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Administration of slow release exogenous melatonin modulates oxidative stress profiles and *in vitro* fertilizing ability of the cryopreserved mithun (*Bos frontalis*) spermatozoa

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ABSTRACT

Mithun (*Bos frontalis*) is a unique domestic free range bovine species of North Eastern hilly regions of India. The present study was designed to assess the seasonal effect of slow release exogenous melatonin (MT) implant on semen quality parameters (SQP) and *in vitro* zona binding ability (IVZ) of spermatozoa. The experimental animals were divided into Gr I: Control (n = 5) and Gr II: Treatment (n = 5; melatonin implant @ 18mg/50 kg bwt). A total of 20 semen samples/group in winter, spring, autumn and summer seasons (n = 160), twice per week were collected. Following cryopreservation, samples were evaluated for motility parameters (forward progressive, mobility & velocity by computer assisted sperm analyser (CASA), viability, acrosome integrity, plasma membrane and nuclear abnormality, functional status of mitochondria, enzymatic, antioxidant and oxidative profiles, and IVZ. The study revealed significant ($p < 0.05$) improvement in total motility, viability, acrosome-, plasma membrane-, and nuclear-integrity, and antioxidant profiles; with highest values in spring and lowest in summer season in the fresh semen in Gr II than the Control. A significant ($p < 0.05$) improvement in motility parameters, membrane potential of mitochondria, antioxidant profiles and reduction in sperm and nuclear abnormalities, leakage of intracellular enzymes and oxidative stress and IVZ index & binding percentage in post-thaw semen samples in melatonin supplemented than in un-supplemented control group was observed. It can be concluded from the study that slow-release melatonin supplementation can be effectively utilized to improve the antioxidant profiles and reduction of oxidative stress, with cascading beneficial effects on semen quality parameters and fertility status of the mithun bull.

1. Introduction

North-Eastern Hilly region of India has vast natural resources and great cultural diversity. Among the livestock of NEH region, mithun is an unique domestic bovine species, primarily used for meat production, and is considered an economically important species of the region. Mithun is not yet endangered, but suffers from severe non-cyclical population fluctuations on a national basis as per the livestock censuses of Government of India [1]. Several studies on reproductive pattern revealed mithun population suffers from intensive inbreeding and lack of suitable breeding bulls & breeding management system [2]. Mithuns are reared under extensive free-range system with natural service as preferred breeding practice which has limitation of disease transmission, inbreeding depression, poor body confirmation of the calves born, and therefore loss of productive and reproductive performances. The limitations of natural services could be easily overcome by following artificial insemination (AI) under field condition.

There are various studies on semen collection, preservation and field fertility trials in mithun species conducted with varied success rate. These investigations covered semen collection methods [3,4], storage of liquid semen at 4 °C for 72 h using Tris–egg yolk diluent [5], cryopreservation of semen [6–8], effect of additives such as reduced glutathione [9], bovine serum albumin [10], low density lipoprotein [11] instead of 20% egg yolk on seminal and biochemical profiles of preserved mithun semen. Further, Perumal et al. [12,13]

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identified the spring and winter as suitable seasons for collection and preservation of mithun semen. Though studies point to detrimental effects of free radicals on semen quality parameters (SQPs), and fertility of spermatozoa [14], perusal of available literature on similar line of investigation reveal insufficient information in mithun species.

Melatonin (N-acetyl-5-methoxytryptamine with MW = 232), an indoleamine, synthesized from tryptophan via serotonin, and secreted by the pineal gland in the brain [15], is responsible for regulating the circadian rhythm [16] and seasonal reproduction in mammals [17]. Melatonin appears to be involved in the regulation of gonadal function by influencing the hypothalamic-pituitary-gonadal axis. Animal studies indicate that melatonin can modify the firing frequency of the hypothalamic gonadotropin-releasing hormone pulse generator, thereby altering the release of gonadotropins (luteinizing hormone and follicle-stimulating hormone) from the anterior pituitary [18] and stimulating testicular testosterone production and release [19]. There was also evidence from clinical studies that a relationship existed between melatonin and male reproductive hormones [20]. Earlier studies demonstrated that melatonin and its metabolites are indirect antioxidants and powerful direct scavengers of free radicals [21] besides its multiple actions on various physiological functions. Melatonin is also multifunctional and universal unlike other scavengers which are function-specific [22]. Melatonin is an effective antioxidant because of its bio-physical (amphophilic, soluble in water as well as in lipids) and functional properties, which make it an effective antioxidant. Owing to its bio-physical property, melatonin acts as a hydrophilic and hydrophobic antioxidant. Since melatonin stimulates the enzymes responsible for metabolising ROS (reactive oxygen species), and thereby helps in preservation of the cell membrane fluidity and integrity; this functional property makes it an effective anti-oxidative defense mechanism in both *in vitro* and *in vivo* situations [23]. Melatonin directly scavenges the highly toxic OH^- to form cyclic 3-hydroxymelatonin (3-OHM), a stable metabolite of melatonin [24]. Melatonin has potency as twice as vitamin E in removing peroxyl radicals [25] and is more effective on scavenging of hydroxyl radicals than glutathione and mannitol [26]. Accumulated reports from earlier studies show melatonin as an additive in semen extender to protect sperm of ram, boar, bull and mithun against the harmful effects of ROS during liquid or in frozen state for extended period of time [27–31]. Thereafter, application of slow-release melatonin implant was shown to significantly improve fertility parameters in both male and female livestock species (sheep: [32], rodents: [33], cattle: [34], goat: [35], buffalo: [36], cat [37]) by improving the reproductive performance in general and sperm & oocyte quality in particular.

Earlier, significant improvement in the SQPs, antioxidant profiles and reduction of intracellular enzymes leakage when melatonin was used as an additive in semen extender in mithun was reported [12,27,38,39]. Melatonin has short half-life (<30 min) and its application in injectable form such as intramuscular injection for quick release does not justify its intended use [40]. In animal models, any beneficial effect on protracted semen production could only be achieved by continuous release of melatonin in the system for the sperm production is a protracted process (the period required to form the sperm from spermatogonium A is 59–60 days in bulls, 38 days in buffalo bulls, 47 days in ram and 55 days in stallion). Therefore, it was hypothesized that application of slow-release subcutaneous melatonin implant (with oil base or bee wax base) could be more beneficial on *in vitro* sperm functional parameters and zona binding profiles in mithun. With this, the objective of the present study was to assess the effect of slow-release subcutaneous melatonin implant on SQPs, oxidative stress profiles and *in vitro* fertility indexes of the cryopreserved mithun semen.

2. Materials and methods

2.1. Location of the study

The proposed study was conducted at the mithun breeding farm, ICAR-National Research Centre on Mithun, Medziphema, Nagaland, India. It is located between 25°54′30″ North latitude and 93°44′15″ East longitude, at an altitude range of 250–300 m above mean sea level. The temperature humidity index (THI) during the period of study was calculated from meteorological factors such as ambient temperature and relative humidity values obtained from the meteorology station of ICAR Research Complex for NEH region, Nagaland Centre, located at close proximity of the experimental station. Experimental animals were kept in semi-intensive system of rearing. Seasons wise THI was calculated for five whole calendar years, which formed basis for the division of the year in to four season 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). Temperature Humidity Index was estimated by applying the following formula [41], $THI = 0.72 (W + D) + 40.6$, where W stands for wet bulb temperature (°C) and D for dry bulb temperature (°C).

2.2. Experimental animals

Ten apparently healthy (body condition score 5–6, classified as good) mithun bulls of 4–6 years of age were selected. The average body weight of the bulls was 510 kg (495–520 kg). Experimental animals were maintained under uniform feeding (farm schedule), lighting, housing and other management conditions. Experimental animals were offered *ad libitum* potable drinking water, 30 kg mixed jungle forages (18.40% and 10.20% dry matter and crude protein, respectively) and 4 kg concentrates (87.10% and 14.50% dry matter and crude protein, respectively) fortified with mineral mixture and salt.

2.3. Experimental groups

The experimental animals (n = 10) were selected and divided into two groups, Gr I: Control (n = 5) and Gr II: Treatment (n = 5; melatonin implant @ 18mg/50 kg b.wt). The selection of the experimental bulls was based on the previous history and records of semen production and SQPs; with non-significant variation in their semen production and its quality profiles over a period of the time. As melatonin implants are not available in India [42,43]. Crystalline melatonin powder (analytical grade, Sigma Chemicals, USA) was dissolved in refined corn oil (Coronola containing refined corn oil, Sangrur Agro Ltd., Sangrur, Punjab, India) in quantities sufficient to make a final concentration of 18 mg/mL at room temperature. Once dissolved, the suspension was used on the same day [36,43,44]. Control animals were administered the corn oil as placebo.

The blood samples were collected by venipuncture of jugular vein in heparin tubes (20 IU of heparin/mL of blood) from the experimental mithun bulls at 0400 h interval throughout the day at 4-day interval from day −8 to 40 (day 0: day of melatonin implantation) during the different seasons. The blood samples were centrifuged at 1200 × *g* for 15 min at 4 °C. The plasma samples were separated rapidly, labelled properly and preserved at −80 °C in deep freezer for melatonin estimation. Melatonin (analytical sensitivity: 2.3 pg/ml, intra- and inter assay coefficients of variation: 10.89% and 10.85%, respectively) was estimated by RIA based diagnostic kits (Immunotech, France) by using a gamma counter (PC-RIA MAS; Stretec, Germany). We observed increase in the melatonin concentration to reach peak on day 8, which was