Buffalo Bulletin (July-September 2023) Vol.42 No.3
https://doi.org/10.56825/bufbu.2023.4233571

ASSESSMENT OF PLASMA MELATONIN PROFILE IN SUMMER ANESTROUS BUFFALOES EXHIBITING DIFFERENTIAL FERTILITY FOLLOWING MELATONIN IMPLANTS TREATMENT

Sarvpreet Singh Ghuman[1,*], Mrignak Honparkhe[2] and Bilawal Singh[3]

Received: 02 May 2020
Accepted: 29 September 2023

ABSTRACT

Plasma melatonin *vis-a-vis* anestrus or estrus status of buffaloes during summer or winter season (n=50 in each group) suggested its impact on reproductive axis as plasma melatonin activity tended to be higher (P>0.05) in buffaloes exhibiting estrus compared to their anestrous counterparts irrespective of season. The differential reproductive status of these buffaloes was confirmed by history as well as differences (P<0.05) in diameter of largest follicle and luteal profile. Further, for evaluating the impact of melatonin treatment during summer season on reproductive axis, 132 anestrous buffaloes were subcutaneously inserted 2x4 mm absorbable slow-release melatonin implants (18 mg/50 kg b wt) at the base of left ear and 60 buffaloes were used as control. In these buffaloes, ovarian ultrasonography and jugular vein blood sampling was carried out at 7-day interval till day 35 post-treatment or till ovulation, whichever was earlier. Control and implanted buffaloes were subjected to artificial insemination (AI) at overt or induced estrus followed by pregnancy diagnosis at day 90 post-AI. In treatment group, an increase (P<0.05) in plasma melatonin was recorded in all the buffaloes, exhibiting differential fertility status, during post-treatment study period compared to their pre-treatment and control group values. However, within treatment group, there was no difference (P>0.05) in plasma melatonin between ovulatory or non-ovulatory as well as between pregnant or non-pregnant counterparts. Moreover, plasma melatonin within control buffaloes remained similar (P>0.05) throughout the study period irrespective of differential exhibition of fertility. It can be concluded that factors other than circulating melatonin are also involved in the display of differential fertility in terms of initiation of ovarian cyclicity/ovulation or ability to conceive in summer anestrous buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, anestrus, fertility, melatonin, season

[1]Department of Teaching Veterinary Clinical Complex, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India, *E-mail: ghuman_s@yahoo.co.in
[2]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India
[3]Department of Veterinary and Animal Husbandry Extension Education, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India

INTRODUCTION

In buffaloes, the seasonal decline in fertility is subjective to exogenous (photoperiod, climate, nutrition, management) and endogenous (hormones, genotype) causes (D'Occhio *et al*., 2020). In rural Punjab, the proportion of buffaloes exhibiting anestrus (no ovulatory activity) during summer is appreciably greater (74 to 86%) compared to winter (22 to 26%; Singh *et al*., 1989). In addition, the ability of buffaloes to conceive during summer is markedly hampered by an increase in early embryonic mortality from 20% in winter to 45% in summer (Abdoon *et al*., 2001). These interpretations necessitate strategies that can relieve the buffaloes from seasonal decline in fertility.

During the dark phase of photoperiod, melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland and plays a major role in a variety of physiological functions, including regulation of circadian rhythms associated with reproductive and neuroendocrine actions (Reiter, 1998). The decline in daylength was associated with a longer period of melatonin secretion, which was stimulatory to reproductive axis in ewe, a short-day breeder (Malpaux *et al*., 1997). In fact, in buffaloes during summer season, the pregnancy maintenance was favored following melatonin implant treatment (Pandey *et al*., 2019). Thus, it can be hypothesized that melatonin implants can be used as an important medication to not only induce ovarian cyclicity in anestrous buffaloes but can also have beneficial impact on conception rate during summer season (Ghuman *et al*., 2010).

Nevertheless, the literature is not available regarding the circulating melatonin concentrations and exhibition of differential fertility status in terms of initiation of ovarian cyclicity/ovulation or ability to conceive in buffaloes. Hence, we investigated plasma melatonin in anestrus and estrus buffaloes during summer and winter season as well as in melatonin-treated summer anestrous buffaloes exhibiting differential fertility.

MATERIALS AND METHODS

The present study was conducted on buffaloes (mean age: 52.11±4.22 months, mean body weight: 435.21±30.92 kg) of various smallholder dairy farmers near to Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (latitude: 30°56'N, longitude: 75°52'E), India, during winter (November to January) and hot-humid months (April to August, referred to as the 'summer season' with maximum ambient temperatures and relative humidity ranging from 36 to 45°C and 30 to 80%, respectively). For the confirmation of estrus and anestrus, all the buffaloes were subjected to three observations per day before the start of experiment.

Study design

The preliminary study aimed at assessment of plasma melatonin *vis-a-vis* reproductive status of buffaloes during summer and winter season used the buffaloes showing anestrus or estrus during summer or winter season (n=50 in each group). The experimental study aimed at melatonin implants treatment in which summer anestrous buffaloes were randomly allocated to non-implanted control (n=60) or implanted Treatment (n=132) group. Using an implanter, Treatment group buffaloes were administered 2×4 mm size absorbable slow release melatonin implants (18 mg melatonin/ implant, Regulin, CEVA Animal Health Limited,

France) at the base of left ear. Total implants inserted to each buffalo were calculated on basis of their body weight (one implant/50 kg, Ghuman *et al*., 2010).

Ultrasonography

In preliminary study, the buffaloes on day of overt estrus were subjected once to ovarian ultrasonography with a battery-operated B-mode ultrasound scanner equipped with linear-array rectal transducer (Sonosite Vet M Turbo, USA) for measuring the diameter of largest ovarian follicle and corpus luteum (CL). The similar procedures were followed for anestrous buffaloes presented on any random day. In experimental study, summer anestrous buffaloes starting from the day of implants-insertion till day 35 or till initiation of ovarian cyclicity/ovulation, whichever earlier, were subjected to ovarian ultrasonography at weekly interval. The similar procedures were followed for non-implanted controls. A buffalo was considered to have ovulated or not if at subsequent examination(s) the dominant follicle either disappeared (ovulatory follicle) or became atretic (dominant non-ovulatory follicle), respectively. Ovulation was verified based upon the subsequent emergence of a corpus luteum (CL) on a site previously occupied by the disappeared dominant follicle (Ghuman *et al*., 2010). During the study period, in the Control group, 43 buffaloes failed to initiate ovarian cyclicity (non-ovulatory) and 17 buffaloes resumed cyclicity (ovulatory), whereas, Treatment group had 59 non-ovulatory and 73 ovulatory buffaloes.

Blood sampling and hormone analysis

In the preliminary and experimental study, the blood samples collected from the jugular vein in a heparinized vial after each scan were centrifuged at 3000 rpm for 15 minutes. Plasma was separated and kept in two aliquots at -20°C for analysis. Plasma progesterone was assayed with a solid-phase radioimmunoassay using antisera raised in our laboratory (Ghuman *et al.,* 2009). Sensitivity of the assay was 0.1 ng ⁄ ml; intra- and inter- assay variation coefficients were 5.4% and 9.2%, respectively. Plasma melatonin was estimated using a commercially available Direct Immunoenzymatic Assay Kit (Bovine MT ELISA Kit, China). The sensitivity of the assay was 6.9 pg/ml. The intra-assay and inter-assay coefficient of variation was <10% and <12%, respectively.

Artificial insemination (AI) and conception rate

After the start of experimental study, in the control and Treatment group, all the buffaloes were subjected to three observations per day for the detection of overt estrus. Buffaloes exhibiting overt estrus were inseminated using frozen semen, and the others failing to show estrus were subjected to fixed time AI protocol (FTAI) on day 35 of experiment. This protocol involved administration (i/m) of gonadotropin releasing hormone (GnRH, 0.02 mg) on day 0 and 9, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 500 µg cloprostenol sodium) on day 7. In addition, buffaloes were inserted per-vaginally a controlled internal drug release (CIDR, 1.38 g progesterone) device for 7 days (day 0 to 7). On day 10 of protocol, the fixed time AI was done using frozen semen. Per-rectal pregnancy diagnosis of all the inseminated buffaloes was carried on day 90 post-insemination. By the end of experiment, the control group had 39 non-pregnant and 21 pregnant buffaloes, whereas, Treatment group had 56 non-pregnant and 76 pregnant buffaloes.

Statistical analysis

Numerical data are represented as

mean±SEM, and differences were considered to be significant at P<0.05. Two samples of Student's t-test was employed on the preliminary study data of plasma melatonin, largest ovarian follicle diameter and luteal profile (plasma progesterone, CL diameter) of buffaloes exhibiting estrus or anestrus during summer and winter. Similarly, the differences in plasma melatonin data of experimental study were assessed with regard to concentrations in: (a) pre- *vs*. post-treatment period, (b) Control *vs*. Treatment group, and (c) ovulatory *vs.* non-ovulatory and pregnant *vs.* non-pregnant buffaloes within and between groups. Statistical analyses were performed using MINITAB release 13.2 statistical software (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSIONS

The data of preliminary study in buffaloes exhibiting estrus, during summer as well as winter, revealed higher plasma melatonin compared to their anestrous counterparts (119.22±10.97 *vs.* 81.04±8.33 pg/ml; P>0.05, Table 1), thus, suggesting the association of circulating melatonin with reproductive axis activity. Similarly, the buffaloes in which reproductive activity was uniformly distributed around the year had persistently high plasma melatonin and low seasonal ovulatory activity was associated with low plasma melatonin in these buffaloes (Parmeggiani *et al*., 1994). The Mediterranean buffaloes showing seasonal reproductive trend had highest plasma melatonin in early winter corresponding to the start of reproductive axis activity and lowest in summer (Borghese *et al*., 1995).

Furthermore, during summer as well as winter, the largest follicle attained ovulatory diameter in buffaloes exhibiting estrus (12.81±0.26 mm) or anestrus (10.02±0.41 mm), although the same was greater in the estrus group (P<0.05; Table 1). It was revealed earlier that buffaloes during summer anovulatory period display dominant follicles that even after attaining ovulatory size (10.1 to 15.7 mm) undergo atresia without ovulation (Ghuman *et al.,* 2010). Moreover, the anestrous condition in buffaloes during summer season was referred as 'superficial' in view of the presence of follicles of ovulatory size, and these animals might have required little push to already existing gonadotropin stimulus to start the ovulatory activity (Ghuman *et al*., 2010).

In addition, the luteal activity (CL and plasma progesterone) in anestrous buffaloes was higher (P>0.05) irrespective of season in comparison to their estrus exhibiting counterparts (Table 1). This could be due to the fact that the ultrasonography and blood sampling in estrus buffaloes was carried out on the day of estrus when luteal activity is known to be at minimal level. Whereas, in anestrous buffaloes, the same procedures were carried out on a random day and buffaloes with persistent CL or silent estrus might have contributed to active luteal profile.

In summer anestrous buffaloes, following melatonin implants treatment, about three-fold increase (P<0.05) in plasma melatonin concentrations was recorded on day 7 post-treatment (from 94.9±8.7 to 253.7±23.4 pg/ml), which continued till the end of study period (day 35: 303.8±28.4 pg/ml, Table 2). In addition, on all post-treatment days, plasma melatonin was higher (P<0.05) in treatment group compared to their control group counterparts ranging between 96.9±13.3 and 113.9±16.7 pg/ml (Table 2). The melatonin implants used in the study were designed to induce high plasma melatonin for at least 60

Table 1. Plasma melatonin, largest ovarian follicle diameter and luteal profile of buffaloes (n=200) exhibiting anestrus or estrus activity during summer or winter season.

Reproductive status	Season	Plasma melatonin, pg/ml	Largest ovarian follicle, mm	Luteal Profile	
				Corpus luteum, mm	Plasma progesterone, ng/ml
Anestrus	Overall, n=100	81.04±8.33	10.02±0.41[a]	6.01±0.66[a]	0.59±0.05[a]
	Summer, n=50	73.18±10.81	10.11±0.45[c]	6.29±0.89[c]	0.64±0.07[c]
	Winter, n=50	88.47±12.59	10.06±0.30[e]	5.73±0.99[e]	0.55±0.08[e]
Estrus	Overall, n=100	119.22±10.97	12.81±0.26[b]	2.27±0.39[b]	0.21±0.02[b]
	Summer, n=50	102.08±14.12	11.87±0.35[d]	2.21±0.54[d]	0.25±0.03[d]
	Winter, n=50	136.83±16.54	13.75±0.33[f]	2.34±0.56[f]	0.17±0.03[f]

[a] *vs* b, c *vs* d, e *vs* [f] $P<0.05$, within a column.

Table 2. Plasma melatonin profile in control and melatonin-implanted buffaloes *vis-a-vis* reproductive outcome during post-treatment period. Day 0 = Insertion of melatonin implants.

Day	**Control, n=60**		**Melatonin-implanted, n=132**	
Plasma melatonin, pg/ml				
0	113.9±16.7		94.9±8.7[a]	
7	113.1±13.6[c]		253.7±23.4[b,d]	
14	96.9±13.3[c]		271.9±23.1[b,d]	
21	105.1±13.9[c]		271.1±24.4[b,d]	
28	100.8±14.0[c]		284.1±24.1[b,d]	
35	108.1±13.7[c]		303.8±28.4[b,d]	
Plasma melatonin, pg/ml vs. Dominant follicle outcome				
Day	**Non-Ovulatory, n=43**	**Ovulatory, n=17**	**Non-Ovulatory, n=59**	**Ovulatory, n=73**
0	102.3±16.5	131.8±34.4	91.8±9.6[a]	96.9±12.9[a]
7	102.2±14.4[c]	137.9±30.0[e]	259.1±33.6[b,d]	249.5±32.7[b,f]
14	96.6±15.5[c]	98.4±18.6[e]	310.5±35.3[b,d]	237.4±29.6[b,f]
21	110.0±15.7[c]	74.2±20.9[e]	304.5±35.3[b,d]	232.0±30.9[b,f]
28	100.9±15.3[c]	99.6±4.4[e]	302.5±32.3[b,d]	255.1±35.5[b,f]
35	109.9±15.5[c]	95.3±10.7[e]	290.8±31.7[b,d]	351.6±64.9[b,f]
Plasma melatonin, pg/ml vs. Artificial Insemination outcome				
Day	**Non-Pregnant, n=39**	**Pregnant, n=21**	**Non-Pregnant, n=56**	**Pregnant, n=76**
0	100.4±17.6	137.6±34.2	93.9±12.4[a]	95.8±12.3[a]
7	104.2±14.6[c]	128.6±27.7[e]	256.9±30.0[b,d]	251.1±35.1[b,f]
14	83.7±14.4[c]	126.8±27.2[e]	278.3±32.8[b,d]	266.5±32.8[b,f]
21	90.3±15.8[c]	136.9±25.2[e]	285.9±33.5[b,d]	256.8±34.8[b,f]
28	93.3±16.9[c]	115.7±25.5[e]	296.0±32.4[b,d]	271.2±36.3[b,f]
35	89.3±12.2[c]	156.5±32.5[e]	315.9±36.1[b,d]	286.0±46.8[b,f]

[a] *vs* [b]$P<0.05$, within a column; [c] *vs* [d], [e] *vs* [f]$P<0.05$, within a row.

days, although their functionality can extend to more than 100 days in ewes (Forcada *et al*., 2002). In a similar study on Murrah anestrous buffaloes in summer season, plasma melatonin concentrations ranged between 149.5±6.7 to 343.0±9.0 pg/ml during post-melatonin implant period as compared to the values between 29.0±1.3 to 32.0±2.0 pg/ml in non-implanted controls (Pandey *et al*., 2019). In another study, summer anestrous buffaloes administered with single subcutaneous injection of melatonin (18 mg/50 kg b wt) dissolved in sterilized corn oil displayed higher plasma melatonin compared to controls (Kumar *et al*., 2016). In post-partum cows, serum melatonin remained considerably high till 4th week following subcutaneous administration of melatonin dissolved in beef tallow (Sharpe *et al*., 1986).

Nevertheless, within Treatment group, with regard to an increase ($P<0.05$) in plasma melatonin in anestrous buffaloes during post-treatment period, there was no difference ($P>0.05$) in plasma melatonin between ovulatory or non-ovulatory as well as between pregnant or non-pregnant counterparts (Table 2). Moreover, plasma melatonin within control buffaloes remained similar ($P>0.05$) throughout the study period irrespective of differential exhibition of fertility. This suggested a buffalo-to-buffalo variation in requirement of melatonin-induced gonadotrophin stimulus for attaining threshold level necessary to activate reproductive axis for the induction of ovulation or for getting conceived. Moreover, the factors other than circulating melatonin can also be involved in the display of differential fertility in terms of initiation of ovarian cyclicity/ovulation or ability to conceive in summer anestrous buffaloes. In addition, heat-stress induced oxidative stress can provoke alterations in follicle and CL development and thus can increase embryonic mortality (Willard *et al*., 2003). Oxidative stress status may have a role as melatonin is an important indirect anti-oxidant (stimulates anti-oxidative and inhibits pro-oxidative enzymes) and powerful direct free radical scavenger known to provide protection against ionizing radiation-induced toxic effects (Vijayalaxmi *et al*., 2004). Moreover, melatonin implants were able to induce estrus and alleviate oxidative stress in summer anestrous buffaloes (Singh *et al*., 2016). Nevertheless, majority of the observed plasma metabolites were not altered following melatonin implants treatment of buffaloes (Singh *et al*., 2010).

In conclusion, the present study indicated that slow-release melatonin implants treatment in anestrous buffaloes during summer season have a role in exhibition of differential fertility in terms of initiation of ovarian cyclicity/ovulation or ability to conceive, however, the role of factors other than melatonin cannot be ruled out.

REFERENCES

Abdoon, A.S., O.M. Kandil, T. Otoi and T. Suzuki. 2001. Influence of oocyte quality, culture media and gonadotropins on cleavage rate and development of *in vitro* fertilized buffalo embryos. *Anim. Reprod. Sci*., **65**(3-4): 215-223. DOI: 10.1016/s0378-4320(01)00079-3

Borghese, A., V.L. Barile, G.M. Terzano, A.M. Pilla and A. Parmeggiani. 1995. Melatonin trend during season in heifers and buffalo cows. *Bubalus Bubalis*, **1**: 61-65.

D'Occhio, M.J., S.S. Ghuman, G. Neglia, G. Valle, P.S. Baruselli, L. Zicarelli, J.A. Visintin, M. Sarkar and G. Campanile G. 2020. Exogenous and endogenous factors in seasonality of reproduction in buffalo:

A review. *Theriogenology*, **150**: 186-192. DOI: 10.1016/j.theriogenology.2020.01.044

Forcada, F., J.A. Abecia, O. Zuniga and J.M. Lozano. 2002. Variation in the ability of melatonin implants inserted at two different times after the winter solstice to restore reproductive activity in reduced seasonality ewes. *Aust. J. Agri. Res*., **53**(2): 167-173. DOI: 10.1071/AR00172

Ghuman, S.P.S., D. Dadarwal, M. Honparke, J. Singh and G.S. Dhaliwal. 2009. Production of polyclonal antiserum against progesterone for radioimmunoassay. *Indian Vet. J*., **86**(9): 909-911.

Ghuman, S.P.S., J. Singh, M. Honparkhe, D. Dadarwal, G.S. Dhaliwal and A.K. Jain. 2010. Induction of ovulation of ovulatory size non-ovulatory follicles and initiation of ovarian cyclicity in summer anoestrous buffalo heifers (*Bubalus bubalis)* using melatonin implants. *Reprod. Domest. Anim*., **45**: 600-607. DOI: 10.1111/j.1439-0531.2008.01310.x

Kumar, A., S. Mehrotra, G. Singh, V.P. Maurya, K. Narayanan, A.S. Mahla, R.K. Chaudhari, M. Singh, Y.K. Soni, B.L. Kumawat, S.K. Dabas and N. Srivastava. 2016. Supplementation of slow-release melatonin improves recovery of ovarian cyclicity and conception in summer anoestrous buffaloes (*Bubalus bubalis*). *Reprod. Domest. Anim*., **51**(1): 10-17. DOI: 10.1111/rda.12639

Malpaux, B., C. Viguie, D. Skinner, J.C. Thiery and P. Chemineau. 1997. Control of the circannual rhythm of reproduction by melatonin in the ewe. *Brain Res. Bull*., **44**(4): 431-438. DOI: 10.1016/s0361-9230(97)00223-2

Pandey, A.K., P. Gunwant, N. Soni, Kavita, S. Kumar, A. Kumar, A. Magotra, I. Singh, J.B. Phogat, R.K. Sharma, Y. Bangar, S.P.S. Ghuman and S.S. Sahu. 2019. Genotype of MTNR1A gene regulates the conception rate following melatonin treatment in water buffalo. *Theriogenology*, **128**(1): 1-7. DOI: 10.1016/j.theriogenology.2019.01.018

Parmeggiani, A., R. Di Palo, L. Zicarelli, G. Campanile, L. Esposito, E. Seren, P.A. Accorsi and S.M. Soflai. 1994. Melatonin and reproductive seasonality in the buffalo cow. *Agricoltura Recerca*, **153**: 41-48.

Reiter, R.J. 1998. Melatonin and human reproduction. *Ann. Med*., **30**(1): 103-108. DOI: 10.3109/07853899808999391

Sharpe, P.H., D.R. Gifford, P.F. Flavel, M.B. Nottle and D.T. Armstrong. 1986. Effect of melatonin on postpartum anestrus in beef cows. *Theriogenology*, **26**(5): 21-29. DOI: 10.1016/0093-691X(86)90169-X

Singh, B., S.P.S. Ghuman, R.S. Cheema and A.K. Bansal. 2016. Melatonin implant induces estrus and alleviates oxidative stress in summer anestrus buffalo. *Indian J. Anim. Reprod*., **37**: 28-32.

Singh, J., S.P.S. Ghuman, D. Dadarwal, M. Honparkhe, G.S. Dhaliwal and A.K. Jain. 2010. Estimations of blood plasma metabolites following melatonin implants treatment for initiation of ovarian cyclicity in true anestrus buffalo heifers. *Indian J. Anim. Sci*., **80**(3): 229-231.

Singh, G., G.B. Singh and G.S. Dhaliwal. 1989. Studies on reproductive status of rural buffaloes in summer. *Indian J. Anim. Reprod*., **10**: 151-153.

Vijayalaxmi, R.J. Reiter, D. Tan, T. Herman and C.R. Thomas. 2004. Melatonin as a radioprotective agent: A review. *Int.*

J. Radiat. Oncol., **59**(3): 639-653. DOI: 10.1016/j.ijrobp.2004.02.006

Willard, S., S. Gandy, S. Bowers, K. Graves, A. Elias and C. Whisnant. 2003. The effects of GnRH administration postinsemination on serum concentrations of progesterone and pregnancy rates in dairy cattle exposed to mild summer heat stress. *Theriogenology*, **59**(8): 1799-1810. DOI: 10.1016/s0093-691x(02)01232-3