Original Article

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THE RESPONSE OF BLOOD PLASMA METABOLITE CONCENTRATIONS OF ANESTRUS LACTATING BUFFALO TO CIDR-eCG WITH OR WITHOUT MELATONIN TREATMENT

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ABSTRACT

The objective of this study was to compare the efficiency of CIDR-eCG protocol with or without melatonin treatment on blood plasma metabolites in anestrus Murrah buffaloes during out-of-breeding season. Twelve lactating Murrah buffaloes were allotted to control and treated groups each of six animals. Treated buffaloes were implanted with melatonin (18 mg melatonin / 50 kg body weight) for 45 days, then animals of both groups received CIDR for 9 days. All animals received 500 IU eCG intramuscularly one day before CIDR removal and 10 µg GnRH on the day of AI. The animals were subjected to estrus detection daily as per farm routine. At weekly intervals, blood samples were collected to determine the concentration of blood plasma metabolites over 84 days' period between June and September. All buffaloes exhibited estrus after CIDR removal. CIDR-eCG preceded with melatonin treatment increased plasma total protein, albumin, cholesterol and HDL-cholesterol ($P<0.05$) in comparison to only CIDR treated control. Buffaloes treated with melatonin plus CIDR exhibited increased ($P<0.01$) plasma glucose on days 28, 53 and 55 and reduction ($P<0.01$) in HDL and AST at time of pregnancy diagnosis, while plasma concentration of phosphorus was the highest ($P<0.05$) at day 84. In conclusion, CIDR-eCG treatment preceded with melatonin implantation improved reproductive performance and altered blood metabolite composition of lactating buffaloes during out-of-breeding season as compared to CIDR-eCG only.

Keywords: *Bubalus bubalis*, buffaloes, melatonin, CIDR-eCG protocol, blood metabolites, lactating buffalo

INTRODUCTION

Although buffaloes (*Bubalus bubalis*) are able to breed throughout the year in tropical regions, they show a seasonal breeding pattern off the equator (Perera, 2011; Singh *et al*., 2000), that is determined by melatonin secretion in response to short-day length (Zicarelli, 1997). In

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general, buffaloes' reproductive activity is mainly determined by day length, climate (Ambient temperature and relative humidity) and nutrition (Singh *et al*., 2000). In summer, poor nutrition coupled with high ambient temperature were implicated in anestrus condition in buffaloes (Shah, 1988). Also, heat stress in the hot summer months is an important driver of anestrus in buffalo, whose effects are mediated by increased blood concentration of prolactin (Perera, 2011), leading to decreased progesterone (P_4) secretion and, consequently, extended calving to conception intervals due to repeat breeding and, in general, reduced reproductive performance (Zicarelli, 2007). Reduced sexual activity coincided with the increase in ambient temperature and day length, both in heifers as well as mature buffaloes (Tailor *et al*., 1990). The proportion of buffaloes exhibiting estrus during the period of short-day length was significantly greater than that in the long-day period (74% versus 26%) (Tailor *et al*., 1990). Also, the conception rates are usually lower between February and August and the numbers of services per conception are higher for animals calving in summer compared to those calving in other seasons (Madan, 1988). Decreased day length is a strong determinant of the onset of puberty and postpartum ovarian activity, while ambient temperature and relative humidity have relatively lesser impact (Singh, 1993). Hormonal treatments have been designed to control follicular and luteal functions induce and synchronize estrus or ovulation and, more importantly, eliminate estrus detection by timed artificial insemination. In deep anestrous buffaloes, out-of-season breeding requires melatonin, a hormone produced and stored in the pineal gland during the day and secreted during the dark. It controls the reproductive rhythm in diverse ruminant species. Its mediated pathways regulate GnRH pulsatility and, therefore, the activity of the reproductive neuroendocrine axis. It also modulates prolactin secretion by acting on the hypophysis. Alternative choice of controlled internal drug release (CIDR) device, supplying natural P_4 in conjunction with equine chorionic gonadotrophin (eCG) (Hamra *et al*., 1989) that increases estrous response and conception rate, is also employed for inducing cyclicity in different livestock species. In addition to melatonin administration, concomitant application of estrus / ovulation induction protocols suggests that CIDR gave better results in anestrus buffaloes (Ramadan *et al*., 2014; 2016).

Excessive heat causes decreased food intake and disturbances in protein and energy metabolism, mineral balance, enzymatic reactions, hormones and metabolites secretion in the blood (Delfino *et al*., 2012). Metabolic disorders caused by thermal stress lead to reduced milk production, growth, and reproductive rates and increase the susceptibility of animal to diseases causing economic loss (Nardone *et al*., 2010). Climate changes leading to increases in average temperature and reduced rainfall put the sustainability of the livestock production systems to risk (Scholtz *et al*., 2013). In lactating animals, blood biochemical variables including total protein, triglycerides, FFA, and thyroid hormones are important indicators of metabolic activity (Karapehlivan *et al*., 2007). In addition, some blood metabolites including total proteins and glucose are significantly correlated with milk yield in goats (Mohy El-Deen *et al*., 1985; Hassan *et al*., 1986). Milk production depends on mammary gland development in addition to the availability of nutrients, especially glucose (Ingvartsen and Friggens, 2005) that can promote milk production by enhancing lactose synthesis (Janicek *et al*., 2007), the major osmoregulator of the mammary gland which ultimately impacts milk yield (Rigout *et al*., 2003). In addition, the high energy requirement for milk synthesis during lactation is associated with high serum total protein (Bremmer *et al*., 2000) whereas; using glucose for milk lactose synthesis reduces serum glucose during lactation (Antunovic *et al*., 2011).

This study was performed to evaluate the response of blood plasma metabolite concentrations in true anestrus lactating buffaloes receiving melatonin implants in conjunction with CIDR-eCG protocol for preventing summer-induced decline in ovarian activity.

MATERIALS AND METHODS

The experiment was done at the Central Institute for Research on Buffaloes, Hisar, India (29°10′N, 75°41’E) on anestrus lactating buffaloes during the out-of-breeding season from June to September. Buffaloes were observed for visual signs of estrus twice daily for one hour each as per the standard farm management routine. All procedures and experimental protocols were conducted in accordance with the “Guide for the Care and Use of Agricultural Animals in Research and Teaching”, Federation of Animal Science Societies (2010).

Animals and management

Total of 12 lactating Murrah buffalo weighing 400 to 500 kg in parities 2 to 4 having body condition score between 4-5; yielding 7 to 9 kg of milk per day at day 65 to 70 of lactation were experimented in the current study. The study was carried out during the hot-humid months of June to September. The range of ambient temperature and relative humidity were 35 to 45°C and 35 to 80% respectively. Daily maximum and minimum temperatures and relative humidity were recorded professionally in Agriculture Meteorology department, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana. Records of temperature and humidity were collected from a nearby station about 5 kms away from the experimental farm. The temperature humidity index (THI) was calculated according to the equation of Kendall and Webster (2009):

$$THI = (1.8*AT+32)-[(0.55-0.0055*RH)\times(1.8*AT-26)],$$

Where AT = average temperature (°C), RH = Relative humidity (%). The mean values of temperature, humidity and THI for different weeks are depicted in Figure 1. The experimental period was divided into: low (70<); moderate (70-80) or high (>80) THI. Lactating buffaloes were confined for the entire period of study to a barn with access to an open sheltered space. They were subjected to teasing twice daily before allocation for the experiment for estrus detection, but failed to exhibit estrus signs. Animals were fed on roughages and concentrate supplement according to their body weight requirements (NRC, 2001). Chaffed green fodder and wheat straw were also offered. Water was freely accessibly at all times. Animals were free from diseases and were clinically normal with a healthy appearance. Lactating buffaloes were milked twice a day; morning (4 am) and evening (3 pm). They were also subjected to gynecological examination before inclusion in the study, and those diagnosed with any pathological condition of the reproductive tract were excluded. The ovarian activity was assessed ultrasonographically before commencement of the trial and based on the absence of a functional corpus luteum (CL), the animals were categorized as non-cycling.

Experimental design

Lactating buffaloes were randomly allocated to melatonin non-implanted (Control) and implanted (Treated) groups (n = 6 each). In melatonin-treated group, animals were administered 2×4 mm absorbable melatonin implants (18 mg melatonin / implant, Regulin, CEVA Animal Health Limited, Chesham, Buckinghamshire, UK) at the base of left ear using an implanter. Total implants inserted to each animal were calculated on the basis of body weight (one implant/50 kg,) (Papachristoforou *et al*., 2007; Ramadan *et al*., 2019; 2020). These implants were designed to release melatonin for at least 60 days, although their functionality can extend to more than 100 days without disturbing the endogenous secretion of melatonin as seen in ewes (Forcada *et al*., 1995; 2002). On day 45 after melatonin implantation, all animals were treated with Eazi-Breed CIDR (1.38 g of progesterone; Pfizer Animal Health, New Zealand) for 9 days (removed on day 54), and were intramuscularly treated with 500 IU eCG (Folligon; Intervet, International, Boxmeert, Netherlands) on the day before CIDR removal (day 53). All animals were subjected daily for estrus detection and were also, intramuscularly treated with 10 µg GnRH (Receptal; Intervet, International, Boxmeert, Netherlands) at the second insemination which was carried out on days 55 / 56.

Blood sampling

After each scan, blood samples were collected at 6 am from the jugular venipuncture into a heparinized vial. Plasma was separated immediately by centrifugation and kept in two aliquots at -20°C until analysis. Blood sampling were taken from the treated and control buffaloes once a week throughout the first 6 weeks of the experiment (Days 0, 7, 14, 21, 28, 35 and 42) then on the day of CIDR insertion (Day 45) and removal (Day 54) and on day of GnRH injection (Day 56). Also, blood samples were collected on days 65, 75 and 84 (Days 10, 21, and 30 after AI) to determine the blood plasma metabolites and diagnose pregnancy.

Plasma biochemical parameters

Plasma total protein was measured by the Biuret method as described by Armstrong and Carr, (1964), total albumin concentration by the method of Doumas *et al.* (1972) and Glucose according to Barham and Trinder (1972). Total cholesterol concentration was measured calorimetrically as described by Watson (1960). High density lipoproteins-Cholesterol (HDL-cholesterol) was measured by phosphotungstic acid method as described by Burstein *et al.* (1970). Transaminase activities [aspartate aminotransferase (AST) and alanineaminotransferase (ALT)] were measured colorimetrically (Reitaman and Frankel, 1957). Alkaline phosphatase (ALP) and Phosphorous (P) were measured as performed by Zilva and Pannall (1979). Calcium (Ca) was measured as described by Beeler and Catrou (1983). Calcium: Phosphorous ratio was calculated.

Statistical analysis

All data records were tested for normality with the Shapiro-Wilk (W) test from the UNIVARIATE procedure (SAS, 2000) and results indicated that all data were distributed normally (W >0.90). Data for testing the effect of melatonin was analyzed using PROC MIXED of SAS (SAS Inst., Inc., Cary, NC) for repeated measures. Treatment and days were used as fixed effects and individual lactating buffalo as random. To compare the effect of melatonin followed by CIDR *virsus* CIDR only on blood plasma metabolites, the following model was used:

$$Y_{ijk} = \mu + T_i + D_j + (T \times D)_{ij} + b(THI)_{ijk} + e_{ijk},$$

Where Y_{ijk} is the observed value of the dependent variable determined from a blood sample taken from each animal, μ is the overall mean, T_i is the fixed effect of the i^{th} melatonin + CIDR or CIDR only treatments (i = 1, 2), D_j is the fixed effect of the j^{th} day (j = Day 0: Day 84), $(T \times D)_{ij}$ is the first order interaction between treatments and days and b is the regression of each observation on the corresponding $(THI)_{ijk}$ and e_{ijk} is the residual error. Significant differences among means within each classification were tested using least square differences 0.05.

RESULTS

Plasma metabolites concentrations for anestrus lactating buffalo as affected by melatonin implantation in conjunction with CIDR-eCG treatment are displayed in (Table 1). Melatonin treatment increased ($P<0.05$) plasma total protein, albumin, cholesterol and HDL-cholesterol concentrations. Concentrations of other blood plasma components were not affected by melatonin treatment. The effect of days after hormonal treatments caused increases ($P<0.05$) in glucose at days 28, 53 and 55. Increased ($P<0.05$) concentrations of plasma cholesterol at days 14 and 21 tended to decrease thereafter. Hormonal treated buffaloes showed reduction ($P<0.05$) in plasma concentration of HDL-cholesterol and AST after AI, while phosphorus recorded the highest concentration ($P<0.05$) at the end of experiment. None of the regressions of plasma metabolites on THI were significant.

DISCUSSION

The increased blood serum total protein concentration by the administration of melatonin and CIDR was advantageous than CIDR only to lactating cows as reported in sheep (El-Mokadem *et al*., 2019). High blood serum protein enhances the induction of reproductive activities and maintains colloid osmotic pressure, blood pressure and acid-base balance which may help in the intiation of reproductive activity of domestic animals (Swenson, 1977). The high value of serum total protein during lactation is necessary for milk synthesis (Bremmer *et al*., 2000) and also for reactivation of ovarian function after delivery. However, continuous protein equilibrium should exist between plasma and follicular fluids (Jindal *et al*., 1997).

The high levels of plasma albumin concentration due to melatonin treatment in postpartum buffaloes are in agreement with results reported by Singh *et al*. (2010); Ramadan *et al*. (2015) on buffalo heifers treated with melatonin. Plasma albumin is characterized by its small molecular weight and large residual negative charge at pH 7.4, which makes it very hydrophilic. These characteristics make albumin responsible for 75 to 80% of the colloidal osmotic pressure in blood and an important factor in keeping equilibrium with tissue fluids (Swenson, 1977). With advancement of melatonin implantation, serum glucose concentration increased in agreement with that reported previously in buffalo heifers (Ramadan *et al*., 2015). The reduction in serum glucose concentration during early pregnancy (Days 65, 84 of study) could possibly be due to the incremental requirement for glucose as the fetus and uterus utilize glucose as a major energy source (Lindsay, 1973). In addition, low glucose levels in pregnancy are associated with foetus development and mobilization of maternal glucose to foetal blood circulation (Jacob and Vadodaria, 2001). Glucose is the principal source of energy for the mammalian cells. All cells require a constant supply of this indispensable nutrient and only small changes in serum level of glucose are tolerated without adverse effects on the health of the animal (Kaneko *et al*., 1997).

Buffaloes enrolled in the present study exhibited an increase in plasma cholesterol subsequent to melatonin implantation. Advantageously, cholesterol has a significant role in the ovarian function as it is the precursor molecule for steroid biosynthesis (Arshad *et al*., 2005). The lower cholesterol concentration in reproductively acyclic buffaloes indicates the contribution of particular nutrients to the development of reproductive cyclicity (Das and Khan, 2010). Previous studies in cows and sheep (Marcos *et al*., 1990; Nazifi *et al*., 2002) reported a strong reduction in lipogenesis during the lactation period. During the late-pregnancy and lactation periods the number of total insulin receptors decrease and the insulin stimulation of lipogenesis becomes inefficient (Guesnet *et al*., 1991). That effect was more prominent on large-sized follicles of reproductively acyclic buffaloes and could be the reason for decreased estradiol synthesis leading to an inability to stimulate preovulatory LH surge and subsequent ovulation (Bhat *et al*., 2015). It is noteworthy to mention that cholesterol in the follicular fluid is a part of the high density lipoprotein derived from blood and transported across the blood-follicle barrier (Brantmeier *et al*., 1987).

Minerals are required for both physiological and biochemical functions. Many of the body disorders are associated with the altered serum mineral levels. The mean blood plasma calcium / phosphorus ratio in the present study was found slightly higher in anestrus lactating buffalo treated with melatonin and CIDR compared to CIDR only. In general, the level of blood calcium alone might not affect the normal physiology of animal directly, but alteration in calcium / phosphorus ratio do affect the ovarian activity through blocking the action of pituitary gland resulting in prolongation of first post-partum estrus and ovulation, also influence the ability of animals to utilize other micro-minerals, certain enzyme systems and reproductive efficacy (Dhoble and Gupta, 1996). The altered calcium / phosphorus ratio may affect the ovarian function through a blocking action on pituitary gland, which results in prolongation of first estrus and ovulation ultimately reducing the reproduction efficiency (Satish, 2003).

Ali *et al*. (1991) reported that calcium and phosphorus play intermediate role in the action of hormones and enzymes at sub cellular level in an integrated fashion for initiation of estrus in animals. Kumar *et al*. (2016) also reported significantly lower calcium / phosphorus ratio in post-partum anoestrus than normal cyclic buffaloes. The disturbed calcium / phosphorus ratio in post-partum animals was also reported by Kumar *et al*., (2010); Roginosu *et al*., 2011.

In conclusion, buffaloes exhibit negligible reproductive activities during out-of-breeding season. Melatonin and CIDR treatment achieved significant increase in blood plasma concentrations of total protein, albumin, cholesterol and HDL-cholesterol, which might be involved in altered ovarian activity in response to the treatment, as well as establishment of early pregnancy.

Table 1. Effect of controlled internal drug release (CIDR; n = 6) device and CIDR device preceded with melatonin (Mel + CIDR; n = 6) treatments on blood biochemical constituents of lactating buffaloes during out-of-breeding season (Least square means ± SEM).

Parameter	**Treatment**	
	CIDR	**Melatonin + CIDR**
Total protein (g/dl)	8.65± 0.10[b]	9.11±0.09[a]
Albumin (g/dl)	3.34± 0.04[b]	3.48±0.02[a]
Glucose (mg/dl)	54.52±1.75	56.28±1.76
Cholesterol (mg/dl)	128.87±2.96[b]	138.2±2.06[a]
HDL-cholesterol (mg/dl)	76.03±1.40[b]	80.28±1.21[a]
AST (IU/L)	181.20±3.17	173.89±4.03
ALT (IU/L)	63.02±1.19	65.43±1.41
ALP (IU/L)	166.13±6.41	151.63±7.31
Ca (mg/dl)	7.68±0.25	7.76±0.19
Pho (mg/dl)	6.30±0.26	6.50±0.18
Ca Pho ratio	1.25±0.01[a]	1.20±0.01[b]

HDL Cholesterol: High Density Lipoproteins Cholesterol (mg/dl);
AST: Aspartate Aminotransferase (IU/L); ALT: Alanine Aminotransferase (IU/L);
ALP: Alkaline Phosphatase (IU/L); Ca: Calcium (mg/dl); Pho: Phosphorous (mg/dl).
[a-b]Within a row, means without a common superscript differ ($P<0.05$).

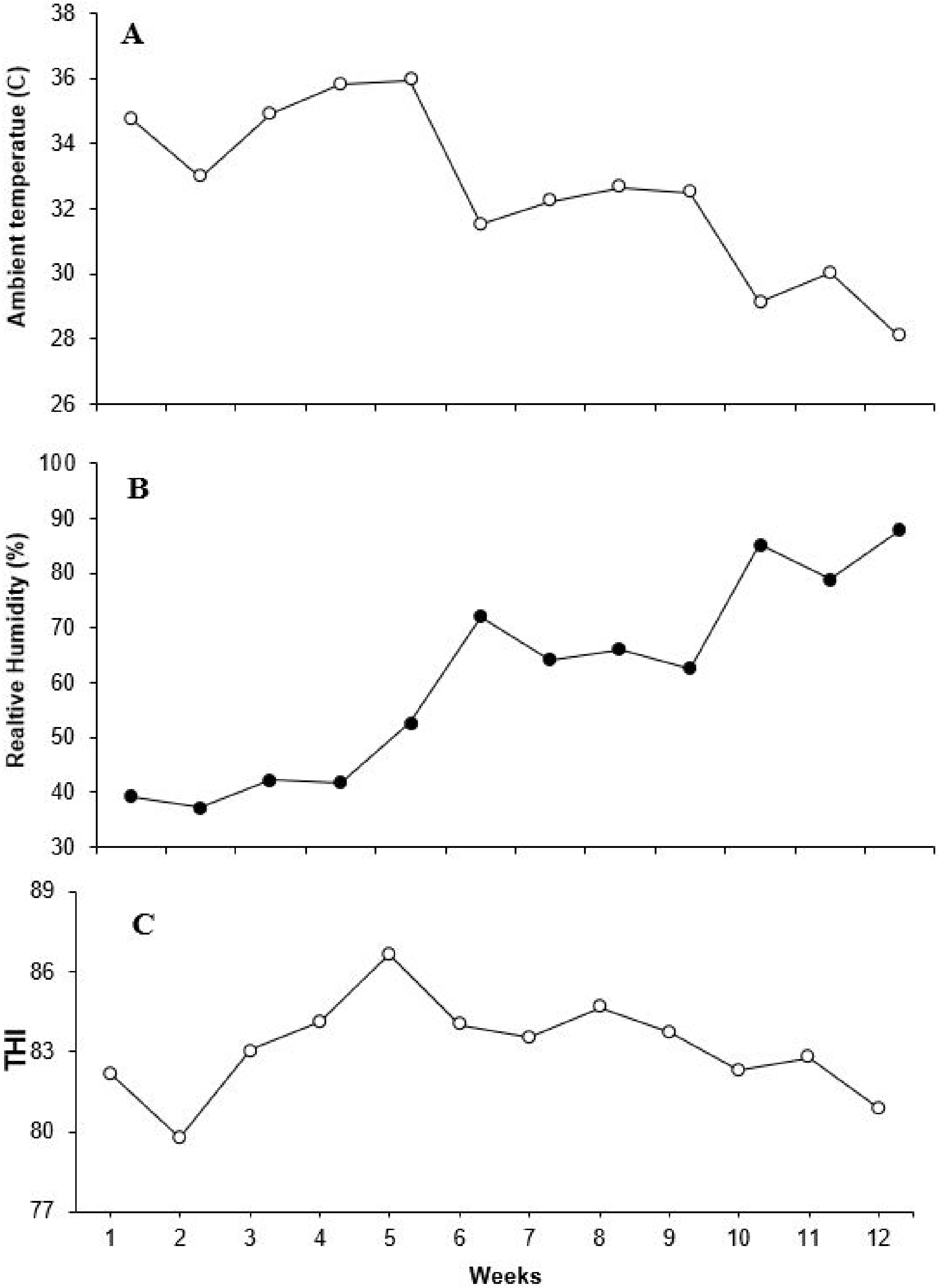

Figure 1. The weekly average of ambient temperature (A), relative humidity (B) and (THI, C, Kendall and Webster, 2009) over the experimental period.

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