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Inclusion of Melatonin in Semen Extender Modulates Post Thaw Motility and Velocity Pa-rameters of Mithun Sperm

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Abstract

Melatonin (MT) has significant function on seasonal reproduction and circadian clock regulation in the mammals. It also a powerful antioxidant and anti-apoptotic compound in semen cryopreservation. Environmental parameters like day-length, temperature and relative humidity are important factors to determine the variation in semen production, its quality profiles and fertilizing ability of the sperm. Therefore, a study was designed to assess MT effect on mobility and velocity parameters by computer assisted sperm analyser (CASA) at different seasons in mithun. Total of 80 ejaculates (20 ejaculates per season) were collected from mithun twice per week by transrectal massage method and ejaculates were grouped into six equal, processed and diluted with standard Tris-egg yolk-citrate-glycerol-extender (TEYCGE). Gr1: extender without additives (control), Gr 2 to 6: extender extended with MT 1, 2, 3, 4 and 5 mM, respectively. CASA profiles were measured in frozen thawed semen. Addition of MT in the extender has significantly (p<0.05) minimized the static velocity or dead spermatozoa, simultaneously significant improvement was in sperm total motility (TM), forward progressive motility (FPM), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and rapid velocity in treatment as compared to control group at different seasons of the year. The present study result clearly indicated that melatonin protected the post thaw CASA parameters in different seasons and also semen quality varied from different seasons and was significantly higher in spring and lowest was in summer season.

Keywords: Melatonin, Mithun, semen, CASA parameters, cryopreservation

1. Introduction

In India, North-Eastern Hilly (NEH) region has great cultural diversity and vast natural resources among the different states. Mithun is a magnificent beautiful, unique domestic cattle species among the hilly livestock species and is primarily used for meat production. Mithun is an economically important species in states of NEH region. Mithun is affected with severe non-cyclical fluctuations, however, still is not yet endangered according to the livestock censuses of Government of India (Livestock Census, 2012). Based on the available research studies, it is clearly indicated that mithun severely affected from various problems

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like intensive inbreeding depression and lack of proper breeding bulls and breeding management programmes (Dhali et al., 2008). Mithuns are maintained under extensive free-range system with available natural breeding practices as preferred method with limitation of disease transmission control, inbreeding depression, poor body confirmation of bulls in field condition and leads to loss of productive as well as reproductive performances in future generations. These limitations of natural breeding could be easily controlled by proper planning, semen collection, cryopreservation and artificial insemination under field condition.

Semen production and its quality profiles were affected by various factors like vaccination (Perumal et al., 2013a; Perumal et al., 2013b; Perumal, 2014a; Perumal, 2014b; Perumal, 2018a), seasons (Perumal et al., 2015; Rajoriya et al., 2016; Perumal et al., 2017a; Perumal et al., 2017b), pathological condition of reproductive system (Perumal, 2013; Perumal et al., 2013c; Perumal et al., 2016a), inclusion of additives (Perumal et al., 2012a; Perumal et al., 2012b; Perumal et al., 2013e; Perumal, 2019), glycerol (Baruah et al., 2016) and hereditary and congenital causes (Perumal et al., 2012c) are. Photoperiod, temperature and relative humidity are the most important factors influence the semen production, its quality parameters and fertilization capacity (Perumal et al., 2017a). Thermo tolerance and tolerance on disease infection of the indigenous cattle (*Bos indicus)* is significantly higher exotic bovine species like *Bos taurus* as characterized by reduced percentage of total sperm abnormalities and higher progressive motility (Rajoriya et al., 2013). Higher semen production and its quality were reported during spring season in different species of livestock. Higher THI (Humid hot season) is not acceptable for highly mobile and fertile semen production. Buffalo bulls ejaculate good quality semen during winter and also in spring season, however, zebu (*Bos indicus*)bulls release ejaculate with higher sperm motility during spring and summer seasons (Rajoriya et al., 2013). Effect of season on freezability of semen clearly demonstrated that season has considerable effect on sperm post-thaw motility and were considerably highest in samples collected and cryopreserved during winter and lowest was in summer season in buffaloes (Sagdeo et al., 1991).

Mammalian semen contains antioxidants to protect the sperm from harmful free radicals and reactive oxygen species (Perumal et al., 2013f; Perumal et al., 2014c). However, the level of these antioxidants is reduced during the different stages of extension and preservation in liquid and in frozen stage (Kumar et al., 2011; Perumal, 2017). Therefore, addition of antioxidants (Perumal et al., 2013f; Perumal, 2014c) or dietary supplementation of the natural and/or synthetic antioxidants (Jayaganthan et al., 2013; Jayaganthan et al., 2015) are needed to minimize the harmful effects of oxidative as well as cryo stress in different process of semen cryopreservation (Perumal et al., 2011a; Perumal et al., 2011b). MT is one among the antioxidants, derivatives of natural indole endogenous compound secreted and released by the endocrine pineal gland rhythmically in brain and acts in circadian clock regulation and also seasonal reproduction in different species (Reiter, 1991). It has multiple functions on different physiological mechanisms. MT and its metabolites are also the potential powerful antioxidants and also powerful direct free radical scavengers (Reiter et al., 1998). Melatonin is the universal and multifunctional antioxidants unlike other free radical scavengers studied (Tomas-Zapico and Coto-Montes, 2005). MT is an amphophilic nature (soluble in water and lipids). Further, melatonin also induces the enzymes related with metabolising free radicals as well as reactive oxygen species which inturn stabilize the cell membrane fluidity with sperm of higher fertility rate. MT has scavenging capacity as twice as vitamin E in scavenging the peroxyl radicals (Pieri et al., 1994) and also it has higher effective function on removing the hydroxyl radicals than other standard antioxidant like reduced glutathione or mannitol (Hardeland et al., 1993). MT also minimizes or prevents the *in-vitro* sperm capacitation and inhibits apoptotic like changes indicates MT has a direct action on spermatozoa to protect from free radicals. Addition of MT in the semen preservation extender has been reported in different species like in ovine (Ashrafi et al., 2011), porcine (Hyun-Yong et al., 2006), bovine (Ashrafi et al., 2013) and mithun (Perumal et al., 2013d) was reported indicates it protects the sperm against the adverse effects of ROS or free radicals and showed significant improvement in the sperm motility, intactness of membrane and ferility in the frozen thawed sperm.

Analysis of literatures revealed scanty information available on effect of MT on the CASA parameters during ultralow temperature cryopreservation of mithun semen during different seasons. Therefore, this study was designed with the objectives of to assess the melatonin effect on post thaw CASA parameters at different seasons in mithun to pursuit semen collection and preservation programme in future.

2. Materials and Methods

2.1. Experimental animals

Eight healthy adult mithun bulls of 4 to 6 yr of age with good body condition (score 5-6) were selected from the herd of mithun derived from various hilly tracts of the NEH region of India and maintained under uniform feeding, housing and lighting conditions in ICAR-National Research Centre on Mithun, Medziphema, Nagaland, India. Semen was collected from the mithun bulls through trans-rectal massage method. In shortly, seminal vesicles were massaged centrally and backwardly for 5 min followed by the gentle milking of ampullae one by one for 3–5 min, which resulted into erection and ejaculation (Palmer et al., 2004). An assistant collected the semen as it was emitted from the preputial orifice into a plastic bag suspended in a thermos containing warm water at the bottom, resulting in a temperature of 35-38ºC in the plastic bag. During collection, the initial transparent secretions

were discarded and neat semen drops were collected in a graduated test tube with the help of a funnel.

2.2. Semen collection and processing

The collection seasons were divided into four based on the temperature humidity index viz. spring (February to April; THI: 63.51±1.85), summer (May to July; THI: 76.06±1.74), autumn (August to October; THI: 74.00±1.77) and winter (November to January; THI: 54.41±1.09).A total numbers of 40 ejaculates (10 ejaculates from each season) were collected from the mithun. Immediately after collection, the samples were kept in a water bath at 37 °C and evaluated for volume, colour, consistency, mass activity and pH. After the preliminary evaluations, samples were subjected to the initial dilution with pre-warmed (37 °C) Tris egg yolk citrate glycerol extender (TEYCGE). The partially diluted samples were then brought to the laboratory in an insulated flask containing warm water (37 °C) for further processing.

Each ejaculate was split into six equal aliquots and diluted with the TEYCG extender with melatonin. Gr1: semen without additives (control), Gr2 to 6: semen with 1 mM, 2 mM, 3 mM, 4 mM and 5 mM of melatonin, respectively. However, pH of diluents was adjusted to be 6.8–7.0 by using phosphate buffer solution. Diluted semen samples of control and treatment groups were cooled simultaneously from 37 to 5 °C at a rate of 0.2 - 0.3 °C per min in a cold cabinet (IMV, L'Aigle, France) and maintained at 5 °C for 2 h. Polyvinyl chloride (PVC) straws (0.5 mL) (IMV, L'Aigle, France) were filled and maintained in a cold cabinet at 5 °C for 2.5 h. Subsequently, these straws were wipe-cleaned, dried and spread over the freezing rack. The rack containing straws was kept in a biological programmable freezer for freezing (final temperature maintained at -124 °C, 12 min) followed by plunging of straws into the liquid nitrogen (-196 °C) and was stored therein. At the time of evaluation, the stored semen straws were taken out of the cryo-cans and thawed in water bath at 37 °C for 30s.The CASA parameters such as TM, FPM, VAP, VCL, VSL, ALH, BCF, STR, LIN and rapid & static velocity of sperm were measured as per standard procedure.

2.3. Computer assisted sperm analysis (CASA)

The sperm concentration and motility parameters were evaluated by Hamilton Thorne Sperm Analyser, version IVOS 11 (HTM-IVOS, Version 10.8, Hamilton Thorne Research, Beverly, MA, USA). This CASA system consists of a phase-contrast microscope, camera, mini-therm heating stage, image digitizer and computer saving & analyzing the data. The software settings are shown in the following Table 1.

After semen collection, the sperm concentration was first estimated using a microscope. 25 µL of semen was diluted into 50-100 µL of Tris (formulated for bull semen) and 5 µL of this diluted semen was loaded into a pre-warmed dual chamber disposable Leja slide and was allowed to settle on the mini-therm heating stage (38 °C) before the analysis.

Table 1: Software settings of HTR IVOS 2 used in the study

Parameters	Value
Chamber type	Leja 4
Temperature of analysis (°C)	37.0
Fields acquired	10
Frame rate (Hz)	60
No of frames	30
Minimum static contrast	35
Minimum cell size (pixels)	5
Straightness (STR), thresholds (%)	70
VAP cut–off (µm s^{-1})	30
Progressive minimum VAP (µm s^{-1})	50
VSL cut-off (µm s^{-1})	15
Cell intensity	80
Magnification	1.89

The following parameters were measured: concentration (Con), percentage of total motile spermatozoa (TM), percentage of spermatozoa with a forward progressive motility (FPM), velocity average pathway (VAP), velocity straight line (VSL) (VSL, um/s; VSL in mm/s is the average path velocity of the spermatozoa head along a straight line from its first to last position), curvilinear velocity (VCL) (VCL in mm/s is the average path velocity of the spermatozoa head along its actual trajectory), amplitude of lateral head displacement (ALH in mm/s is the average value of the extreme side-to-side movement of the spermatozoa head in each beat cycle), beat cross-frequency (BCF, Hz), straightness (STR, ratio of VSL/VAP, (%)) and linearity of the curvilinear trajectory (LIN, (%), LIN is the ratio between VSL and VCL).

According to the low VAP cut-off and medium VAP cut-off, the sperm population was additionally divided into four categories: Rapid, Medium, Slow, and Static. A minimum of 200 spermatozoa from at least two different drops of each sample were analyzed from each specimen. The number of objects incorrectly identified as spermatozoa were manually removed and final analysis was done for each sample.

2.4. Statistical analysis

The statistical analysis of the data was performed as per standard procedures (Snedecor and Cochran, 1994). Analysis of variance (ANOVA) was performed using a generalized liner model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001) and treatment means were separated using Student–Newman-Keuls (SNK) multiple range test. Tables present the non-transformed data. The data used in the study were tested for normality before analysis using Shapiro–Wilk statistics. Means were analyzed by two way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences between the different seasons with treatment or without treatment on

these sperm parameters using the SAS software /PC computer program. The per cent data were subjected to arcsine (angular) transformation before proceeding to general linear model. Differences with values of $p<0.05$ were considered to be statistically significant after arcsine transformation of percentage data.

3. Results and Discussion

Effects of melatonin on CASA parameters such as TM (Table 2), FPM (Table 3), rapid and static velocity (Table 4), VAP (Table 5), VSL (Table 6), VCL (Table 7), ALH (Table 8), BCF (Table 9), STR (Table 10) and LIN (Table 11) in the frozen (-196 ^{o}C) thawed semen from different seasons were presented in tables. The TM and PFM were significantly ($p<0.05$) higher in MT added group as compared to untreated control group (Table 1). Furthermore, MT 3 mM included samples have significantly ($p<0.05$) higher velocity and motility parameters. Proportionally the motility parameters were significantly ($p<0.05$) higher in spring season in the MT3mM treated group. Out of the five groups of MT treated, MT 4mM and MT 5 mM has significantly reduced TM and PFM. The motility parameters were increased gradually and significantly ($p<0.05$) from control to MT 3mM group and then reduced in the MT 4 and MT 5 groups. The proportion of reducing TM and PFM were higher in MT 4 mM and MT 5 mM treated group as compared to other MT treated groups.

In the present experiment, rapid velocity revealed that MT included group has significantly higher percentage than untreated control group (Table 2). MT 3 mM 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 in summer season. The rapid velocity was positively and significantly correlated with PFM in all the experimental groups. On the other hand, static velocity was significantly lesser in spring and winter than in season and in MT 3mM treated group followed by 2 mM and 1 mM whereas it was increased in MT 4 and 5 mM treated group. The result revealed that there was a no significant difference among the experimental groups and among the seasons for VAP and VCL. For VSL, significant difference was observed between the experimental groups in spring season and between control and MT 1mM for different seasons. However, non-significantly higher values were observed in MT 3 mM than other MT treated and untreated control groups.

The result of ALH revealed that there was a significant ($p<0.05$) difference among the experimental groups in summer season and control, MT 1, 4 and 5 groups revealed significant

Table 2: Mean (±S.E.) total motility of post thawed mithun semen treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	39.15 ±1.98^{abBC}	41.25±1.79^{bcC}	33.80 ±2.06^{abA}	37.30±2.02^{bcdB}
MT 1 mM	40.70±1.73^{bcB}	42.05±1.88^{Cb}	35.70±2.10^{bcA}	36.60±1.91^{abcB}
MT 2 mM	42.60±1.69^{cB}	43.50±1.71^{cdB}	37.45±2.21^{bcA}	38.80±1.65^{cdA}
MT 3 mM	43.50±1.77^{cB}	45.70±1.62^{dB}	39.15±1.66^{cA}	40.25± 1.81^{dA}
MT 4 mM	37.55±2.22^{abAB}	38.40±1.86^{abB}	34.75±1.95^{abA}	35.30±1.80^{abAB}
MT 5 mM	36.55±1.96^{aBC}	37.80±1.87^{aC}	31.25±2.00^{aA}	33.50±1.85^{aAB}

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 (±S.E.) forward progressive motility of post thawed of mithun semen treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	19.50±2.55^{bB}	19.60 ±2.47^{abB}	13.50±1.79^{aA}	15.75±2.50^{abAB}
MT 1 mM	19.55±2.93^{bB}	20.25±2.43^{abB}	14.40±2.27^{abA}	16.15±1.76^{abAB}
MT 2 mM	20.25±2.41^{bB}	21.70±2.50^{bB}	15.85±2.19^{abA}	18.70±1.58^{bcAB}
MT 3 mM	21.95±2.17^{bB}	22.55±2.48^{bB}	17.05±2.33^{bA}	20.95±2.31^{cAB}
MT 4 mM	17.15±2.42^{abAB}	19.65±2.07^{abB}	14.70±1.91^{abA}	16.35±2.25^{abAB}
MT 5 mM	12.95±1.73^{aAB}	15.45±1.89^{aB}	13.50±1.96^{aAB}	12.50±1.91^{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 4: Mean (±S.E.) different degree of velocity of post thawed mithun sperm treated with melatonin at different seasons

Degree of Velocity	Additives	Seasons			
		Winter	Spring	Summer	Autumn
Rapid	Control	31.80±2.79^{abcAB}	33.90±2.43^{abB}	26.85± 2.61^{abA}	27.20± 2.41^{abA}
	MT 1 mM	34.20±2.42^{bcC}	34.85±3.37^{abB}	27.15± 2.56^{abA}	30.90±2.42^{bcAB}
	MT 2 mM	35.45±2.36^{cB}	35.85±2.57^{abB}	27.95± 2.50^{abA}	31.15±2.59^{bcAB}
	MT 3 mM	35.55±2.96^{cAB}	38.15± 2.34^{bB}	30.85± 2.56^{bA}	34.70± 2.18^{cAB}
	MT 4 mM	28.80±2.86^{abAB}	32.10±3.54^{abB}	26.90± 2.41^{abA}	27.40± 2.69^{abA}
	MT 5 mM	26.60±2.42^{aAB}	31.10 ±2.70^{aB}	23.65±2.36^{aA}	24.15±3.01^{aA}
Static	Control	39.25±3.58^{ab}	37.60±3.87	47.55±3.44^{ab}	40.50±4.80
	MT 1 mM	37.30±3.98^{abAB}	34.55±3.39^{A}	43.75± 3.11^{abB}	38.15±3.92^{AB}
	MT 2 mM	34.95±3.39^{aA}	34.70±3.20^{A}	43.45± 3.94^{abB}	37.40±2.99^{AB}
	MT 3 mM	39.65±3.89^{ab}	34.40±3.32^{A}	41.85± 3.68^{a}	36.85±3.48
	MT 4 mM	39.55±3.89^{abA}	35.35±3.98^{A}	51.00± 2.82^{abB}	45.85±3.14^{AB}
	MT 5 mM	45.70±3.08^{bAB}	39.50±3.66^{A}	52.40±2.79^{bB}	46.80±3.09^{AB}

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 5: Mean (±S.E.) average path velocity (VAP) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	97.06±4.35	98.24±5.79	90.38±4.07	94.20±5.37
MT 1 mM	99.63±4.54	101.07±4.27	91.43±3.78	95.70±4.20
MT 2 mM	100.40±6.29	101.25±4.59	93.83±5.59	96.94±4.47
MT 3 mM	101.10±3.60	102.62±4.24	94.74±5.37	98.68±4.87
MT 4 mM	94.37±4.85	102.11±3.85	91.46±4.56	91.70±4.53
MT 5 mM	88.62±5.35	93.13±4.12	83.62±4.54	86.24±3.63

Table 6: Mean (±S.E.) straight line velocity (VSL) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	70.82±4.03^{AB}	75.40±4.20^{abB}	62.34±3.59^{A}	68.24±4.83^{AB}
MT 1 mM	74.56±4.31^{AB}	75.99±3.64^{abB}	64.69±3.82^{A}	68.53±3.66^{AB}
MT 2 mM	74.64±3.85	76.13±4.09^{ab}	66.09±4.48	69.30±3.95
MT 3 mM	76.63±6.16	79.50±3.94^{b}	68.33±4.94	70.91±4.03
MT 4 mM	70.28±5.26	74.71±4.19^{ab}	64.10±4.21	66.20±4.14
MT 5 mM	61.97±3.61	66.78±3.77^{a}	57.07±4.18	59.89±4.72

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)

difference between the seasons. In different seasons, MT 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 winter season and MT 1 and MT 2 showed significant (p<0.05) difference among the seasons.

Percentage of straightness revealed that there was a significant difference among the experimental groups at different seasons, but between the experimental groups,

Table 7: Mean (±S.E.) curve linear velocity (VCL) of post thawed mithun treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	171.53±6.19	179.10±5.55	169.83±6.67	169.55±6.33
MT 1 mM	180.95±7.00	186.64±4.14	170.64±6.98	178.15±6.19
MT 2 mM	185.90±5.99	193.13±5.63	174.34±6.25	178.60±6.57
MT 3 mM	193.74±4.95	196.04±4.32	176.60±5.28	182.65±6.90
MT 4 mM	179.12±4.74	181.55±5.93	171.11±5.72	176.12±6.22
MT 5 mM	172.54±6.44	175.74±7.04	164.83±5.67	165.10±5.02

Table 8: Mean (±S.E.) amplitude of lateral head displacement (ALH) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	8.38±1.44^{AB}	9.43±1.82^{B}	7.70±1.12^{abAB}	7.46±1.31^{A}
MT 1 mM	8.79±1.42^{AB}	9.55±2.01^{B}	7.77±1.22^{abA}	8.13±1.31^{AB}
MT 2 mM	9.83±2.09	11.80±3.81	8.03±1.09^{ab}	8.18±1.22
MT 3 mM	9.18±1.97	11.82±3.93	8.25±1.29^{b}	8.29±1.45
MT 4 mM	8.44±0.98^{AB}	9.44±1.81^{B}	7.61±1.13^{abA}	8.08±1.32^{AB}
MT 5 mM	8.24±1.35^{AB}	9.32±2.04^{B}	7.09±1.31^{aA}	8.03±1.24^{AB}

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 9: Mean (±S.E.) beat cross frequency (BCF) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	26.89±2.49^{a}	27.17±2.39	24.15±2.05	25.98±2.22
MT 1 mM	28.06±2.38^{abAB}	29.16±2.00^{B}	24.67±2.13^{A}	26.24±2.22^{AB}
MT 2 mM	28.84±2.31^{abAB}	30.89±2.11^{B}	25.26±2.13^{A}	26.63±2.46^{A}
MT 3 mM	30.96±2.49^{b}	32.63±5.21	25.88±2.35	27.10±2.24
MT 4 mM	26.93±2.13^{a}	27.95±2.51	24.70±2.64	26.06±2.45
MT 5 mM	26.33±2.17^{a}	26.98±2.17	24.80±2.95	25.52±2.23

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)

significant difference was observed in the autumn season. Similar to STR, LIN was significantly (p<0.05) differed among the seasons in control and MT 1 (Table 5) and significant difference was observed between the experimental groups in spring, summer and autumn seasons. The WOB revealed that there was a no significant difference was observed among the experimental groups in different seasons and between the seasons.

The results of present study revealed that inclusion of MT in the semen extender has enhanced the motility and velocity parameters of sperm in mithun. Available literature revealed that scanty reports available on addition of MT on CASA parameters in the mountain cattle species and also it is clearly stated that this is the first report on post thaw CASA parameters as per our knowledge. Previous workers in other species observed that significantly higher benefit in MT treated and preserved sperm in refrigerated condition, which has enhanced the velocity and mobility parameters in the current 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., 2013f; Perumal et al., 2015; Perumal, 2019). Season or temperature relative humidity index is one of the pivotal

Table 10: Mean (±S.E.) straightness (STR) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	71.45±2.51^{B}	71.90±2.65^{B}	66.15±2.12^{A}	69.45±2.69^{abAB}
MT 1 mM	71.60±3.00^{AB}	73.57±2.48^{B}	66.95±2.11^{A}	69.90±2.63^{bAB}
MT 2 mM	72.90±2.60^{AB}	75.10±2.74^{B}	67.70±3.02^{A}	70.40±2.25^{bAB}
MT 3 mM	73.10±2.25^{AB}	75.20±2.47^{B}	68.80±2.66^{A}	70.55±2.59^{abAB}
MT 4 mM	70.30±2.59^{AB}	71.80±3.06^{B}	66.25±2.29^{A}	67.55±2.60^{bAB}
MT 5 mM	69.40±2.53^{AB}	70.30±2.21^{B}	64.80±2.63^{A}	65.30±2.68^{aAB}

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 11: Mean (±S.E.) linearity (LIN) of post thawed mithun sperm treated with melatonin at different seasons

Additives	Seasons			
	Winter	Spring	Summer	Autumn
Control	40.55±2.29^{B}	40.60±2.56^{abB}	35.65±2.01^{abA}	37.65±2.30^{abAB}
MT 1 mM	41.50±2.96^{AB}	43.60±2.52^{abB}	36.80±2.16^{abA}	40.00±2.79^{abAB}
MT 2 mM	42.25±2.94	44.15±2.48^{ab}	40.05±2.33^{b}	40.40±2.20^{b}
MT 3 mM	42.70±2.90	44.90±3.00^{b}	40.10±3.20^{b}	40.80±2.54^{b}
MT 4 mM	41.10±2.56	43.05±3.06^{ab}	38.45±2.44^{ab}	39.65±2.62^{ab}
MT 5 mM	39.35±2.59	39.30±2.32^{a}	35.25±2.74^{a}	35.85±2.67^{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)

parameter which has capacity to alter the difference in CASA motility & velocity parameters and fertilization rate. In the present study, seminal mobility and velocity profiles were significantly influenced by seasons. Indigenous cattle (*Bos indicus*) have higher heat tolerance and disease resistance capacity as compared to exotic (*Bos taurus*) bulls expressed as lower percentage of total sperm abnormalities in their semen ejaculates (Rajoriya et al., 2013).

MT has expressed its functionality in dose depended method (Casao et al., 2010; Perumal et al., 2013f; Perumal et al., 2015) as like 3 mM MT is optimum and most suitable dosage in mithun semen preservation. In boar, it was reported similarly that MT had stimulated the spermatozoa to a hyperactive functional state (Martin-Hildago et al., 2011) as because it has elevated ATP synthesis and promotion of mitochondrial complex efficiency to a higher level for ATP production and energy utilization (Martin et al., 2000). MT has increased the velocity and mobility parameters because of its interaction with second messenger calmodulin in the sperm in the current study (Benitez-King and Anton-Tay, 1993) and which inturn induce the cytoskeletal structures of sperm leads to increased sperm velocity and motility profiles MT is not only antioxidant but also antiapoptotic in semen preservation extender and it protects the sperm against free radicals generation, caspase-3 and -9 activities, phosphatidylserine externalization, apoptosis and finally sperm death (Espino et al., 2011) and also it protects the sperm mitochondrial function for energy production for progress in forward direction.

Summer months cause thermal as well as hot stress to the mithun bulls resulted significant harmful effect on Leydig cell function caused reduction of testosterone production and accessory sex glands function mainly epididymis and seminal vesicles causes production of antioxidants are decreased from epididymis (cauda; Fouchecourt et al., 2000) and seminal vesicle (Tramer et al., 1998)as because the epididymis, accessory sex glands are thermo sensitive and androgen dependent (Saeed et al., 1994).

MT improved the ATPase production (Chen et al., 1994), source energy for the sperm and for the sperm movement to activate sperm motility and velocity profiles (Burger et al., 1991). MT also induces cellular influx of $Ca^{2+}$for enhancing sperm motility (Delgadillo et al., 1994). Further, Si (1997) observed that $Ca^{2+}$is responsible for regulation of the flagella movement & forward motility and calmodulin, a second messenger have been detected in spermatozoa and flagella of the sperm for sperm movement (Tash and Means, 1983). Similarly, Ahmad et al. (1996) reported that calmodulin antagonist caused a decrease of VCL and ALH and functional membrane potential of mitochondria. MT also has acts on the cAMP (Yung et al., 1995) causes higher stimulation on the

velocity (Lindamann, 1978) and also acts on the secondary messenger, calmodulin (Garbers and Kopf, 1980).

Final data of the present study indicated that addition of MT @ 3 mM in the extender has increased mobility and velocity profiles of mithun sperm cryopreserved at -196 °C. Whereas, on the other hand, reducing rate of motility and velocity rate were increased significantly in the semen samples treated with MT4 to 5 mM or without MT, control group. However, addition of MT3 mM caused increased the velocity and mobility profiles as compared to untreated control group (Du Plessis et al., 2010). MT various effects on various semen quality parameters were described by various authors (Ashrafi et al., 2011; Shoae and Zamiri, 2008; Perumal et al., 2013f; Perumal et al., 2015), revealed the excessive amount of MT than threshold concentration causes higher plasma membrane fluidity, creating the environment as it damages to the plasma as well as the acrosomal membrane and also addition of higher dosage causes deleterious effect on spermatozoa as because it alters the physiological as well as physical function of the diluent. However, the concentration of antioxidant is higher than optimum amount is deleterious and toxic to spermatozoa (Maxwell and Watson, 1996; Perumal et al., 2013f; Perumal et al., 2015). Similarly, reduced concentration also influenced the sperm quality parameters and structures at cryopreserved semen. Therefore, according to the present study, mobility and velocity profiles were increased gradually reached maximum to 3 mM then decreased to 5 mM.

In the previous study, addition of exogenous MT in the semen extender has improved semen quality profiles such as sperm motility, intactness of acrosomal membrane and viability (Perumal et al., 2016b; Perumal, 2019), same kind of results was also observed in other studies (Casao et al., 2009; Ashrafi et al., 2011) and by previous researchers in different livestock species, bull (Ashrafi et al., 2013), mithun (Perumal et al., 2013f; Perumal et al., 2015, Perumal, 2019) and boar (Hyun-Yong et al., 2006). MT also protects sperm plasma membrane, mitochondrial membrane intactness, intactness of acrosomal membrane and functional structure of flagella of sperm and cytoskeleton structure as cell protecting effects in the sperm (Leon et al., 2005).

MT also protects and stimulates various functional enzymes of antioxidant such as superoxide dismutase, glutathione and catalase (Karbownik and Reiter, 2000), which maintains the intactness of the sperm membrane and membrane transportation process (Alvarez and Storey, 1992) and finally the fertilizing capacity of the sperm cells. As it was reported that it reduces indirectly the free radicals and ROS generation and it enhances the generation of sperm protecting components against oxidative and peroxidative stresses. The velocity and motility profiles of sperm were increased significantly through these various mechanisms by MT in the present study. From this study, it was concluded that supplementation of MT in the extender has enhanced the CASA parameters MT at 3 mM.

4. Conclusion

Significantly higher CASA profiles are due to MT inhibits generation of ROS as a potent antioxidant in dose dependent manner and enhanced fertility at 3 mM. Melatonin effect on CASA profiles was significantly higher in spring than in summer season. In conclusion, MT in extender has enhanced mobility and velocity parameters assessed by CASA. Semen collection and preservation need to conduct during winter and spring season and MT at 3 mM is more suitable and optimum for mithun semen cryopreservation.

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