RESEARCH ARTICLE

Estrus Induction with Exogenous Melatonin in Anestrus Pubertal Jaffarabadi Buffalo Heifers during Summer Season

Urvashi B. Ramoliya[1*], Hardipsinh P. Vijyeta[1], Ramesh J. Padodara[2], Karshan B. Vala[1], Jignesh V.Vadalia[3], Nirbhay R. Nandaniya[1]

Abstract

The present study evaluated the effect of exogenous melatonin on ovarian follicular dynamics, hormonal profile, oxidative stress, and conception rate in pubertal Jaffarabadi buffalo heifers during the summer season. Twelve buffalo heifers (32-36 months; 300-350 kg) were randomly divided into two equal groups: Control (Group I; corn oil 1 mL/50 kg s/c) and Melatonin-treated (Group II; melatonin 18 mg/50 kg b.wt. in corn oil s/c). Ovarian activity was monitored using ultrasonography, and blood samples were analyzed for melatonin, progesterone, lipid peroxidation (LPO), and total antioxidant capacity (TAC). Melatonin significantly ($p<0.05$) enhanced follicular recruitment and follicle diameter, reduced oxidative stress, increased TAC, and improved estrus induction and conception rate. The study demonstrates melatonin's beneficial role in alleviating heat stress-induced reproductive suppression and improving fertility in buffalo heifers.

Key words: Buffalo heifers, Estrus induction, Follicular dynamics, Melatonin, Oxidative stress, Progesterone.

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Introduction

Buffaloes are one of the most valuable livestock species in Asia, contributing significantly to milk, meat, and draught power. India, home to nearly 97% of the world's buffalo population, leads in buffalo genetic improvement and reproductive biotechnology (Deb *et al.,* 2016; Mota-Rojas *et al.,* 2021). Despite their adaptability, buffaloes suffer from poor reproductive efficiency due to factors such as delayed puberty, silent estrus, and prolonged inter-calving intervals-problems that become more pronounced during summer when temperature–humidity index exceeds 75 (Dash *et al.,* 2015; Warriach *et al.,* 2015). Seasonal anestrus remains a major limitation to realizing the productive potential of buffaloes (Das and Khan, 2010).

Reproductive seasonality in buffalo is photoperiod dependent. During shorter days (October-March), ovarian cyclicity and fertility are enhanced, whereas during longer days, estrus expression and conception rates decline (Zicarelli, 1997). This cyclicity is largely mediated by melatonin, a hormone secreted by the pineal gland in response to darkness, which regulates the hypothalamo-pituitary-gonadal axis (Malpaux *et al.,* 2001). Melatonin has been reported to improve follicular development, luteal function, and oocyte quality by increasing GnRH and gonadotropin secretion (Ghuman *et al.,* 2010). It also exhibits potent antioxidant activity-more effective than vitamins E or C by scavenging reactive oxygen species and enhancing key antioxidant enzymes such as SOD, CAT, and GPx (Bashandy *et al.,* 2021).

Exogenous melatonin administration has been shown to restore cyclicity and improve conception in summer anestrus buffaloes (Ramadan *et al.,* 2014; Kumar *et al.,* 2016). It not only supports hormonal balance but also reduces lipid peroxidation, thereby maintaining the ovarian microenvironment conducive for follicular growth (Venegas *et al.,* 2012). Moreover, subcutaneous melatonin injection offers a simple, practical alternative to slow-release implants (Yang *et al.,* 2017; Ratchamak *et al.,* 2022).Considering the strong regulatory and antioxidant role of melatonin, the present study was undertaken to evaluate its effect on estrus induction, ovarian activity, hormonal and oxidative stress

[1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Junagadh-362001, Gujarat, India.

[2]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Junagadh-362001, Gujarat, India.

[3]Department of Veterinary Surgery and Radiology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh-362 001, Gujarat, India

Corresponding Author: Dr. Urvashi B. Ramoliya, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Kamdhenu University, Junagadh-362 001, India. e -mail: urvashiramoliya1@gmail.com

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markers, and conception rate in pubertal Jaffarabadi buffalo heifers during the summer season, comparing melatonin treatment with untreated control animals.

Materials and Methods

The study was carried out on twelve pubertal anestrus Jaffarabadi buffalo heifers (32-36 months, 300-350 kg) maintained at the Cattle Breeding Farm, Kamdhenu University, Junagadh. The animals were divided randomly into two groups (n=6 each): Group I (Control; corn oil 1 mL/50 kg b.wt., s/c) and Group II (Melatonin @18 mg/50 kg b.wt. in corn oil, s/c). Estrus signs were observed twice daily, and animals showing estrus were inseminated artificially with frozen thawed semen.

Ovarian structures were examined ultrasonographically on days 0, 10, on the day of estrus/AI, and day 25 post-AI. Blood samples were collected on day 0, 4, 10, on the day of estrus/AI, day 12 and 25 post-AI for estimation of plasma melatonin and progesterone by using standard ELISA kits of Fine Biotech Company. Lipid peroxidation (MDA production, nmol/mL) and total antioxidant capacity (TAC; µmol/L) were assessed by using commercially available ELISA kits of HiMedia Lab Pvt. Ltd., Mumbai.

Data obtained was statistically analyzed by one-way analysis of variance with Duncan's *post-hoc* test to find significant differences between periods, and student's t-test was employed to compare same blood and USG parameters between treatment and control group. The observations on estrus responses and conception rates were recorded and compared between different groups statistically by using Chi-square test (Snedecor and Cochran, 1994).

Results and Discussion

Effect of Melatonin on Ovarian Follicular Dynamics

The mean number and diameter of small, medium and large follicles in group I (control) and group II (melatonin-treated) buffalo heifers are presented in Table 1. Melatonin supplementation significantly increased ($p<0.05$) the number and size of small and medium size follicles on day 10 compared with the control group as well as from the day 0 in the treated group, indicating enhanced follicular recruitment and early growth, and this status continued till the day of estrus and AI. This improvement supports the antioxidant and endocrine-modulating roles of melatonin (Reiter *et al.,* 2009; El-Raey *et al.,* 2011). By day 25 post-AI, melatonin-treated buffaloes retained larger follicular diameters, reflecting sustained follicular health and better ovarian activity under heat stress (Tamura *et al.,* 2012). This dynamics in medium size follicular number and diameter is further attributed to melatonin's stimulatory action on the hypothalamo-pituitary-gonadal axis and its strong antioxidant effect, which protects granulosa cells and supports steroidogenesis under summer heat stress. Similar findings of improved folliculogenesis following melatonin therapy have been reported by Ghuman *et al.* (2010), Kumar *et al.* (2016), and Abulaiti *et al.* (2023).

Melatonin also significantly enhanced large follicle development, particularly on day of estrus, compared to the control group and other days of treatment (Table 1). There was no such significant change in the number and diameters of small, medium or even large follicles in control group over the monitoring period from day 0, estrus to day 25 post-AI. This improved preovulatory follicle size in melatonin-treated group suggests enhanced LH surge sensitivity and ovulatory readiness (Ghuman *et al.,* 2010). Larger follicles were also

Table 1: Effect of exogenous melatonin on number and diameter of small, medium and large follicles compared to control group of pubertal Jaffarabadi buffalo heifers (Mean ± SE, n=6)

		Number of follicles		Diameter of follicle (mm)	
Follicle size	**Days of USG**	**Group I (Control)**	**Group II (Melatonin)**	**Group I (Control)**	**Group II (Melatonin)**
Small	0 Day	3.67 ± 0.49^{A}	3.83 ± 0.47^{A}	2.34 ± 0.09^{A}	2.45 ± 0.17^{A}
	10 Day	4.40 ± 1.07^{aA}	7.20 ± 0.58^{bB}	2.51 ± 0.31^{aA}	3.72 ± 0.10^{bB}
	Estrus/AI	6.50 ± 1.50^{A}	6.25 ± 0.48^{B}	2.92 ± 0.28^{A}	3.05 ± 0.30^{A}
	25 Day PAI	4.50 ± 1.50^{A}	5.50 ± 1.19^{A}	1.76 ± 0.05^{aB}	2.25 ± 0.15^{bA}
Medium	0 Day	1.33 ± 0.33^{A}	1.25 ± 0.25^{A}	5.55 ± 0.49^{A}	5.23 ± 0.58^{A}
	10 Day	1.00 ± 0.00^{aA}	2.50 ± 0.88^{bB}	5.24 ± 0.61^{aA}	7.00 ± 0.39^{bA}
	Estrus	1.50 ± 0.50^{A}	2.33 ± 0.33^{B}	6.20 ± 1.10^{A}	7.26 ± 0.27^{B}
	25 Day PAI	1.50 ± 0.50^{A}	2.00 ± 0.41^{A}	5.60 ± 0.30^{aA}	7.62 ± 0.19^{bB}
Large	0 Day	1.00 ± 0.00^{A}	1.25 ± 0.25^{A}	8.35 ± 0.15^{A}	8.72 ± 0.34^{A}
	10 Day	1.50 ± 0.50^{A}	1.67 ± 0.33^{A}	8.32 ± 0.02^{A}	8.70 ± 0.20^{A}
	Estrus	1.00 ± 0.00^{aA}	2.25 ± 0.25^{bB}	9.20 ± 0.80^{aA}	11.85 ± 0.30^{bB}
	25 Day PAI	1.33 ± 0.33^{A}	1.67 ± 0.33^{A}	9.05 ± 0.65^{A}	9.60 ± 0.32^{A}

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

associated with greater luteal development and progesterone secretion post-ovulation (Phogat *et al.,* 2018). Melatonin-treated heifers (group II) exhibited consistently higher follicular numbers and diameters across all follicle categories (small, medium, large) and stages (day 10, estrus, 25 days post-AI). This demonstrates its dual action - direct antioxidative protection to the ovary and indirect hormonal modulation via the hypothalamo-pituitary-gonadal axis (Tamura *et al.,* 2012).

Effect of Melatonin on Hormonal Levels and Oxidative Stress

The concentrations of melatonin at various periods in melatonin-treated (group II) compared to control (group I) pubertal anestrus buffalo heifers are depicted in Table 2. Single subcutaneous injection of melatonin markedly elevated circulating melatonin levels almost 6-fold by day 4, and 5-fold by day 10 compared to the control group, while in control group it did not vary appreciably between days 0, 4 and 10. This confirms efficient absorption and sustained bioavailability of exogenous melatonin. The increased circulating melatonin may enhance gonadotropin release and follicular growth through its action on the hypothalamo-pituitary axis. Similar trends were observed in summer anestrus buffaloes by Ghuman *et al.* (2010) and Kumar *et al.* (2016), where melatonin injections restored cyclicity and improved ovarian activity during heat stress.

Progesterone concentration showed a gradual rise in melatonin-treated heifers (group II) after treatment, with non-significantly higher levels on day 10 and significantly higher on day 12 post-AI compared to control and on other days of sampling, while in control group it was more or less constantly lower on all days (Table 2). The increase in progesterone reflects improved luteal function and successful ovulation induced by melatonin. Higher levels in conceived animals further support melatonin's luteotrophic effect, likely mediated through enhanced LH secretion and antioxidant protection of luteal cells (Phogat *et al.,* 2018; Kumar *et al.,* 2016).

Overall, these hormonal findings confirm that melatonin not only increases circulating melatonin but also improves luteal progesterone synthesis, promoting better reproductive efficiency under summer heat stress.

The lipid peroxidation (MDA production) levels at various periods in melatonin-treated (group II) and control (group I) pubertal anestrus buffalo heifers are depicted in Table 3. Malonaldehide levels (MDA), an indicator of oxidative damage, were significantly lower in melatonin-treated buffaloes (group II) on days 4 and 10 compared to control group of animals as well as from day 0, *i.e.* beginning of treatment, demonstrating its strong antioxidant property. This reduction reflects melatonin's ability to neutralize free radicals and protect ovarian cells from oxidative stress during high ambient temperature conditions. These findings agreed with Zarezadeh *et al.* (2022), who described melatonin as a superior lipid-phase antioxidant compared to vitamins C and E.

Melatonin treatment also significantly increased total antioxidant capacity (TAC) of animals on days 4 and 10 post-treatment compared to control as well as from day 0 (Table 3), indicating activation of the body's endogenous antioxidant defense. This rise in TAC suggests enhanced activities of enzymatic antioxidants such as SOD, GPx, and catalase. The improvement in oxidative balance corresponds with better follicular health and hormonal stability observed in treated heifers. Similar results were reported by Benot *et al.* (1998) and Kumar *et al.* (2015), confirming melatonin's systemic antioxidant effect in livestock during heat stress.

Table 2: Peripheral concentration of melatonin and progesterone during different periods of melatonin-treated and control group of pubertal anestrus buffalo heifers (Mean ± SE, n=6)

Days of sampling	Melatonin (pg/mL)		Progesterone (ng/mL)	
	Group I (Control)	Group II (Melatonin)	Group I (Control)	Group II (Melatonin)
0 Day	69.70 ± 5.92^{A}	67.70 ± 17.50^{A}	0.28 ± 0.02^{A}	0.92 ± 0.42^{A}
4 Day	70.90 ± 10.00^{aA}	409.00 ± 26.10^{bB}	--	--
10 Day	78.40 ± 8.43^{aA}	373.00 ± 31.30^{bB}	0.32 ± 0.05^{A}	1.07 ± 0.39^{A}
Day of AI	--	--	0.08 ± 0.05^{A}	0.25 ± 0.13^{A}
12 Day PAI	--	--	0.51 ± 0.34^{aA}	2.39 ± 0.81^{bB}
25 Day PAI	--	--	0.21 ± 0.21^{A}	1.06 ± 0.79^{A}

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).

Table 3: Lipid peroxidation (MDA; µmol/L) and total antioxidant capacity (TAC) in melatonin-treated and control groups of buffalo heifers during different periods of sampling (Mean ± SE, n=6)

Day	MDA (µmol/L)		TAC (µmol/L)	
	Group I (Control)	Group II (Melatonin)	Group I (Control)	Group II (Melatonin)
0 Day	3.90 ± 0.41^{A}	4.54 ± 0.29^{B}	2.30 ± 0.20^{A}	2.13 ± 0.19^{A}
4 Day	4.34 ± 0.30^{bA}	2.46 ± 0.71^{aA}	2.35 ± 0.30^{aA}	3.98 ± 0.31^{bB}
10 Day	4.27 ± 0.42^{bA}	2.72 ± 0.26^{aA}	2.50 ± 0.51^{A}	2.90 ± 0.46^{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).

Table 4: Estrus induction rate (%) and conception rate at first insemination (%) in melatonin-treated compared to control group of buffaloes heifers

Groups	No. of animal induced estrus	Estrus induction rate (%)	No. of animal conceived	Conception rate (%)
Group I (Control)	2/6	33.33	0/2	0
Group II (Melatonin treated)	4/6	66.66	1/4	25.00

Estrus Response and Conception Rate

Estrus induction rate (%) was calculated based on animals came in estrus post-corn oil/ melatonin administration in group I (control) and group II (melatonin), respectively, and then first insemination conception rate (%) was calculated as given in Table 4. Melatonin-treated buffaloes showed higher estrus response and improved conception rate at first insemination, confirming its beneficial role in inducing ovarian cyclicity during summer anestrus.

The results presented in Table 4 demonstrate that melatonin supplementation had a positive impact on estrus induction and conception rate in buffaloes during the summer anestrus period. In Group I (Control), only 33.33% of animals exhibited estrus, and none conceived at first insemination. In contrast, in Group II (Melatonin-treated), the estrus induction rate increased to 66.66%, with a conception rate of 25.00% at first insemination. These findings clearly indicate that melatonin administration enhances reproductive performance under heat-stressed conditions. These observations were in agreement with earlier reports by Kumar *et al*. (2016), and Phogat *et al*. (2016), who also reported a significant improvement in estrus induction and conception rates following melatonin treatment in anestrus buffaloes during summer months.

The improved estrus response in melatonin-treated buffaloes could be attributed to its neuroendocrine action on the hypothalamic-pituitary-gonadal axis. Melatonin is known to stimulate GnRH secretion, which in turn enhances the release of FSH and LH, promoting follicular growth and ovulation. During periods of high ambient temperature, melatonin counteracts the inhibitory effects of heat stress on gonadotropin secretion, thereby restoring ovarian activity. Additionally, melatonin possesses potent antioxidant and anti-stress properties, which help maintain physiological homeostasis and improve follicular health and oocyte quality, ultimately leading to better conception rates. The current findings thus reaffirm the beneficial role of melatonin in inducing ovarian cyclicity and enhancing reproductive efficiency in buffaloes experiencing thermal stress.

Conclusion

Melatonin administration (18 mg/50 kg b.wt. s/c) during summer effectively improved follicular growth, peripheral hormone melatonin and progesterone profile, antioxidant status, and fertility in pubertal anestrus Jaffarabadi buffalo heifers. The findings demonstrate that exogenous melatonin at a given dose rate can be a practical therapeutic tool to overcome summer anestrus and enhance reproductive efficiency in pubertal Jaffarabadi buffalo heifers.

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