal of Livestock Research, 10*(12), 61-67.

Crespilho, A.M., Sá Filho, M.F., Dell'Aqua Jr, J.A., Nichi, M., Monteiro, G.A., Avanzi, B.R., Martins, A., & Papa, F.O. (2012). Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin-based extenders. *Livestock Science*, *149*(1-2), 1-6.

Dash, R., Acharya, C., Bindu, P.C., & Kundu, S.C. (2008). Antioxidant potential of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts. *BMB Reproduction, 41,* 236-241.

Demra, R., Gandotra, V.K., Singh, A.K., & Kumar, A. (2017). Validation of Sericin concentration in semen freezing media and its effect on post-thaw quality of buffalo bull semen. *Veterinary Practitioner, 18*(1), 42-44.

Dhami, A.J., Chaturvedi, D., Thesia, M.J., Chaudhari, D.V., & Patel, B.S. (2020). Role of extender additives and AI technicians in improving first service conception rate with frozen semen in cattle and buffaloes under field conditions. *Indian Journal of Animal Reproduction, 41*(2), 45-50.

Gordon, I. (1996). *Controlled Reproduction in Cattle and Buffaloes*. CAB International. Willingford, UK, p. 432-466.

Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., & Zaneveld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Reproduction*, *70*(1), 219-228.

Kadirvel, G., Kumar, S., & Kumaresan, A. (2009). Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Animal Reproduction Science, 114*(1-3), 125-134.

Kato, N., Sato, S., Yamanaka, A., Yamada, H., Fuwa, N., & Nomura, M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Bioscience, Biotechnology and Biochemistry*, *62*(1), 145-147.

Khan, D.R., Ahmad, N., Anzar, M., & Channa, A.A. (2009). Apoptosis in fresh and cryopreserved buffalo sperm. *Theriogenology, 71*(5), 872-876.

Kumar, P., Kumar, D., Sikka, P., & Singh, P. (2015). Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. *Animal Reproduction Science*, *152,* 26-31.

Kutty, I.C., Joseph, M., & Iyer, C.P.N. (1996). A simplified nigrosine-eosin-Geimsa staining technique to distinguish acrosome damaged live and dead spermatozoa. *Indian Journal of Animal Research*, *17*(2), 122-123.

Masakazu, T., Kazuhisa, T., Hideyuki, Y., Hiroshi, T., & Shigeru, N. (2003). The silk protein, sericin, protects against cell death caused by acute serum deprivation in insect cell culture. *Biotechnology Letters, 25,* 1805-1809.

Patel, T.M., Dhami, A.J., Chaudhari, D.V., & Pathan, M.M. (2019). Influence of antioxidant sericin in tris extender on oxidative markers during cryopreservation (–196ºC) of bovine semen. *The Indian Journal of Veterinary Sciences & Biotechnology*, *15*(2), 34-37.

Sasaki, M., Kato, Y., Yamada, H., & Terada, S. (2005). Development of a novel serum-free freezing medium for mammalian cells using the silk protein sericin. *Biotechnology and Applied Biochemistry*, *42*(2), 183-188.

Snedecor, G.W., & Cochran, W.G. (1994). *Statistical Methods*. 6th edn., Oxford and IBH Publishing Company, Calcutta, India.

Terada, S., Nishimura, T., Sasaki, M., Yamada, H., & Miki, M. (2002). Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including hybridoma. *Cytotechnology, 40,* 3-12.