Effect of melatonin on follicular development parameters in a fixed-timed artificial insemination programme in water buffalo

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Abstract

It is well known that buffaloes have seasonal anoestrus and that the use of melatonin reduces the effects of season in reproduction, particularly among sheep and goats. This study evaluates the use of melatonin on water buffaloes to increase pregnancy rates in a fixed-time insemination programme. The study was conducted on an Argentinian buffalo herd, located in the Corrientes Province, during an unfavourable reproductive season. Artificial insemination cycles were performed in September and December 2018. Sixty animals were selected, and melatonin was administered to 29 females at a dose of 18 mg/50 kg. Ten days after the melatonin implant, ovulation synchronization was started. Animals were inseminated with frozen semen of a single bull. Pregnancy was evaluated by ultrasound 35 days after insemination. The pregnancy rate of the two repetitions was 34.62% for September and 6.4% for December (P=0.007). No significant differences were found in the parameters associated with follicular development and formation of the *corpus luteum*. Analysis of the effects of melatonin on the two replicates showed that there was no statistically significant effect of ovarian response regarding the analysed follicular development parameters. There was also no effect on pregnancy rates (17.85% *vs*. 20.68%; P=0.15), or on follicular development for treatment and control. In conclusion, this study was not able to demonstrate an effect of melatonin on the ovarian response of buffaloes as reported by other authors. Other factors, such as environmental conditions of subtropical areas and species specificities, may have a more significant effect on buffalo endocrinology. These factors should be evaluated to improve the results of fixed-timed artificial insemination programmes.

Key words: *melatonin; buffaloes; follicular development; artificial insemination: reproduction*

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Introduction

The Argentinean buffalo population is 87,711 head, distributed virtually throughout the national territory in 20 of 23 provinces. Eighty percent of the buffalo population is found in northeastern Argentina, with the highest population densities and significant annual population increases in the Formosa and Corrientes Provinces. This population growth shows the future possibilities of bubaline production as an emerging activity for the 8 million hectares of suitable areas to support a load of 4 million buffalo in the country, to meet the growing interest in buffaloes (*Bubalus bubalis*). To fill the suitable land and to ensure year round production of meat or milk, new strategies to manage reproduction are required, especially to break the seasonality of reproduction (Crudeli et al., 2014).

Despite being polyoestric, buffaloes are reproductively seasonal. Both endogenous factors (insulin-like growth factor 1, insulin, prolactin, thyroid hormones, melatonin, progesterone, genotype), and exogenous factors (photoperiod, nutrition, temperature, and humidity) are associated with this seasonality in the species (D'Occhio et al., 2020).

In Argentina, despite the lack of a stationary mount, calving is typical between February and June, with a peak between March and April. Rare pregnancies outside this season occur in heifers that reach proper body development in September or October. For the second farrowing, heifers regularize their reproduction for the autumn months like the rest of the herd. Reproductive efficiency increases when daylight hours begin to decline and darkness hours increase (Crudeli, 2011). Hassan et al. (2017) showed that in subtropical regions north of the equator, animals born in autumn had fewer open days, calving interval, and services per conception compared to those born in spring.

Photoperiod is the number of hours of light to which animals are exposed in a 24-hour period. A long photoperiod is when an animal is exposed to light from 16 to 18 hours a day with 6 to 8 hours of darkness, while a short photoperiod is when the amount of light is 8 hours and darkness 16 hours (Dahl et al., 2012). As a consequence, animals can generate biological adaptations since they develop an ability to measure and respond to the amount of daylight they are exposed to, and this stimulus is called a biological calendar. According to data from the Argentinean Meteorological Service, the length of day in Corrientes city, where this study was carried out, varies throughout the year. In 2018, the shortest day was June 20, with 10 hours and 24 minutes of natural light. The longest day is December 21, with 13 hours and 53 minutes of daylight.

In addition to the quantity of light, in tropical or subtropical areas where variation in daylight hours is less pronounced, it has been suggested that temperature, relative humidity, and precipitation influence the presentation of seasonality. All the above parameters contribute to the calculated temperature and humidity index (ITH), and values of >75 have been reported to have a negative effect on reproductive function (Vale, 2007).

It has been accepted that the molecule responsible for photoperiod effect on reproduction in organism is melatonin (N-acetyl-5-methoxytryptamine): an indolamine synthesized periodically in the pineal gland from tryptophan, which is associated with the regulation of reproduction in species affected by photoperiod (Reiter and Fraschini, 1969). Other actions of melatonin as a powerful antioxidant and free radical scavenger have recently been reported, protecting corpus luteum cells from radicals produced during steroidogenesis (Chew et al., 1984).

The use of melatonin to re-establish ovarian activity during the unfavourable season has been widely described in sheep and mares (Malpaux et al., 2001; Guillaume et al., 2010). It has been used with the same objective in buffalo ovulation induction protocols. The use of melatonin implants with the addition of vaginal progesterone devices, for the treatment of buffalo in winter anaesthesia in subtropical regions, resulted in produced 100% ovulation (Kumar et al., 2011). Kavita et al. (2018) used melatonin implants in Murrah breed heifers with gonadotropin-releasing hormone (GnRH) as an ovulation inducer. They reported that 100% of the animals presented oestrus and ovulation, higher progesterone levels, and a better conception rate in the treated buffaloes compared with the control. Ramadan *et al*. (2016) reported that in India, the use of melatonin implants not only improved pregnancy rate but significantly increased (P<0.01) superoxide dismutase activity, corpus luteum diameter and plasma concentrations of progesterone. It is important to note that in most buffalo reports, the melatonin used is a commercial implant designed for sheep with an adjusted dose, due to the difficulty of obtaining specific formulations for this species.

Due to the lack of information for Argentina and subtropical regions, the objective of this study was to evaluate the use of melatonin on follicular development, ovulation, and the formation of the corpus luteum in buffaloes synchronized outside the reproductive season.

Materials and methods

The study was conducted during the unfavourable season for reproduction in 2018/2019 (August to February) at a buffalo breeding farm in Paso Florentín, Corrientes, Argentina (27º20′33″S, 58º08′27″W). Animals were diagnosed by ultrasound as being in anoestrus by the lack of observation of corpus luteum. Melatonin was administered using subcutaneous implants in the ear (Melovine™, CEVA, Barcelona, Spain), at a dose of 18 mg/50 kg body weight. Buffalo were synchronized and inseminated at fixed times in September and December. All animal procedures were reviewed and approved by the Animal Welfare Committee (Universidad Nacional del Nordeste, Corrientes, Argentina), and conducted in accordance with ethical standards approved by the Institution.

A group of 60 buffaloes females were evaluated, three animals were excluded because one presented a cyst and two were uncooperative at the end 57 buffalo females of proven fertility of the Murrah and Mediterraneo breed, between 4 and 8 years old, without alterations in their internal genitalia, and an average weight of 499 kg (range 415-616 kg), were included in the study. Animals were randomly divided into two groups. One received melatonin implants (N=29, 500.68±54.98 kg), and the other (N=28, 499.64±50.73 kg) was left as a control. Two repetitions in September (n=28) and December (n=29) were performed. On the day of the start of the experiment, all animals in the experimental group received IM 150 µg d-Cloprostenol (Arsaprost®, Arsa, Argentina) and the melatonin implant. On day 10, they received a vaginal progesterone device (DIB®, Sintex, Argentina) plus 2 mg IM estradiol benzoate (Sintex, Argentina), while the device was removed on day 17. Animals were then injected with 400 IU Ecg (Novormon®, Sintex, Argentina), the same dose of d-Cloprostenol as above and 0.5 mg estradiol cypionate (Cipiosyn, Sintex, Argentina). At 30 hours after the application of the last medication, animals were inseminated (AI) with the semen of a bull of proven fertility, and all inseminations were performed by the same technician. Follicular development was evaluated using ultrasound at the start of the experiment, at the administration of the second dose of prostaglandin, at AI, seven days after ovulation, and 35 days after AI, using ultrasound equipment (Pie Medical S-100 with a 5.0-7.5 MHz sector probe, Maastrich, Netherlands). The entire experiment lasted 57 days. The duration of daylight was 12.58 hours in September and 13.52 hours in December. Animals were kept at pasture, and supplemented with minerals and water *ad libitum*.

All data was entered into a separate database, and the two repetitions, September and December, were initially compared. Later, all animals grouped according to treatment (melatonin vs. control) were analysed. Analysis was conducted using the chi-square test on qualitative variables and follicle sizes using the Mann-Whitney test. Significant differences were considered at values of $P<0.05$.

Results

The time elapsed between the administration of melatonin and insemination was 19 days. The average weight of the animals at the end of the study was 500.95 ± 51.52 kg. Data were collected for 57, animals from September and December are not the same.

Pregnancy rates in the two repetitions were 34.62% and 6.4% ($P=0.007$) for September and December, respectively. Table 1 shows the parameters associated with follicular development and pregnancy in the two replicates. A significant difference was found only for in the pregnancy rate between the two

Table 1. Comparison of follicular parameters and pregnancy in the two repetitions of the experiment

	September	December	*P* value
Number of animals	26	31	
Number of follicles at the beginning (mean±SEM)	6.4 ± 0.5	6.9 ± 0,4	0.182
Number of follicles at ovulation (mean±SEM)	6.8 ± 0.5	6.2 ± 0.5	0.087
Animals with ovulatory follicle > 9mm	61.5%	74.2%	0.4665
Animals with corpus luteum	46.2%	61.3%	0.2677
Pregnancy rate	34.6%	6.4%	0.0078
Size of dominant follicle (mm) (mean±SEM)	9.8 ± 0.4	9.2 ± 0.3	0.0934

Table 2. Effect of melatonin on follicle development

	Melatonin	Control	*P* value
Animals	29	28	
Number of follicles at the beginning, mean ± SE	6.4 ± 0.5	7 ± 0.5	0.3185
Number of follicles at ovulation, mean ± SE	5.4 ± 0.4	7.5 ± 0.6	0.016
Animals with an ovulatory follicle > 9 mm, n (%)	24 (82.75)	23 (82.14)	0.976
Animals with corpus luteum, n (%)	14 (58.33)	17 (73.91)	0.07
Pregnancy rate, n (%)	5 (20.68)	6 (17.85)	0.15
Size of follicle development (mm), mean ± SE	9.5 ± 0.4	9.4 ± 0.3	0.801

Table 3. Associated events of follicular development in treated and control animals

	Melatonin	Control	*P* value
N	29	28	
Ovulation, n (%)	19 (65.52)	20 (71.42)	0.6349
Ovulation and corpus luteum, n (%)	11/19 (57.89)	15/22 (68.18)	0.8325
Ovulation and no corpus luteum, n (%)	8/19 (42.10)	7/22 (31.81%)	0.5005
Ovulation, corpus luteum and pregnancy, n (%)	4/11 (36.36)	3/15 (20.0)	0.3622
Ovulation, corpus luteum and no pregnancy, n (%)	7/11 (63.64)	12/15 (80.0)	0.3622
Ovulation, no corpus luteum and pregnancy, n (%)	1/8 (12.5)	1/7 (14.28)	0.9209
Ovulation, no corpus luteum and no pregnancy, n (%)	7/8 (87.5)	6/7 (85.71)	0.7261
No ovulation, n (%)	10 (34.48)	8 (28.57)	0.795
No ovulation and corpus luteum, n (%)	3/10 (30.0)	2/8 (25.0)	0.8191
No ovulation and no corpus luteum, n (%)	7/10 (70.0)	6/8 (75.0)	0.8191
No ovulation, corpus luteum and pregnancy, n (%)	0/3 (0)	1/2 (50.0)	0.2207
No ovulation, corpus luteum and no pregnancy, n (%)	3/3 (100)	1 /2 (50.0)	0.2207
No ovulation, no corpus luteum and pregnancy, n (%)	1/7 (14.28)	0/6 (0)	0.3546
No ovulation, no corpus luteum and no pregnancy, n (%)	6/7 (85.71)	6/6 (100)	0.3546

repetitions, while no other parameters presented statistically significant results.

Since the differences observed in the pregnancy rate between the two repetitions are most likely associated with uncontrolled factors in this experiment, this parameter will not be discussed. Only the results associated with the effects of melatonin on follicular development and ovulation for each repetition are shown in Tables 2 and 3. Table 3 describes the events associated with follicular development of the treated and control animals during the experiment.

Discussion

The discussion will focus on two separate aspects: the significant difference in the pregnancy rate between the two repetitions, and the effect of melatonin on follicular development. The use of progesterone implants associated with eCG has been shown to produce better follicular development than other protocols for ovulation synchronization in out-of-season buffaloes (Carvalho et al., 2013), additionally with a statistically significant improvement on the hemodynamic parameters of the *corpus luteum* (Samir et al., 2019).

The pregnancy rates obtained in September are comparable with the results during the favourable season in Argentina (Crudeli, 2011). However, the lower results for December are difficult to explain, and it should be mentioned that the successful pregnancies in this study in that month all correspond to the group of animals treated with melatonin.

Precipitation and temperature were 0.53 mm, 2.04 mm, and 21.4°C, 25.3°C in September and December, respectively, and both precipitation and temperature were lower in the period with the highest pregnancy rates. Due to the low number of observations, the synergistic effect of these two environmental factors as the cause of the observed differences cannot be ruled out. The thick epidermis of the buffalo contains high amounts of melatonin that give it a black colour, and these animals are known to have a superior ability to adapt to hot and humid lands. Therefore, it has been postulated that environmental changes frequently affect them and consequently their reproduction (Vale, 2007).

There were variations in daylight hours between the repetitions, i.e. from 11.53 hours to 12.38 hours in September, and from 13.50 to 13.52 hours in December. Additionally, the average insolation was 185 w/m^2 in September, and 283 w/m^2 in December. It is evident that there was a difference of 40 minutes of daylight during the experiment in September and December, it was almost constant. The nearly two fewer hours of daily, precipitation of 1.51 mm and temperature of 3.9°C in September appears to be the only external factor to explain the differences in pregnancy rates. Consequently, in the first experiment, animals had lower levels of circulating melatonin than in December due to the shorter days. Another explanation of the observed differences could be attributed to embryonic mortality, and higher embryonic mortality has been reported during long days (Campanile et al., 2010). Melatonin is secreted during darkness (Earl et al., 1990), and thus is higher during short days than on long days. However, it cannot be ruled out that these results are derived from other known effects of melatonin other than photoperiod (D'Occhio and Suttie, 1992).

Another very important aspect to mention are the particularities of melatonin secretion in buffaloes, e.g. that Mediterranean breed heifers produce less melatonin than adult buffaloes (Barile, 2005). Additionally, seasonality is a known trend in buffaloes, but not all buffaloes alter their reproductive behaviour during the season, only those that decrease their melatonin level. This pattern is repeated in other animals, suggesting a genetic component to the appearance of the phenomenon that could be used in selection, in addition to polymorphism in the melatonin receptor MT1 (MTRN1A) (Li et al., 2018).

The number of follicles at the time of prostaglandin injection obtained here was similar to other reports of 8.2 follicles/animal (Samir et al., 2019), but less than in some reports, 17.6 follicles/animal (Gimenes et al., 2015) and 12.5 follicles/animal (Ferraz et al., 2015), where melatonin-treated buffaloes had fewer follicles than the control animals (*P*=0.016). The responses to ovulation induction were 65.51% and 71.42% in treated and control buffaloes, respectively. Among the ovulated females, 57.89% and 68.18% formed a corpus luteum respectively, and consequently, only 37.99% of treated and 53.57% of control animals responded as physiologically expected with medication, i.e. induction of ovulation, corpus luteum formation.

Something very particular in this experiment was that 12.5% of ovulated buffaloes that did not show a corpus luteum and 14.68% of non-ovulated buffaloes that did not have a corpus luteum became pregnant. No less strange is the observation that in 30% of buffaloes, no dominant follicle was observed, though subsequently appeared with a corpus luteum, one female of this category in the control group became pregnant at the end of the experiment, which may be due to late follicular development after the performed sonography. Since the protocols applied were adapted from cattle, one possibility for the observed results is that the buffaloes have a different chronology that requires adjustment of the protocol. But beyond these particularities, which can be attributed to the species, it should be mentioned that most females responded as expected to treatment, with 82.14% and 82.75% of the treated and control buffaloes had a dominant follicle. But as mentioned above, about half of them ovulated and formed a corpus luteum. Variation is very common in buffaloes.

Although the results were not as expected, other researchers have also been surprised in research on buffalo reproduction. Ferraz et al. (2015) evaluating the effect of somatotropin on the number of follicles and the production of embryos, and found that although the number of follicles increased, there was an adverse effect on the quantity and quality of the blastocysts obtained, contrary to that observed in other species.

Under the conditions of this study, using progestogens and melatonin, it could not be confirmed that melatonin improves the reproductive function in fixed-time artificial insemination programmes in buffalo as reported by Ramadan et al. (2016). A decrease in the number of animals responding to treatment was observed, which will necessarily affect the final result of synchronization. Subsequent experiments should assess hormone levels. Of the evaluated parameters, day length had the expected effect in explaining the results of the experiment. To improve the efficiency of protocols, it is proposed that breeders should apply ultrasound monitoring of follicular development during insemination protocols.

Acknowledgments

The authors wish to express their gratitude to the Universidad Nacional de Colombia, Sede Medellín, the Universidad Nacional del Nordeste, the Austral del Chaco University, A CONYCET, Argentina and MINCIENCIAS, Colombia, for the financial support to the researchers for the development of this investigation. In addition to the Pedro Antonio Silva Cottage, Little Punjab, owner of the animals in the experiment.

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Učinak melatonina na parametre razvoja folikula u programu umjetnog osjemenjivanja bizona u točno određenom vremenu

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Dobro je poznat sezonski anestrus u bizona, kao i da je uporaba melatonina pokazala smanjenje učinaka sezone na reprodukciju, posebice u ovaca i koza. Cilj je ovog rada bio procijeniti uporabu melatonina u vodenih bizona za povećanje postotka gravidnosti u programima umjetnog osjemenjivanja u točno određenom vremenu. Ovaj rad je proveden na stadu argentinskih bizona koji se nalaze u provinciji Corrientes tijekom nepovoljne reproduktivne sezone; u rujnu i prosincu 2018. godine obavljeni su ciklusi umjetnog osjemenjivanja, a 29 u ženki implantiran je melatonin u dozi od 18 mg/50 kg. Deset dana nakon implantacije melatonina, započeta je sinkronizacija ovulacije. Životinje su osjemenjene smrznutim sjemenom jednog bika. Gravidnost je dijagnosticirana ultrazvukom 35 dana nakon osjemenjivanja. Postotak gravidnosti dva ponavljanja bila je 34,62 % te 6,4 % (*P*=0,007), u rujnu, odnosno prosincu. Nije otkrivena značajna razlika u parametrima povezanima s razvojem folikula i formiranjem *corpus luteum*. Analiza rezultata o učinku melatonina tijekom dva ponavljanja pokazala je da nema statistički značajnog učinka odgovora jajnika na analizirane parametre razvoja folikula. Isto tako, nije bilo učinka na postotke gravidnosti, 17,85 % u usporedbi s 20,68 % (*P*=0,15) te na razvoj folikula u pokusnoj, odnosno kontrolnoj skupini. Zaključno, u uvjetima ove studije nije bilo moguće dokazati učinak melatonina na odgovor jajnika u bizona o kakvom su izvijestili drugi autori. Pretpostavljamo da drugi čimbenici, poput uvjeta okoliša u suptropskim područjima te posebnosti vrste, imaju značajniji učinak na endokrinologiju bizona. Potrebno je procijeniti točno određeno vrijeme da bi poboljšali rezultate programa umjetnog osjemenjivanja.

Ključne riječi: *melatonin, bizoni, razvoj folikula, umjetno osjemenjivanje, reprodukcija*