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Melatonin supplementation enhances uterine hemodynamics in buffaloes exposed to summer heat stress

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Abstract

Heat stress-induced reproductive dysfunction is a major factor limiting fertility in buffaloes during the summer season. Melatonin, a potent antioxidant and reproductive regulator, has been reported to improve reproductive performance; however, its effects on uterine hemodynamics remain poorly understood. The present study was conducted to evaluate the influence of melatonin supplementation on middle uterine artery blood flow in buffaloes exposed to summer heat stress. Eighteen healthy buffaloes were randomly allocated into two groups: melatonin-treated (n = 9) and control (n = 9). Animals in the treatment group received a single subcutaneous injection of melatonin (18 mg/50 kg body weight), whereas control animals received corn oil alone. Spectral Doppler ultrasonography was performed at five-day intervals to assess pulsatility index (PI), resistance index (RI), middle uterine artery diameter (MUAD), time-averaged maximum velocity (TAMV), and blood flow volume (BFV). Environmental conditions indicated severe heat stress, with an average temperature-humidity index (THI) of 84.5. Melatonin supplementation significantly improved uterine blood flow characteristics. PI and RI decreased significantly following treatment, indicating reduced vascular resistance, whereas MUAD, TAMV, and BFV increased significantly compared with the control group. The lowest PI (0.82 ± 0.30) and RI (0.36 ± 0.05) values and the highest TAMV (40.41 ± 5.13 cm/s) and BFV (270.35 ± 36.77 mL/min) were observed on Day 10 post-treatment. In conclusion, melatonin enhanced uterine vascular perfusion under heat stress conditions by reducing vascular resistance and increasing blood flow.

Keywords: Buffalo, melatonin, heat stress, uterine blood flow, doppler ultrasonography, middle uterine artery

Introduction

Buffaloes (*Bubalus bubalis*) are among the most important dairy livestock species in developing countries, particularly India, which ranks first globally in milk production (247.87 million tonnes) and possesses the largest buffalo population (109.85 million head), accounting for nearly half of the world's buffaloes. Buffaloes contribute approximately 43.89% of India's milk production and 15.14% of global dairy output, highlighting their considerable economic significance (FAOSTAT, 2025) [7]. Despite their high productive potential, reproductive inefficiency remains a major constraint in buffalo production systems. Buffaloes are short-day breeders that exhibit pronounced seasonal reproductive activity, with fertility declining during the long-day summer season. In tropical and subtropical regions, elevated temperature, humidity, and fluctuations in forage availability adversely affect reproductive performance, resulting in summer anestrus characterized by poor estrus expression, reduced ovarian activity, and low conception rates (Das and Khan, 2010; Zicarelli, 2017) [5, 7]. Melatonin modulates the hypothalamic-pituitary-gonadal axis and possesses potent antioxidant and cytoprotective properties that can mitigate heat stress-induced oxidative damage. Several studies have demonstrated beneficial effects of melatonin supplementation on reproductive performance. In buffaloes, melatonin administration during summer anestrus enhanced antioxidant status, reduced malondialdehyde and nitric oxide

concentrations, improved estrus induction and conception rates, and increased oocyte recovery and blastocyst yield (Kumar *et al.*, 2015; Singh *et al.*, 2016; Ghuman *et al.*, 2023; Abulaiti *et al.*, 2023) [9, 13, 8, 1]. Although the effects of melatonin on ovarian activity, oxidative stress, and fertility have been extensively studied, its influence on reproductive hemodynamics remains largely unexplored. Uterine blood perfusion plays a crucial role in reproductive function, and Doppler ultrasonography provides a reliable tool for evaluating vascular dynamics. However, information regarding the effect of melatonin supplementation on uterine artery blood flow during the summer season in buffaloes is lacking. Therefore, the present study was undertaken to investigate the effect of melatonin supplementation on uterine blood flow dynamics using color Doppler ultrasonography and to elucidate its relationship with oxidative status and reproductive performance in summer-exposed buffaloes.

Materials and Methods

Experimental Animals and Treatment

A total of eighteen clinically healthy buffaloes were selected and randomly allocated into two experimental groups: a melatonin-treated group and a control group, comprising nine animals ($n = 9$) in each group. Melatonin (Cat. No. M5250, Sigma-Aldrich, USA) was dissolved in corn oil (Cat. No. C8267-500ML, Sigma-Merck) according to the method described by Critser *et al.* (1987) [4]. Buffaloes in the treatment group received a single subcutaneous injection of melatonin at a dose rate of 18 mg per 50 kg body weight as described by Kumar *et al.* (2015) [9], whereas animals in the control group received an equivalent volume of corn oil alone. To assess the influence of climatic conditions on reproductive performance, ambient temperature and relative humidity were recorded throughout the experimental period. Meteorological data were obtained from the Indian Meteorological Department (IMD), Government of India.

Ultrasonographic Evaluation of Uterine Blood Flow

Ultrasonographic examinations were performed at five-day intervals throughout the study period using spectral Doppler ultrasonography to evaluate middle uterine artery (MUA) blood flow characteristics. The abdominal aorta was first identified by positioning the transrectal ultrasound transducer in a transverse orientation facing dorsally and tracing the vessel caudally up to its bifurcation (Singh *et al.*, 2019) [14]. The MUA was subsequently identified within the mesometrium using colour Doppler ultrasonography as a movable arterial structure. Following identification of the vessel, the ultrasound system was switched to spectral Doppler mode and the Doppler sample gate was positioned centrally within the lumen of the artery and adjusted according to vessel diameter. Spectral Doppler recordings were obtained from both the right and left middle uterine arteries. Only recordings exhibiting at least three similar consecutive waveforms were considered acceptable for analysis. Measurements obtained from both sides were averaged to generate a single value for each animal at each examination. All ultrasonographic examinations were performed by the same operator under standardized conditions, and each scanning session lasted approximately 30–45 min. The procedure was carried out according to Debertolis *et al.* (2016) [6] with suitable modifications. The

following spectral Doppler parameters were evaluated to assess uterine blood flow (Sahu *et al.*, 2026) [12]:

1. Resistance Index (RI): $RI = (PSV - EDV)/PSV$, where PSV is peak systolic velocity and EDV is end-diastolic velocity.
2. Pulsatility Index (PI): $PI = (PSV - EDV)/TAMV$, where TAMV represents time-averaged maximum velocity.
3. Time-Averaged Maximum Velocity (TAMV): TAMV was recorded directly from the spectral Doppler waveform and represented the average maximum blood flow velocity during the cardiac cycle.
4. Blood Flow Volume (BFV): $BFV = TAMV \times \pi \times (D/2)^2 \times 60$, where BFV is blood flow volume (mL/min), TAMV is time-averaged maximum velocity, and D is arterial diameter (cm).
5. Middle Uterine Artery Diameter (MUAD): Following spectral Doppler assessment, the transducer was rotated on its transverse axis without linear displacement to obtain a circular cross-sectional image of the vessel in B-mode ultrasonography. The diameter of the middle uterine artery was measured from frozen B-mode images at three different locations corresponding to at least three similar and consecutive waveforms. The mean of these measurements was used for statistical analysis.

Statistical Analysis

All data were analyzed using GraphPad Prism software version 10.5.0.774 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean $\pm$ standard error (SE). The effects of treatment, day of observation, and their interaction on Doppler ultrasonographic parameters were evaluated using two-way repeated-measures analysis of variance (ANOVA), with treatment group (melatonin vs. control) as the between-subject factor and day of examination as the within-subject repeated factor. When significant main effects or interactions were detected, Tukey's multiple comparison *post hoc* test was applied for pairwise comparisons among means. Statistical significance was considered at $p < 0.05$.

Results

The maximum ambient temperature ranged from 26.4 °C to 36.3 °C, with a mean value of approximately 31.6 °C, whereas the minimum temperature varied between 24.4 °C and 28.4 °C, averaging 26.3 °C. Relative humidity ranged from 57% to 100%, with an average value of approximately 76.9%. Consequently, the calculated temperature-humidity index ranged from 77.4 to 87.4, with a mean value of approximately 84.5, indicating that the experimental buffaloes were exposed to moderate to severe heat stress throughout the study period. The effects of melatonin supplementation on middle uterine artery blood flow characteristics are presented in Table 1 and 2; Fig. 1. No significant differences were observed between the melatonin-treated and control groups during the pretreatment period (Day -5) and on the day of treatment (Day 0) for any of the evaluated Doppler parameters. Following melatonin administration, significant alterations in uterine hemodynamics were observed. PI was significantly lower ($p < 0.05$) in the melatonin-treated group on Days 5, 10, and 20 compared to the control group. The lowest PI value was recorded on Day 10 (0.82 ± 0.30).

Similarly, RI decreased progressively following treatment, reaching its minimum value on Day 10 (0.36 ± 0.05), and remained significantly lower than the control group on Day

20 post-treatment. In contrast, parameters associated with vascular perfusion increased significantly following melatonin supplementation.

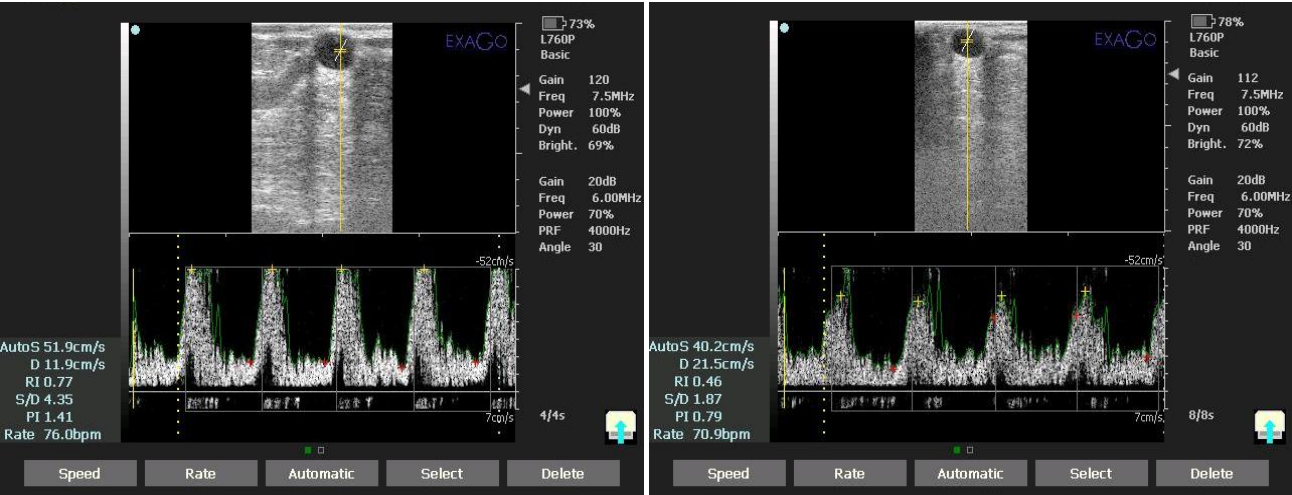


Fig 1: Representative spectral Doppler ultrasonographic images of the middle uterine artery in donor buffaloes showing (A) control animals and (B) melatonin-treated animals.

The MUAD was significantly higher ($p<0.05$) in the melatonin-treated group on Days 5, 10, 15, and 20 compared with the control group. The highest MUAD value was observed on Day 15 (12.73 ± 0.65 mm), followed by a slight decline on Day 20. TAMV increased significantly after melatonin treatment and remained higher than the control group throughout the experimental period. Significant differences were observed on Days 5, 10, and 20, with the

highest TAMV value recorded on Day 10 (40.41 ± 5.13 cm/s). Likewise, BFV was significantly greater ($p<0.05$) in the melatonin-treated group on Days 5, 10, 15, and 20. The maximum BFV was recorded on Day 10 (270.35 ± 36.77 mL/min), after which a gradual decline was observed; however, values remained significantly higher than those of the control group until the end of the study.

Table 1: Peak systolic velocity (PSV; cm/sec), end-diastolic velocity (EDV; cm/sec), pulsatility index (PI) and resistance index (RI) in experimental buffaloes recorded at different time points

Parameter	Days	-5	0	5	10	15	20
PI	Control	3.07±0.47 ^{Aa}	2.76±0.47 ^{Aa}	2.25±0.56 ^{Ab}	1.11±0.31 ^{Bc}	1.81±0.48 ^{Bb}	1.60±0.56 ^{Bb}
	Melatonin	2.76±0.35 ^{Aa}	2.53±0.42 ^{Aa}	1.60±0.49 ^{Bb}	0.82±0.30 ^{Bc}	1.57±0.45 ^{Bb}	1.07±0.33 ^{Bc}
RI	Control	0.82±0.05 ^{Aa}	0.78±0.05 ^{Aa}	0.55±0.09 ^{Ab}	0.38±0.06 ^{Bc}	0.58±0.08 ^{Bb}	0.53±0.09 ^{Bb}
	Melatonin	0.78±0.05 ^{Aa}	0.81±0.05 ^{Aa}	0.53±0.09 ^{Ab}	0.36±0.05 ^{Bc}	0.53±0.08 ^{Bb}	0.42±0.07 ^{Bc}

Data are expressed as mean±SEM. Means bearing different lowercase superscripts (^{a-b}) within the same column differ significantly between groups, whereas means bearing

different uppercase superscripts (^{A-B}) within the same row differ significantly among experimental days within a group ($p<0.05$).

Table 2: Middle uterine artery diameter (MUAD; mm), time-averaged maximum velocity (TAMV; cm/sec) and blood flow volume (BFV; mL/minute) in experimental buffaloes recorded at different time points

Parameter	Days	-5	0	5	10	15	20
MUAD (mm)	Control	8.48±0.13 ^{Aa}	8.42±0.13 ^{Aa}	9.09±0.20 ^{Ab}	10.77±0.26 ^{Ac}	10.62±0.55 ^{Ac}	9.33±0.38 ^{Ab}
	Melatonin	8.37±0.14 ^{Aa}	8.34±0.13 ^{Aa}	9.59±0.18 ^{Bb}	11.88±0.39 ^{Bc}	12.73±0.65 ^{Bd}	11.25±0.48 ^{Bc}
TAMV (cm/sec)	Control	18.51±2.42 ^{Aa}	19.24±2.48 ^{Aa}	23.68±4.06 ^{Ab}	34.99±5.01 ^{Bc}	29.22±4.81 ^{Bc}	31.03±5.52 ^{Bc}
	Melatonin	18.49±1.76 ^{Aa}	17.74±2.09 ^{Aa}	30.38±5.18 ^{Bb}	40.41±5.13 ^{cc}	31.62±5.13 ^{Bc}	34.94±6.12 ^{cc}
BFV (mL/minute)	Control	61.35±8.10 ^{Aa}	67.45±8.59 ^{Aa}	91.90±18.11 ^{Ab}	187.77±31.38 ^{Bc}	139.06±22.37 ^{Bc}	131.40±27.25 ^{Bb}
	Melatonin	64.61±6.95 ^{Aa}	62.53±7.35 ^{Aa}	130.93±20.53 ^{Bb}	270.35±36.77 ^{cc}	230.05±38.61 ^{cd}	207.64±41.95 ^{cc}

Data are expressed as mean±SEM. Means bearing different lowercase superscripts (^{a-b}) within the same column differ significantly between groups, whereas means bearing different uppercase superscripts (^{A-B}) within the same row differ significantly among experimental days within a group ($p<0.05$).

Discussion

The environmental conditions recorded during the study confirmed that the experimental buffaloes were exposed to

substantial thermal stress. The average temperature-humidity index (THI) remained above 84 throughout the experimental period, with values ranging from 77.4 to 87.4, indicating moderate to severe heat stress conditions. Buffaloes are particularly susceptible to thermal stress because of their dark skin pigmentation, sparse sweat glands, and limited capacity for evaporative heat dissipation. Previous studies have reported that reproductive and physiological disturbances begin to occur at THI values above 68, whereas severe heat stress is experienced when

THI exceeds 77 (Umar *et al.*, 2021) ^[15]. Furthermore, elevated relative humidity aggravates thermal stress by reducing the efficiency of body heat dissipation and inducing endocrine, metabolic, and cellular alterations that adversely affect reproductive performance (Ximenes *et al.*, 2024) ^[16]. Under such conditions, impaired ovarian activity, reduced follicular development, compromised oocyte quality, and lower fertility are commonly observed in buffaloes (Das and Khan, 2010) ^[5]. Despite exposure to severe heat stress, melatonin supplementation markedly improved uterine hemodynamics as evidenced by significant reductions in PI and RI, accompanied by increases in MUAD, TAMV, and BFV. These findings indicate reduced vascular resistance and enhanced uterine perfusion following melatonin administration. Lower PI and RI values are indicative of improved blood supply and reduced impedance to blood flow, facilitating efficient delivery of oxygen, nutrients, hormones, and growth factors to reproductive tissues (Bollwein *et al.*, 2016) ^[3]. Enhanced uterine and ovarian perfusion has been closely associated with improved follicular growth, steroidogenesis, oocyte competence, and reproductive performance in cattle and buffaloes. In the present study, the greatest improvement in blood flow parameters was observed around Day 10 post-treatment, coinciding with the first OPU session, suggesting a positive association between enhanced vascularization and ovarian response. Increased blood flow likely improved the follicular microenvironment by facilitating nutrient supply and metabolic exchange required for follicular growth and maturation. The beneficial effects of melatonin on reproductive blood flow may be attributed to its potent antioxidant, anti-inflammatory, and vasoregulatory properties. Melatonin has been reported to protect vascular endothelial cells from oxidative damage, improve endothelial function, and regulate nitric oxide bioavailability, thereby promoting vasodilation and tissue perfusion (Reiter *et al.*, 2018) ^[11].

Conclusion

The present study demonstrated that melatonin supplementation significantly improved uterine hemodynamics in buffaloes exposed to severe summer heat stress. Melatonin treatment reduced vascular resistance, as evidenced by lower PI and RI values, while significantly increasing middle uterine artery diameter, blood flow velocity, and blood flow volume. These findings suggest that melatonin may serve as an effective strategy to mitigate heat stress-induced reproductive impairment and improve fertility in buffaloes during the summer season.

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Competing interest's disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or conflict with the subject matter or materials discussed in the manuscript.

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