Theriogenology 157 (2020) 498–502

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Treatment with estradiol cypionate at progesterone withdrawal reduces handling without compromising the pregnancy rate to timed-AI in buffalo

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ARTICLE INFO

Article history:
Received 2 June 2020
Received in revised form
19 August 2020
Accepted 20 August 2020
Available online 24 August 2020

Keywords:
Buffalo
Estradiol cypionate
Ovulation synchronization
Pregnancy
Timed-AI

ABSTRACT

The aim of this study was to determine if treatment with estradiol cypionate (EC) at the time of P4 withdrawal induced ovulation in a synchronization/timed-AI (TAI) protocol in buffalo. In Experiment 1, 56 buffaloes received an intravaginal P4 device (1.0 g) plus estradiol benzoate (EB, 2.0 mg im) on Day 0 (D0). On Day 9, the P4 device was removed and buffaloes were given $PGF_{2\alpha}$ (0.53 mg im sodium cloprostenol) plus eCG (400 IU im). Buffaloes were then randomly allocated to one of two groups: Group GEC (n = 29), treated with EC (1.0 mg im) at P4 device removal; Group GEB (n = 27), treated with EB (1.0 mg im) 24 h after P4 device removal. Ovarian ultrasound was undertaken on: D0, to ascertain general ovarian status; D9 to D11 (every 24 h), to measure diameter of the largest follicle (LF) and follicular growth rate; D11 to D13 (every 12 h for 72 h), to determine the time of ovulation and ovulation rate. Following P4 device removal, Groups GEC and GEB had a similar follicular growth rate (0.9 ± 0.1 and 1.1 ± 0.1 mm/day, respectively; P = 0.15) and similar LF diameter on D11 (11.4 ± 0.6 and 12.5 ± 0.5 mm; P = 0.12). Groups GEC and GEB also had a similar diameter of the ovulatory follicle (13.0 ± 0.5 and 13.4 ± 0.6 mm; P = 0.52), interval from P4 device removal to ovulation (68.2 ± 2.8 and 71.1 ± 1.4 h; P = 0.41) and ovulation rate (62.1% and 70.4%; P = 0.44). In Experiment 2, 199 buffaloes were assigned to the two treatments in Experiment 1 (GEC, n = 100; GEB, n = 99). All animals underwent TAI 56 h after P4 device removal and pregnancy diagnosis was preformed on D41. The pregnancy rate was similar for Groups GEC and GEB (50.0 and 45.5%, respectively; P = 0.45). The findings indicate that treatment with EC at the time of P4 withdrawal induces ovulation and achieves the same pregnancy rate to TAI as treatment with EB 24 h after P4 removal. The use of EC requires one less handling which is highly important in facilitating practical adoption of TAI in assisted breeding and genetic improvement in buffalo.

1. Introduction

The induction of ovulation and timed-AI (TAI) has important application in assisted reproduction in Water buffalo (*Bubalus bubalis*). It allows buffalo to be bred during both the breeding and nonbreeding seasons [1] and it increases the service and pregnancy rates [1,2]. Ovulation induction with TAI also allows buffalo to be

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https://doi.org/10.1016/j.theriogenology.2020.08.024
0093-691X/

bred without the need to detect estrus [1,3–6]. In a typical P4-based + estrogen synchronization protocol, buffalo are required to be handled three times for treatments, before a fourth handling for TAI [7–10]. The first handling is to commence treatment with P4 and estrogen, the second handling is to withdraw P4 and administer $PGF_{2\alpha}$ and eCG, the third handling is to treat with estrogen, and the forth handling is for TAI. This amount of handling can be considered impractical and restricts the adoption of assisted reproduction in buffalo [1,10]. A protocol that involved less handling, but did not reduce the pregnancy rate, would be more practical and would facilitate greater use of assisted breeding in buffalo.

Treatment with estrogen is used to synchronize the time of ovulation in conjunction with TAI in buffalo [8] and cattle [11–17]. Esters of estradiol-17β (E-17β), which require metabolism to E-17β for biological activity, are typically used as they extend the period of elevated E-17β in blood after treatment. The latter increases the likelihood of a biological effect and synchronized ovulation [18]. Different esters are metabolized at different rates and produce different profiles of E-17β in circulation [19,20]. The ester, estradiol benzoate (EB), undergoes relatively fast metabolism and is associated with an early peak in blood E-17β followed by a fairly quick decline [19]. A second ester, estradiol cypionate (EC), is metabolized slower than EB and, when administered at the same dose as EB, is associated with a delayed peak in blood E-17β, a lesser peak height, and slower decline in blood E-17β [19]. Given the pharmacokinetics of EC, the present study sought to determine if EC could be administered at the time of P4 withdrawal in buffalo. This could potentially replace the need to treat with EB 24 h after the removal of P4 [8,10,16,21,22]. The rationale was that blood E-17β would remain elevated for sufficient time after treatment with EC to induce a follicular response that synchronized ovulations. The hypothesis tested was that EC (animals handled three times) and EB (animals handled four times) would have similar efficacy in the induction of ovulation and pregnancy rate in a P4-based + estrogen TAI protocol in buffalo.

2. Materials and methods

2.1. Experiment 1 - Ovarian follicular dynamics in buffalo treated with EC or EB

2.1.1. Animals and management

The study was conducted at the Research and Development Unit of Registro/Diversified Animal Science Research Center/Institute of Animal Science (Registro, São Paulo, Brazil). Lactating Murrah buffalo (*Bubalus bubalis*) cows (n = 56) at 7.2 ± 0.3 kg of milk/day (mean ± SEM), 3.1 ± 0.4 lactations, 152 ± 13 days in milk, 7.5 ± 0.8 years old and body condition score (BCS) 3.4 ± 0.1 (scale 1–5, where 1 = very thin and 5 = very fat) were used. Cows were milked once daily and had contact with their calves only during milking. Buffaloes grazed tropical grasses and were supplemented with fresh tropical grasses and grain mix containing ground corn, soybean meal, citrus pulp, whole cottonseed, minerals and vitamins.

2.1.2. Experimental design

Buffaloes (*Bubalus bubalis)* received an intravaginal progesterone device (P4; 1.0 g; Sincrogest®, Ourofino Saúde Animal, Brazil) plus EB (2.0 mg im; Sincrodiol®, Ourofino Saúde Animal) on Day 0 (D0). On Day 9, the P4 device was removed and buffaloes received an injection of $PGF_{2\alpha}$ (0.53 mg im sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal) plus eCG (400 IU im; equine chorionic gonadotropin; SincroeCG®, Ourofino Saúde Animal). Buffaloes were then allocated to one of two groups based on age, lactations, milk production, days in milk, BCS, general ovarian status on D0, and diameter of the largest follicle on D9. The two groups were: Group GEC (n = 29), 1.0 mg im estradiol cypionate (EC; SincroCP®, Ourofino Saúde Animal) given at P4 device removal (D9); and Group GEB (n = 27), 1.0 mg im estradiol benzoate (EB; Sincrodiol®, Ourofino Saúde Animal) given 24 h after P4 device removal (D10) (Fig. 1). The experiment was undertaken during the nonbreeding season for buffalo, which coincides with an increase in day length (spring to summer).

2.2. Ultrasonographic examinations

Ovaries were scanned by ultrasonography using a 7.5-MHz linear-array transrectal transducer (Mindray DP-2200Vet; Shenzhen, Guangdong, China). Scans were performed on: D0, to ascertain general ovarian status; D9 to D11 (every 24 h), to measure diameter of the largest follicle (LF) and establish follicular growth rate; D11 to D13 (every 12 h for 72 h), to verify the interval from P4 device removal to ovulation and ovulation rate (Fig. 1). The time of ovulation was defined as the time of disappearance of a previously identified LF from one ultrasound examination to the next. The maximum diameter of the LF was defined as the greatest diameter of the largest follicle recorded over 108 h after P4 device removal.

2.3. Experiment 2 – Pregnancy per AI in buffalo treated with EC or EB

2.3.1. Animals and management

Experiment 2 was conducted at five commercial farms (Farm A, n = 52; Farm B, n = 27; Farm C, n = 18, Farm D, n = 34 and Farm E, n = 68) located in the State of São Paulo, Brazil. Lactating crossbred Murrah x Mediterranean buffalo (*Bubalus bubalis)* cows (n = 199) with BCS 3.5 ± 0.1 and 132 ± 6 days in milk were used. The treatments were equally distributed among the Farms. Buffaloes were maintained on a *Brachiaria spp* pasture with free access to water and mineralized salt. This experiment was also undertaken during the nonbreeding season for buffalo.

2.3.2. Experimental design

All animals received the P4-based + estrogen TAI synchronization protocol as described in Experiment 1 and were assigned using the same criteria to one of two groups: Group GEC, n = 100 and Group GEB, n = 99 (Fig. 1). Buffaloes underwent TAI 56 h after P4 device removal. Inseminations were performed by the same technician who had no knowledge of treatment group. Frozen-thawed semen straws from two buffalo bulls of proven fertile were distributed equally between the two treatments and the five farms.

2.3.3. Pregnancy diagnosis

Pregnancy diagnosis was performed by ultrasonography (Mindray DP-2200Vet, Shenzhen, Guangdong, China) on D41 (30 days after TAI). The pregnancy per TAI protocol (P/TAI) was defined as the number of pregnant buffalo divided by the total number of buffalo mated by TAI. The detection of an embryonic vesicle with a viable embryo (presence of a heartbeat) was used as an indicator of pregnancy.

2.4. Statistical analyses

Statistical analyses were performed using Statistical Analysis System for Windows-SAS. In Experiment 1, the variables evaluated were diameter of the largest follicle (D9, D10, D11), diameter of the ovulatory follicle, time from P4 device removal to ovulation, and ovulation rate. Continuous data were tested for normality of the residues and analyzed by the UNIVARIATE procedure (transformed when necessary) and subjected to the Bartlett's test to assess the

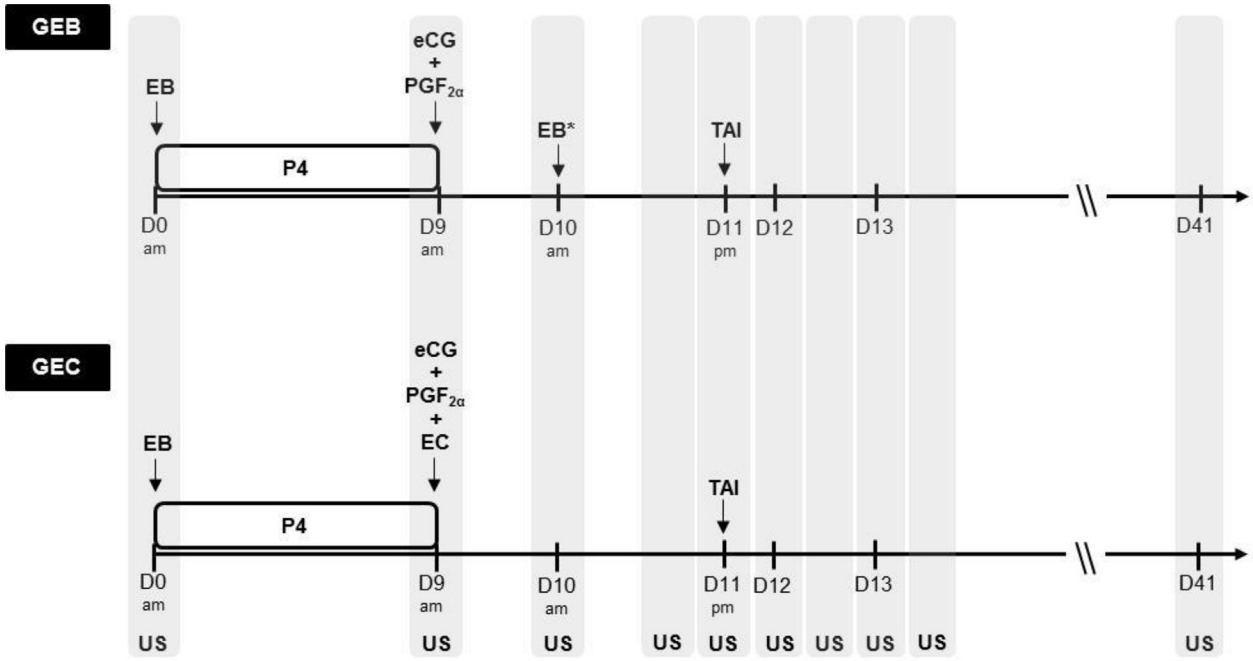

Fig. 1. Diagram of the experimental design and treatments. Buffalo cows were subjected to a P4-based + estrogen TAI synchronization protocol and treated with either estradiol cypionate (GEC) at the time of P4 withdrawal (Day 9) or estradiol benzoate (GEB) 24 h after P4 withdrawal (Day 10). EB = 2.0 mg estradiol benzoate; P4 = 1.0 g progesterone; eCG = 400 IU equine chorionic gonadotropin; $PGF_{2\alpha}$ = 0.53 mg sodium cloprostenol; EC = 1.0 mg estradiol cypionate; EB* = 1.0 mg estradiol benzoate; TAI = timed artificial insemination; US = ovarian ultrasound examination.

homogeneity of variances. All variables followed a normal distribution, except for the time of ovulation that was evaluated by lognormal. The GLIMMIX procedure was used to determine significant differences between groups. All values are expressed as mean ± SEM. Binomial variable ovulation rate was analyzed using PROC GLIMMIX of SAS and expressed as percentages (%). In Experiment 2, the model included the fixed effects of treatment, BCS at Day 0, parity, farm, days-in-milk, number and size of follicles at Day 0, bull and the random effects of buffaloes. All two-way interactions were tested in logistic regression models. Data were analyzed by a multivariate logistic regression using the LOGISTIC procedure of SAS. Variables were removed by backward elimination, based on the Wald statistics criterion when $P > 0.20$ to form the final model. Variables included in the final model for analysis of P/TAI were treatment (GEC and GEB) and BCS at Day 0. The P/TAI was analyzed using the GLIMMIX procedure of SAS. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

3. Results

3.1. Experiment 1

The results for Experiment 1 are summarized in Table 1 together with the statistical analyses. The diameter of the LF during and after synchronization treatment, and the diameter of the ovulatory follicle, was similar for Group GEC and Group GEB. The interval from P4 device removal to ovulation (GEC = 68.2 ± 2.8 h and GEB = 71.1 ± 1.4 h) and the ovulation rate (GEC = 62.1% and GEB = 70.4%) was also similar for Group GEC and Group GEB. Group GEB showed lower ($P = 0.001$) variability in the time of ovulation than Group GEC (Fig. 2).

3.2. Experiment 2

In experiment 2, the pregnancy rate (P/TAI) did not differ ($P = 0.45$) for Group GEC (50/100, 50%) and Group GEB (45/99, 45.5%). There was no interaction between group and BCS at D0 on P/TAI ($P = 0.19$).

4. Discussion

The hypothesis tested in the present study was that treatment with EC on Day 9 of a P4-based TAI protocol would result in similar ovulation and pregnancy rates as treatment with EB on Day 10 in buffalo. It was found that EC and EB produced comparable outcomes in terms of follicular growth, ovulation, and pregnancy rate. Based on these findings, the hypothesis can be accepted that EC and EB have similar efficacy in the induction of ovulation and pregnancy rate in buffalo subjected to a P4-based TAI protocol. The study was conducted during the nonbreeding season which indicated that the administration of EC at the time of P4 withdrawal induces ovulation in a high proportion of acyclic buffalo. The reduced handling in a P4-based + EC protocol is highly important for facilitating greater adoption of assisted breeding in buffalo.

The diameter of the LF at TAI did not differ between buffalo treated with EC or EB. The mean interval from P4 device removal to ovulation, and the ovulation rate, also did not differ. This would have been anticipated as the pre-ovulatory peak in LH occurs approximately 24 h after treatment with EB in buffalo [23,24] and cattle [16,25,26], and approximately 52 h after treatment with EC in cattle [16]. Buffalo treated with EC did, however, show greater variability in the timing of ovulation. This may have been due to differences in blood E-17β profiles after treatment with EC and EB. Treatment with EB is associated with an earlier and higher peak in blood E-17β compared with treatment with EC [27]. Whilst these differences in the pharmacokinetics of EC and EB appeared to influence the synchrony of ovulation, there were no apparent effects

Table 1
Ovarian follicular responses (mean ± sem) in a P4-based + estrogen TAI synchronization protocol in buffalo cows. Buffalo were treated with estradiol cypionate (GEC) at the time of P4 withdrawal (Day 9) or with estradiol benzoate (GEB) 24 h after P4 withdrawal (Day 10).

	Treatment[a]		
	GEB	GEC	*P* value
Number of animals	27	29	
Diameter of LF on D9 (mm)	9.7 ± 0.5	9.3 ± 0.4	0.53
Diameter of LF on D10 (mm)	10.9 ± 0.5	10.2 ± 0.5	0.23
Diameter of LF on D11 a.m. (mm)	11.9 ± 0.5	10.9 ± 0.5	0.14
Diameter of LF on D11 p.m. (mm)	12.5 ± 0.5	11.4 ± 0.6	0.12
Diameter of the OF (mm)	13.4 ± 0.6	13.0 ± 0.5	0.52
Ovulation rate, % (n/n)	70.4 (19/27)	62.1 (18/29)	0.44
Interval from P4 device removal to ovulation (h)	71.1 ± 1.4	68.2 ± 2.8	0.41
Growth rate of LF (mm/d)	1.1 ± 0.1	0.9 ± 0.1	0.15

Abbreviations: LF, largest follicle (mm); OF, ovulatory follicle (mm)
[a] Information on the synchronization protocol is given in Fig. 1, and other details on experimental design are given in Section 2.

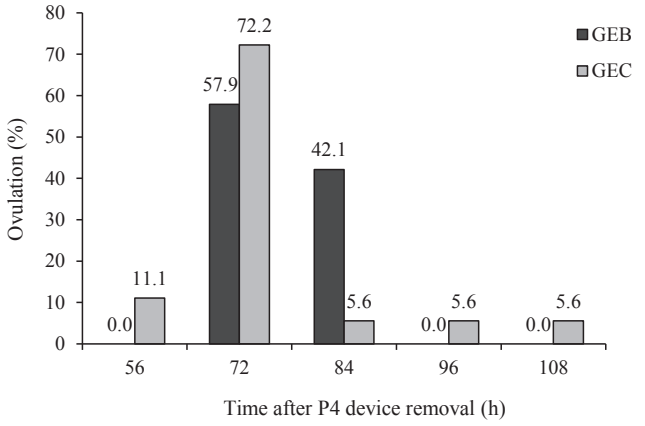

Fig. 2. Distribution of the time of ovulation (h) after P4 device removal in buffalo cows subjected to a P4-based + estrogen TAI synchronization protocol and treated with either estradiol cypionate (GEC) at the time of P4 withdrawal (Day 9) or estradiol benzoate (GEB) 24 h after P4 withdrawal (Day 10). Bartlett's test P = 0.001.

on ovulation rate and pregnancy rate. Treatment with EC on Day 9 would have exposed buffalo to elevated blood E-17β for a longer time than treatment with EB on Day 10 and this may have outweighed the differences in metabolism and clearance rates of EC and EB. In a previous study, treatment with EC at the time of P4 withdrawal was less effective than GnRH in synchronizing the LH surge in cattle [13]. Cattle also showed more variability in the timing of ovulation after treatment with EC compared with EB [22,28].

The similarity in size of the ovulatory follicle for buffalo treated with EC and EB was likely important for functionality of the CL after ovulation [29]. Relationships between size of the ovulatory follicle and CL size, CL size and blood P4 concentrations, and blood P4 concentrations and pregnancy rate, have been reported for buffalo [7,30,31] and cattle [32–34]. Greater concentrations of blood P4 support embryonic development [35] and the maintenance of pregnancy in buffalo [31] and cattle [36–39]. It could be concluded that, in the present study, the size of the ovulatory follicle was perhaps more important in influencing pregnancy outcome, than the absolute synchrony of ovulation after P4 withdrawal.

In summary, the present study has shown that treatment with EC at the time of P4 withdrawal in a P4-based + estrogen TAI protocol results in comparable ovulation and pregnancy rates in buffalo as treatment with EB 24 h after P4 withdrawal. Hence, the use of EC reduces the number of times that animals are handled in a P4-based + estrogen synchrony protocol. This makes the synchrony protocol more practical which is highly important in facilitating the adoption of assisted breeding for reproductive management and genetic improvement in buffalo.

CRediT authorship contribution statement

Nelcio Antonio Tonizza de Carvalho: Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Júlia Gleyci Soares de Carvalho:** Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization. **José Nélio de Sousa Sales:** Software, Validation, Formal analysis, Visualization, Data curation. **Bruna Martins Guerreiro:** Validation, Resources. **Bruno Gonzalez de Freitas:** Validation, Resources, Funding acquisition. **Michael J. D'Occhio:** Writing - original draft, Writing - review & editing, Visualization, Supervision. **Pietro Sampaio Baruselli:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Acknowledgments

The authors are grateful to the Institute of Animal Science (Registro-SP, Brazil) and the buffalo producers for the generous provision of animals and facilities for this study. The research was supported by Ourofino Saúde Animal (Cravinhos, SP, Brazil) and FAPESP (Process 2017/50.339-5).

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