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Melatonin modulates daily and seasonal rhythmic secretory pattern of endocrinological profiles in Mithun bull

P PERUMAL[1✉], A K DE[1], R VIKRAM[2], M H KHAN[2], A K NAHAK[3] and P C MISHRA[3]

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

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ABSTRACT

Present study was designed to evaluate the effect of slow release exogenous melatonin on endocrinological profiles for 24h in adult mithun bulls during different seasons (winter, spring, summer and autumn). Mithun bulls (n=12) were selected and divided into two groups, viz. Gr I: Control (n=6) and Gr II: Treatment (n=6; Melatonin implant @ 18 mg/50 kg body weight). Endocrinological profiles such as follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, cortisol, thyroxine (T4), insulin like growth factor-1 (IGF-1), prolactin and melatonin (MT) were estimated at 4 h interval (8, 12, 16, 20, 24 and 4 h) for one whole day in four seasons. Melatonin treated group had significantly higher FSH, LH, testosterone, IGF-1 and MT than control group; and MT significantly reduced the level of T4, cortisol and prolactin in different seasons at different collection times. The study concludes that MT significantly improved the endocrine profiles in different seasons and collection times. MT treatment and spring season had significantly greater beneficial effects on reproduction and artificial breeding programmes in mithun species in the semi-intensive management in the present location.

Keywords: Endocrinological profiles, Melatonin, Mithun, Seasonality, 24-h profiles

Mithun is a domestic free-range bovine species of NEH region of India reared for its protein rich meat. Several studies on reproductive pattern revealed that mithun suffers from intensive inbreeding depression, and lack of suitable breeding bulls and breeding management system (Perumal 2018). Mithuns are reared under extensive free-range system with natural service as the preferred breeding practice with various limitations.These limitations of natural services could be overcome by following artificial insemination under the field condition.

Melatonin is synthesized from tryptophan via serotonin, secreted by the pineal gland in the brain and is responsible for regulating the circadian rhythm (Reiter 1993) and seasonal reproduction in mammals (Revel *et al.* 2009). Melatonin appears to be involved in the regulation of gonadal function by influencing the hypothalamic-pituitary-gonadal axis. There was also evidence from the clinical studies that a relationship exists between melatonin and male reproductive hormones (Caglayan *et al.* 2001). Thereafter, slow-release exogenous melatonin implant improved the fertility parameters significantly in both male and female animals (sheep: El-Battawy and Sckalcki 2015, cattle: Aggarwal *et al.* 2005, goat: Jimenez *et al.* 2014, buffalo: Kumar *et al.* 2015) by improving the reproductive performance. Melatonin has short half-life (<30 min) and its application in injectable form such as intramuscular or intravenous injection for quick release does not justify its intended use (Nowak and Zawilska 1998). In animal models, any beneficial effect on protracted semen production through alteration of endocrinological profiles could only be achieved by continuous release of melatonin because the sperm production is a protracted process (period required to form the sperm from A spermatogonium is 59-60 days in bulls). Therefore, it was hypothesized that application of slow-release subcutaneous melatonin implant could be more beneficial in influencing the endocrine profiles which in turn favours or enhances the semen production and its quality profiles, *in vitro* sperm functional parameters and fertility rate in mithun. Information on effect of exogenous slow release melatonin on secretary pattern and rhythmic changesof the hormones over 24 h is lacking in adult mithun for different seasons. Therefore, the present study was designed to evaluate the effect of exogenous slow release melatonin on specific characteristics of 24 h profiles of the hormones in adult mithun at different seasons and to examine their temporal relationships throughout the sampling period.

MATERIALS AND METHODS

Area of the study: Present study was conducted at ICAR-NRC on Mithun, Medziphema, Nagaland (located between 25°54”30” North latitude and 93°44”15” East longitude)

Present address: [1]ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands. [2]ICAR-National Research Centre on Mithun, Medziphema, Nagaland. [3]OUAT-College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha. ✉Corresponding author email: perumalponraj@gmail.com

and at an altitude range of 250-300 m MSL.The experiment was conducted in four seasons, viz. winter (November to January; THI: 54.41±0.28; sunshine hours:4.11±0.36), spring (February to April; THI: 63.51±0.48; sunshine hours: 4.81±0.28), summer (May to July; THI: 76.06±0.45; sunshine hours: 6.55±0.15) and autumn (August to October; THI: 74.67±0.38; sunshine hours: 6.32±0.28) with an average THI of 66.89±0.39 and sunshine hours of 5.45±0.34.

Experimental animals: Twelve apparently healthy mithun bulls of 5-6 years of age were selected for the present study. Experimental animals were maintained under uniform feeding, lighting, housing and other standard managemental practices as per the farm schedule. Each experimental animal was fed with 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 and was offered *ad lib.* potable drinking water.

Experimental groups: Experimental animals (n = 12) were randomly divided into two groups, viz. Gr I: Control (n = 6) and Gr II: Treatment (n = 6; melatonin implant @ 18 mg/50 kg b.wt.). Crystalline melatonin powder (analytical grade, Sigma Chemicals, USA) was dissolved in refined corn oil at room temperature to make a final concentration of 18 mg/mL. Once dissolved, the suspension was used immediately on the same day. Control animals were administered with corn oil as placebo via subcutaneous route. MT supplementation was done once in a month during the experimental period and blood samples were collected and the endocrinological profiles analysed for each season.

Collection of blood and estimation of hormone profiles: Blood samples were collected from the experimental mithun bulls by jugular venipuncture into heparin tubes (20 IU of heparin per mL of blood) at 4 h interval throughout the day (6 collections per day per season) during different seasons. Blood samples were centrifuged at 1200 × g for 15 min at 4°C. The plasma samples were separated, labelled and preserved in deep freezer for further analysis.

FSH (analytical sensitivity: 0.1 mIU/mL; intra- and inter-assay coefficients of variation: 6.47 and 9.54%, respectively), LH (analytical sensitivity: 13 nmol/L; intra- and inter-assay coefficients of variation: 5.15 and 8.31%, respectively) and prolactin (analytical sensitivity: 1.0 ng/mL; intra- and inter-assay coefficients of variation: 4.23 and 6.42%, respectively) were estimated using commercially available bovine ELISA kits (MyBiosource, San Diego, CA, USA) at λ 450 nm optical density in 96-well clear polypropylene microplate using microplate reader (MRC Laboratory Instruments, Holon, Israel). Testosterone (Immunotech, France; analytical sensitivity: 0.04 ng/mL; intra- and inter-assay coefficients of variation: 5.13 and 9.27%, respectively), cortisol (Immunotech, France; analytical sensitivity: 5 nM; intra- and inter-assay coefficients of variation: 5.78 and 9.66%, respectively), T4 (Immunotech, France; analytical sensitivity: 10.63 nmol/L; intra-and inter-assay coefficients of variation: 5.33 and 9.79%, respectively) and MT (LDN Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany; analytical sensitivity: 2.3 pg/ml, intra- and inter assay coefficients of variation: 10.89% and 10.85%, respectively) were estimated by radio immuno assay based diagnostic kits by using a gamma counter (PC-RIA MAS; Stretec, Germany).

Statistical analysis: The statistical analysis of the data was performed as per standard procedures. Repeated Measure ANOVA was applied using PROC Mixed of SAS 9.3.1. Software (SAS, Version 9.3.1; SAS Institute, Inc., Cary, NC) and for multiple comparison, Tukey test was applied. The mean values are expressed as mean ± SEM. Differences were considered significant if $p<0.05$. Associations between different hormones in different seasons in control and treatment were analysed for statistical significance using Pearson's correlation coefficient. If the r-value is greater than 0.50, thecorrelation is considered as large, 0.50–0.30 is considered as moderate, 0.30–0.10 is considered as small.

RESULTS AND DISCUSSION

Endocrinological profiles differed significantly among the seasons and collection times in both treatment and control groups as well as between treatment and control groups in different collection times. FSH, LH, Testosterone, T4, IGF-1 and MT concentrations were highest in spring and winter, and lowest in summer season. On the contrary, cortisol and prolactin were significantly higher in summer than winter and spring seasons. FSH, LH, testosterone, IGF-1 concentrations were higher and cortisol, prolactin and T4 were lower significantly in MT treated than in untreated control group in different seasons. Endocrinological profiles clearly revealed that these hormones have a 24 h circulating pattern in blood circulation with phase specific or time specific relationships in mithun bulls. Endocrinological profiles of mithun differed significantly among the seasons and among the times of collection within the season.

Role of MT and its metabolites in reproductive physiology or endocrinology of mithun is less studied. Although beneficial effects of exogenous MT on endocrinological profiles and reproductive performances were reported in different livestock species (sheep: El-Battawy and Sckalcki 2015, cattle: Aggarwal *et al.* 2005, goat: Jimenez *et al.* 2014, buffalo: Kumar *et al.* 2015), similar line of studies were lacking in mithun. MT release from the implant is continuous and does not follow the diurnal rhythm of endogenous MT secretion. Moreover, MT estimation in blood plasma of implanted mithun bulls indicated that MT implants were capable to maintain the supra-physiological plasma levels of this hormone several times higher than the endogenous concentration which indicated that the constant prolonged supply of MT from the implanted implant did not block the endogenous secretion of MT (Forsberg and Madej 1990).

Day length and THI are the two important factors which influence production of hormones and their functions. Concentration of prolactin was elevated during the summer season (Peirce *et al.* 1987). Hyperprolactinemia inhibited GnRH secretion which in turn impaired the LH release from the pituitary gland, thus, disturbed the hypothalamic-pituitary-gonadal axis (Koike *et al.* 1991). This in turn creates hormonal imbalance or induces improper secretion of the hormones. Improper secretion of these hormones leads to reduced production of testicular testosterone, poor sex libido, increased reaction time, poor semen production and its quality profiles and ultimately end-up with infertility or subfertility or sterility in breeding male (Perumal *et al.* 2017). In the present study, FSH, LH and Testosterone were higher in nocturnal than in day time blood collections. In the present study, highest level of FSH and LH was observed at 20, 4, 16 and 24 h in control and at 4, 24, 4 and 20 h in treatment in winter, spring, summer and autumn seasons, respectively. On the contrary, lowest value of FSH and LH were observed at 12 h in different seasons in both control and treatment groups (Fig. 1). Similarly, testosterone concentration was highest at 4 h in winter, spring summer and autumn seasons in control group; and at 4 h in winter and autumn seasons and at 24 h in spring and summer seasons in treatment group. Further, lowest testosterone concentration was observed at 12 h in both control and treatment groups in different seasons in mithun species (Fig. 1). Average daily temperature and sunshine hours showed significant negative correlation with LH concentration as summer heat stress and longer day length had reduced the secretion of LH and testosterone in mithun (Vinogradova *et al.* 2010). Similar observation was reported in both men and male rhesus macaques as testosterone level was peak during nocturnal than in day time (Sitzmann *et al.* 2010). The physiological importance of a nocturnal testosterone peak is not clear, however, owing to its involvement in anabolic processes, if this nocturnal rhythm is perturbed, there may be severe physiological adverse consequences. Testosterone secretion frequency and its concentration mainly depends on the endogenous LH secretary pattern (Katongole *et al.* 1971), IGF-1 concentration (Barth *et al.* 2008) and other metabolic and anabolic hormones (Schneider 2004). FSH, LH, testosterone and MT were significantly higher and T4, cortisol and prolactin were significantly lower in MT treated than in untreated control group. Increased plasma MT concentration stimulated the hypothalamic-pituitary-gonadal axis which led to increased concentration of gonadotropins which in turn triggered the testes to secrete higher testosterone in mithun (Misztal *et al.* 2002). Melatonin acts directly on GnRH neurons of hypothalamus (Roy *et al.* 2001); this in turn increases GnRH pulse secretion and immediate increase of LH pulsatile secretion

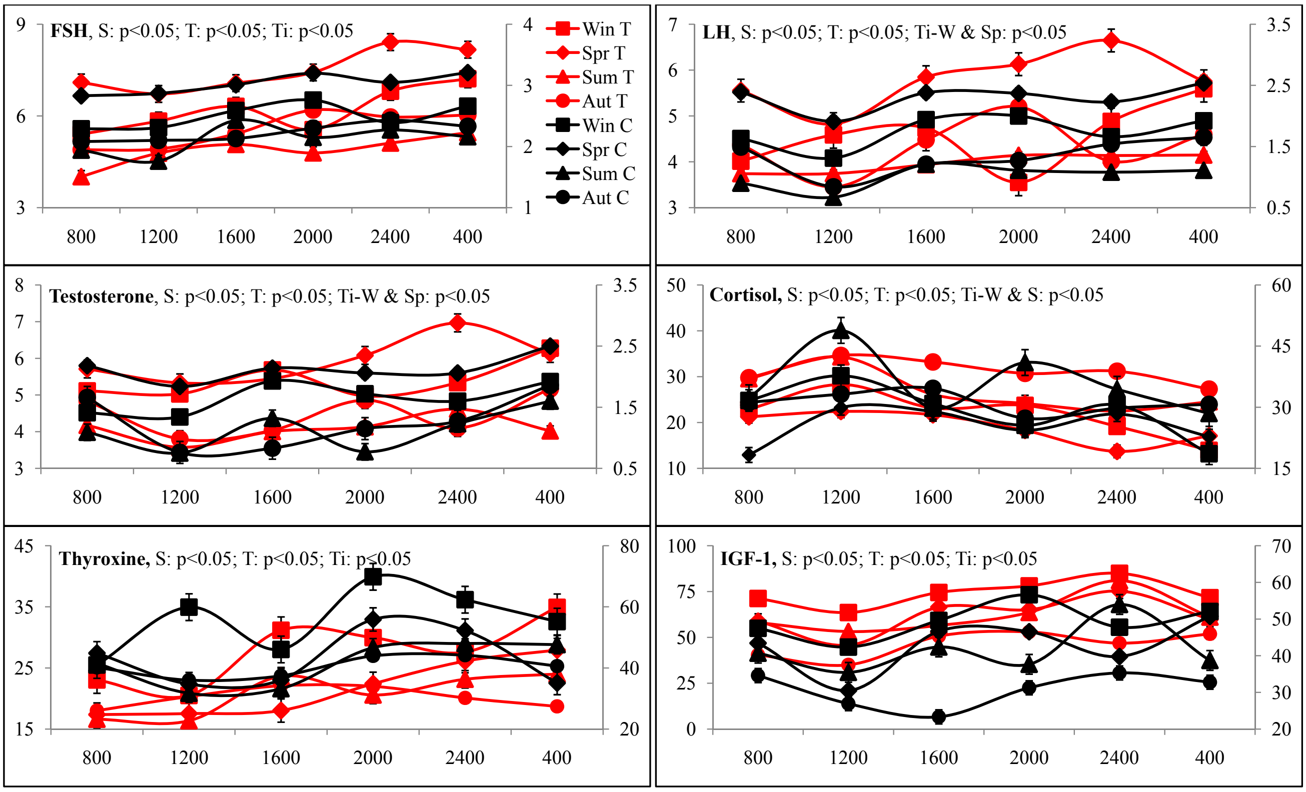

Fig. 1. Effect of exogenous melatonin on rhythmic changes of serum endocrinological profiles in mithun bull. FSH, Follicle Stimulating Hormone (mIU/mL); LH, Luteinizing Hormone (mIU/mL); Testosterone (ng/mL), Cortisol (nmol/L), Thyroxine (nmol/L) and IGF-1, Insulin like Growth Factor-1 (ng/mL). Win, Winter (Nov-Jan); Spr, Spring (Feb-Apr); Sum, Summer (May-July) and Aut, Autumn (Aug-Oct) Season (S). C, Control and T, Treatment groups (T). Collection Time on Horizontal Axis (Ti). Vertical bar on each point represents standard error of mean. Average age: 53.03 months.

(Viguie *et al.* 1995), which causes increased testosterone release (Rosa and Bryant 2003). In addition, MT also has significant effects on male accessory glands and testis as MT receptors are prevalent in mithun male reproductive system as in other species (Gilad *et al.* 1998). Therefore, MT has increased testosterone secretion, followed by development of the reproductive system and gonads.

Cortisol, a stress hormone, is generally used to assess the stress of animals (Schmidt *et al.* 2010). Significantly highest concentration of cortisol was observed at 12 h in different seasons in both control and treatment groups. Significantly lowest cortisol concentration was observed at 4, 24, 24 and 20 h in winter, spring, summer and autumn seasons, respectively in both control and treatment groups (Fig. 1). In marked contrast to melatonin and testosterone, cortisol secretion was higher in day time and at 12 h indicated that plasma cortisol peaks in the morning (08 to 12 h) when the animals wake up and a nadir late in the evening when they are going to sleep. Similar result was observed in mithun bulls; cortisol level increased during day time (mid-day) and decreased in the night time (mid-night). Activity rhythms are closely linked to the circadian rhythm of cortisol and they show parallel phase shifts when the photoperiod is changed in mithun as reported in other species (Lemos *et al.* 2009). Our results in mithun, therefore, agree with findings whereby cortisol levels are low in the evening (or around mid-night) and high in the morning (or around mid-day) as in other ungulates and that they are determined by the light-dark cycle. Higher level of cortisol in dry summer season may be due to activation of the hypothalamic–pituitary–adrenal cortical axis and sympatho-adrenal medullary axis which in turn increases circulatory cortisol and corticosterone levels (Minton 1994). Concentration of cortisol is not only altered by acute and chronic heat exposure (Christison and Johnson 1972) but also changes in photoperiod (Leining *et al.* 1980); similar result was obtained in the present study. Similar observations were also reported in Sahiwal cattle (Alhussien and Dang 2017) and Murrah buffaloes (Bombade *et al.* 2017) in which higher levels of plasma cortisol was observed in hot summer season than in cool winter season. The seasonal changes of cortisol levels might also be associated with the remarkable alterations in the secretion of reproductive hormones. Slow release MT has significantly reduced the concentration of cortisol in mithun as it inhibits adreno cortico tropic hormone stimulated cortisol production (Frank *et al.* 2004). MT has been reported to decrease the aggressive behaviour in dogs and improved their quality of life (Rosado *et al.* 2010). Similarly, supplementation of MT decreased cortisol and its side effects before, during and after surgery in canine species (Desborough 2000).

Prolactin acts as a peripheral regulatory key factor for reproductive function in males and is involved in a feedback mechanism that signals the CNS centers controlling the sexual arousal as well as the sex behaviour. Prolactin has inhibitory actions on the activity of the hypothalamic-pituitary-adrenal axis and the neurohypophysial oxytocin system (Torner and Neumann 2002). In the present study, prolactin concentration decreased from 12 to 24 h and increased from 04 to 12 h in both control and treatment groups. Significantly highest and lowest prolactin levels were observed at 12 and 24 h, respectively in both control and treatment groups in different seasons in mithun species (Fig. 2). Heat stress and a longer photoperiod in summer seasons increase plasma prolactin level in cattle and buffaloes (Peirce *et al.* 1987). Hyperprolactinemia in males induces hypogonadism by inhibiting GnRH pulsatile secretion (Koike *et al.* 1991) and consequently, FSH, LH and testosterone release. This leads to spermatogenic arrest, adverse effects on semen production, impaired motility and viability, results in morphologic alterations of the testes similar to those observed in prepubertal testes (De Rosa *et al.* 2003) and finally led to infertilityin breeding male.

Gustafson (1994) reported that the plasma prolactin concentration was lower during dark than light hours in dairy cows. In the present study, similar result was obtained in the mithun species where prolactin reached peak level in mid-day and lower level was observed in mid-night. MT regulates the prolactin secretion by multiple complex mechanisms which depend on the physiological status of animals (Villauna *et al.* 2007). MT alters lactotrophs sensitivity through its direct actions at pituitary level and also has direct effect on the hypothalamic site (Villauna *et al.* 2007) to modify the prolactin release. Similarly, Lincoln and Clarke (1995) reported that MT primarily influenced

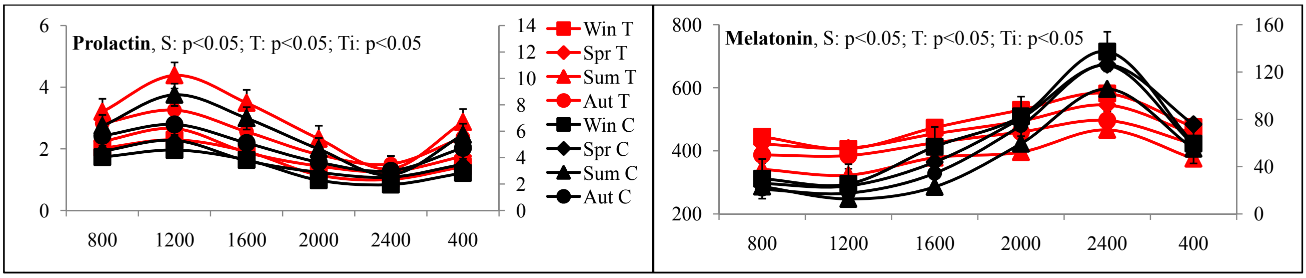

Fig. 2. Effect of exogenous melatonin on rhythmic changes of serum prolactin (ng/mL) and melatonin (pg/mL) profiles in mithun bull. Win, Winter (Nov-Jan); Spr, Spring (Feb-Apr); Sum, Summer (May-July) and Aut, Autumn (Aug-Oct) Season (S). C, Control and T, Treatment groups (T). Collection Time on Horizontal Axis (Ti). Vertical bar on each point represents standard error of mean. Average age: 53.03 months.

the pituitary gland to regulate the prolactin secretion and development of partial refractoriness to control the prolactin secretion. Moreover, prolactin stimulates the adrenal corticoid's hypersecretion and inhibits the GnRH secretion through its receptors on dopaminergic neurons which inturn causes hyperprolactinemia, which is one of the major key causes of infertility in male. In our study, prolactin concentration was significantly reduced in MT treated group and this result was expected due to the well-known suppressive effect of MT on prolactin secretion and its concentration. Further, higher concentration of MT acts directly on pars tuberalis (Santiago-Moreno *et al.* 2000) to decrease the prolactin secretion. Similarly, in our study, MT treated bulls had significantly lower prolactin.

In male reproductive system, Leydig and sertoli cells of testis have IGF1 receptors (Tan *et al.* 2007). IGF1 functions as a paracrine/ autocrine factor in the developmental and functional activity of interstitial cells in the fetus. In the present study, significantly high IGF-1 was observed at 20 h in winter, 4 h in spring and 24 h in summer and autumn seasons in control; and at 24 h in winter, spring and summer seasons and at 20 h in autumn season in treatment group. Similarly, significantly lowest IGF-1 concentration was observed at 12 h in both control and treatment groups in different seasons in mithun bulls (Fig. 1). Mehrzadi *et al*. (2016) reported that IGF-1 concentration was increased in bulls with advancement of age. It was also described that there was bull difference within breed with regards to secretion of IGF1. Increased IGF1 concentration which in turn stimulates the LH secretary pattern (Barth *et al.* 2008); consequently increases testosterone production; such bulls reveal earlier sexual maturity and high quality semen production (Bahrami *et al.* 2018). Moreover, size and weight of the testis and scrotal circumference in breeding bulls has been dependent on the level of the circulatory reproductive and metabolic hormones (Ashrafi *et al.* 2013). Plasma IGF1 secretion is positively correlated with LH concentration, besides, LH and testosterone secretion is also regulated by IGF1 concentration (Horikawa *et al.* 1989). Further, Palacin *et al*. (2008) advised that the bulls should be screened for IGF1 for selection of breeding bulls as IGF1is highly associated with testicular parameters, scrotal circumference and sperm total as well as the progressive forward motilities.

MT concentration was significantly higher in night and at 24 h collections in the present study. Similar result was reported in heifers (Critser *et al.* 1987) and adult cows (Skrzypczak 1998). Crister *et al*. (1987) in Friesian pre-pubertal heifers also observed that during light exposure, MT concentrations were low (40-60 pg/ml), whereas during dark period, MT levels were high (80-120 pg/ml). These MT concentrations were comparable to the present study in mithun. A first candidate that influences the MT secretion is day length or photo period and MT secretion was reported as highest during the night time and winter and spring seasons (Todini *et al.* 2011) as observed in the present study also. Moreover, MT takes active part in circadian rhythm regulation (Pevet and Challet 2011) and induces direct inhibitory actions on adrenal responses to ACTH (Torres-Farfan *et al.* 2008) as increased MT concentration has significantly reduced the concentration of cortisol in the present study and also both the MT and cortisol are inversely correlated. Similar results were reported in sheep (Torres-Farfan *et al.* 2008) and goats (Sharma *et al.* 2013). Similarly, Lincoln and Clarke (1995) also reported that MT influenced primarily the pituitary gland to alter the prolactin secretion and development of partial refractoriness to regulate the prolactin secretion.

Thyroxine concentration was significantly decreased during dry summer than cool winter and pleasant spring seasons. In the present study, significantly high T4 value was observed at 20 h in winter and spring, and at 24 h in summer and autumn in control mithun group; and at 4 h in winter, spring and summer and at 20 h in autumn season in treatment group. Similarly, lowest T4 concentrations were recorded at 8 h in winter, at 12 h in spring, summer and autumn seasons in control group and at 12 h in winter, spring and summer and at 8 h in autumn season in treatment group (Fig. 1). Similar results were reported in buffaloes (Silva *et al.* 2014) and cattle (Magdub *et al.* 1982) in which, reason behind lower T4 level in dry summer season was attributed to heat stress and longer photo period. Further, lower concentration of T4 during the dry summer season may be due to direct effect of heat stress on thyroid gland activity (Banerjee *et al.* 2015). Temperature plays a dual role; it exerts direct effect on TRH, subsequently reduces plasma T4 and indirect effect on decreasing appetite which in turn decreases thyroid hormone level (Valtorta *et al.* 1982).

Association study revealed that FSH, LH, testosterone, T4, IGF-1 and MT had significant positive correlation with each other whereas these hormones had significant negative correlation with cortisol and prolactin in control group. FSH, LH, testosterone, IGF-1 and MT had significant positive correlation with each other and these were significantly negatively correlated with cortisol, prolactin and thyroxine in treatment groups (Fig. 3). During the short day photo period and reduced temperature, concentration of MT increases and protects the testicular tissue which in turn increases the testosterone production. Significant

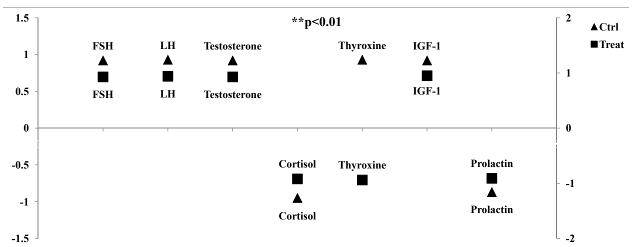

Fig. 3. Correlation coefficients among the endocrinological profiles in melatonin treated mithun bulls. FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone and IGF-1, Insulin like Growth Factor-1. **Correlation coefficient was highly significant, p<0.01.

seasonal variation in MT secretion was observed in mithun that might partly explain the variation in hormonal profiles observed between the different seasons in this species (Othman *et al.* 2016). Variation in concentration of MT in different seasons may be the cause of greater values of FSH, LH, T4 and testosterone obtained in winter and spring seasons as compared to summer season. Exogenous MT reduced the concentration of T4 in the present study. It was reported that MT injections led to a reduction in blood TSH and T4 concentrations (Oztürk *et al.* 2000) as MT decreased the thyroid gland growth and its function (Lewinski 2002). Similar suppressive effect of MT on thyroid gland and level of blood TSH was reported in cattle (Aggarwal *et al.* 2005). Such a suppressive effect could result from inhibition of TRH secretion from hypothalamus as well as direct rapid effect of MT on thyroid gland (Mess and Peter 1975).

The study concludes that season and time of blood collection have significant effect on the endocrinological profiles of mithun bulls. Exogenously administered slow release MT and spring and winter seasons increased the pulsatile secretion of hypothalamic GnRH, which inturn increased gonadotropin (LH and FSH) and gonadal hormone (testosterone) secretion. Thus exogenous slow release MT and spring and winter seasons have significantly greater beneficial effects on reproduction and artificial breeding programme in mithun species reared under semi-intensive management in the present location.

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