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Effect of melatonin administration on hormonal profile and oxidative stress status in pubertal Jaffarabadi buffalo heifers during summer season

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ABSTRACT

Reproductive inefficiency during the summer season is a major challenge in buffaloes due to heat stress–induced anestrus and hormonal imbalance. The present study aimed to evaluate the effect of exogenous melatonin administration on plasma melatonin and progesterone concentrations, as well as oxidative stress markers in pubertal Jaffarabadi buffalo heifers during summer. A total of 24 buffalo heifers were divided into four equal groups: T_0 (control, Corn oil), T_1 (Corn oil + Ovsynch), T_2 (Corn oil + Melatonin), and T_3 (Corn oil + Melatonin + Ovsynch). Blood samples were collected on days 0, 4, 10 and 28 post-treatments for estimation of plasma melatonin, progesterone, lipid peroxidation (LPO) and total antioxidant capacity (TAC). The results revealed a significant rise ($p<0.05$) in plasma melatonin levels on day 4 in T_2 (409 ± 26.10 pg/ml) and T_3 (451 ± 24.00 pg/ml) compared to control groups, followed by a gradual decline by day 28. Progesterone level increased non-significantly on day 10 in melatonin-treated groups and was highest post-AI in T_3, indicating stronger luteal support. Melatonin supplementation significantly ($p<0.05$) reduced LPO and elevated TAC on days 4 and 10 in T_2 and T_3, indicating mitigation of heat-induced oxidative stress. The study concluded that melatonin improved endocrine function and antioxidant status, making it beneficial for managing summer anestrus in buffalo heifers.

Keywords: Buffalo heifers, Hormonal profile, Lipid peroxidation, Melatonin, Progesterone, TAC

Buffaloes are an economically valuable livestock resource in India, contributing significantly to milk, meat and draft power. India accounts for the majority of the world's buffalo population (Deb *et al.* 2016). However, their reproductive efficiency is considerably influenced by environmental stressors, particularly during the summer season, during which reproductive cyclicity is markedly suppressed. This seasonal reproductive failure, commonly referred to as summer anestrus, is characterized by poor estrus expression, delayed ovulation, reduced conception rate and prolonged calving interval, ultimately affecting herd productivity and farmer profitability (Michael *et al.* 2020). The reproductive seasonality in buffaloes is closely linked with changes in photoperiod and ambient temperature, where ovarian activity is more pronounced during shorter day lengths and declines markedly during long, hot summer days. Increased temperature-humidity index (THI) during summer leads to compromised follicular development and reduced reproductive hormone secretion, resulting in weak or silent estrus (Dash *et al.* 2015). One of the key physiological pathways influenced during this period is the hypothalamus-pituitary-gonadal (HPG) axis, which becomes less responsive under heat stress, causing irregular or absent ovulation.

The pineal gland produces and stores the hormone melatonin during the day, and releases at night, beginning after sunset and ending at morning. Particularly at higher latitudes, melatonin regulates the reproductive rhythm in a variety of ruminant species, including horses (long-day species) and goats and sheep (short-day species) (Chemineau *et al.* 1992). In Chinese crossbred buffaloes, injection of melatonin initiating the synchronization protocol improved the ovulation, ovulatory follicle diameter and pregnancy rates (Abulaiti *et al*. 2023). Nevertheless, there is little data on Indian buffaloes to confirm the function of melatonin in Murrah and Jaffarabadi buffalo's or heifer's reproduction. Melatonin has been shown in numerous studies to both increase the quality and accelerate the meiotic maturation of oocytes (Takasaki *et al.* 2003, Kang *et al.* 2009, Manjunatha *et al.* 2009).

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Apart from its endocrine function, melatonin is recognized as a potent antioxidant that neutralizes reactive oxygen species (ROS) and supports cellular defense mechanisms. Buffaloes under heat stress experience elevated oxidative stress, reflected by increased lipid peroxidation (LPO) and reduced total antioxidant capacity (TAC) in body tissues and fluids (Bashandy *et al.* 2021). High oxidative stress impairs ovarian follicular development, oocyte maturation and luteal function, contributing further to reproductive inefficiency. Melatonin, due to its ability to cross cell membranes and enter mitochondria, protects ovarian and luteal cells from oxidative damage and maintains metabolic homeostasis more efficiently than conventional antioxidants (Reiter *et al.* 2018). Therefore, evaluating the hormone dynamics and oxidative stress modulation under melatonin administration is crucial to understanding its mechanistic role in restoring reproductive physiology during heat stress.

MATERIALS AND METHODS

Location: The study was carried out during summer season (March to June) on twenty-four pubertal anestrus Jaffarabadi buffalo heifers (32-36 months, 300-350 kg) maintained at the Cattle Breeding Farm, Kamdhenu University, Junagadh. The animals were randomly divided into four groups (n=6 each).

Ethical approval: The study was approved by Institutional Animal Ethical Committee (IAEC), an experimental protocol No. KU-JVC-IAEC-LA-154-25.

Animals, experimental design and samples: The study was carried out on twenty-four pubertal anestrus Jaffarabadi buffalo heifers. The study followed a complete randomized design with three treatments.

Control Group (T_0; Corn Oil), the heifers of this group were administered only with single injection of sterile filtered corn oil subcutaneously @ 1 ml/50 kg b. wt.

Treatment Group-I (T_1; Corn Oil + Ovsynch), the heifers of this group were administered with single injection of sterile filtered corn oil subcutaneously @ 1 ml/50 kg b. wt. (Kumar *et al.* 2016) and were subjected to Ovsynch protocol from 28th day. Under this protocol each animal received 20 µg GnRH analogue inj. ConciMate (5 ml) intramuscularly, on the day of the start (day 0), followed by the intramuscular injection of 500 µg, synthetic PGF2α analogue inj. Pragma (2 ml) on day 7 and a second injection of 20 µg GnRH analogue inj. ConciMate (5ml) on day 9 (48 h after PGF2α injection), and AI was performed twice on day 10.

Treatment Group-II (T_2; Corn Oil + Melatonin), the heifers of this group were injected with melatonin @ 18 mg/50 kg b. wt. dissolved @ 18 mg per ml of sterile filtered corn oil and then administered in heifers @ 1 ml/50 kg b. wt. as a single subcutaneous injection on day 0. The corn oil was filtered using a Syringe filter of 0.22 µm before dissolving the melatonin.

Treatment Group-III (T_3; Corn oil + Melatonin + Ovsynch), the heifers of this group were injected with melatonin mixed with sterilized corn oil as in group-II, and then were subjected to Ovsynch protocol from 28th day as in group-I.

Blood samples were collected on days 0, 4, 10 and 28 for estimation of melatonin, progesterone, LPO and TAC. Plasma melatonin and progesterone concentrations were estimated using ELISA kits from Fine Biotech Company. Plasma Lipid Peroxidation (LPO) and Total Antioxidant Capacity (TAC) were estimated using TBARS and antioxidant assay ELISA kits, respectively.

Statistical analysis: Qualitative variables were analysed with contingency tables and data were statistically analyzed using ANOVA with GraphPad Prism software (version 9.0).

RESULTS AND DISCUSSION

Plasma melatonin levels: The concentrations of melatonin at various periods in all four groups T_0, T_1, T_2 and T_3 are depicted in Table 1. The administration of sustained release melatonin in anestrous buffalo heifers significantly (p<0.05) increased the melatonin concentration in treated buffalo heifers compared to control group. Melatonin supplementation resulted in a significant increase (p<0.05) in plasma melatonin on day 4 in T_2 (409 ± 26.10 pg/ml) and T_3 (451 ± 24.00 pg/ml), followed by a decline by day 28, but still above baseline levels. This confirmed rapid absorption and short-term elevated circulatory concentration of melatonin.

Abulaiti *et al.* (2023) evaluated the melatonin effect and found that an increment in melatonin level (p<0.05) was detected in single melatonin treated group at 1st day of treatment as compared to the control group but in the present study, concentration was increased at 4th day of treatment. Ghuman *et al.* (2023) reported that plasma melatonin levels significantly increased (p<0.05) in all the treated buffaloes as compared to their pre-treatment levels and those of the control group similar pattern was observed in present study.

Plasma progesterone (P_4) assay: The concentrations of

Table 1. Group wise Melatonin concentration during different periods of sampling in buffalo heifers (Mean ± SE, n=6)

Day	Melatonin (pg/ml)			
	T_0	T_1	T_2	T_3
0 Day	69.70 ± 5.92^{aA}	43.10 ± 4.25^{aA}	67.70 ± 17.50^{aA}	78.70 ± 11.40^{aA}
4 Day	70.90 ± 10.00^{aA}	62.90 ± 9.57^{aA}	409 ± 26.10^{bB}	451 ± 24.00^{bC}
10 Day	78.40 ± 8.43^{aA}	64.20 ± 10.20^{aA}	373 ± 31.30^{bB}	315 ± 29.70^{bB}
28 Day	-	63.10 ± 14.10^{aA}	-	279 ± 31.30^{bB}

Means with different small superscripts (a, b) within the row, and those with capital superscripts (A, B, C) within the column differ significantly (p<0.05).

Table 2. Group wise progesterone concentration during different periods of sampling in buffalo heifers (mean ± SE, n=6)

Day	Progesterone (ng/ml)			
	T_0	T_1	T_2	T_3
0 Day	0.28 ± 0.02^{aA}	0.36 ± 0.11^{aA}	0.92 ± 0.42^{aA}	0.62 ± 0.22^{aA}
10 Day	0.32 ± 0.05^{aA}	0.39 ± 0.21^{aA}	1.07 ± 0.39^{aA}	0.85 ± 0.22^{aA}
28 Day	-	1.61 ± 0.49^{bB}	-	0.76 ± 0.33^{aA}
35 Day	-	2.98 ± 0.47^{aB}	-	2.59 ± 0.36^{aB}
AI	0.08 ± 0.05^{aA}	0.45 ± 0.21^{aA}	0.25 ± 0.13^{aA}	0.53 ± 0.16^{aA}
12 PAI	0.51 ± 0.34^{aA}	2.31 ± 0.60^{abB}	2.39 ± 0.81^{abB}	3.66 ± 0.60^{bB}
25 PAI	0.21 ± 0.21^{aA}	1.83 ± 0.93^{aAB}	1.06 ± 0.79^{aA}	3.35 ± 0.99^{aB}

Means with different small superscripts (a, b) within the row, and those with capital superscripts (A, B) within the column differ significantly (p<0.05).

progesterone at various periods of study are presented in Table 2. Melatonin treatment did not influence progesterone concentrations and remained at basal level at day 0 in treatment groups T_2 and T_3 (<0.6 ng/ml), and very low in groups T_0 and T_1 (<0.4 ng/ml). On day 10th, it was non-significantly (p>0.05) higher (1.07 ± 0.39 ng/ml and 0.85 ± 0.22 ng/ml, respectively) in treatment groups T_2 and T_3, as compared to T_0 & T_1 (0.32 ± 0.05 ng/ml & 0.39 ± 0.21 ng/ml, respectively). On day 28-35 it rose significantly (p<0.05) in T_1 (1.61 ± 0.49 ng/ml to 2.98 ± 0.47 ng/ml) and T_3 (0.76 ± 0.33 ng/ml to 2.59 ± 0.36 ng/ml), due to improvement in luteal phase.

On the day of induced estrus in group T_0 only 2 animals revealed low level of progesterone (0.08 ± 0.05 ng/ml), while T_2 group had 4 animals with induced estrus and found low level of progesterone (0.25 ± 0.13 ng/ml) which indicated strong estrus response and estrogen dominancy. In group T_3, highest level of progesterone, found on day 12th post AI (3.66 ± 0.60 pg/ml) was significantly (p<0.05) higher above the other groups, which indicates strong luteal support. On day 25th post-AI, group T_3 maintained high concentration of progesterone (3.35 ± 0.99 ng/ml), as compared to T_1 (1.83 ± 0.93 ng/ml), T_2 (1.06 ± 0.79 ng/ml) which indicated maximum number of conceptions and strong luteal support and in T_0 with negligible level of progesterone (0.218 ± 0.216 ng/ml), which indicated no conception or luteal phase due to lysis of CL with cycle progression. Here, Melatonin + Ovsynch (T_3) showed the most consistent and highest progesterone levels post-AI, especially at 12 and 25 days post AI, indicating improved luteal function and pregnancy support. Corn Oil + Ovsynch (T1) group also showed increased progesterone, but less strongly. Melatonin alone group (T_2) showed intermediate response and P4 in control group (T_0) remained minimal throughout cycle.

Phogat *et al.* (2018) recorded similar pattern of increase in progesterone level (p<0.05) but on days 15 and 22 post-melatonin treatment while in the present study, it was recorded on day 10th post treatment in T_2 and T_3 groups. Other days like 12 and 25 post AI had similar pattern of higher progesterone concentration to pregnancy and luteal support in treatment group compared to control.

Plasma lipid peroxidation factor (LPO): Lipid peroxidation (LPO) refers to oxidative degradation of lipids, primarily polyunsaturated fatty acids, in cell membranes. This process is initiated by reactive oxygen species (ROS), which cause oxidative damage, leading to the formation of lipid radicals and, eventually, harmful byproducts like malondialdehyde (MDA). MDA, a byproduct of LPO, is widely used as a biomarker to assess oxidative stress (Georgieva, 2005).

Plasma lipid peroxidation (LPO) level at various periods in all four groups T_0, T_1, T_2 and T_3 are depicted in Table 3. The administration of sustained release melatonin in anestrus buffalo heifers significantly (p<0.05) decreased the concentration of LPO on day 4th and 10th in treatment group T_2 and T_3. In treatment group-II (T_2), the level of LPO significantly (p<0.05) decreased to 2.46 ± 0.71 µmol/L on 4th day following melatonin administration compared to 0 day (4.54 ± 0.29 µmol/L), and on day 10th (2.72 ± 0.26 µmol/L) it significantly (p<0.05) increased as compared to day 4th post-melatonin administration, but reduced as compared to day 0. In treatment group-III (T_3), LPO level significantly (p<0.05) reduced to 1.91 ± 0.33 µmol/L on day 4th following melatonin administration compared to day of administration (4.49 ± 0.30 µmol/L), and on day 10th (2.70 ± 0.17 µmol/L) it significantly (p<0.05) increased than day 4th post melatonin administration. However, the LPO level increased to 3.70 ± 0.33 µmol/L on 28th day which was significantly (p<0.05) higher than the concentration found on 4th and 10th day, But the level of LPO on 28th day was significantly (p<0.05) lower than that of day 0 day.

Table 3. Group wise LPO level (MDA; µmol/L) during different periods of sampling in buffalo heifers (Mean ± SE, n=6)

Day	LPO (µmol/L)			
	T_0	T_1	T_2	T_3
0 Day	3.90 ± 0.41^{aA}	4.34 ± 0.28^{aA}	4.54 ± 0.29^{aB}	4.49 ± 0.30^{aB}
4 Day	4.34 ± 0.30^{bA}	4.07 ± 0.36^{bA}	2.46 ± 0.71^{aA}	1.91 ± 0.33^{aA}
10 Day	4.27 ± 0.42^{bA}	3.83 ± 0.48^{bA}	2.72 ± 0.26^{aA}	2.70 ± 0.17^{aA}
28 Day	–	4.24 ± 0.37^{aA}	–	3.70 ± 0.33^{aB}

Means with different small superscripts (a, b) within the row, and those with capital superscripts (A, B) within the column differ significantly (p<0.05).

On day 0, no significant differences were found between groups (all ~4 µmol/L) and at day 4th post melatonin treatment, treatment groups T_2, T_3 show a significant (p<0.05) reduction in LPO compared to T_0 and T_1, and then on day 10th in melatonin groups (T_2, T_3) it remain significantly lower than control while at day 28th, T_3 stayed low (3.70 ± 0.33 µmol/L), andT_1 remained high (4.24 ± 0.37 µmol/L).

Strong antioxidant like melatonin has an unusual capacity to dissolve in both lipids and water, which allows it to work in a variety of settings, including organelles, body fluids, membranes, and cell interiors (Zarezadeh *et al.* 2022). Kumar *et al.* (2015) studied that sustained delivery of exogenous melatonin influences biomarkers of oxidative stress and total antioxidant capacity in summer-stressed anestrus water buffalo (*Bubalus bubalis*). On days 8 and 12, the serum MDA concentration was considerably (p<0.05) higher than in the treatment group but, here in present study on day 4th and 10th post treatment of melatonin in T_2 and T_3 groups, MDA concentration remarkably decreased plausibly due to effect of exogenous melatonin. In present study, similar effect was observed as reported by Perumal *et al.* (2021), in Andaman local anestrus buffalo, wherein the dry (summer) season caused infertility. They evaluated the impact of a subcutaneous slow-release exogenous melatonin (MT) implant on antioxidant profiles, which were higher and oxidative stress profile was lower in MT treated group than in control group.

Total antioxidant capacity (TAC): Plasma TAC concentration at various periods in all four groups T_0, T_1, T_2 and T_3 are depicted in Table 4. The administration of sustained release melatonin in anestrous buffalo heifers significantly (p<0.05) increased TAC concentration in treated buffalo heifers compared to control group.

In treatment group T_2, TAC concentrations significantly (p<0.05) increased at 3.98 ± 0.31 µmol/L on day 4th following melatonin administration compared to day of administration (2.13 ± 0.19 µmol/L), and on day 10th (2.90 ± 0.46 µmol/L), it significantly (p<0.05) decreased than day 4th post-treatment value. In treatment group T_3, TAC concentrations significantly (p<0.05) reached highest levels of 4.45 ± 0.78 µmol/L on day 4 following melatonin administration compared to day of administration (2.17 ± 0.24 µmol/L), and on day 10th (3.00 ± 0.26 µmol/L), it significantly (p<0.05) decreased than day 4th value. However, the TAC concentration reduced to 2.70 ± 0.42 µmol/L on 28th day which was significantly (p<0.05) lower than the concentration found on 10th day. But the concentration of TAC on 28th day (2.7 ± 0.42 µmol/L) was non-significantly (p<0.05) higher than that of day 0. On day 0, all groups had similar baseline values of TAC (~2-2.5 µmol/L), and were lower compared to day 4th values. On day 4th, the values of T_2 (3.98± 0.31 µmol/L) and T_3 (4.45 ± 0.78 µmol/L) increased significantly (p<0.05) compared to T_0/T_1 (~2.3-2.7 µmol/L). On day 10th all groups converged (~2.5-3.0 µmol/L), here with minimal difference found, and on day 28th TAC, remained elevated (3.4 ± 0.60 µmol/L) in T_1, while it dropped closer to 2.7 ± 0.42 µmol/L in T_3.

Prior research done by Benot *et al.* (1998) found that exogenous MLT raised TAC in rat serum, indicating that MLT might be important for its role in antioxidative capacity. A similar effect of exogenous MLT on plasma TAC level was observed in the present study which was raised significantly (p<0.05) when level of plasma MLT concentration was raised.

Estrus Response and Conception Rate (CR): The estrus induction rate varied markedly among the different treatment groups (Table 5). In the control group (T_0), only 2 out of 6 heifers exhibited estrus, corresponding to a low estrus induction rate of 33.33%, which reflected the limited effect of corn oil on ovarian activity. In contrast, estrus was successfully induced in all six heifers of treatment group-I T_1 and treatment group-III T_3, yielding an induction rate of 100%. This indicates that both the ovsynch protocol alone and in combination with melatonin were highly effective in achieving reliable estrus induction. In treatment group-II T_2, where melatonin was administered without synchronization, estrus was induced in 4 out of 6 heifers (66.67%). Although this response was considerably higher than the control, it was still lower than the ovsynch and melatonin + ovsynch groups, suggesting that while melatonin enhanced ovarian activity, its effect was less consistent when used alone.

The first insemination conception rate (%) was calculated after animal came in estrus post oil/melatonin

Table 4. Group wise TAC level (µmol/L) during different periods of sampling in buffalo heifers (Mean ± SE, n=6)

Day	TAC (µmol/L)			
	T_0	T_1	T_2	T_3
0 Day	2.30 ± 0.20^{aA}	2.52 ± 0.26^{aA}	2.13 ± 0.19^{aA}	2.17 ± 0.24^{aA}
4 Day	2.35 ± 0.30^{aA}	2.74 ± 0.51^{abA}	3.98 ± 0.31^{bcB}	4.45 ± 0.78^{cB}
10 Day	2.50 ± 0.51^{aA}	2.72 ± 0.41^{aA}	2.90 ± 0.46^{aAB}	3.00 ± 0.26^{aB}
28 Day	-	3.40 ± 0.60^{aB}	-	2.70 ± 0.42^{aA}

Means with different small superscripts (a, b, c) within the row, and those with capital superscripts (A, B) within the column differ significantly (p<0.05).

Table 5. Estrus induction rate (%) in different groups (T_0, T_1, T_2 and T_3)

Particulars	Treatment groups			
	T_0 (n=6)	T_1 (n=6)	T_2 (n=6)	T_3 (n=6)
Estrus induced animal (no.)	2/6	6/6	4/6	6/6
Estrus induction rate (%)	33.33	100	66.67	100

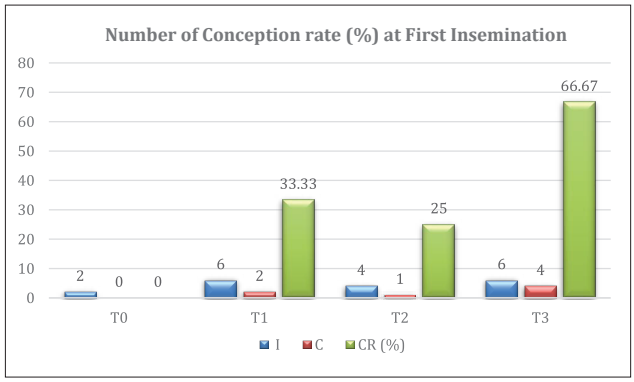

Fig. 1. Conception rate (%) at first insemination in four different groups (T_0, T_1, T_2 and T_3); I= No. of First Insemination, C=No. of Conception, CR (%) = CR on First AI

Table 6. First insemination conception rate in different groups (T_0, T_1, T_2 and T_3)

Particulars	T_0	T_1	T_2	T_3
Insemination no.	2	6	4	6
Conception no.	0	2	1	4
Conception rate (%)	0	33.33	25.00	66.67

administration in control group (T_0) and treatment group T_2 and in treatment group T_1 and T_3 after fixed time artificial insemination (FTAI) as given in Table 6 and Figure 1. The first insemination conception rate varied considerably among the groups. In the control group T_0, no conception was recorded, as both inseminated animals failed to conceive (0%). In contrast, the conception rate improved in all treatment groups. In treatment group T_2, where estrus was induced following melatonin administration, the conception rate was 25% (1/4 animals conceived). A higher conception rate of 33.33% was achieved in treatment group-T_1, where estrus was synchronized using the Ovsynch protocol, suggesting improved timing of insemination. The most favorable results were obtained in Treatment Group T_3, where melatonin pretreatment was combined with Ovsynch synchronization, achieving a conception rate of 66.67% (4/6 animals conceived). This indicated a synergistic effect of melatonin and synchronization in improving both estrus expression and conception outcomes.

Phogat *et al*. (2018) observed a 20% conception rate in Ovsynch-only animals and 50% in melatonin + Ovsynch animals. In the present study, T_1 (33.33%) showed a higher CR than this Ovsynch control (20%), while T_3 (66.67%) exceeded their reported melatonin + Ovsynch rate (50%). This indicated a stronger positive effect of melatonin in combination with Ovsynch in Jaffarabadi heifers in present study as compared to their findings.

Thus, the present study confirmed that while conception rates remained poor in untreated buffaloes, melatonin in combination with Ovsynch protocol provides the best reproductive outcome, highlighting its potential as a management tool for improving fertility during the non-breeding season or summer season. Melatonin administration improved hormonal balance and oxidative stress resilience in buffalo heifers, with the combination of melatonin + Ovsynch proving most effective.

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