Journal of Experimental Biology and Agricultural Sciences

<http://www.jebas.org>

ISSN No. 2320 – 8694

EFFECT OF MELATONIN ON MOBILITY AND VELOCITY PARAMETERS OF MITHUN (*Bos frontalis*) SEMEN PRESERVED IN LIQUID STATE (5°C)

Perumal P^{1,*}, Chang S¹, Sangma CTR², Savino N² and Khate K¹

¹ICAR- National Research Centre on Mithun, Jharnapani, Nagaland- 797 106, India

²NU-School of Agricultural Science and Rural Development, Medziphema, Nagaland- 797 106, India

Received – August 8, 2016; Revision – October 25, 2016; Accepted – October 26, 2016

Available Online – October 30, 2016

DOI: [http://dx.doi.org/10.18006/2016.4\(Spl-3-ADPCIAD\).S95.S102](http://dx.doi.org/10.18006/2016.4(Spl-3-ADPCIAD).S95.S102)

KEYWORDS

Melatonin

Mithun

Computer assisted sperm analysis

Liquid semen preservation

ABSTRACT

Present study was conducted to evaluate the effect of melatonin (MEL) on velocity and motility of mithun semen by computer assisted sperm analyser. Semen ejaculates (n=25) were collected from matured mithun bulls (n=10) using trans-rectal massage method and were divided and grouped into six aliquots equally, extended with the standard Tris Egg Yolk Citrate semen diluent. Six groups for various treatments were prepared, these groups were control - semen without MEL (Gr 1), 1mM (Gr 2), 2mM (Gr 3), 3mM (Gr 4), 4mM (Gr 5) and 5mM (Gr 6). Various parameters such as total motility, forward progressive motility, straight line velocity, curvilinear velocity, average path velocity, wobble, linearity, straightness, beat/cross frequency, amplitude of lateral head displacement and velocity of rapid, medium, slow and static were measured for 0-30h at 6 hrs interval at 5°C. The results observed that these mobility and velocity parameters were varied significantly (p<0.05) among the experimental periods and among the experimental groups. Further, MEL at 3 mM has significant (p<0.05) improvement in the mobility and velocity parameters than MEL at 1, 2, 4 or 5 mM stored *in-vitro* for up to 30 h of incubation. It was concluded that MEL 3 mM treated sperm has increased functional sperm structures faster to move and forward direction, probably improves the fertilization rate.

* Corresponding author

E-mail: perumalponraj@gmail.com (Perumal P)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI]
(<http://www.horizonpublisherindia.in/>).
All rights reserved.

All the article published by Journal of Experimental Biology and Agricultural Sciences is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License Based on a work at www.jebas.org.

1 Introduction

Mithun is a pride of North-Eastern hill region of India and is well adopted anatomically and physiologically at an altitude ranged from 300 –3000 meter MSL (Simoons, 1984). Latest livestock census (Livestock Census of India, 2012) revealed that population of mithun is reducing gradually due to lack of scientific strategy on reproduction and production areas. Required efforts should be undertaken from all quarters to preserve the germ plasm of mithun. Therefore, implementation of artificial breeding will enhance and improve the breeding strategy in mithun species.

For minimizing sperm metabolism and to maintain quality of the sperm, it was preserved at 5°C till the completion of study. But the sperm mobility and velocity parameters are reduced in the storage period. Antioxidants are present in semen to protect the sperm against free radical (Bilodeau et al., 2001; Bucak et al., 2008; Akhter et al., 2011). However, these antioxidants level are decreased during the process of extension and preservation process in liquid as well as in frozen state (Kumar et al., 2011). Therefore, supplementation (Shoae & Zamiri, 2008; Perumal et al., 2013) or feeding of the natural/synthetic antioxidants (Jayaganthan et al., 2013) could able to reduce the deleterious effect of oxidative and cryo stress during the process of semen preservation (Perumal et al., 2011a; Perumal et al., 2011b).

MEL is rhythmically secreted from the pineal gland and has an essential function in the circadian clock as well as the seasonal reproduction in mammalian species (Reiter, 1991). MEL and its metabolites also act as indirect powerful antioxidants to scavenge the free radicals (Reiter et al., 1998). Moreover, MEL has also potency two times as like of vitamin E in reaction against free radicals and reactive oxygen species (Pieri et al., 1994). The effect of anti-oxidant, MEL on ram (Casao et al., 2009; Ashrafi et al., 2011), boar (Hyun-Yong et al., 2006; Martin-Hildago et al., 2011), bull (Ashrafi et al., 2013); mithun (Perumal et al., 2013; Perumal et al., 2015), human (Du Plessis et al., 2010) and buffalo sperm (Li et al., 2012) revealed that it improves sperm mobility and velocity parameters of sperm in liquid storage. Perusal of literatures revealed that no information on the effect of MEL on the mobility and velocity parameters measured by CASA during liquid storage for extended period in mithun species. Hence, the objective of this study was designed to assess these parameters in semen samples extended with MEL at different concentration in liquid preservation for different incubation periods.

2 Material and Methods

2.1 Experimental Animals

Healthy matured mithun bulls (n=10) were selected. The experimental animals were maintained under same housing, feeding, watering and management systems. The feeding methods and feeds were followed uniformly as per the farm

schedule. Semen ejaculates was collected through trans-rectal massage method from the matured mithun bulls. All the experimental protocols strictly followed the regulation proposed by Institutional Animal Care and Use Committee.

2.2 Semen collection and processing

The semen were ejaculates (n=25) and collected from the matured mithun bulls (n=10) but it should not more be than twice per week. The semen samples were placed in a water bath (37°C) and analysed the routine semen quality parameters immediately after collection. The partially extended samples were then carried to the andrology laboratory in an insulated thermo flask filled with warm water (37°C) for further processing. The ejaculates which have concentration >500 million / ml, individual motility >70%, mass activity >3+ and total abnormality <10% were accepted for further investigation.

Individual ejaculates were divided and grouped into six aliquots equally and extended with the TEYC extender with MEL. The groups are Gr 1: control; semen without MEL, Gr 2: 1mM, Gr 3: 2mM, Gr 4: 3mM, Gr 5: 4mM and Gr 6: 5mM. Extended semen samples were placed in the cold cabinet for 30 hrs at 5°C. The velocity and motility parameters were estimated by CASA analyzer as per standard procedure in samples during storage of semen at 5°C for 30 h.

2.3 Computer assisted sperm analysis

The casa parameters were measured by Hamilton Thorne Sperm Analyser. The sperm concentration was estimated with a phase-contrast microscope (Nikon, Eclipse 80i; 400× magnification) after the semen collection. 25 µL of semen was extended into 50-100 µL of TEYC and 5 µL of this extended semen was pipetted and loaded into a pre-warmed (37°C) dual chamber disposable Leja slide and was left to some time to settle on the mini-therm heating stage before the analysis. Parameter, total motility, forward progressive motility, straight line velocity, curvilinear velocity, average path velocity, wobble, linearity, straightness, beat/cross frequency, amplitude of lateral head displacement and velocity of rapid, medium, slow and static were measured for 0-30 h at 6 hrs interval at 5°C. Minimum of 200 spermatozoa were assessed from at least different two drops of individual sample from each semen ejaculates. The objects were incorrectly observed as spermatozoa were manually deleted and final assessment was done for each semen sample. The values were analyzed using the computer software program. Significant differences were expressed at the values, p<0.05.

3 Results

The TM and PFM were significantly (p<0.05) higher in MEL added group as compared to untreated control group (Table 1). Furthermore, MEL 3 mM included samples have significantly (p<0.05) higher velocity and motility parameters.

Table 1 Mean ($\pm$ S.E.) total motility (TM) and forward progressive motility (FPM) percentage for mithun semen following storage at 5°C for different storage time.

CASA parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Total Motility	Control	70.45 $\pm$ 2.30 ^{abC}	65.90 $\pm$ 2.53 ^{bcC}	47.95 $\pm$ 2.56 ^{abB}	35.55 $\pm$ 2.08 ^{aA}	31.95 $\pm$ 2.37 ^{aA}
	MEL 1 mM	72.90 $\pm$ 2.61 ^{abE}	68.95 $\pm$ 1.80 ^{cdD}	50.80 $\pm$ 1.96 ^{bcC}	41.00 $\pm$ 1.72 ^{bB}	36.90 $\pm$ 1.88 ^{bA}
	MEL 2 mM	75.20 $\pm$ 2.16 ^{Bd}	70.85 $\pm$ 2.09 ^{dD}	53.20 $\pm$ 2.22 ^{cdC}	44.50 $\pm$ 2.48 ^{bB}	39.90 $\pm$ 2.25 ^{bA}
	MEL 3 mM	82.70 $\pm$ 2.11 ^{cE}	75.50 $\pm$ 2.00 ^{cD}	56.75 $\pm$ 2.19 ^{dC}	51.65 $\pm$ 2.18 ^{cB}	45.40 $\pm$ 2.33 ^{cA}
	MEL 4 mM	72.85 $\pm$ 2.74 ^{abD}	62.30 $\pm$ 1.90 ^{abC}	46.15 $\pm$ 1.89 ^{abB}	35.15 $\pm$ 1.85 ^{aA}	31.80 $\pm$ 1.84 ^{aA}
	MEL 5 mM	68.75 $\pm$ 1.79 ^{aE}	61.70 $\pm$ 1.80 ^{aD}	46.25 $\pm$ 1.90 ^{aC}	33.35 $\pm$ 2.25 ^{aB}	27.95 $\pm$ 1.62 ^{aA}
Forward Progressive Motility	Control	30.05 $\pm$ 3.03 ^{abB}	22.10 $\pm$ 2.52 ^{aA}	19.85 $\pm$ 2.33 ^{abA}	19.10 $\pm$ 2.16 ^{abA}	17.50 $\pm$ 2.15 ^{bcA}
	MEL 1 mM	29.70 $\pm$ 2.61 ^{abB}	27.15 $\pm$ 2.10 ^{abB}	21.65 $\pm$ 2.47 ^{abA}	19.70 $\pm$ 1.88 ^{bA}	17.50 $\pm$ 2.30 ^{bcA}
	MEL 2 mM	31.75 $\pm$ 1.96 ^{bB}	31.40 $\pm$ 2.54 ^{bB}	19.90 $\pm$ 1.92 ^{abA}	19.00 $\pm$ 2.30 ^{abA}	19.30 $\pm$ 2.13 ^{cA}
	MEL 3 mM	41.00 $\pm$ 2.24 ^{cB}	38.65 $\pm$ 3.63 ^{cB}	23.70 $\pm$ 2.36 ^{bA}	19.60 $\pm$ 2.25 ^{bA}	19.15 $\pm$ 3.11 ^{cA}
	MEL 4 mM	26.10 $\pm$ 2.29 ^{aD}	24.70 $\pm$ 2.27 ^{abCD}	20.35 $\pm$ 2.64 ^{abBC}	17.30 $\pm$ 2.21 ^{abB}	12.45 $\pm$ 1.98 ^{abA}
	MEL 5 mM	29.10 $\pm$ 2.10 ^{abD}	24.05 $\pm$ 2.32 ^{aC}	18.35 $\pm$ 1.81 ^{aB}	15.00 $\pm$ 1.86 ^{aB}	11.05 $\pm$ 1.45 ^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$)

Table 2 Mean ($\pm$ S.E.) different degree of velocity (percentage) of mithun sperm following storage at 5°C for different storage times.

Degree of Velocity	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Rapid Velocity	Control	59.10 $\pm$ 2.99 ^{abC}	51.25 $\pm$ 3.85 ^{aC}	36.20 $\pm$ 2.74 ^{aB}	28.05 $\pm$ 2.70 ^{abAB}	25.50 $\pm$ 2.66 ^{abA}
	MEL 1 mM	56.70 $\pm$ 3.00 ^{aC}	55.05 $\pm$ 3.96 ^{abC}	40.85 $\pm$ 2.94 ^{abB}	34.20 $\pm$ 2.67 ^{bcAB}	30.70 $\pm$ 2.21 ^{bcA}
	MEL 2 mM	67.05 $\pm$ 2.19 ^{bc}	64.55 $\pm$ 3.47 ^{bcC}	42.55 $\pm$ 3.50 ^{abB}	35.15 $\pm$ 2.54 ^{cAB}	32.50 $\pm$ 3.12 ^{cA}
	MEL 3 mM	81.35 $\pm$ 2.80 ^{cD}	69.95 $\pm$ 2.74 ^{cC}	47.50 $\pm$ 3.14 ^{bB}	43.75 $\pm$ 3.25 ^{dB}	33.80 $\pm$ 2.98 ^{cA}
	MEL 4 mM	59.70 $\pm$ 3.38 ^{abC}	54.60 $\pm$ 2.71 ^{abC}	35.25 $\pm$ 2.90 ^{aB}	27.20 $\pm$ 2.42 ^{aA}	23.15 $\pm$ 2.29 ^{aA}
	MEL 5 mM	62.85 $\pm$ 3.14 ^{abD}	53.80 $\pm$ 2.52 ^{aC}	36.60 $\pm$ 2.84 ^{aB}	24.30 $\pm$ 2.68 ^{aA}	19.25 $\pm$ 2.13 ^{aA}
Medium Velocity	Control	11.35 $\pm$ 2.37 ^{abBC}	15.15 $\pm$ 3.04 ^{aC}	12.45 $\pm$ 2.33 ^{BC}	8.25 $\pm$ 2.37 ^{abAB}	5.90 $\pm$ 1.79 ^{aA}
	MEL 1 mM	18.30 $\pm$ 3.88 ^{bB}	11.90 $\pm$ 3.03 ^{bcAB}	9.95 $\pm$ 2.71 ^A	6.06 $\pm$ 2.20 ^{aA}	6.70 $\pm$ 1.73 ^{aA}
	MEL 2 mM	10.55 $\pm$ 2.75 ^{abB}	3.60 $\pm$ 2.04 ^{aA}	12.90 $\pm$ 2.45 ^B	9.65 $\pm$ 2.99 ^{abAB}	10.05 $\pm$ 2.68 ^{abB}
	MEL 3 mM	5.55 $\pm$ 2.19 ^{aA}	12.65 $\pm$ 2.90 ^{bcB}	9.30 $\pm$ 2.95 ^{AB}	9.80 $\pm$ 2.43 ^{abAB}	11.65 $\pm$ 2.36 ^{bB}
	MEL 4 mM	13.15 $\pm$ 2.59 ^{abB}	7.90 $\pm$ 2.69 ^{abA}	11.05 $\pm$ 2.71 ^{AB}	8.55 $\pm$ 2.21 ^{abA}	8.85 $\pm$ 2.14 ^{abA}
	MEL 5 mM	7.20 $\pm$ 2.84 ^{aA}	7.95 $\pm$ 2.40 ^{abA}	10.10 $\pm$ 2.49 ^{AB}	12.65 $\pm$ 2.61 ^{bB}	8.45 $\pm$ 2.34 ^{abAB}
Slow Velocity	Control	15.45 $\pm$ 2.71 ^{bAB}	18.40 $\pm$ 2.44 ^{bAB}	20.30 $\pm$ 3.11 ^{abB}	16.75 $\pm$ 3.29 ^{AB}	12.45 $\pm$ 2.43 ^{aA}
	MEL 1 mM	13.60 $\pm$ 2.65 ^{bAB}	15.75 $\pm$ 2.70 ^b	18.15 $\pm$ 3.31 ^{ab}	16.85 $\pm$ 4.26	19.10 $\pm$ 3.17 ^{ab}
	MEL 2 mM	13.39 $\pm$ 2.49 ^{bAB}	8.35 $\pm$ 1.83 ^{aA}	20.15 $\pm$ 2.54 ^{abC}	16.35 $\pm$ 2.87 ^{BC}	17.85 $\pm$ 3.01 ^{abBC}
	MEL 3 mM	6.80 $\pm$ 1.83 ^{aA}	8.40 $\pm$ 2.41 ^{aAB}	15.35 $\pm$ 2.72 ^{aBC}	20.95 $\pm$ 3.50 ^C	19.00 $\pm$ 3.18 ^{abC}
	MEL 4 mM	14.60 $\pm$ 2.48 ^{bA}	16.20 $\pm$ 2.72 ^{bAB}	15.15 $\pm$ 2.74 ^{aA}	20.00 $\pm$ 3.23 ^{AB}	21.60 $\pm$ 3.52 ^{abB}
	MEL 5 mM	11.55 $\pm$ 2.45 ^{abA}	14.55 $\pm$ 2.61 ^{bAB}	24.80 $\pm$ 3.36 ^{bcC}	22.45 $\pm$ 4.10 ^{BC}	22.40 $\pm$ 3.68 ^{bcBC}
Static Motility	Control	14.15 $\pm$ 2.25 ^{bcA}	15.15 $\pm$ 1.98 ^{abA}	31.10 $\pm$ 3.78 ^{abB}	46.85 $\pm$ 3.79 ^{bcC}	55.95 $\pm$ 2.86 ^{cd}
	MEL 1 mM	12.75 $\pm$ 2.49 ^{abA}	15.85 $\pm$ 2.83 ^{bA}	31.95 $\pm$ 3.38 ^{abB}	46.40 $\pm$ 2.61 ^{bcC}	43.10 $\pm$ 3.39 ^{abC}
	MEL 2 mM	12.60 $\pm$ 3.16 ^{abA}	20.75 $\pm$ 2.40 ^{bcB}	24.30 $\pm$ 2.73 ^{abB}	37.70 $\pm$ 3.25 ^{abC}	38.55 $\pm$ 3.20 ^{abC}
	MEL 3 mM	7.10 $\pm$ 2.31 ^{aA}	9.50 $\pm$ 2.37 ^{aA}	25.50 $\pm$ 3.64 ^{abB}	27.85 $\pm$ 2.78 ^{abAB}	38.85 $\pm$ 3.36 ^{aC}
	MEL 4 mM	11.80 $\pm$ 1.99 ^{abA}	21.40 $\pm$ 3.05 ^{bcB}	37.65 $\pm$ 3.14 ^{bcC}	44.20 $\pm$ 3.26 ^{bcC}	46.45 $\pm$ 3.78 ^{abC}
	MEL 5 mM	18.55 $\pm$ 2.26 ^{aA}	23.90 $\pm$ 2.73 ^{aA}	28.60 $\pm$ 3.21 ^{abA}	39.65 $\pm$ 4.22 ^{bbB}	49.05 $\pm$ 3.88 ^{bcB}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$), Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$)

Table 3 Mean ($\pm$ S.E.) average path velocity (VAP), straight line velocity (VSL) and curve linear velocity (VCL) of mithun sperm following storage at 5°C for different storage times.

Velocity Parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Average Path Velocity (VAP)	Control	122.33 $\pm$ 5.04 ^{abC}	106.51 $\pm$ 4.79 ^{aBC}	90.04 $\pm$ 4.04 ^{aAB}	89.35 $\pm$ 3.98 ^{AB}	87.88 $\pm$ 4.44 ^{aA}
	MEL 1 mM	126.80 $\pm$ 4.67 ^{abC}	118.79 $\pm$ 5.19 ^{abBC}	102.35 $\pm$ 4.53 ^{abAB}	99.75 $\pm$ 4.62 ^{AB}	89.70 $\pm$ 4.24 ^{abA}
	MEL 2 mM	131.88 $\pm$ 4.79 ^{bcC}	118.82 $\pm$ 5.57 ^{abBC}	100.97 $\pm$ 4.41 ^{abAB}	100.27 $\pm$ 5.21 ^{AB}	95.04 $\pm$ 4.42 ^{abA}
	MEL 3 mM	149.20 $\pm$ 4.42 ^{cC}	130.40 $\pm$ 5.35 ^{bBC}	109.15 $\pm$ 5.03 ^{bAB}	106.76 $\pm$ 5.29 ^A	98.10 $\pm$ 4.20 ^{bA}
	MEL 4 mM	110.83 $\pm$ 4.78 ^{aB}	98.67 $\pm$ 3.99 ^{aAB}	92.40 $\pm$ 4.39 ^{abA}	90.38 $\pm$ 4.08 ^A	87.36 $\pm$ 4.21 ^{abA}
	MEL 5 mM	110.03 $\pm$ 4.68 ^{aB}	98.18 $\pm$ 4.05 ^{aAB}	89.59 $\pm$ 4.36 ^{aAB}	88.55 $\pm$ 6.51 ^{AB}	83.95 $\pm$ 4.09 ^{aA}
Straight Line Velocity (VSL)	Control	85.47 $\pm$ 3.96 ^{abC}	76.27 $\pm$ 4.00 ^{abBC}	68.66 $\pm$ 4.32 ^{AB}	64.05 $\pm$ 4.16 ^{abAB}	58.80 $\pm$ 3.31 ^{aA}
	MEL 1 mM	85.98 $\pm$ 4.38 ^{abC}	79.23 $\pm$ 4.48 ^{abBC}	72.83 $\pm$ 4.01 ^{A^{BC}}	67.97 $\pm$ 3.84 ^{abAB}	63.31 $\pm$ 4.36 ^{abA}
	MEL 2 mM	93.97 $\pm$ 4.95 ^{bcB}	80.57 $\pm$ 4.79 ^{abAB}	72.81 $\pm$ 4.77 ^A	71.95 $\pm$ 4.24 ^{bA}	64.52 $\pm$ 3.93 ^{abA}
	MEL 3 mM	103.18 $\pm$ 4.06 ^{cB}	92.78 $\pm$ 4.18 ^{bB}	75.39 $\pm$ 4.69 ^A	72.33 $\pm$ 4.01 ^{bA}	69.04 $\pm$ 3.89 ^{bA}
	MEL 4 mM	77.88 $\pm$ 4.14 ^{abB}	74.70 $\pm$ 4.24 ^{aAB}	66.22 $\pm$ 3.98 ^{AB}	65.20 $\pm$ 3.27 ^{abAB}	62.00 $\pm$ 3.25 ^{abA}
	MEL 5 mM	75.01 $\pm$ 4.25 ^{aB}	66.93 $\pm$ 4.14 ^{aAB}	64.90 $\pm$ 6.24 ^{AB}	59.31 $\pm$ 3.46 ^{aA}	57.35 $\pm$ 3.54 ^{aA}
Curve Linear Velocity (VCL)	Control	216.38 $\pm$ 7.84 ^{abB}	197.58 $\pm$ 7.48 ^{abAB}	191.38 $\pm$ 6.16 ^{abAB}	172.54 $\pm$ 5.03 ^A	169.73 $\pm$ 5.32 ^A
	MEL 1 mM	272.11 $\pm$ 5.26 ^{cB}	200.30 $\pm$ 6.37 ^{abA}	195.48 $\pm$ 5.92 ^{abA}	177.41 $\pm$ 8.19 ^A	175.06 $\pm$ 6.57 ^A
	MEL 2 mM	242.48 $\pm$ 6.36 ^{bcC}	224.64 $\pm$ 6.79 ^{bBC}	197.83 $\pm$ 5.55 ^{abAB}	187.81 $\pm$ 5.58 ^A	178.22 $\pm$ 5.79 ^A
	MEL 3 mM	242.65 $\pm$ 6.19 ^{bcB}	230.94 $\pm$ 7.27 ^{bB}	212.63 $\pm$ 6.75 ^{bAB}	188.24 $\pm$ 6.47 ^A	188.21 $\pm$ 4.63 ^A
	MEL 4 mM	206.05 $\pm$ 6.66 ^{abB}	191.29 $\pm$ 5.83 ^{abAB}	182.44 $\pm$ 6.00 ^{abAB}	176.85 $\pm$ 6.00 ^{AB}	166.58 $\pm$ 5.07 ^A
	MEL 5 mM	196.64 $\pm$ 7.54 ^{aB}	183.48 $\pm$ 5.01 ^{aAB}	173.90 $\pm$ 6.76 ^{aAB}	173.37 $\pm$ 5.81 ^{AB}	162.09 $\pm$ 7.70 ^A

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

Proportionally the motility parameters were significantly ($p < 0.05$) higher till 30 hrs of experimental period in the MEL 3mM treated group. Out of the five groups of MEL treated, MEL 4mM and MEL 5mM has significantly reduced TM and PFM. The motility parameters were increased gradually and significantly ($p < 0.05$) from control to MEL 3mM group and then reduced in the MEL 4 and MEL 5 groups. The proportion of reducing TM and PFM were higher in MEL 4mM and MEL 5mM treated group as compared to other MEL treated groups.

In the present experiment, rapid velocity revealed that MEL included group has significantly higher percentage than untreated control group (Table 2). MEL 3mM treated group has significantly ($p < 0.05$) higher rapid velocity than other treatment groups. Rapid velocity was increasing from 1 mM to 3 mM at maximum and reducing from 4 mM to 5 mM. Moreover, similar to TM and PFM, rapid velocity was reducing proportionally upto the experimental period (30 hrs of incubation).

The rapid velocity was positively and significantly correlated with PFM in all the experimental groups. The result revealed that there was a significant ($p < 0.05$) difference among the experimental groups with regards to the VAP, VSL and VCL at different periods of incubation except at 24 hrs for VAP, 12 hrs of incubation for VSL and 24 and 30 hrs of incubation for VCL (Table 3). The velocity parameters (VAP, VSL and VCL) were significantly ($p < 0.05$) higher in MEL 3mM treated group

than the other treatment groups. These velocity parameters were significantly increased from 1mM to 3 mM and then decreased in 4 mM treated followed by MEL 5 mM. These velocity parameters were reduced over a period of the time during the experimental period. But the proportion was significantly higher in MEL high concentrated experimental groups (MEL 4 and 5 mM).

The result of ALH revealed that there was a significant ($p < 0.05$) difference among the experimental groups in 6, 12 and 24 hrs of incubation (Table 4). Incubation period from 0 to 30 hrs, MEL 3mM was showing higher value than other treatment groups irrespective of significant or non-significant among the experimental groups. BCF revealed that there was a significant ($p < 0.05$) difference among the experimental groups in 0, 24 and 30 hrs of incubation and significantly ($p < 0.05$) higher in MEL 3mM followed by MEL 2 mM and least was in MEL 5 mM. The BCF value was increased from 1 mM to 3 mM and then decreased to 5 mM (Table 4).

Percentage of straightness revealed that there was a significant difference among the experimental groups at 6 hrs of incubation (Table 4). The MEL 3 mM was significantly ($p < 0.05$) higher among the experimental groups in 6 hrs of incubation. STR was significantly ($p < 0.05$) differed among the experimental periods for all experimental groups except MEL 1 mM. But reduction of STR from 0 to 30 hrs of incubation was observed.

Table 4 Mean ($\pm$ S.E.) amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) of mithun sperm following storage at 5°C for different storage times.

CASA Parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Amplitude of lateral head displacement (ALH)	Control	12.28 $\pm$ 3.85 ^B	8.60 $\pm$ 1.25 ^{aAB}	8.09 $\pm$ 1.24 ^{aAB}	8.07 $\pm$ 1.29 ^{aAB}	7.49 $\pm$ 1.10 ^A
	MEL 1 mM	9.01 $\pm$ 1.23 ^B	8.96 $\pm$ 1.28 ^{abB}	8.18 $\pm$ 1.26 ^{aAB}	8.09 $\pm$ 1.53 ^{aAB}	7.58 $\pm$ 1.33
	MEL 2 mM	10.58 $\pm$ 0.87 ^B	9.15 $\pm$ 1.29 ^{abA}	8.87 $\pm$ 1.07 ^{abA}	8.33 $\pm$ 1.20 ^{aA}	8.22 $\pm$ 1.27
	MEL 3 mM	10.90 $\pm$ 2.31 ^B	9.92 $\pm$ 1.20 ^{abB}	9.17 $\pm$ 1.47 ^{abAB}	9.11 $\pm$ 1.25 ^{abAB}	7.29 $\pm$ 1.23
	MEL 4 mM	11.57 $\pm$ 3.67	9.93 $\pm$ 1.43 ^{ab}	9.29 $\pm$ 1.14 ^{ab}	8.99 $\pm$ 1.22 ^{ab}	7.90 $\pm$ 1.33
	MEL 5 mM	13.21 $\pm$ 4.20	10.14 $\pm$ 1.24 ^b	10.01 $\pm$ 2.18 ^b	9.95 $\pm$ 2.08 ^b	7.72 $\pm$ 1.18
Beat cross frequency (BCF)	Control	28.32 $\pm$ 2.03 ^{aB}	26.96 $\pm$ 2.02 ^B	25.21 $\pm$ 2.38 ^{AB}	24.34 $\pm$ 2.32 ^{abcAB}	22.54 $\pm$ 2.30 ^{abA}
	MEL 1 mM	30.79 $\pm$ 2.71 ^{aC}	27.89 $\pm$ 2.35 ^{BC}	25.49 $\pm$ 2.30 ^{AB}	25.47 $\pm$ 1.96 ^{abcAB}	22.64 $\pm$ 1.79 ^{abA}
	MEL 2 mM	33.49 $\pm$ 6.71 ^{bC}	28.32 $\pm$ 2.19 ^B	27.59 $\pm$ 2.34 ^A	26.74 $\pm$ 1.99 ^{bcA}	24.32 $\pm$ 1.78 ^{bA}
	MEL 3 mM	43.31 $\pm$ 7.46 ^{cB}	29.54 $\pm$ 1.68 ^A	27.85 $\pm$ 2.41 ^A	27.30 $\pm$ 2.17 ^A	25.79 $\pm$ 2.34 ^{bA}
	MEL 4 mM	28.26 $\pm$ 2.23 ^{aB}	26.56 $\pm$ 2.73 ^{BC}	24.98 $\pm$ 3.06 ^{AB}	23.18 $\pm$ 1.95 ^{abAB}	22.45 $\pm$ 1.95 ^{abA}
	MEL 5 mM	27.02 $\pm$ 2.29 ^{aC}	26.41 $\pm$ 2.20 ^C	23.60 $\pm$ 2.21 ^{BC}	21.91 $\pm$ 1.88 ^{aAB}	19.79 $\pm$ 1.54 ^{aA}
Straightness (STR)	Control	69.90 $\pm$ 2.81 ^B	69.40 $\pm$ 2.75 ^{abAB}	66.25 $\pm$ 2.46 ^{AB}	65.15 $\pm$ 2.29 ^{AB}	62.50 $\pm$ 3.36 ^A
	MEL 1 mM	69.95 $\pm$ 2.30	69.45 $\pm$ 2.81 ^{ab}	66.60 $\pm$ 2.26	65.30 $\pm$ 2.45	64.96 $\pm$ 3.89
	MEL 2 mM	72.00 $\pm$ 2.79 ^B	69.55 $\pm$ 2.82 ^{abAB}	68.45 $\pm$ 2.94 ^{AB}	64.80 $\pm$ 1.47 ^A	63.80 $\pm$ 2.31 ^A
	MEL 3 mM	72.50 $\pm$ 2.66 ^B	72.30 $\pm$ 2.70 ^{bB}	68.50 $\pm$ 2.59 ^{AB}	66.25 $\pm$ 1.88 ^A	64.20 $\pm$ 2.39 ^A
	MEL 4 mM	69.00 $\pm$ 3.13 ^B	66.50 $\pm$ 2.49 ^{abAB}	64.85 $\pm$ 2.58 ^{AB}	66.50 $\pm$ 2.36 ^{AB}	63.30 $\pm$ 1.88 ^A
	MEL 5 mM	68.95 $\pm$ 2.72 ^B	65.20 $\pm$ 2.43 ^{aA}	64.80 $\pm$ 2.13 ^A	64.35 $\pm$ 2.25 ^A	64.30 $\pm$ 2.03 ^A

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

Similar to STR, LIN was significantly ($p < 0.05$) differed among the experimental groups at 6 and 12 hrs of incubation at 5°C (Table 5). But the incubation period from 0 to 30 hrs, the MEL 3 mM group was significantly (6 and 12 hrs) higher among the experimental groups. The value of LIN was reduced significantly ($p < 0.05$) from 0 to 30 hrs of incubation and it was lesser proportion in the MEL 3 mM or 2 mM treated groups and higher in MEL 4 mM or 5 mM treated groups. The WOB

revealed that there was a significant difference was observed among the experimental groups in 0 and 6 hrs of incubation periods. Moreover, control and MEL 5 mM groups were significantly ($p < 0.05$) differed in 0 hr of incubation. The value of WOB was reduced from 0 to 30 hrs of incubation for the group MEL 2 mM and MEL 4 mM whereas significant ($p < 0.05$) difference was observed in other experimental groups (Table 5).

Table 5 Mean ($\pm$ S.E.) linearity (LIN) and wobble (WOB) of mithun sperm following storage at 5°C for different storage times.

CASA parameters	Experimental groups	Storage Period				
		0 h	6h	12h	24h	30h
Linearity (LIN)	Control	41.00 $\pm$ 3.08 ^B	37.75 $\pm$ 2.69 ^{aAB}	37.65 $\pm$ 2.60 ^{abAB}	36.35 $\pm$ 2.46 ^{AB}	35.40 $\pm$ 2.87 ^A
	MEL 1 mM	42.25 $\pm$ 3.91 ^B	39.60 $\pm$ 2.62 ^{aAB}	38.95 $\pm$ 2.70 ^{abAB}	37.50 $\pm$ 2.04 ^{AB}	35.35 $\pm$ 2.16 ^A
	MEL 2 mM	42.35 $\pm$ 2.58 ^C	41.45 $\pm$ 2.43 ^{bBC}	37.40 $\pm$ 1.59 ^{abAB}	37.05 $\pm$ 2.40 ^{AB}	36.25 $\pm$ 2.03 ^A
	MEL 3 mM	43.50 $\pm$ 2.93 ^B	42.00 $\pm$ 3.31 ^{bAB}	40.10 $\pm$ 2.88 ^{bAB}	38.15 $\pm$ 2.46 ^{AB}	36.35 $\pm$ 2.05 ^A
	MEL 4 mM	38.75 $\pm$ 2.66 ^B	38.15 $\pm$ 2.56 ^{aB}	37.75 $\pm$ 2.17 ^{abAB}	35.80 $\pm$ 2.32 ^{AB}	34.25 $\pm$ 1.76 ^A
	MEL 5 mM	38.45 $\pm$ 3.40 ^B	35.80 $\pm$ 2.36 ^{aAB}	35.75 $\pm$ 1.96 ^{aAB}	34.35 $\pm$ 2.12 ^{AB}	33.70 $\pm$ 2.34 ^A
Wobble (WOB)	Control	81.82 $\pm$ 11.29	65.71 $\pm$ 8.05	51.40 $\pm$ 3.86	51.83 $\pm$ 2.12	52.45 $\pm$ 3.39
	MEL 1 mM	47.02 $\pm$ 3.04	61.06 $\pm$ 3.92	53.76 $\pm$ 2.26	59.65 $\pm$ 4.62	59.74 $\pm$ 6.56
	MEL 2 mM	54.47 $\pm$ 1.78	52.40 $\pm$ 1.98	51.63 $\pm$ 1.72	53.05 $\pm$ 2.91	53.35 $\pm$ 2.10
	MEL 3 mM	63.51 $\pm$ 4.03	57.40 $\pm$ 3.38	51.30 $\pm$ 1.70	58.43 $\pm$ 4.00	51.99 $\pm$ 2.47
	MEL 4 mM	54.09 $\pm$ 1.92	51.78 $\pm$ 1.61	53.08 $\pm$ 3.94	53.04 $\pm$ 3.76	52.28 $\pm$ 2.38
	MEL 5 mM	78.11 $\pm$ 10.50	53.36 $\pm$ 1.85	55.57 $\pm$ 4.61	58.60 $\pm$ 7.63	59.21 $\pm$ 5.22

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$); Within rows means with different letters (A, B, C, D) differ significantly ($P < 0.05$).

4 Discussions

The results showed that addition of MEL has improved the motility and velocity parameters of mithun semen. Based on the perusal of literature, no reports on inclusion of MEL on mobility and velocity parameters in mithun species and this is the primary report to our the best of knowledge. Earlier workers reported that MEL has significantly higher benefit on refrigerated preservation mammalian sperm and also enhanced the velocity and mobility parameters in the present study (Ashrafi et al., 2011; Ashrafi et al., 2013; Du Plessis et al., 2010) and also on semen quality parameters (Casao et al., 2009; Ashrafi et al., 2011; Hyun-Yong et al., 2006; Ashrafi et al., 2013; Du Plessis et al., 2010; Perumal et al., 2013; Perumal et al., 2015).

The MEL functioned on dose depended method (Casao et al., 2010; Perumal et al., 2013; Perumal et al., 2015) as 3 mM MEL is the most suitable and optimum dosage. Similar observation was reported that MEL had induced boar spermatozoa to a hyperactive state (Martin-Hildago et al., 2011) as the result of an elevated synthesis of ATP; MEL is known to promote mitochondrial complex efficiency and ATP production (Martin et al., 2000). MEL has improved the velocity and mobility parameters in the current study as because of its interaction with second messenger calmodulin in the sperm (Benitez-King & Anton-Tay, 1993) and which in turn stimulate the cytoskeletal structures of sperm leads to higher sperm velocity and motility. Moreover, the MEL acts as an antioxidant and an antiapoptotic agent in sperm storage medium and it protects the sperm through inhibition of ROS generation, caspase-3 and caspase-9 activities, phosphatidylserine externalization, apoptosis and sperm death (Espino et al., 2011) and through which it protects the sperm and its mitochondrial potential for energy production to progress forward direction.

Improved actions of MEL is due to it increases the ATPase production (Chen et al., 1994), which is the source energy and used by the sperm and activate motility and velocity (Burger et al., 1991). According to Delgadillo et al. (1994) MEL also stimulates cellular influx of Ca^{2+} and enhancing motility. Further, Si (1997) suggested that Ca^{2+} regulate the flagella movement and calmodulin have been identified in the spermatozoa and flagellar (Tash & Means, 1983). According to Ahmad et al. (1996) calmodulin antagonist caused a reduction in VCL and ALH and mitochondrial membrane potential. Moreover, MEL has improved the parameters act on the cAMP (Yung et al., 1995) and stimulates velocity (Lindemann, 1978) and/or acting on secondary messenger (Garbers & Kopf, 1980).

The results of the present study revealed that inclusion of MEL @ 3 mM has improved the keeping quality, mobility and velocity parameters of mithun sperm preserved at 5°C for 30 hrs of incubation. Motility and velocity parameters of the sperm were decreased during the time of storage and remedially maintained above 50% for upto 30 h period of time.

In contrarily, decreasing rate in the motility percentage and velocity rate were higher in the ejaculate treated with 4 to 5 mM MEL or without MEL. However, inclusion of 3 mM MEL, the velocity and motility parameters were higher as compared to untreated control group in the present study (Du Plessis et al., 2010). Various effects of MEL at various might be described according to the observation reported by Ashrafi et al. (2011), Shoaie & Zamiri (2008), Perumal et al. (2013) and Perumal et al. (2015) revealed the excessive amount of MEL than optimum in turn to higher fluidity of plasma membrane of sperm, creating the sperm are more prone to plasma membrane and acrosomal damages and also inclusion of high dosage leads to deleterious effect on the spermatozoa as because alteration in physiological and physical condition of diluent. But the antioxidant concentration higher than required amount was deleterious and toxic to spermatozoa (Maxwell & Watson, 1996; Perumal et al., 2013; Perumal et al., 2015). However, reduced concentration also altered the sperm parameters and structures. Therefore based on the present study, mobility and velocity parameters were increased maximum upto 3 mM then reduced to 5 mM.

Inclusion of exogenous MEL improved semen quality, motility, acrosomal membrane quality and viability of semen, similar types of results was also reported by Casao et al. (2009) and Ashrafi et al. (2011) and by various researchers in various organism such as bull sperm (Ashrafi et al., 2013), mithun (Perumal et al., 2013; Perumal et al., 2015) and boar sperm (Hyun-Yong et al., 2006). Furthermore, MEL protects plasma membrane, mitochondrial membrane integrity, acrosomal membrane and functional structure of flagella of sperm, cytoskeleton structure as cell protecting effects (Leon et al., 2005).

MEL has also protects and stimulates the functions of antioxidant enzymes like SOD, GSH and CAT (Karbownik & Reiter, 2000), which helps to maintain membrane integrity, membrane transportation process (Alvarez & Storey, 1992) and fertility rate of the sperm cells. Further it reduces the number of free radicals, ROS indirectly, and it also may enhance the production of sperm protecting molecules against oxidative and peroxidative stress. Through these mechanisms, the velocity and motility parameters of sperm were increased significantly by using MEL in the current study.

It was concluded that the possible protective effects of MEL supplementation were it enhanced the mobility and velocity parameters assessed by computer assisted sperm analyser at 3 mM in future, cryopreservation studies is needed to confirm the present research findings.

Acknowledgement

This research work was supported by a grant from the Department of Biotechnology, Government of India, New Delhi for the project entitled "Evaluation of MEL as fertility marker in Mithun (*Bos frontalis*) bulls: Effect on circadian rhythm and seasonal variation in semen quality parameters".

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References

Ahmad K, Bracho GE, Wolf DP, Tash JS (1996) Regulation of human sperm motility and hyperactivation components by calcium, calmodulin and protein phosphatases. *Archives of Andrology* 35: 187-208. DOI: <http://dx.doi.org/10.3109/01485019508987871>

Akhter S, Rakha BA, Ansari MS, Andrabi SMH, Ullah N (2011) Storage of Nili-Ravi buffalo (*Bubalus bubalis*) semen in skim milk extender supplemented with ascorbic acid and α -tocopherol. *Pakistan Journal of Zoology* 43 : 273-277.

Alvarez JG, Storey BT (1992) Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a model of sub lethal cryo damage to human sperm during cryopreservation. *Journal of Andrology* 13 : 232-241. DOI: 10.1002/j.1939-4640.1992.tb00306.x

Ashrafi I, Kohram H, Naijian H, Bahreini M, Poorhamdollah M (2011) Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5°C. *African Journal of Biotechnology* 10 : 6670-6674. DOI: 10.5897/AJB11.1020

Ashrafi I, Kohram H, Ardabili FF (2013) Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Animal Reproduction Science* 139 : 25–30. DOI: 10.1016/j.anireprosci.2013.03.016.

Benitez-King G, Anton-Tay F (1993) Calmodulin mediates melatonin cytoskeletal effects. *Experientia* 49: 35–41. DOI: 10.1007/BF01923944

Bilodeau JF, Blanchette S, Gagnon C, Sirard MA (2001) Thiols prevent H₂O₂-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* 56: 275–286. DOI: [http://dx.doi.org/10.1016/S0093-691X\(01\)00562-3](http://dx.doi.org/10.1016/S0093-691X(01)00562-3)

Bucak MN, Atessahin A, Yuce A (2008) Effect of antioxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research* 75: 128-134. DOI: <http://dx.doi.org/10.1016/j.smallrumres.2007.09.002>

Burger B, VanderHorst G, Menkveld R, Maritz GS, de Villiers A, Conradie E, Kruger TF (1991) Relationship between biochemical markers and fertilization in vitro. Presented at the Annual Reproductive Biology Work Seminar, Pretoria, South Africa.

Casao A, Cebrián I, Assumpcao ME, Pérez-Pé R, Abecia JA, Forcada F, Cebrián-Pérez JA, Muino-Blanco T (2010)

Seasonal variations of melatonin in ram seminal plasma are correlated to those of testosterone and antioxidant enzymes. *Reproductive Biology and Endocrinology* 8 : 59. DOI: 10.1186/1477-7827-8-59, 2010.

Casao A, Mendoza N, Perez-Pe R, Grasa P, Abecia JA, Forcada F, Cebrián-Perez JA, Muino-Blanco T (2009) Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. *Journal of Pineal Research* 48: 39–46. DOI: 10.1111/j.1600-079X.2009.00722.x.

Chen L, Kumar P, Reiter RJ, Tan D, Manchester LC, Chambers JP, Poeggeler B, Saarela K (1994) Melatonin prevents the suppression of cardiac Ca²⁺ stimulated ATPase activity induced by Alloxan. *American Journal of Physiology* 267: E57-E62.

Delgadillo LH, Tay FAA, King GB (1994) Effect of melatonin on microtubule assembly depends on hormone concentration: Role of melatonin as a calmodulin antagonist. *Journal of Pineal Research* 17: 50-58. DOI: 10.1111/j.1600-079X.1994.tb00114.x

Du Plessis SS, Hagenaar K, Lampiao F (2010) The in vitro effects of melatonin on human sperm function and its scavenging activities on NO and ROS. *Andrologia* 42 : 112–116. DOI: 10.1111/j.1439-0272.2009.00964.x.

Espino J, Ortiz A, Bejarano I, Lozano GM, Monllor F, Garcia JF, Rodriguez AB, Pariente JA (2011) Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. *Fertility and Sterility* 95: 2290–2296. DOI: 10.1016/j.fertnstert.2011.03.063.

Garbers DL, Kopf GS (1980) The regulation of spermatozoa by calcium and cyclic nucleotides. In: Greengard P, Robinson GA (Eds.) "Advances in Cyclic Nucleotide Research., New York, Raven Press, pp. 251-306.

Hyun-Yong J, Sung-Gon K, Jong-Taek K, Choon-Keun P, Hee-Tae C, Hak-Kyu L, Boo-Keun Y (2006) Effects of antioxidants on sperm motility during in vitro storage of boar semen. *Korean Journal of Gerontology* 16 : 47-51.

Jayaganthan P, Perumal P, Balamurugan TC, Verma RP, Singh LP, Pattanaik AK, Kataria M (2013) Effects of *Tinospora cordifolia* supplementation on semen quality and hormonal profile of ram. *Animal Reproduction Science* 140 : 47-53. DOI: 10.1016/j.anireprosci.2013.05.003.

Karbownik M, Reiter RJ (2000) Antioxidative effects of melatonin in protection against cellular damage caused by ionizing radiation. *Proceedings of the Society for Experimental Biology and Medicine* 225 : 9–22. DOI: 10.1046/j.1525-1373.2000.22502.x

- Kumar R, Jagan Mohanarao G, Arvind R, Atreja SK (2011) Freeze-thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. *Molecular Biology Report* 38 : 1499-1506. DOI: 10.1007/s11033-010-0257-1.
- Leon J, Acuna-Castroviejo D, Escames G, Tan DX, Reiter RJ (2005) Melatonin mitigates mitochondrial malfunction. *Journal of Pineal Research* 38 : 1–9. DOI: 10.1111/j.1600-079X.2004.00181.x
- Li XX, Yang XG, Lu YQ, Lu SS, Zhang M, Yao HL, Meng LJ, Lu KH (2012) Protective effects of melatonin against oxidative stress in flow cytometry-sorted buffalo sperm. *Reproduction in Domestic Animals* 47: 299–307. DOI: 10.1111/j.1439-0531.2011.01858.x.
- Lindemann CB (1978) A cAMP-induced increase in the motility of dimembrated bull sperm models. *Cell* 13: 9-18.
- Livestock Census of India (19th) (2012) Department of Animal Husbandry, Dairying & Fisheries, Ministry of Agriculture, Government of India, New Delhi, India.
- Martin M, Macias M, Escames G, Reiter RJ, Agapito MT, Ortiz GG, Acuna-Castroviejo D (2000) Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. *Journal of Pineal Research* 28: 242–248. DOI: 10.1034/j.1600-079X.2000.280407.x
- Martin-Hildago D, Baron FJ, Bragado MJ, Carmona P, Robina A, Garcia-Marin LJ, Gil MC (2011) The effect of melatonin on the quality of extended boar semen after long-term storage at 17 °C. *Theriogenology* 75: 1550–1560. DOI: 10.1016/j.theriogenology.2010.12.021.
- Maxwell WMC, Watson PF (1996) Recent progress in the preservation of ram semen. *Animal Reproduction Science* 42 : 55–65. DOI:10.1016/0378-4320(96)01544-8
- Perumal P, Selvaraju S, Barik AK, Mohanty DN, Das S, Mishra PC (2011a) Role of reduced glutathione in improving post-thaw seminal parameters in poor freezable Jersey crossbred bull semen. *Indian Journal of Animal Sciences* 81 : 807–810.
- Perumal P, Chamuah JK, Rajkhowa C (2013) Effect of catalase on the liquid storage of mithun (*Bos frontalis*) semen. *Asian Pacific Journal of Reproduction* 2 : 209-214. DOI: 10.1016/S2305-0500(13)60148-7
- Perumal P, Nahak AK, Vupru K, Khate K, Balamurugan TC, Prakash Krupakaran R (2015) Effect of addition of bovine serum albumin on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Journal of Cell and Tissue Research* 15 : 4795 – 4800.
- Perumal P, Selvaraju S, Selvakumar S, Barik AK, Mohanty DN, Das S, Das RK, Mishra PC (2011b) Effect of pre-freeze addition of cysteine hydrochloride and reduced glutathione in semen of crossbred jersey bulls on sperm parameters and conception rates. *Reproduction in Domestic Animals* 46 : 636-641. DOI: 10.1111/j.1439-0531.2010.01719.x.
- Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F (1994) Melatonin: A peroxy radical scavenger more effective than vitamin E. *Life Science* 55 : 271–276. DOI:10.1016/0024-3205(94)00666-0
- Reiter RJ, Tan DX, Kim SJ, Qi W (1998) Melatonin as a pharmacological agent against damage to lipids and DNA. *Proceedings of Western Pharmacology Society* 41: 229–236.
- Reiter RJ (1991) Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. *Endocrine Reviews* 12 : 151–180. DOI:10.1210/edrv-12-2-151
- Shoae A, Zamiri MJ (2008) Effect of butylated hydroxytoluene on bull sperm frozen in egg yolk-citrate extender. *Animal Reproduction Science* 104 : 414-418. DOI: <http://dx.doi.org/10.1016/j.anireprosci.2007.07.009>
- Si Y (1997) Temperature-dependent hyperactivated movement of hamster spermatozoa. *Biology of Reproduction* 57: 1407 - 1412. DOI: 10.1095/biolreprod57.6.1407
- Simoons FJ (1984) Gayal or mithun. In: Manson IL (Ed.), *Evolution of Domesticated Animals*. Longman, London, pp. 34–36.
- Tash JS, Means AR (1983) Cyclic adenosine 3', 5' monophosphate, calcium and protein phosphorylation in flagellar motility. *Biology of Reproduction* 28: 75-104. DOI: 10.1095/biolreprod28.1.75
- Yung LY, Tsim ST, Wong YH (1995) Stimulation of cAMP accumulation by the cloned *Xenopus* melatonin receptor through G1 AND G2 proteins. *FFBS Letters* 372: 99-102.