



Ovarian dynamics of buffalo (*Bubalus bubalis*) synchronized with different hormonal protocols

Jorge Alonso Peralta-Torres¹ · Oswaldo Margarito Torres-Chablé¹ · José Candelario Segura-Correa² · Nadia Florencia Ojeda-Robertos¹ · Alfonso Juventino Chay-Canul¹ · Carlos Luna-Palomera¹ · Víctor Hugo Severino-Lendecky³ · Jesús Ricardo Aké-Villanueva⁴

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Abstract

The study evaluates the effect of three hormonal protocols on ovarian dynamics and progesterone (P4) secretion of buffalo (*Bubalus bubalis*). Twenty-nine pluriparous Murrah buffaloes were used. The protocols were as follows: OVSYNCH ($n = 10$): 100 µg of gonadorelin (day 0), 500 µg of cloprostenol (day 7), and 100 µg of gonadorelin (day 9). CIDR+EB (intravaginal device (CIDR®) + estradiol benzoate; $n = 10$): CIDR plus 2 mg of EB (day 0), withdrew of CIDR, 500 µg of cloprostenol (day 7) and 1 mg of EB (day 8). CIDR+eCG ($n = 9$): CIDR plus 2 mg of EB (day 0), withdrew of CIDR, 500 µg of cloprostenol and 400 IU of eCG (day 7). Follicles were counted with an ultrasound and measured at 0, 24, and 54 h. The maximum follicle diameter and ovulation were evaluated at 70, 80, and 94 h after CIDR withdrew. Estrous was detected per 1 h three times daily. Blood samples were collected on days 0, 7, 10, 15, and 22 to determine P4 concentration. In CIDR+EB protocol, 50% of buffaloes presented estrous, at 69.6 h. All buffaloes ovulated. CIDR+eCG group had the shortest (69 h) ovulation time. No treatment differences for follicular population, maximum follicle diameter, and P4 concentration on days 7 and 10 ($P > 0.05$) were found. The P4 concentration in OVSYNCH and CIDR+eCG protocols were > 1 ng/ml, on days 15 and 22 ($P < 0.05$). There was no difference in ovarian activity; however, the P4 secretion was normal in the OVSYNCH and CIDR+eCG protocols compared to the CIDR+EB protocol.

Keywords Estrous · Follicular population · Maximum follicle diameter · Ovulation · Progesterone

Introduction

There has been a marked increase in the population of water buffalo (*Bubalus bubalis*) in Latin America in recent years

(Zava 2013). Water buffaloes have been introduced into the hot and humid regions of Mexico, mainly in Veracruz, Tabasco, Chiapas, and Campeche states, because these entities have large wetland areas, which are the natural habitat of this species. Domesticated water buffaloes are broadly classified into 2 groups (river and swamp buffalo) based on their morphology, behavior, and karyotype (Perera 2011). The subspecies of river buffaloes are further divided into multiple breeds, such as Murrah breed, which has undergone selection for more milk production.

The water buffalo is an option to produce meat and milk under extensive management conditions, because it poses characteristics of hardiness, adaptation to humid tropical conditions, and better feed efficiency, compared to cattle (Cervantes et al. 2010; Borghese 2011). However, the productivity of buffalo is affected by the animal's reproductive performance, which is impaired by the late maturity of females, poor estrous expression, longer calving intervals, and reduced ovarian activity, during the hot season (Madan et al. 1996;

✉ Jorge Alonso Peralta-Torres
japt83@hotmail.com; jorge.peralta@ujat.mx

¹ División Académica de Ciencias Agropecuarias, Universidad Juárez Autónoma de Tabasco, Carretera Villahermosa-Teapa Km. 25. R/A La Huasteca 2a Sección, CP 86280 Villahermosa, Tabasco, Mexico

² Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán, Carretera Xmatkuil Km. 15.5, Mérida, Yucatán, Mexico

³ Centro de Estudios Etnoagropecuarios, Universidad Autónoma de Chiapas, Blvd. Javier López Moreno S/N, San Cristóbal de las Casas, Chiapas, Mexico

⁴ Instituto Eloísa Patrón de Rosado, Licenciatura en Administración Ganadera, C. 17 # 101e, Col. Itzimmá, Mérida, Yucatán, Mexico

Singh et al. 2000). Artificial insemination (AI) combined with estrous synchronization is now an integral part of water buffalo production systems.

Various estrous synchronization protocols use progesterone (P4), progestogens (Carvalho et al. 2017), PGF_{2α}, and its synthetic analogues with GnRH (Neglia et al. 2016), to facilitate the use of AI. However, due to poor estrous (30–40%) detection (Barkawi et al. 1993), heterogeneous duration of estrous (4–64 h) (Ohashi 1994), and the difficulty encountered in predicting the time of ovulation, AI in buffalo is poorly used. Therefore, the establishment of synchronization protocols is important to increase the reproductive performance in this species. Implementation of estrous synchronization protocols, which allows the control and modulation of follicular dynamics, is important to know the right time when estrous and ovulation occur, and to estimate the appropriate time for fixed time artificial insemination programs (FTAI). In addition, the knowledge of the P4 secretion of the subsequent corpus luteum (CL) will enable the use of those synchronization protocols without the need of estrous detection in buffaloes under the tropical conditions of Mexico. The objective of this study was to evaluate the effect of three synchronization hormonal protocols on the ovarian dynamics and P4 secretion in water buffalo (*Bubalus bubalis*) in the hot-humid tropics of Mexico.

Material and methods

Study area

The study was carried out in Coatzacoalcos, Veracruz, Mexico (17° 55' N, 94° 06' W) at an altitude of 19 m. The climate of the region is hot-humid with rain in summer and average annual temperature and rainfall of 25 °C and 2750 mm, respectively (INEGI 2017).

Animals and handling

During the reproductive season (November to December), 29 pluriparous Murrah buffaloes of 6.27 ± 3.62 years of age, 3.79 ± 2.04 parities, 86.52 ± 44.53 days in milk, 471.6 ± 87.2 kg of weight, and 4.1 ± 0.57 body condition score (BCS) were used. BCS was measured on a scale of 1 to 5, where 1 is emaciated and 5 is obese (Anitha et al. 2011). The buffaloes grazed freely in native pastures (*Paspalum fasciculatum*, *Paspalum notatum*, *Paspalum conjugatum*), and they had water ad libitum.

Hormonal treatments

To diagnose buffaloes with CL (OVSYNCH 1.09 ± 0.14 , CIDR+EB 1.10 ± 0.16 , CIDR+eCG 1.08 ± 0.14 mm) and

without ovarian or uterine abnormalities, a real-time ultrasound (Emperor 830®, with a 6.5 MHz linear transducer) was used (Monteiro et al. 2016). Three treatment protocols were compared:

OVSYNCH ($n = 10$): on day 0, all buffaloes received 100 µg of gonadorelin (Gonavet® Bayer); on day 7, they were given 500 µg of cloprostenol (Ciclase®, Lab. Syntex, Argentina); and on day 9, 100 µg of gonadorelin, via intramuscular (Fig. 1a).

CIDR+EB ($n = 10$): on day 0, buffaloes were applied a bovine intravaginal device (CIDR® Lab. Pfizer) containing 1.9 g of natural P4, via intramuscular, plus 2 mg of EB (Estradiol Benzoate® Lab. Syntex, Argentina). The CIDR remained in situ 7 days and then it was administered 500 µg of cloprostenol, and 24 h later (day 8) 1 mg of EB, both via intramuscular (Fig. 1b).

CIDR+eCG ($n = 9$): similar to previous treatment, except that at the CIDR withdrawal the females received 500 µg of cloprostenol and 400 IU of eCG (Folligon®, Intervet) (Fig. 1c).

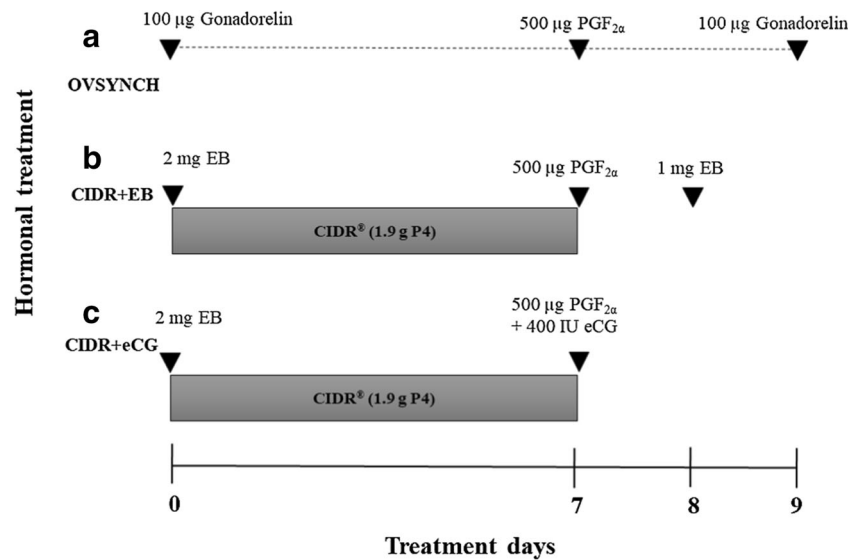
Estrous and ovarian dynamics

The estrous was detected by visual observation (the buffalo was allowed to mount and remained immobile for 3 s), 1 h, three times a day (06:00, 12:00, and 18:00 h), starting 24 h after the withdrawal of the CIDR or application of cloprostenol, finishing on day 5. The follicles of both ovaries of each buffalo of the three hormonal protocols were counted and measured with the help of an ultrasound, after the CIDR removal or cloprostenol application of PGF_{2α} (hour 0), at 24 and 54 h. The follicles were classified as small (≤ 3 mm), medium (3.1–6 mm), and large (≥ 6.1 mm) as suggested by Vishnu et al. (2017). Subsequently, using an ultrasound, the largest follicle (70, 80, and 94 h) was followed to determine its maximum follicle diameter (MFD) and to determine the ovulation time (disappearance of the follicle), as suggested by Ali and Fahmy (2007).

Progesterone determination

Blood samples (5 ml) of the coccygeal vein were collected in heparinized tubes, on day 0 (insertion of the CIDR or application of 100 µg GnRH), day 7 (withdrawal of the CIDR or the application of 500 µg of cloprostenol), and days 10, 15, and 22 of treatment. The plasma was separated by centrifugation at 1500g by 15 min, and then transferred, in duplicate, in 2-ml vials and stored at -20 °C until analysis. Plasma P4 concentration was measured with a solid phase radioimmunoassay kit (Coat-A-Count®, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The sensitivity of the assay was 0.05 ng/ml, and the coefficient of variation intra assay was 5.8%.

Fig. 1 a OVSYNCH, b CIDR+EB, and c CIDR+eCG synchronization protocols used in buffalo. PGF_{2α} prostaglandin F_{2α}, cloprostenol sodium, EB estradiol benzoate, CIDR controlled internal drug release, P4 progesterone, eCG equine chorionic gonadotropin



Statistical analysis

All data were analyzed using SAS procedures (SAS 2015). Buffaloes were distributed in a completely randomized design. The data corresponding to the time (h) of CIDR withdrawal to estrous and ovulation presentation were analyzed using the GLM procedure, and mean comparison was performed using the Tukey test. The estrous and ovulation data were analyzed by chi-square tests, using the PROC FREQ procedure. The statistical model that described the number of follicles and the MFD included the effects of treatment, time, and the interaction, considering time data as repeated measures. The P4 data were analyzed with the GLM procedure and treatment means compared using the Tukey test.

Results

Estrous was only observed in the CIDR+EB group, in 50% of buffaloes with average of 69.6 h after the withdrawal of the CIDR (Table 1). However, 100% of the buffaloes of the three treatments ovulated. The shortest time of device withdrawal to ovulation was observed in the CIDR+eCG group (69.11 h) compared to the OVSYNCH group (82 h) ($P < 0.05$); no

difference was found with the CIDR+EB treatment. In addition, no difference was found in number of follicles ($P > 0.05$; Table 2). On average, 9 small (≤ 3 mm), 3 medium (3.1–6 mm), and 1 large follicle (≥ 6.1 mm) were recorded from 0 to 54 h after CIDR withdrawal or the application of cloprostenol. The MFD means, prior to ovulation, in the OVSYNCH, CIDR+EB, and CIDR+eCG groups were 8.25 ± 1.10 mm, 7.97 ± 0.95 mm, and 7.72 ± 0.66 mm, respectively ($P > 0.05$). The plasma concentration of P4 on day 7 of the CIDR withdrawal or application of 500 µg of cloprostenol was > 1 ng/ml ($P > 0.05$). On day 10, P4 levels were ≤ 1 ng/ml ($P > 0.05$) in all treatments. For days 15 and 22, the P4 concentrations in the OVSYNCH and CIDR+eCG groups were > 1 ng/ml (Fig. 2; $P < 0.05$).

Discussion

The fact that estrous was only observed in buffaloes of the CIDR+EB group differs from the result obtained by Khan et al. (2018), who reported 81 and 98% of buffaloes showing estrous in the CIDR+EB and the CIDR+eCG groups, respectively. Neglia et al. (2003) report 88% of buffaloes in estrous at AI (18–24 h after the second application of GnRH).

Table 1 Mean \pm SE by hormonal protocol for estrous, ovulation, and ovulation time of synchronized water buffalo

Variable	OVSYNCH	CIDR+EB	CIDR+eCG
Number of buffalo	10	10	9
Estrous (%)	0	50	0
Time of CIDR withdrawal to estrous (h)	.	69.6 \pm 8.22	.
Ovulation (%)	100a	100a	100a
Time of cloprostenol or CIDR withdrawal to ovulation (h)	82.40 \pm 4.15b	77.60 \pm 4.15ab	69.11 \pm 4.37a

Different letters between columns indicate statistical difference of means ($P < 0.05$)

Table 2 Follicular population of small (≤ 3 mm), medium (3.1–6 mm), and large follicles (≥ 6.1 mm) \pm SE in buffaloes synchronized with different hormonal treatments

Follicle size	OVSYNCH PGF _{2α} application (h)			CIDR+EB CIDR withdrawal (h)			CIDR+eCG CIDR withdrawal (h)		
	0	24	54	0	24	54	0	24	54
≤ 3 mm	9.0 \pm 2.7a	8.7 \pm 1.8a	7.7 \pm 2.6a	9.4 \pm 2.1a	8.2 \pm 2.5a	6.4 \pm 2.5a	10 \pm 2.1a	9.7 \pm 1.4a	5.2 \pm 3.1a
3.1–6 mm	2.1 \pm 1.3a	2.1 \pm 1.8a	2.0 \pm 0.9a	2.9 \pm 1.4a	3.2 \pm 2.7a	4.3 \pm 2.9a	3.7 \pm 1.3a	2.8 \pm 1.4a	2.6 \pm 1.6a
≥ 6.1 mm	1.0 \pm 0.7a	1.3 \pm 0.7a	1.7 \pm 0.8a	0.7 \pm 0.7a	1.2 \pm 1.0a	1.3 \pm 0.7a	0.7 \pm 0.7a	1.3 \pm 0.5a	1.2 \pm 0.7a

Different letters between rows within treatment indicate significant difference ($P < 0.05$)

Probably, estrous behavior was only observed in the CIDR+EB group because the EB was applied 24 h after CIDR withdrawal, which has the pharmacological effect of inducing estrous manifestations (Sumano and Ocampo 2006). The low proportion of buffaloes with estrous in the present study, probably was due to the fact that visual observation was used as a method to determine estrous expression. Awasthi et al. (2007) reported that buffaloes show silent estrous, which may be due to the reduced diameter of the ovulatory follicle and reduced secretion of 17β -estradiol in the granulosa cell (Ginther et al. 2001). Other authors used other physiological criteria to determine estrous in buffaloes, such as vulvar edema, presence of vaginal mucus secretion, increased uterine tone, and the presence of a follicle > 9 mm (Awasthi et al. 2007; Yousuf et al. 2015; Khan et al. 2018).

In the present study, 100% of buffaloes ovulated, 69 to 78 h after the withdrawal of the CIDR. These results differ from those of Khan et al. (2018), who obtained ovulation rates of 74 and 93% ($P < 0.05$) at 72.2 and 68.1 h ($P < 0.05$) with the CIDR+EB and CIDR+eCG protocols, respectively. In the OVSYNCH protocol, 100% of the buffaloes ovulated at 82 h after the application of cloprostenol or 34 h after the

second application of gonadorelin, being different from the groups synchronized with the CIDR. This result agrees with those of Paul and Prakash (2005), who reported 90% ovulation rate at 23.3 h after the second application of GnRH. The proportion of females that ovulate and the time that occurs during estrous synchronization is of great importance for the implementation of FTAI programs. The optimal AI time is estimated between 48 and 60 h after removal of the intravaginal device (Haider et al. 2015) or 16–20 h after the second application of GnRH in the OVSYNCH protocol (De Rensis et al. 2005), to get a possible fertilization.

The follicular populations recorded in this study were equal among the synchronization groups evaluated. Similar results were reported by Neglia et al. (2016), who used OVSYNCH protocols to synchronize pluriparous Italian Mediterranean river buffaloes. However, most of the research only evaluate the dominant follicle at different stages and synchronization protocols (Carvalho et al. 2017; Rathore et al. 2017; Monteiro et al. 2018). In this sense, in the present study, no effect of the MFD was found at 54 h after the withdrawal of the CIDR. Carvalho et al. (2013) found diameters of the dominant follicle of 12.06 and 13.07 mm with the CIDR+EB and CIDR+eCG protocols, respectively. The average MFD in the OVSYNCH protocol here used differs from the results of Oropeza et al. (2010), who report 15.2 mm for the dominant follicle at AI. The poor MFD here reported agree with Neglia et al. (2016) results, who found that the area of the largest follicle was smaller ($P < 0.05$) for the buffaloes that ovulated compared to those that did not do it (1.08 and 1.29 mm, respectively). The importance of determining the follicular population and the MFD in the synchronization protocols is that the proportion of follicles during this event must be known, and that a dominant follicle be present, which should reach its largest diameter to guarantee that the ovulated oocyte and the CL are of good quality (Vecchio et al. 2012; Monteiro et al. 2016). Probably a similar behavior occurs in cattle, where large diameter of the dominant follicle increases the probability of pregnancy in FTAI programs (Sá Filho et al. 2010, 2011).

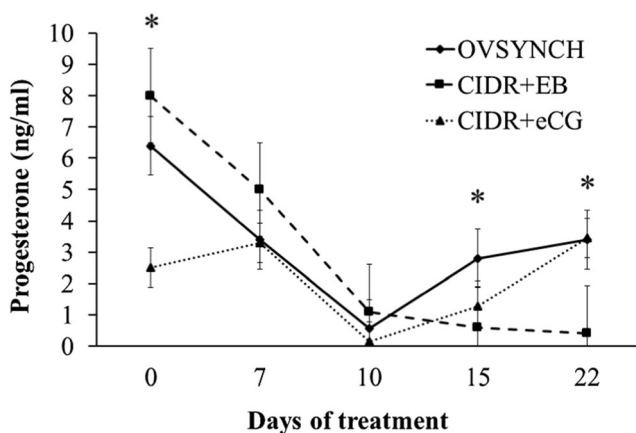


Fig. 2 Progesterone concentration (ng/ml) of buffaloes at the beginning of treatment (day 0; insertion of the CIDR or application of gonadorelin), day 7 (removal of the CIDR or application of cloprostenol), and days 10, 15, and 22 days of treatment. *Significant differences between groups ($P < 0.05$)

The main difference in P4 concentration was recorded in the CIDR+EB group on days 15 and 22 of treatment. This result is similar to that of Khan et al. (2018), who report a higher concentration of P4 on days 15 (2.86 vs 1.16 ng/ml), 18 (3.91 vs 1.68 ng/ml), and 21 (5.94 vs 2.33 ng/ml) of the diestrous, in the CIDR+eCG and CIDR+EB treatments, respectively. In addition, the greater P4 concentration was related to a larger diameter of the CL. In relation to the OVSYNCH protocol, the results here found agree with those of Paul and Prakash (2005), who report concentrations ≥ 1 ng/ml of P4 from days 16 to 20 after hormonal treatment. The P4 levels of the present study corroborate the ovulations observed by ultrasonography (levels < 1 ng/ml; day 10), the formation of CL and its functionality (levels > 1 ng/ml; day 15 and 22). This indicates that the OVSYNCH and CIDR+eCG treatments had a better response forming and developing a functional CL, compared to the CIDR+EB treatment. This suggests that those CL were not of short life and this could have favored a pregnancy. In conclusion, there was no difference in ovarian activity of synchronization protocols; however, during the diestrous, the secretion of P4 was normal in the OVSYNCH and CIDR+eCG protocols compared to the CIDR+EB protocol in synchronized buffaloes in the hot-humid tropics of Mexico.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal rights All of the authors declare that during the experiment, no animals or people were maltreated or injured. The procedures were performed in accordance with the Mexican Official Standard guideline 051-ZOO-1995 and the Mexican Official Standard of technical specifications for production, care, and use of experimental animals. All of the listed authors have participated in the development of this research and wrote the document.

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