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Equine chorionic gonadotropin improves the efficacy of a timed artificial insemination protocol in buffalo during the nonbreeding season

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ABSTRACT

Two experiments were conducted to evaluate the effects of equine chorionic gonadotropin (eCG) treatment on ovarian follicular response, luteal function, and pregnancy in buffaloes subjected to a timed artificial insemination (TAI) protocol during the nonbreeding season. In experiment 1, 59 buffalo cows were randomly assigned to two groups (with and without eCG). On the first day of the synchronization protocol (Day 0), cows received an intravaginal progesterone (P4) device plus 2.0 mg estradiol benzoate im. On Day 9, the P4 device was removed, all cows were given 0.150 mg $PGF_{2\alpha}$ im, and half were given 400 IU eCG im. On Day 11, all cows were given 10 μg of buserelin acetate im (GnRH). Transrectal ultrasonography of the ovaries was performed on Days 0 and 9 to determine the presence and diameter of the largest follicle; between Days 11 and 14 (12 hours apart), to evaluate the dominant follicle diameter and the interval from device removal to ovulation; and on Days 16, 20, and 24 to measure CL diameter. Blood samples were collected on Days 16, 20, and 24 to measure serum P4. In experiment 2, 256 buffaloes were assigned to the same treatments described in experiment 1, and TAI was performed 16 hours after GnRH treatment. Pregnancy diagnosis was performed by ultrasonography 30 days after TAI. Treatment with eCG increased the maximum diameter of dominant follicles ($P = 0.09$), ovulation rate ($P = 0.05$), CL diameter ($P = 0.03$), and P4 concentrations ($P = 0.01$) 4 days after TAI, and pregnancy per AI (52.7%, 68/129 vs. 39.4%, 50/127; $P = 0.03$). Therefore, eCG improved ovarian follicular response, luteal function during the subsequent diestrus, and fertility for buffalo subjected to a TAI synchronization protocol during the nonbreeding season.

1. Introduction

Buffalo exhibit seasonality in breeding activity and become sexually active in response to a decreasing day length in late summer to early autumn [1]. As latitude decreases, calving is more concentrated during the breeding season, providing rapid re-establishment of postpartum ovarian activity and conception [2]. Therefore, during the nonbreeding season, buffalo often exhibit a high anestrous incidence, which extends the calving to conception interval and, consequently, reduces reproductive performance [3]. Consequently, hormonal treatments have been designed to control both luteal and follicular functions, providing exciting possibilities for synchronization of follicular growth and ovulation to enable the use of timed artificial insemination (TAI) during the nonbreeding season [4–8].

Satisfactory pregnancy rates (approximately 40% to 60%) [7,9–12] have been achieved with the Ovsynch protocol

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(Day 0, GnRH; Day 7, PGF2α; Day 9, GnRH; TAI 16 hours after the second GnRH injection) [13] in cycling buffalo synchronized during the breeding season. However, anestrous buffalo respond poorly to the Ovsynch protocol [8,12,14,15] and have lower pregnancy rates after TAI during the nonbreeding season [16].

In previous studies in postpartum anestrous cows, exogenous progesterone (P4) increased LH pulse frequency during and after the treatment period [17]. In addition, in comparison with untreated animals, this increased follicular fluid and circulating estradiol concentrations, LH pulsatile release, and numbers of LH receptors of granulosa and theca cells in preovulatory follicles [18].

Treatment with intravaginal P4 devices combined with eCG at device removal has been extensively used in *Bos indicus* cattle (reviewed in [19]) and increased ovulation rates, plasma P4 concentrations, and pregnancy rates for suckled beef cows with a high prevalence of anestrous or a low body condition score (BCS) [19–24]. Similar to bovine cows, eCG treatment has been suggested as an effective tool for increasing pregnancy outcomes using TAI protocols in buffalo herds with a high incidence of postpartum anestrous [4,14,25,26]. However, there are few reports of ovarian follicle response and luteal function in the subsequent diestrus of lactating buffalo cows treated with eCG and subjected to a TAI protocol during the nonbreeding season.

Thus, the aim of the present study was to evaluate the effects of eCG on ovarian response, luteal function, and pregnancy outcomes in buffalo (*Bubalus bubalis)* subjected to P4 plus estradiol benzoate treatment for ovulation synchronization using TAI protocol. Our hypothesis was that the eCG treatment would effectively increase ovulation rates, CL diameter, and circulating P4 concentrations in the subsequent diestrus and pregnancy per TAI during the nonbreeding season in buffalo.

2. Materials and methods

2.1. Experiment 1

2.1.1. Animals and management

Experiment 1 was conducted at the Santa Helena farm (Sete Barras, SP, Brazil) during the nonbreeding season (spring through summer; November 2009 to March 2010). In this period, the minimum and maximum temperature were 22.0 °C and 31.9 °C and rainfall was 28.2 mm. Fifty-nine lactating buffalo (*Bubalus bubalis*) were assigned into one of two treatment groups (with eCG, N = 29, and without eCG, N = 30) according to parity (2.2 ± 0.4), days after parturition (170.0 ± 15.4), ovarian activity (ultrasonographic examinations performed to confirm the absence of a CL on Day 0) and BCS (3.0 ± 0.1; scale of 1 to 5, where 1 = very thin and 5 = very fat). Cows had contact with their calves only during milking. These buffaloes were maintained on a *Brachiaria decumbens* pasture with free access to water and mineralized salt.

2.1.2. Experimental design

All buffaloes received an intravaginal P4 device (1.0 g P4; DIB; MSD Animal Health, Sao Paulo, SP, Brazil) plus 2.0 mg of estradiol benzoate (EB; im; Gonadiol; MSD Animal Health) at random stages of the estrus cycle (designated Day 0). On Day 9, the DIB was removed, a luteolytic dose of $PGF_{2\alpha}$ im (0.150 mg days-cloprostenol; Preloban; MSD Animal Health) was given, and the buffaloes were allocated into two groups. One group received 400 IU of equine chorionic gonadotropin (eCG Folligon; im; MSD Animal Health) whereas the other group (without eCG) did not receive any additional treatment. After 2 days (Day 11, approximately 48 hours after device removal), buffalo of both groups received 10 μg of buserelin acetate (GnRH; im; Conceptal; MSD Animal Health; Fig. 1). Blood samples were collected from all cows to measure (RIA) serum P4 concentrations on Days 16, 20, and 24.

2.1.3. Ultrasonographic examinations

Transrectal ultrasonography of the ovaries was performed using a 7.5 MHz linear-array transducer (Mindray DP-2200Vet; Shenzhen, China) on Days 0 and 9 to assess the diameter of the largest follicle, between Days 11 and 14 (12-hour intervals) to evaluate dominant follicle diameter and the interval from the device removal to ovulation, and on Days 16, 20, and 24 to measure CL diameter. The growth rate of the dominant follicle (mm/d) was defined as the difference between the dominant follicle recorded on Day 11 and on Day 9, divided by two. Ovulation was considered to have occurred when a large follicle, previously observed, was no longer present at the subsequent ultrasonographic examination.

2.1.4. Blood sampling and P4 assays

Blood samples were collected by jugular venipuncture after the ovulation synchronization by GnRH administration (Days 16, 20, and 24 from the start of treatment). The samples were refrigerated (4 °C) for 4 to 5 hours after

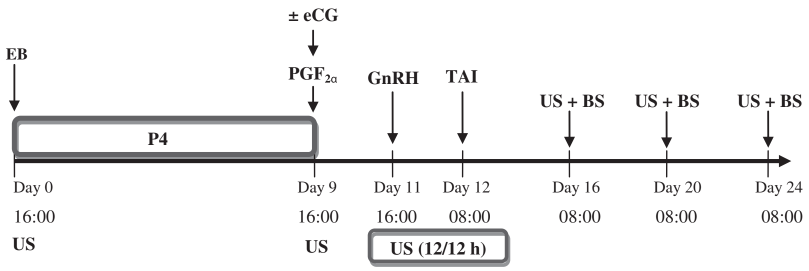

Fig. 1. Schematic diagram of the treatments with or without eCG to synchronize ovulation in buffaloes. BS, blood sample; EB, 2.0 mg estradiol benzoate; eCG, 400 IU equine chorionic gonadotropin; GnRH, 10 μg buserelin acetate; $PGF_{2\alpha}$, 0.150 mg days-cloprostenol; US, ultrasonographic examination.

collection, then centrifuged (3000 × *g* for 20 minutes), and serum stored in cryotubes at −20 °C until assayed. Serum P4 concentrations were evaluated using an antibody-coated tube RIA kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA), which had been validated [27]. The intra-assay coefficient of variation was 2.8%, and assay sensitivity was 0.006 ng/mL.

2.2. Experiment 2

2.2.1. Animals and management

Experiment 2 was conducted on seven farms (farm A, N = 26; farm B, N = 60; farm C, N = 64; farm D, N = 48; farm E, N = 22; farm F, N = 11; and farm G, N = 25), located in the state of São Paulo, Brazil, during the nonbreeding season (spring through summer; November, 2009 to March, 2010). A total of 256 lactating buffalo (*Bubalus bubalis*) were assigned to one of two treatment groups (with eCG, N = 127 and without eCG, N = 129) according to postpartum interval (147.0 ± 6.0 days) and BCS (3.0 ± 0.0; scale of 1 to 5, where 1 = very thin and 5 = very fat). These buffaloes were maintained on *Brachiaria decumbens* pastures with free access to water and mineralized salt.

2.2.2. Experimental design

Buffalos were assigned to the same treatments described in experiment 1, and one TAI was performed 16 hours after GnRH treatment. All inseminations were performed by the same technician who had no knowledge of treatment group.

2.2.3. Pregnancy diagnosis

Pregnancy diagnosis was performed by ultrasonography 30 days after the TAI. The pregnancy per artificial insemination (P/AI) was defined as the number of pregnant buffalo divided by the total number of buffalo subjected to TAI in each treatment (with or without eCG).

2.3. Statistical analyses

Statistical analyses were performed using Statistical Analysis System for Windows (SAS, 2001, SAS Institute, Cary, NC, USA). For experiment 1, continuous data were tested for normality of the residues and analyzed using the UNIVARIATE procedure (transformed when necessary) and subjected to Bartlett's test to assess homogeneity of variances. The GLM procedure with a Tukey adjustment was used to determine significant differences among groups. Nonparametric data were analyzed using the NPAR1WAY procedure (Wilcoxon). All values were expressed as mean ± SEM. In experiment 2, variables initially included in models were treatment (with and without eCG), farm, and BCS on the first day of the synchronization protocol. Binomial data were analyzed with a multivariate logistic regression using the LOGISTIC procedure of SAS. Variables were removed by backward elimination based on the Wald statistics criterion when $P > 0.20$. Variables included in the final model for analysis of the P/AI were treatment and farm. The P/AI was analyzed using the GLIMMIX procedure of SAS. Significant differences were indicated by $P < 0.05$.

3. Results

3.1. Experiment 1

The effects of eCG treatment are summarized (Table 1 and Fig. 2). Diameter of the dominant follicle on Days 0 and 9 were similar between groups (P = 0.71 and P = 0.58, respectively). Exogenous eCG increased the maximum diameter of the dominant follicle (P = 0.09) and ovulation rate (P = 0.05; Table 1). Furthermore, eCG increased CL diameter (15.8 ± 0.92 mm vs. 12.7 ± 0.77 mm; P = 0.03) and P4 concentration (0.59 ± 0.08 ng/mL vs. 0.27 ± 0.05 ng/mL; P = 0.01) on Day 16. However, there was no effect of eCG on diameter of the dominant follicle on Day 11 (P = 0.11), growth rate of the dominant follicle between device removal and GnRH treatment (P = 0.24), the interval between device removal and ovulation (P = 0.42), and the interval between GnRH administration and ovulation (P = 0.42; Table 1).

3.2. Experiment 2

There was no interaction between treatment (with or without eCG) and explanatory variables such as BCS (P = 0.72) and farm (P = 0.99) on P/AI. Also, there was no

Table 1
Ovary status and eCG effects on follicular development and/or dynamics, ovulation and CL development (mean ± SEM) and risk factors per AI in lactating buffalo cows treated with or without eCG and subjected to a timed AI protocol of intravaginal progesterone plus 2 mg estradiol benzoate estradiol.

	Treatment		P
	Without eCG	With eCG	
Experiment 1			
Cows, N	29	30	
Diameter of DF on Day 0 (mm)	8.9 ± 0.4	9.0 ± 0.4	0.71
Diameter of DF on Day 9 (mm)	7.9 ± 0.4	8.3 ± 0.4	0.58
Diameter of DF on Day 11 (mm)	10.0 ± 0.7	11.3 ± 0.5	0.11
Growth rate of DF between device removal and GnRH (mm/d)	1.3 ± 0.2	1.5 ± 0.2	0.24
Maximum diameter of DF (mm)	12.6 ± 0.6	13.7 ± 0.4	0.09
Interval between device removal and ovulation (h)	70.0 ± 3.7	69.8 ± 2.1	0.42
Interval between GnRH and ovulation (h)	25.3 ± 4.2	28.6 ± 2.6	0.42
Ovulation rate, % (N/N)	44.8 (13/29)	66.7 (20/30)	0.05
Experiment 2			
Risk factors per AI, % (N/N)	39.4 (50/127)	52.7 (68/129)	0.03

Abbreviation: DF, dominant follicle.

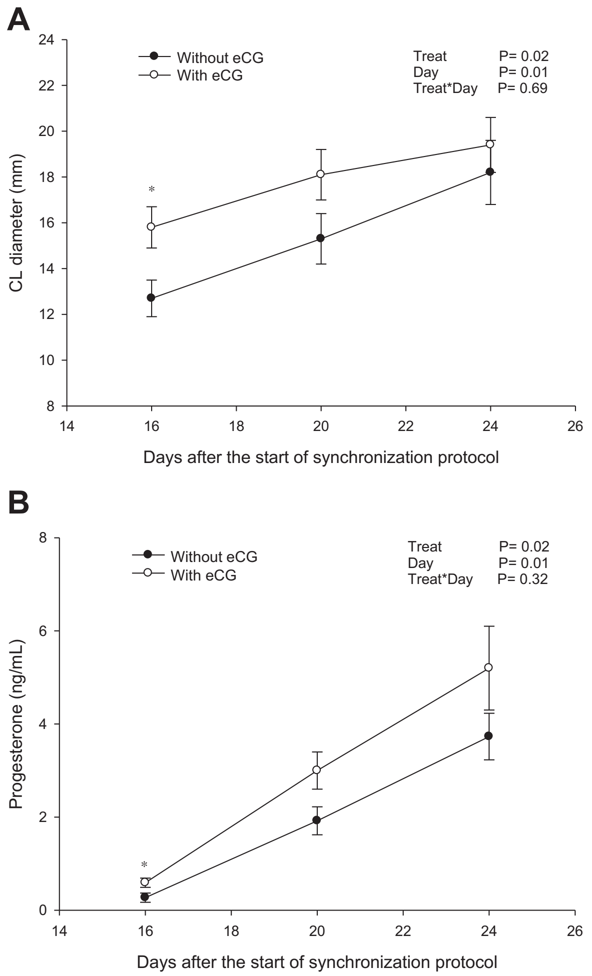

Fig. 2. Mean ± SEM effect of eCG treatment on CL diameter (A) and on the serum progesterone (P4) concentrations (B) during diestrus after synchronized ovulation in lactating buffalo (N = 59). * Effect of eCG treatment on the CL diameter and P4 concentrations ($P < 0.05$).

significant effect of farm (P = 0.09) and BCS (P = 0.71) on P/AI. However, treatment with eCG increased the P/AI (52.7% vs. 39.4%; P = 0.03; Table 1) in buffaloes subjected to TAI.

4. Discussion

This study provided important information regarding the effectiveness of eCG in a TAI protocol in lactating buffalo during the nonbreeding season. Giving eCG at removal of the P4 device increased the diameter of the dominant follicle and ovulation rate. Furthermore, this treatment also increased CL growth rate, the initial P4 concentration, and pregnancy rate after the TAI. Therefore, exogenous eCG improved ovarian responses and P/AI ratios, thereby enhancing reproductive performance of lactating buffalo cows synchronized for TAI during the nonbreeding season and supporting our initial hypothesis.

It has been suggested that anestrous cows treated with the current TAI protocols might ovulate smaller follicles that produce low P4 concentrations after ovulation, altering the synthesis or release of uterine $PGF_{2\alpha}$ and compromising pregnancy recognition and maintenance [28]. In the present study, eCG-treated buffalo had increased maximum diameters of ovulatory follicles at the end of the synchronization protocol. These results corroborated data obtained in anestrous beef cows treated with eCG, which increased the growth of the dominant follicle and the diameter of the largest follicle at TAI [20,23,24]. As previously reported, diameter of the ovulatory follicle appeared to be an indicator of ovarian and fertility responses in *Bos taurus* [29–31] and *Bos indicus* [32–36] cattle.

In the present study, eCG increased ovulation rate in buffalo subjected to TAI, consistent with previous investigations in which eCG increased ovulation rates in buffalo [4,37,38], *Bos indicus* beef heifers [19,23], and suckled beef cows [19,20,24]. In buffalo, Murugavel et al. [25] reported a greater ovulation rate in controlled intravaginal drug release (CIDR) with eCG (81.0%) compared with CIDR (47.4%) treatment in noncyclic buffalo cows. Anestrous cows have insufficient LH pulsatile release to support the final stages of ovarian follicular development and ovulation. The efficiency of eCG is related to its FSH- and LH-like activities [39], which ensure continuation of ovarian follicular growth in cows with compromised gonadotropin secretion. In this regard, Baruselli et al. [21] confirmed that the effect of eCG was more pronounced with deeper anestrous in *Bos indicus* breeds. Thus, improved fertility after eCG supplementation observed in the present study might have been because of an increase in diameter of the ovulatory follicle and the percentage of cows that ovulated after TAI.

In the present study, eCG treatment enhanced CL diameter and P4 concentrations in the early luteal phase following the TAI protocol. The underlying physiology by which eCG increases fertility seems to be related to changes in follicular growth and CL function [19,40]. Several studies attributed beneficial effects on CL area and consequently on P4 concentrations in the estrous cycle after eCG treatment [19,41,42]. Increased P4 concentrations in eCG-treated cows might be a result of an increase in CL diameter, alteration of cellular machinery involved in luteal P4 synthesis, or a combination of these factors. Treatment with eCG increased expression of cytochrome P450 cholesterol side-chain and 3-b-hydroxysteroid dehydrogenase. In addition, in the CL of eCG-treated cows, there was a significant increase in expression of steroid acute regulatory protein (an important protein for steroid biosynthesis) [42].

Strategies that stimulated dominant follicle growth before ovulation were associated with increased ovulation rate, enhanced CL development, and greater capacity of P4 production, which are related to maintenance of pregnancy and improved fertility in cattle [41,43–45] and buffalo [46]. Also, P4-induced changes in the endometrium and/or embryo during the early luteal phase are responsible for enhanced embryo development during conceptus elongation and the associated increased interferon-tau secretion, leading to a higher pregnancy rate [47]. In the current study, P/AI was greater in eCG-treated compared with nontreated lactating buffalo during the nonbreeding season, confirming the beneficial effects of eCG treatment.

In addition, Murugavel et al. [25] reported greater pregnancy rates after TAI in both cyclic and noncyclic anestrous buffalo subjected to a CIDR with eCG (40.6%) treatment than a CIDR without eCG (27.3%). Furthermore, two protocols for synchronization of ovulation for buffalo TAI were studied during the nonbreeding season (Ovsynch vs. P4 with EB and eCG) [6]; pregnancy rate was greater for buffalo synchronized with P4 with EB and eCG (53.5%) than those given a GnRH/$PGF_{2\alpha}$/GnRH protocol (28.2%).

Improved pregnancy rates after eCG treatment at removal of the P4 device in the present study supported previous results of TAI programs in *Bos indicus* cattle with a high incidence of anestrus [19,24,48]. However, in some studies, there was no effect of eCG treatment on follicular responses and pregnancy rates in buffalo [7] and cattle [40]. Perhaps the incidence of anestrus because of insufficient LH pulses is less of a problem in some herds because of different management and nutritional status.

4.1. Conclusions

Treatment with eCG at removal of the intravaginal P4 device in a TAI protocol increased the maximum diameter of the dominant follicle, ovulation rate, CL growth rate, and functionality after TAI, and pregnancy outcomes in lactating buffalo. Thus, administration of eCG at P4 device removal should be considered to enhance the reproductive efficiency in lactating buffalo (*Bubalus bubalis*) subjected to TAI programs during the nonbreeding season.

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