Chapter 5

Role of Melatonin in Reproductive Seasonality in Buffaloes

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Additional information is available at the end of the chapter

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Abstract

Buffaloes are characterized by seasonal reproductive activity. Anestrus buffalo heifers and lactating buffaloes were used to study the effect of melatonin treatment on the resumption of ovarian activity during out-of-breeding season. Buffaloes of treated group were injected or implanted with melatonin (18 mg melatonin/50 kg body weight). Using CIDR-eCG protocol preceded with melatonin successfully achieved estrus behavior and induced conception rate during out-of-breeding season. Furthermore, the reproductive performance of buffaloes during out-of-breeding season was clearly improved by melatonin implantation in conjunction with CIDR-eCG protocol due to the luteotrophic effect of melatonin expressed as increasing diameter of CL (corpus luteum) and progesterone concentration. This improvement resulted in greater values of conception rate, in melatonin implanted compared to not implanted buffaloes. Melatonin implantation in anestrus buffalo heifers increased the diameter of largest follicles and melatonin concentration but progesterone and luteinizing hormone (LH) concentrations were decreased. In addition, melatonin implantation in anestrus lactating buffaloes increased the SOD (superoxide dismutase) enzyme activity. Sustained release of exogenous melatonin significantly protects against oxidative stress while increasing beneficial total antioxidant capacity (TAC) concentration in summer-stressed anestrus buffaloes. Melatonin implantation in conjunction with CIDR-eCG protocol successfully improved some blood metabolites, in anestrus buffalo heifers during out-of-breeding season under tropical conditions.

Keywords: melatonin, CIDR, buffalo, reproductive seasonality, ovarian activity, oxidative stress
1. Introduction

Even though buffaloes are able to breed throughout the year in tropical regions, but with distance from the equator they show a seasonal breeding pattern [1, 2], that is determined by melatonin secretion in response to short-day length [3]. In general, reproductive activity of buffaloes is mainly determined by day length, climate (ambient temperature and relative humidity) and nutrition [2]. During summer, poor nutrition coupled with high ambient temperature was implicated with anestrus condition in buffaloes [4]. Heat stress in the hot summer months is an important driver of anestrus in buffalo, whose effects are mediated by increased blood concentrations of prolactin [1], leading to decreased progesterone secretion, and, consequently, extended calving to conception intervals due to repeated breeding and, generally, reduced reproductive performance [5].

In buffalo (*Bubalus bubalis*), reduced sexual activity has been reported as coincident with an increase in ambient temperature and day length [6], in either heifers or mature buffaloes. 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%, respectively) [6]. Also, the conception rates are usually lower between February and August [7], and the number of services per conception are higher in animals calving in summer compared to animals calving at all the other seasons [7]. Decreasing day length may be a stronger determinant of the onset puberty and postpartum ovarian activity, whereas ambient temperature and relative humidity may have relatively lesser impact [8]. Nevertheless, the species physiological characteristics adversely affect buffalo dairy industry and result in a typical seasonal calvings that impair milk supply throughout the year [9].

To overcome seasonality and discipline milk production, it is necessary to implement suitable management schemes, particularly for out-of-season breeding [10]. Hormonal therapies to induce estrus and ovulation in anestrus buffaloes became important breeding strategies to achieve these goals. Hormonal treatments, adapted from other seasonal ruminant species, have been designed to control follicular and luteal functions, to synchronize estrus and ovulation and, more importantly, to eliminate estrus detection by scheduled of timed artificial insemination (TAI).

In deep anestrus buffaloes, out-of-season breeding requests the use of melatonin. Melatonin is a hormone produced and stored in the pineal gland during the day and secreted during the dark, starting after sunset and ending at sunrise. Melatonin controls the reproductive rhythm in diverse ruminant species, like goats and sheep (short-day species), and also in horses (long-day species), especially at higher latitudes [11]. Melatonin-mediated pathways regulate GnRH pulsatility and, therefore, the activity of the reproductive neuroendocrine axis. It also modulates prolactin secretion by acting on the hypophysis. In addition to melatonin, the concomitant application of estrus/ovulation protocols suggests that controlled internal drug release systems (CIDR) gave better results for anestrus buffaloes [12]. The priming of hypothalmo-hypophysial-gonadal (HHG) axis with adequate amounts of P4 is beneficial for the recovery of reproductive function after calving and, hence, a better display of estrus behavior at the induced estrus [13]. Furthermore, the sufficient priming of endometrium with
P4 might be necessary to enhance the conception rate [14]. Melatonin implantation in conjunction with CIDR-eCG protocol successfully induced estrus behavior and enhanced conception rate in anestrus heifers and anestrus lactating buffaloes during out-of-breeding season under tropical conditions [12, 15].

The objectives of this study were to revise the efficacy of melatonin-based treatments for alleviation of the summer-induced decline in ovarian activity in anestrus heifers and lactating buffaloes and also to evaluate its effects on blood hormonal levels and metabolites concentrations, as well as those of antioxidant enzyme activities, as indicators of sustainability of buffaloes to the expenditure of melatonin treatment for preventing summer-induced decline in ovarian activity in true anestrus buffaloes.

2. Characterization of the buffalo reproductive cycle

The reproductive patterns of an animal are a result of the interaction of the endogenous regulatory mechanisms, mainly endocrine, with environmental signals. This complex interaction may deeply affect the reproductive function, e.g., females suspend cyclic ovarian activity in some periods of the year, or respond to the presence of a dominant individual on the group, or they can ovulate and come into estrus during the non-reproductive season. Bashir (2006) [16] has reported that buffaloes calving in summer (June–August) had a shorter calving interval than those calving in other seasons. The longest calving interval observed in buffaloes was calving in winter (December–January). This means that the buffaloes that had calved just before the onset of their breeding season (October–November) had more chances of getting bred than those calving after passing their breeding season. The buffalo heifers attaining their proper weight just before their breeding season are more likely to get bred than those passing this period and thus, may have lower age at puberty and consequently at calving than those attaining proper weight after this season. Based on this hypothesis, while raising the replacement buffalo heifers, efforts should be made to keep an eye on both critical weight of buffaloes for attaining the age at puberty and also the season in which this weight is attained. In this regard, adjustments in feeding regime may be required to get the critical weight of buffalo heifers just before their breeding season. Because once this breeding season is over, then feeding for accelerated growth may not reduce the age at their puberty and then one has to wait for their next breeding season to see the puberty in the heifers.

The normal interestrous interval in buffaloes may vary from 16 to 28 days, the estrus lasting for 10–20 h during breeding season [17]. The interval between the onset of estrus and LH surge is 1–12 hours and ovulation occurs 18–40 h after the LH surge [18]. The reduced intensity of heat symptoms recorded in buffaloes as compared to cows is possibly associated with lower estradiol levels due to smaller size pre-ovulatory follicles [17], in a mechanism that is further exaggerated by low P4 during luteal phase, a reduced pulsatile LH secretion, growth of ovulatory follicle and low estradiol production during summer months [19].

The stress and adverse environmental factors exert a direct effect on the neuroendocrine set-up, resulting in hyperprolactinemia, reduced pulsatile gonadotrophin secretion, poor
folicular maturation and poor estradiol production, thereby culminating in poor heat expression and anestrus [20]. The postpartum anestrus has been differentiated into temporary (less than 150 days) and deep anestrus (more than 150 days), according to the time elapsed between calving and conception [3], or into superficial and deep anestrus based upon the presence or absence of follicular turnover [21].

3. Mechanisms regulating seasonal reproduction in buffaloes

The place of origin and gestation length undoubtedly influence the way in which reproductive seasonality occurs. The natural necessity to coincide calving and weaning with the most suitable time of year represents one of the causes of this ‘adaptation’ process [22]. This time of year should satisfy the nutritional requirements of the offspring through a period in which etiologic agents (infectious and parasitic) are less aggressive and/or present. Those born under the most favorable conditions have brought about the natural selection of individuals endowed with a more ideal reproductive seasonality that promotes the survival of the species [23].

In domestic animals, spring calving (March–May), which guarantees good availability of forage to offspring in temperate zones occurs whenever reproduction takes place in autumn in species with 5-month gestation (sheep and goats) or in the previous spring in the case of 11- and 12-month gestation (horses and donkeys). The same calving period, therefore, is conditioned by the neuroendocrine system. The reactivation of the reproductive cycle with regard to the length of gestation [short day breeder (negative photoperiod) or long breeder (positive photoperiod)] is therefore controlled.

Researchers in Italy [24] showed that buffaloes displaying stronger seasonality showed high plasma melatonin concentrations 2 hours after sunset, even when they were moved to another farm where other females showed low plasma melatonin concentrations and less sensitivity toward light stimulation [25]. The plasma melatonin concentrations had a repeatability of 0.733 [26]. If the heredity of plasma melatonin turns out to be high, as expected on the base of the high repeatability, the determination of plasma melatonin could be incorporated into genetic selection programmes for buffalo [22]. Lincoln [27] showed that sheep presenting continuous cyclic activity throughout the year retain this characteristic even if living at latitudes where other genotypes were sensitive to the light:dark ratio.

The differences between night and day concentrations of plasma melatonin in March were lower in heifers (5.0 times) than in adult buffaloes (28.3 times) [28]. It has been shown that buffaloes that calve in spring were more adaptable to out-of-breeding-mating strategy [26] and the heifers were less sensitive to the photoperiod [22, 28]. Heifer fertility is not compromised by season [22]. During the summer and when daylight hours are more than the dark, there is an increase in blood prolactin, but contrary to the assertions by Madan [7], buffalo regularly conceive. It is believed that hyperprolactinemia is secondary to hypothyroidism [29] during the warm months. Hypothyroidism exerts a positive feedback on thyroid stimulating hormone and hence on thyrotropin-releasing hormone, which in turn promotes an increase in prolactin [22, 23].
4. Methods to manipulate the out-of-season breeding in buffaloes

In small ruminants, induction of ‘out-of-season’ estrous cycles may be practiced, enabling spring breeding and, therefore, fall lambing/kidding, resulting in winter production of milk and lambs/kids for the winter markets. Several methods to control the reproduction of small ruminants involve the manipulation of the environmental light (extension of hours of light in a day) [30]. Some others are based on the administration of exogenous hormones that modify the physiologic chain of events involved in the sexual cycle (pharmacologic methods) and ultimately modify the luteal phase of the cycle (progesterone/progestagen and prostaglandins) or the annual pattern of reproduction (melatonin). Synchronization of estrous allows the control and short ending of lambing and kidding, with synchronization of weaning and uniform batching of animals to slaughter; it also allows more efficient use of labor and animal facilities. An appropriate management of reproduction allows ewes and does to breed in the spring to increase the supply of product to the market place on a year-round basis. Pharmaceutical control of reproduction is possible, usually through administration of hormones related to the natural estrous cycle, such as progesterone and/or melatonin [31]. Similar treatments can be used to tame the reproductive cycle in buffaloes.

In buffalo, during the out-of-breeding season (spring and summer), the greater light:dark ratio (long days) suppresses the estrus behavior and the occurrence of ovulation. Anestrus buffaloes have insufficient pulsatile secretion of LH to support the final stages of follicular development, and subsequently, estrus behavior and ovulation do not occur, limiting reproductive efficiency, especially in artificial insemination (AI) programs [32]. Therefore, hormonal therapies to induce estrus and ovulation in anestrus buffaloes became important breeding strategies. These hormonal treatments have been designed to control follicular and luteal functions, to synchronize estrus and ovulation and, more importantly, to eliminate estrus detection by preplanned scheduled of timed artificial insemination (TAI).

In most studies, the success rate was lower when treatment was performed in periods of low breeding activity or during seasonal anestrus, and various modified protocols have been tried to improve pregnancy rates. Among the hormonal therapies developed for cattle, GnRH plus PGF2α-based TAI protocols resulted in a reduced ovulatory response when applied in anestrus buffalo [33]. Also, during the out-of-breeding season, when a high incidence of anestrus is expected, lower pregnancy rates are encountered in buffalo cows synchronized with the Ovsynch protocol for TAI [33]. Recent studies in buffalo have demonstrated that similar pregnancy rates at TAI in both breeding and out-of-breeding seasons can be obtained with the use of progesterone (P4), estradiol (E2) and (eCG)-based protocols [34].

Melatonin implants for subcutaneous application have been commercially available in several countries. These implants have been widely used to advance the breeding season of anestrus ewes and goats. Melatonin implants induce high plasma concentrations of melatonin for 24 h every day, without suppressing the endogenous secretion of the pineal hormone during the night. Thereby, implants cause a short day-like response by lengthening the duration of the melatonin signal [35]. The implants contain 18 mg of melatonin and are designed to maintain...
high plasma melatonin concentrations for at least 60 days, although most of them continue to release the hormone for longer than 100 days [36].

The mechanisms by which melatonin improves reproductive performance are not fully understood, as the pineal hormone can act at different body sites. Effects at hypothalamus-hypophysis level have been previously mentioned, and an effect at ovary level seems to be consistent, either by reducing atresia during late folliculogenesis to increase ovulation rate [16] or by acting as luteotropic agent [37] to improve fertility. The time of treatment is important to guarantee a good efficacy. Melatonin implants inserted around the summer solstice have been widely used as a means of advancing the out-of-breeding season in buffaloes in areas with high latitude.

In buffalo reproduction, few investigations have been made to clarify a relationship between plasma melatonin concentrations and seasonal reproductive pattern. The Mediterranean buffaloes showing seasonal reproductive trend had highest night-time plasma melatonin concentrations in winter and lowest in summer [24]. In another study carried out on heifers and buffaloes, the melatonin levels showed remarkable differences between seasons. In peak summer because of the shortest night, the lowest plasma melatonin with less persistence of melatonin peak were found, whereas the highest concentrations were noted in early winter corresponding to the start of hypothalamic-hypophysial-ovarian axis (HPO) activity [28]. Moreover, low plasma melatonin was associated with a low seasonal ovulatory activity in buffaloes [24]. This decrease in ovulatory activity during long days happens despite the presence of follicles with ovulatory size (12–14 mm) on the ovaries of nulliparous and pluriparous Mediterranean buffaloes [3, 21].

Melatonin appears to act at hypothalamic sites to increase the release of GnRH pulses by modulating the negative feedback potency of estradiol [38], which acts at hypothalamic and hypophysial loci to reduce luteinizing hormone secretion [39]. The photoperiod modulates KiSS-1 expression via melatonin, strongly suggesting that kisspeptin relays photoperiodic information to the HPO axis. Kisspeptin stimulates LH secretion in a GnRH-dependent manner by increasing GnRH secretion into the hypophysial portal blood [40]. Kisspeptin neurons express estrogen and progesterone receptors [41], which are directly regulated by these steroids in a manner consistent with both positive and negative feedback regulation of pulsatile GnRH secretion [41].

The protocol for melatonin application is simple and less demanding than the traditional treatment of induction-synchronization of estrus using progestogens. Administration of melatonin during anestrus seems to improve the fertility. It has been reported that melatonin implants in buffaloes can improve conception rate [12, 15]. Melatonin treatment in buffalo is necessary to induce cyclicity during anestrus in out-of-breeding season in which evidenced an improvement in conception rate [42, 43]. Ramadan et al. [12, 15] found that melatonin implantation alone did not affect the post-treatment reproductive performance in either anestrus buffalo or lactating buffaloes during out-of-breeding season. On the other hand, combined melatonin and CIDR treatments induced cyclicity and enhanced the reproductive performance of anestrus buffalo heifers and anestrus lactating buffaloes during out-of-breeding season [12, 15] (Figure 1).
5. The effect of exogenous melatonin on hormonal levels and blood metabolites in buffaloes

In anestrus lactating buffaloes injected with melatonin, the serum P4 concentrations increased (0.71 ng/ml) after melatonin injection compared with control group (0.28 ng/ml) at days 12 and 16 post-treatment (post-AI), during the summer season, suggesting a luteotrophic effect of melatonin [42]. Also, exogenous melatonin might improve uterine expression of P4 receptors or their binding capacity, which would result in higher reproductive efficiency [42]. Melatonin implants plus CIDR in buffalo heifers and lactating buffalo were able to maintain the corpus luteum at day 21 of estrous cycle [12, 15]. In addition, the plasma P4 was highest at the second ovulation post-treatment than the first ovulation in implanted anestrus heifers in summer [43] (Table 1).

The decrease in P4 concentrations in anestrus lactating buffaloes compared with buffalo heifers was attributed to a prolactin effect. Misztal et al. [44] reported that in lactating ewes the melatonin concentration decreases, whereas prolactin, responsible for the initiation and maintenance of lactation, increases. Prolactin may block the hypothalamic mechanism responsible for episodic release of LH or inhibit the positive feedback of estrogen on LH secretion, and it can even affect ovarian steroidogenesis by altering the number of LH receptors [45].

Lactating buffaloes treated with melatonin alone present higher E2 concentrations recorded (16 pg/ml) after melatonin treatment in comparison to the control group (9.02 pg/ml) [42]. The pattern of serum E2 profile in the induced estrous cycle was highest on the day of estrus and decreased in the second week of the cycle [46]. The elevated levels of E2 4 days before estrus may mediate the LH surge, as demonstrated in cattle [47]. The reducing effect of melatonin on estradiol concentration has also been reported in sheep [38]. Moreover, melatonin decreases the estradiol receptor expression in deep endometrial stroma of ewes, where E2-estradiol receptor complex acts as a luteolytic agent [48]. In ewes, estradiol concentration has also been correlated with the mean response of prostaglandins to oxytocin, via the estrogenic stimulation of uterine oxytocin receptors [49].
Melatonin treatment interferes with the effects of melatonin directly in buffalo’s hypophysis. In anestrus heifers, with the advancement of melatonin treatment, on days 28 and 42, it resulted in a decrease in serum LH (3.44 mlU/mL) [12]. Melatonin had no effect on the frequency of LH pulses [12]. In sheep, the rise in circulating melatonin is responsible for the increase in GnRH and gonadotropins, thus leading to follicular growth and ovulation [50]. However, this finding is inconsistent with the reduction of LH serum concentration observed in the melatonin-treated buffalo heifers. The failure of high concentrations of serum melatonin to increase LH concentration might be explained by the reduction of buffalo estradiol concentration in summer [51]. Estradiol seems to be positively linked with the action of melatonin on the female reproductive activity, where melatonin exerts a modulatory effect on LH secretion, stimulating its release in the presence of estradiol feedback (cyclic animals) and inhibiting it during steroid deprivation (animals in anestrus) [52]. Also, the presence of low concentrations of plasma GnRH and gonadotropins during the summer period in buffaloes [53] might disturb the development of LH receptors and the synthesis of adequate estradiol in the dominant follicle [51] required to induce the ovulatory surge of gonadotropins [54].

The administration of slow-release melatonin (implants) was responsible for an increase in serum melatonin concentration, ranging from 14.34 to 412.31 pg/ml in treated lactating buffaloes [42]. Administration of exogenous slow-release melatonin induced the restoration of the ovarian activity in summer anestrus buffaloes, which can be explained by a ‘cascading effect’ of at least a 10-fold increase on the plasma concentrations of GnRH and gonadotrophins, which provides the necessary boost for follicular growth and ovulation [50]. It has been shown that treatments with melatonin implants tend to originate an increase in serum melatonin concentration (9.10 pg/ml) in anestrus buffalo heifers [12] and in anestrus lactating buffalo (6.80 pg/ml) (Table 1), which could be associated with the antiprolactin action of melatonin, as it was suggested in another species [55].

| Route of administration | Associated treatment | Animal type | P₄ (ng/ml) | E₂ (pg/ml) | LH (mlU/mL) | Melatonin (pg/ml) | Ref(s) |
|------------------------|----------------------|-------------|-----------|------------|-------------|------------------|--------|
| Injection              | -                    | Lactating (n=20) | 0.71      | 16         | -           | 412.3            | [42]   |
| Implantation           | -                    | Heifers (n=12)  | 0.66      | -          | -           | -                | [43]   |
| Implantation CIDR eCG GnRH | Heifers (n=8)    | 0.72      | -          | 3.44       | 9.10        | [12] |
| Implantation CIDR eCG GnRH | Lactating (n=6)  | 0.97      | -          | -          | 6.80        | [15] |

1/(18 mg/50 kg BW).
CIDR, controlled internal drug release device (1.38 g progesterone); eCG, equine chorionic gonadotropin (500 IU Folligon, i.m at day before CIDR removal) and GnRH, gonadotropin-releasing hormone (10 μg Receptal, i.m at day after CIDR withdrawal).

Table 1. Hormonal levels in anestrus Murrah buffalo before synchronization with melatonin during the non-breeding season.
Singh et al. [56] showed that, in anestrus lactating buffalo, melatonin treatments decrease plasma albumin (2.7 g/dl), compared to pre-treatment concentrations. In contrast, Ramadan et al. [57] found higher albumin values (3.41 g/dl) in treated anestrus buffalo heifers implanted with melatonin. Ramadan et al. [57] also reported that the combined treatment of melatonin and CIDR resulted in an increase in plasma concentrations of albumin, glucose, high-density lipoprotein (HDL), alanine aminotransferase (ALT) and reduction in plasma alkaline phosphatase (ALP) compared with control animals (Table 2). Because of the interaction of melatonin with various endocrine systems [58], it was proposed that melatonin treatment may initiate ovarian cyclicity in true anestrus buffalo heifers through its influence on body metabolism [59]. Albumin, being the most abundant plasma protein, could play a major role as an antioxidant in plasma, mediating thiol oxidation and carbonyl formation [60].

Plasma concentrations of glucose (65.16 mg/dl) were increased by melatonin and CIDR treatment [57]. Glucose is the primary energy source for the ovary and it is possibly metabolized in the ovary through anaerobic pathways. It also stimulates the ovarian follicular growth [61]. Furthermore, plasma glucose is a positive metabolic signal for the central control of GnRH release [62].

Increased lipolysis during lactation is hormonally regulated and not an expression of energy deficiency. Plasma concentrations of HDL (62.29 mg/dl) were increased by melatonin treatment combined with CIDR [57]. The concentration of transaminase enzyme ALT (98.72 IU/L) was increased with treatment of melatonin and CIDR in anestrus buffalo heifers during out-of-season breeding [57]. Singh et al. [56] found that exogenous melatonin did not record any alterations in plasma concentration of AST and ALT enzyme activities in anestrus lactating buffaloes. Thus, minor alterations in AST during treatment period in spite of elevated plasma activities of ALT suggested that hepatic functions were not impaired in the buffalo [63]. Follicular growth is a dynamic process in which follicular development is continuous but accelerates during the later stages of the estrous cycle [64]. Plasma concentrations of ALP (97.99 IU/L) were increased by melatonin treatment combined with CIDR in anestrus buffalo heifers [57]. The ALP content in serum may changes during the estrous cycle [64]. ALP is a lysosomal enzyme that catalyzes various reactions in the body, including synthesis of proteins and DNA turnover within the nucleus [65].

| Route of administration | Associated treatment | Animal type | Albumin (g/dl) | Glucose (mg/dl) | HDL (mg/dl) | ALT (IU/L) | ALP (IU/L) | Ref(s) |
|-------------------------|---------------------|-------------|----------------|----------------|-------------|------------|------------|--------|
| Implantation            | -                   | Lactating (n=5) | 2.7            | 62.1           | 35.1        | 56.4       | -          | [56]   |
| Implantation            | CIDR eCG GnRH       | Heifers (n=8) | 3.41           | 65.16          | 62.29       | 98.72      | 97.99      | [57]   |

1(18 mg/50 kg BW).

CIDR, controlled internal drug release device (1.38 g progesterone); eCG, equine chorionic gonadotropin (500 IU Folligon, i.m at day before CIDR removal); GnRH, gonadotropin-releasing hormone (10 μg Receptal, i.m at day after CIDR withdrawal); HDL, high-density lipoprotein; ALT, alanine aminotransferase and ALP, alkaline phosphate.

Table 2. Serum metabolites and some enzyme activities in anestrus Murrah buffalo synchronized with melatonin during out-of-breeding season.
6. The effect of exogenous melatonin on the antioxidant enzyme activity in buffaloes

Metabolic pressures of adaptation to high milk yield and environmental conditions, especially high ambient temperature, exert a retrograde effect on buffalo reproduction. High temperature-humidity index (THI) predisposes buffaloes to develop oxidative stress [66] with anti-gonadotropin and anti-steroidogenic actions [67]. This in turn is involved in modulation of estrous cycle and uterine environment causing defective embryo development and reproductive failure in buffaloes [68] and also contributes to the elongation of the postpartum anestrus [69]. Jan et al. [70] found that overall greater concentrations of biomarkers of oxidative stress coupled with the reduced concentrations of total antioxidant capacity (TAC) in follicular fluid of acyclic buffaloes are indicative of the involvement of oxidative stress in the suspension of the ovarian activity. Further evidence showed that the oxidative stress and the depletion of the antioxidant activity play a significant role in diminished ovarian function, as evidenced by the significant increment in circulatory malondialdehyde (MDA) and simultaneous decline of TAC in buffalo heifers [71].

Melatonin affects membrane fluidity in different cells under conditions of high oxidative stress, indicating that its mechanism of action relates to possible due to an antioxidant activity associated to the directly scavenging of free radicals and the inhibition of lipid peroxidation [72] and thereby the decrease in MDA concentration. A wide range of antioxidant enzymes are also induced [73], counteracting the generation of free radicals due to inhibition of the activity of some pro-oxidant enzymes such as nitric oxide (NO) synthase and lipoxygenase [72]. Moreover, melatonin stimulates the activity of several enzymes related to the antioxidant defense system [74].

Several authors reported that exogenous melatonin increased blood melatonin level, which has a positive correlation with antioxidant capacity [75]. Kumar et al. [76] refer that the total antioxidant capacity (TAC) in the serum of melatonin-treated female buffalos during summer anestrus tends to be higher (2.22 mmol/L) on days 12 and 24 after the onset of melatonin treatment, compared with the control group (1.42 mmol/L), supporting the previous studies of Ahmed et al. [77]. In the same study [76], it was also shown that the concentrations of serum MDA and NO tend to decrease (3.97 mmol/L and 42.41 mmol/L, respectively) (Table 3).

The beneficial effect of a sustained release of melatonin on the increase in TAC and the reduction in MDA and NO concentrations [76] suggests a beneficial effect of melatonin in mitigating the oxidative stress effects on fertility, alike that reported in women [78]. The beneficial effect of exogenous melatonin in the resumption of estrus may be related with the facilitation of cellular functions in the growing follicles, because NO, an important intra-ovarian factor, regulates the process of follicular development through its multifaceted role in angiogenesis, vasodilation, regulation of normal follicular growth and function, steroidogenesis and ovulation [79].

Ramadan et al. [15] observed that melatonin treatment resulted in an increase in blood plasma of SOD (superoxide dismutase, 4.72 U/mg protein) activity in anestrus lactating buffaloes under tropical conditions (Table 3). Therefore, the increased activity of erythrocytic SOD in
treated anestrus bovine heifers could be attributed to the physiological upregulation of this enzyme to mitigate superoxide radical challenge \[80\]. It has been established that, in bovine, the SOD and catalase activities in the CL (corpus luteum) parallel the P4 plasma patterns: the enzymatic activity of catalase and SOD present a sixfold to eightfold increase from day 6 to day 16 of the estrous cycle thereafter decreasing through the luteal regression \[80\]. SOD plays important roles in the maintenance of luteal function, possibly by rescuing the corpus luteum when pregnancy occurs \[81\]. It has been reported that SOD acts protectively against superoxide radicals to stimulate P4 production by the corpus luteum \[82\].

The results of Singh et al. \[83\] are consistent with the results reported by Kumar et al., in summer anestrus buffaloes \[76\], which also documented the potential of exogenous melatonin to augment the antioxidative capacity of summer anestrus buffalo \[76\]. Reactive nitrogen species are another category of potentially destructive substances that react with melatonin. It was suggested that melatonin acts by binding with calmodulin and suppresses the gene transcription of NO synthase enzyme \[84\]. The enzyme is involved in a rate limiting step in the synthesis of nitric oxide; a known oxidant suggested being a major free radical causing follicular damage, thereby resulting in anovulatory condition in summer anestrus buffalo \[84\].

7. The effects of exogenous melatonin on the reproductive performance in buffaloes

7.1. Effect of melatonin alone

Estrus response and fertility in melatonin-treated female is largely dependent on the animal status, the association of a progesterone treatment and of animals’ management at onset of treatment. According to Kumar et al. \[42\], a single subcutaneous injection of melatonin...
(18 mg/50 kg BW in sterilized corn oil) in summer anestrus water buffalo showed a definite influence on the interval to induced estrus (18 days), obtaining a 90% estrus induction rate. In addition, treatments with melatonin implant (18 mg/50 kg BW) in delayed pubertal buf-falo heifers during summer successfully induced estrus within 6–36 days [43]. Also Ghuman et al. [43] reported a successful estrus induction in 100% anestrus buffalo heifers implanted with melatonin in summer season (Table 4). However, in other studies on buffalo, melatonin implants alone failed to induce estrus signs for the first 45 days after implantation in either anestrus heifers or lactating buffaloes during summer season [12, 15].

Ghuman et al. [43] showed that melatonin treatment increased the diameter of the largest follicle (14.55 mm) compared to control group (12.2 mm), which might be due to the inability to ovulate even after attaining normal pre-ovulatory diameter (>9 mm) [43]. This suggests that the follicle diameter itself may not be determinant of ovulation. The failure of ovulation of largest follicles could be a drive of a lack of sufficient LH stimulus. The amplitude and frequency of GnRH pulses and, therefore, those of gonadotropins, required to induce the growth of follicles are different from those required for ovulation [85]. The wide extension of the period until estrus induction might be derived from the ovarian structures presented at the ovary at starting of treatment in summer anestrus buffalo heifers. Early responders might have, on the day of melatonin treatment, dominant follicles or follicles emerging in the ovaries, whereas in the later responders the follicles developing in the ovaries still not reached divergence. This hypothesis implies an individual variation in the requirements for exoge-nous melatonin to attain the threshold level necessary to activate hypothalamus-hypophysial-ovarian axis, as defended by Ghuman et al. [43].

Buffalo heifers in anestrus implanted with melatonin revealed larger CL diameter (15.16 mm) at second ovulation than at first [43]. Ghuman et al. [43] hypothesized that in the absence of a strong negative-feedback effect of estradiol on the hypothalamus, the sustained release of

| Route of administration¹ | Associated treatment | Animal type | EIR (day) | Estrus % | LF (mm) | CL (mm) | CR (%) | Ref(s) |
|--------------------------|---------------------|-------------|----------|----------|--------|---------|--------|-------|
| Injection                | -                   | Lactating (n=20) | 18       | 90       | -      | -       | -      | [76]  |
| Injection                |                     | Lactating (n=20) | 18       | 90       | -      | -       | 32.4   | [42]  |
| Implantation             | -                   | Heifers (n=12)   | 6-36     | 100      | 14.55  | 15.16   | -      | [43]  |
| Implantation             | -                   | Lactating (n=41) | -        | 65.8     | -      | -       | -      | [83]  |
| Implantation             | CIDR eCG GnRH       | Heifers (n=8)    | -        | 100      | 10.04  | 16.40   | 37.5   | [12]  |
| Implantation             | CIDR eCG GnRH       | Lactating (n=6)  | -        | 100      | 13.90  | 18.33   | 100    | [15]  |

¹(18 mg/50 kg BW).
CIDR, controlled internal drug release device (1.38 g progesterone); eCG, equine chorionic gonadotropin (500 IU Folligon, i.m at day before CIDR removal); GnRH, gonadotropin-releasing hormone (10 μg Receptal, i.m at day after CIDR withdrawal); EIR, estrus induction response; LF, largest follicle; CL, corpus luteum and CR, conception rate.

Table 4. Estrus response, ovarian activity and conception rate in anestrus Murrah buffalo synchronized with melatonin during out-of-breeding season.
exogenous melatonin in summer anestrus buffalo heifers could stimulate the hypothalamus-
hypophysial-ovarian axis triggering the early induction of estrus. Lactating Murrah buffaloes treated with single subcutaneous injection of melatonin during summer evidenced an improvement in conception rate (32.4%) compared to untreated animals (0%) [42].

7.2. Effect of melatonin plus CIDR

When melatonin treatments are associated to CIDR and eCG, the estrus rate after the progesterone removal recorded 100% in either the buffalo heifers or cows during out-of-breeding season. Also, the diameter of the follicle at the day of AI is higher after melatonin treatment plus CIDR in anestrus heifers and anestrus lactating buffaloes [12, 15]. Moreover, compared to the use of CIDR alone, the association of melatonin treatment and CIDR presented a superior ability to maintain CL for 21 days post-AI (12.5% vs. 37.5%, respectively) in anestrus heifers or between 21 and 30 days post-AI (33.3% vs. 100%, respectively) in anestrus lactating buffaloes [12, 15]. The increase in CL size paralleled the increased serum progesterone concentration in either treated buffalo heifers or cows [12, 15] (Table 4). Furthermore, melatonin may act directly on the corpus luteum to increase progesterone production [37], instead of decreasing the uterine secretion of prostaglandin F2α [86] or modifying prostaglandin biosynthesis in the hypothalamus [87].

Combined melatonin and CIDR treatments enhanced the reproductive performance of buffalo heifers during out-of-breeding season in comparison with CIDR treatments only [12, 15], which could be explained by the deepness of anestrus. The percentage of conception at day 30 post-AI was threefold higher in the melatonin with CIDR-treated anestrus heifers compared to untreated group (37.5% vs. 12.5%) [12], as well as in anestrus lactating buffaloes (100% vs. 33.3%, respectively in the melatonin treated and non-treated groups) [15].

8. Conclusion

So far, it has been believed that milk yield in buffaloes is determined by genetic and environmental factors. In recent years, a special focus has been placed on melatonin treatment on reproductive seasonality. In farm animals, day light changes play a very important role as they establish their yields. The length of day light, and in particular the melatonin profile, is of special importance in buffaloes as they determine reproductive process, in which lactation is the last stage of reproductive physiology. Experiments carried out in buffaloes demonstrated that buffaloes are characterized by seasonal reproductive activity. They become sexually active in response to a decreasing day length in late summer to early autumn. During the out-of-breeding season, buffaloes often exhibit anestrus, which extends the calving to conception interval and, consequently, reduces reproductive performance resulting in seasonal calving and a dramatic disturbance to milk supply throughout the year. To avoid seasonality-related constraints, it is necessary to implement management schemes to overcome reproductive seasonality and regulate production of milk throughout the year. Thus, hormonal therapies to induce estrus and ovulation in anestrus buffaloes are important strategies to overcome seasonality. These hormonal treatments...
have been designed to control follicular and luteal functions synchronizing estrus and ovulation and, more importantly, to eliminate estrus detection by preplanned scheduled of timed artificial insemination. Melatonin implantation in conjunction with CIDR-eCG protocol successfully induced estrus behavior and enhanced conception rate in anestrus heifers and anestrus lactating buffaloes during out-of-breeding season under tropical conditions.

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