



## Injectable progesterone in timed artificial insemination programs in beef cows

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### Abstract

The aims of this study were I) to compare the follicular diameter, corpus luteum diameter and serum progesterone (P4) concentrations in cows treated with conventional protocol vs. injectable P4 protocol; II) to determine the serum P4 profile in ovariectomized heifers; and III) to compare pregnancy rate between protocols. In experiment I, multiparous cows received a protocol for ovulation synchronization with an intravaginal P4 device (n = 38; device + EB day 0; device removal + PGF2 $\alpha$  + eCG + EC day 8) or injectable P4 (n = 38; injection + EB day 0; PGF2 $\alpha$  + eCG + EC day 8). In experiment II, ovariectomized heifers (n = 8) were treated with injectable P4 and blood samples were collected to determine the serum P4 profile. In experiment III, multiparous cows were timed AI with two different P4 approaches, intravaginal P4 device (n = 48) or injectable P4 (n = 47). In the first experiment, cows treated with P4 device had higher (P < 0.05) diameter of dominant follicle after ovulation induction (11.6  $\pm$  1.8 vs. 10.3  $\pm$  1.8 mm) and ovulation rate (97%, 37/38 vs. 47.3%, 18/38) than cows treated with injectable P4. But, the follicular growth daily was higher (P < 0.05) in cows treated with injectable P4 than intravaginal device (1.3  $\pm$  0.4 vs. 1.0  $\pm$  0.3 mm/day, respectively). In experiment II, the P4 concentration peak occurred within 48 hours (6.54 ng/mL) and decreased after 96 hours (P < 0.05) after P4 injection. In experiment III, cows with P4 device had higher (P < 0.05) pregnancy rate than the injectable P4 group (60.4 vs. 34.0%, respectively). These results demonstrate that although the intravaginal P4 devices showed a higher pregnancy rate, a protocol with injectable P4 represents an easier method and a promising alternative for TAI in cattle.

**Keywords:** follicular diameter, injectable progesterone, pregnancy, synchronization of ovulation.

### Introduction

The timed artificial insemination (TAI) programs have been considered one of the largest biotechnological achievements for breeding cattle. Certainly, this is related to the fact that TAI allows all females receiving hormonal treatment to be inseminated without the need for estrus detection (Baruselli *et al.*, 2004; Lamb *et al.*, 2010).

In this context, the synchronic control of wave emergence, dominant follicle growth and ovulation are the main requirements of a hormonal protocol that allow to AI or embryo transfer at a fixed time (Bo *et al.*, 2003; Baruselli *et al.*, 2004; Carvalho *et al.*, 2008; Sá Filho *et al.*, 2013; Sá Filho *et al.*, 2015). Additionally, this pharmacological strategy optimizes the use of females as well as increases the pregnancy rate and reduces the costs of reproductive programs (Marinho *et al.*, 2012).

To assist reproductive biotechnology, several pharmacological strategies have been proposed (Sales *et al.*, 2012; Campos *et al.*, 2013; Barreiros *et al.*, 2014; Torres *et al.*, 2014; Marques *et al.*, 2015; Pellegrino *et al.*, 2016) and progesterone (P4) has been the main exogenous hormonal basis for estrus synchronization in cattle; treatment can be performed through intravaginal devices (Macmillan *et al.*, 1991; Macmillan and Peterson, 1993), ear implants (Baruselli *et al.*, 2004; Sa Filho *et al.*, 2011), oral formulations (Fike *et al.*, 1999) or injectable sources (Morotti *et al.*, 2013a; Morotti *et al.*, 2013b; Campos *et al.*, 2016a).

Commonly the treatment with P4 includes the insertion of releasing P4 devices for 5-10 days that maintains its plasma concentrations this period (Baruselli *et al.*, 2004). The purpose is to maintain high P4 levels to block estrus manifestation and to suppress the endogenous peak of LH (Kinder *et al.*, 1996). In this way, it is possible avoiding ovulation, but keeping the growth and maturation of the dominant follicle (Savio *et al.*, 1993a; Stock and Fortune, 1993; Rhodes *et al.*, 2002).

The use of injectable P4 for TAI has provided lower pregnancy rates in comparison to protocols with P4 devices (Morotti *et al.*, 2013b; Campos *et al.*, 2016b). However, several positive aspects of injectable P4 have been encouraging new experiments. For example, injectable P4 has been related to low cost of handling, easy management of animals, hygienic benefits and no discard of devices (Morotti *et al.*, 2013a; Morotti *et al.*, 2013b; Campos *et al.*, 2016a; Campos *et al.*, 2016b).

Thus, the objectives of this study were: i) to evaluate the follicular diameter, corpus luteum diameter, and serum P4 concentrations in cows treated with conventional ovulation synchronization protocol vs. injectable P4 protocol; ii) to determine the serum P4 profile in ovariectomized heifers, and iii) to compare pregnancy rate between protocols.

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## Materials and Methods

### *Location, animals and feed management*

The present study was performed in compliance with protocols approved by the Committee of Ethics in Animal Experimentation based on the Federal Law 11.794/ 2008. Three experiments were performed in Nelore (*Bos indicus*) cattle in South America (23° 22' 24" S; 50° 50' 35" W). In this region, the climate is tropical with an average temperature of 23.5°C and rainy season. During the experimental period, the animals were kept continuously grazing on *Urochloa brizantha* and *Urochloa humidicola* pastures and were given *ad libitum* access to a mineralized mixture and water.

### *Experiment I*

A total of 76 multiparous cows, 72 to 96 months of age, from 45 to 70 days postpartum (suckling), with a body condition score (BCS) of  $2.7 \pm 0.3$  on scale of 1 to 5 (Lowman *et al.*, 1976) and weighing  $457 \pm 40$  kg, were divided into two groups, the intravaginal P4 device (control) and injectable P4 groups. In the control group, 38 cows received a conventional protocol for ovulation synchronization using an intravaginal device (first use) containing 1 g of P4 (DIB®, Syntex, Buenos Aires, Argentina) associated with the administration of 2 mg of estradiol benzoate (EB; Syntex®, Syntex, Buenos Aires, Argentina) intramuscularly (i.m.) in a random day of the estrous cycle (Day 0). On Day 8, P4 device removal was followed by i.m. injections of 500 µg of cloprostenol (DL Cyclase®, Syntex, Buenos Aires, Argentina), 300 IU of equine chorionic gonadotropin (eCG; Novormon®, Syntex, Buenos Aires, Argentina) and 1 mg of estradiol cypionate (EC; Cipiosyn®, Syntex, Buenos Aires, Argentina). Cows of the injectable P4 group (n = 38) received 350 mg of injectable P4 (Progressincro®<sup>1</sup>, Campos Laboratory Ltda, Londrina, Brazil) i.m. associated with 2 mg of EB on Day 0. On Day 6, they were given 500 µg i.m. of cloprostenol, 300 IU of eCG and 1 mg of EC. Follicular and CL evaluations were performed by ultrasonography (Aloka® SSD-500, Tokyo, Japan) equipped with a 7.5 MHz transducer. Evaluations were performed in both ovaries and recorded individually in a map, from day 4 (daily, for evaluation of follicular diameter), after ovulation inducer (every 12 hours, for monitoring of ovulation) and 12 days after ovulation (to evaluate the CL size and P4 dosage) (Ginther *et al.*, 1989; Figueiredo *et al.*, 1997; Ruiz-Cortes and Olivera-Angel, 1999). Immediately after CL evaluation, blood samples were collected by coccygeal puncture. The serum P4 concentrations were determined using a commercial solid-phase radioimmunoassay kit (RIA

<sup>1</sup>Progressincro® - contains a formulation with 300 mg of natural progesterone in vehicle sesame and peanut oil (long absorption) and a formulation with 50 mg of natural progesterone in same vehicle (fast absorption).

IM1188 kit; Beckman Coulter®, Immunotech, Czech Republic) in 100 µL samples. The test sensitivity was 0.03 ng/mL, and the intra-trial variance was 0.88 to 1.64 ng/mL. Data processing was performed using the Gamma Wizard Reader Model 1470 (Perkin Elmer) with MultiCalc software in the Laboratory of IGAC - Genese Institute of Scientific Analysis, in São Paulo-SP.

### *Experiment II*

Eight ovariectomized heifers, ranging from 24 to 36 months of age, BCS of  $3.0 \pm 0.5$  on a scale of 1 to 5 (Lowman *et al.*, 1976) and average body weight of  $370 \pm 15$  kg were used in this study. The heifers received 250 mg of injectable P4 source (Progressincro®; Laboratório Campos Ltda, Londrina, Brazil) via i.m. injection. Nine blood samples from each animal were collected by jugular vein puncture in 10 mL vacuum tubes (Vacutainer® - Becton Dickinson Indústrias Cirúrgicas Ltda, Juiz de Fora, Brazil) without anticoagulant at 9 a.m., starting 6 hours after the time of P4 injection (0 hour) until 240 hours. Immediately after each blood collection, the samples were prepared immediately after each blood collection, the samples were prepared and serum aliquots were stored at -20°C until analysis. For P4 concentration assessments in this study, the serums were processed together with the samples from experiment I using the same kit during the same assay.

### *Experiment III*

To evaluate the pregnancy rate after TAI using injectable P4, suckling cows (n = 95) 30 to 60 days postpartum and BCS ranging between 2.5 and 3.5 (Lowman *et al.*, 1976) were randomly allocated to injectable P4 or intravaginal P4 device groups. In the injectable P4 group, 47 cows received 250 mg P4 i.m. with 2 mg of EB on a random day of estrous (Day 0). On Day 7 females received 500 µg of cloprostenol and 300 IU of eCG. On Day 8, cows were given 1 mg of EB i.m.. TAI was performed 36 hours later (Day 9). Control group (n = 48) received the same protocol of intravaginal device as in the experiment I. Cows were inseminated by a single trained inseminator using conventional semen from a single bull with known fertility. The pregnancy diagnosis was performed 60 days after TAI by ultrasonography (Aloka® SSD-500, Tokyo, Japan).

### *Statistical analyses*

Numerical variables were evaluated for presence of a normal distribution using the Kolmogorov-Smirnov test. In experiment I the parameters were evaluated by ANOVA, and results are presented as the mean  $\pm$  standard deviation. The variables that did not meet the assumptions of the parametric tests were analyzed by the Mann-Whitney test. Fisher exact test was used to compare the proportion of ovulated cows. In experiment II we used



ANOVA with repeated measures followed by the Tukey test. The pregnancy rates in experiment III were evaluated by the chi-square test. All analyses were performed using Minitab program - Statistical Analysis Software, and the significance level to reject the null hypothesis was 5%.

### Results

In experiment I, the diameter of the dominant follicle 48 hours after ovulation induction (time that TAI is performed) and ovulation rate were higher ( $P < 0.05$ ) in

cows who received a conventional protocol for synchronization with an intravaginal P4 device than an injectable P4 solution (Table 1). However, cows treated with injectable P4 presented higher ( $P < 0.05$ ) follicular growth per day and different time of ovulation to in comparison with cows treated with intravaginal device. Most of the cows in both groups (device and injectable) ovulated between 60 and 72 hours after the application of the ovulation inducer (Figure 1). However, there was no difference ( $P > 0.05$ ) in the proportion of ovulated cows between groups in the respective periods.

Table 1. Follicular diameter and growth, corpus luteum (CL) diameter and serum progesterone (P4) concentration in Nelore cows synchronized with an intravaginal P4 device (control) or injectable P4 solution.

Variables	P4 device		Injectable P4		P-value
	M $\pm$ SD	(n)	M $\pm$ SD	(n)	
DF 48 h after ovulation induction (mm)	11.6 $\pm$ 1.8	38	10.3 $\pm$ 1.8	38	0.003
Follicular growth (mm/day)	1.0 $\pm$ 0.3	37	1.3 $\pm$ 0.4	22	0.003
Estimated diameter of the PF (mm)	14.9 $\pm$ 2.3	37	14.1 $\pm$ 3.1	22	0.293
Ovulation rate (%)	97.3	37	47.3	18	0.001
Time of ovulation (h)	72.6 $\pm$ 4.0	37	78.4 $\pm$ 10.5	18	0.031
CL diameter on day 22 (mm)	18.2 $\pm$ 4.0	37	16.7 $\pm$ 3.5	24	0.153
P4 concentration on day 22 (ng/mL)	8.5 $\pm$ 1.4	17	10.2 $\pm$ 1.4	17	0.400

DF – dominant follicle; PF – preovulatory follicle.

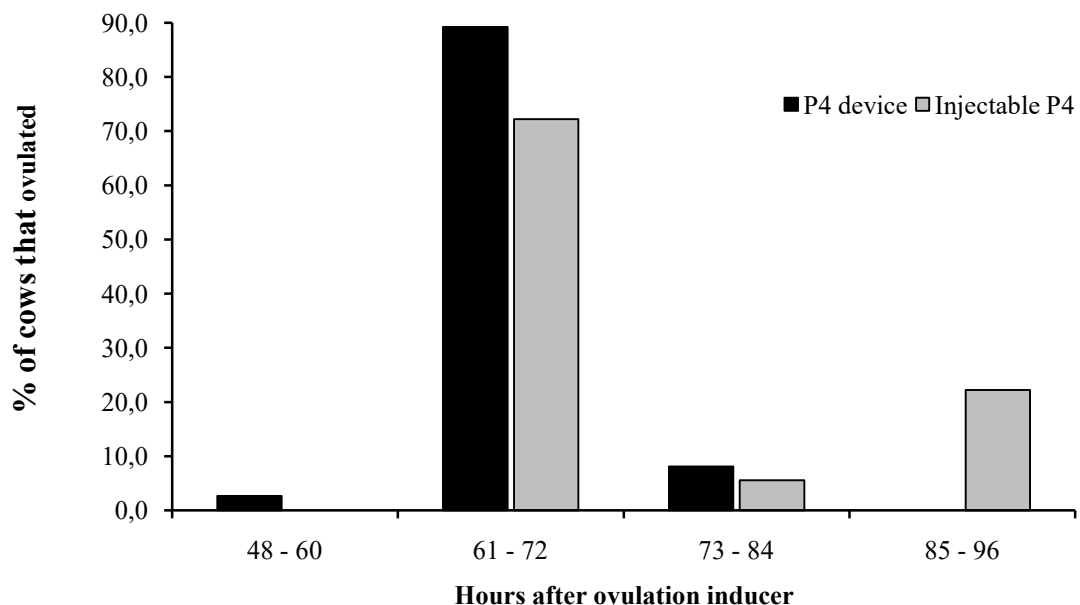


Figure 1. Percentage of ovulation after application of the ovulation inducer in Nelore cows synchronized with an intravaginal P4 device (control) or injectable P4 solution. There was no difference ( $P = 0.478$ ) in the proportion of ovulated cows between groups in the respective periods.

In the experiment II, all heifers had increased serum P4 concentrations after the administration of injectable P4 source on Day 0. Six hours later serum P4 level reached 1.46 ng/mL and eighteen hours after increased from 1.46 to 4.65 ng/mL (within 24 hours;  $P < 0.05$ ; Figure 2), and concentration peak occurred within 48 hours (6.54 ng/mL) from P4 injection ( $P < 0.05$ ). Within 96 hours of P4 application (2.50 ng/mL), the

concentration was lower ( $P < 0.05$ ) in comparison to 48 hours, remaining stable ( $P > 0.05$ ) until the time of 240 hours (1.2 ng/mL).

In experiment III, the pregnancy rate (performed after 60 days of TAI) was higher ( $P = 0.011$ ) in the cows synchronized with an intravaginal P4 device (60.4%, 29/48) compared to those treated with injectable P4 (34.0%, 16/47).

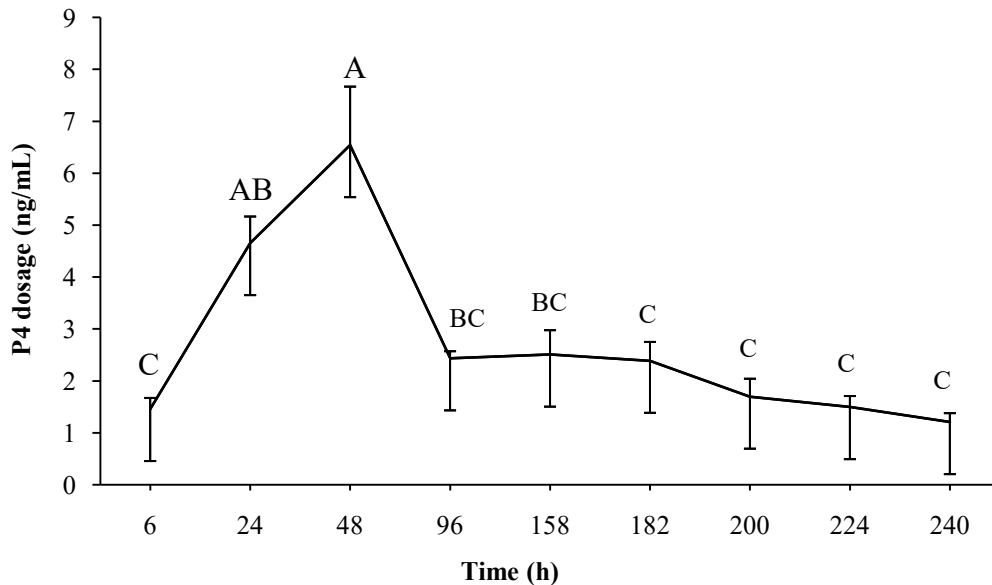


Figure 2. Serum progesterone (P4) profile in ovariectomized Nelore (*Bos indicus*) heifers after receiving an injectable P4 source on Day 0; Capital letters (A, B, C) were different ( $P \leq 0.05$ ) between P4 concentration.

### Discussion

To the best of our knowledge, this is the first study reporting P4 injectable on follicular dynamics, metabolization profile and pregnancy rate in beef cattle subjected on TAI. Despite the lower pregnancy rate obtained with injectable P4 in comparison to intravaginal devices, some advantages were identified favoring injectable P4. For example, rapid and practical management of the animals, no loss of devices and absence of devices on the environment.

Some reproductive parameters remain critical for the fertility of females synchronized with P4 injectable, such as the ovulation and pregnancy rates, but it is valid to highlight important aspects of this pharmacological strategy for reproductive management in cattle. In the present and other studies (Morotti *et al.*, 2013a; Morotti *et al.*, 2013b; Campos *et al.*, 2016a), greater convenience in managing animal synchronization was observed using P4 injectable. Such benefits are due to the convenience of a parenteral application in addition to the hygienic and sanitary advantages of injectable solution compared to intravaginal devices.

In this study, the lower pregnancy rate achieved by the P4 injectable group was possibly due the low ovulation rate (less than 50%) in this group. We believe this lower performance is related to a residual concentration of P4 blocking the ovulation as demonstrated in the serum concentrations of progesterone that remains high 10 days after injection. For example, the P4 function during the TAI protocols is to block the LH peak (Kinder *et al.*, 1996), avoiding the final follicular maturation, oestrus manifestation and ovulation of dominant follicle (Savio *et al.*, 1993b; Stock and Fortune, 1993; Rhodes *et al.*, 2002). However, after the treatment, the P4 concentration should be basal to allow estrus manifestation and ovulation (Baruselli *et al.*, 2004).

The ovulation of the dominant follicle only occurs during the follicular phase after a preovulatory peak in LH secretion (Roche, 1996). The initial stimulus for LH secretion occurs due to the high secretion of estradiol that performs positive feedback with GnRH. In this way, it induces optimal frequency and amplitude in LH pulse during low P4 concentrations (Sunderland *et al.*, 1994; Forde *et al.*, 2011; Sartori and Barros, 2011). This basal P4 concentration (below 1 ng/mL) is usually achieved from 6 and 24 hours after removal of the intravaginal device (Macmillan *et al.*, 1991; Macmillan and Peterson, 1993; Silveira *et al.*, 2012). Perhaps some adjust in the P4 dose and the adoption of more precise inductors of ovulation, like LH, may contribute to solving this problem of present study.

The P4 metabolizing curve showed the peak of the serum P4 concentrations (6.54 ng/mL) at 48 hours (Day 2), and up to 2.3 ng/mL for up to 182 hours (approximately 7.5 days). Beef heifers treated with CIDR devices showed average serum P4 concentrations of 5.6 ng/mL during treatment, varying from 8.7 ng/mL to 2.5 ng/mL at device removal (Macmillan *et al.*, 1991). In general, the serum P4 in the injectable group was close to reports at the literature obtained with P4 devices. This aspect is encouraging for new experiments to adjust injectable P4 for better pregnancy rates, mainly due to the similarity in the CL diameter and P4 concentrations 12 days after ovulation we found with both sources of P4.

Currently, TAI programs in beef cattle have pregnancy rates of approximately 40 to 60% (Baruselli *et al.*, 2004; Ayres *et al.*, 2008; Carvalho *et al.*, 2008; Campos *et al.*, 2013; Barreiros *et al.*, 2014; Marques *et al.*, 2015) depending on several factors, such as the hormonal protocol, BCS, postpartum time, female category, bull fertility, semen quality and general management. Therefore, considering the use of injectable P4 for TAI, the design of this study did not provide favorable efficiency to commercial use, but it is



valid to highlight advances in this pharmacological strategy to timed insemination. The pregnancy rate in this study (34%) was higher than observed in our previous study (18%) with an injectable P4 (Morotti *et al.*, 2013b) and very similar (35.2%, 328/938) to a more recent study (Campos *et al.*, 2016a).

Recently, it was found that cows synchronized with the conventional protocol TAI (Control/CIDR) had a higher ( $P < 0.05$ ) pregnancy rate (60%) than those synchronized with P4 injectable/TAI 36 hours (33.3%). However, the group receiving injectable P4/TAI 48 hours had a similar ( $P > 0.05$ ) pregnancy rate (48.9%) to those treated with either the conventional protocol (Campos *et al.*, 2016b).

In this context, although the present injectable P4 represents a promising strategy, there is a need to continue improving its formulation to increase its efficiency in the synchronization protocols. Certainly, the results achieved so far do not validate it on a commercial scale. However, we emphasize this injectable strategy represents many advantages. First, it facilitates the management of animals due to practical aspects such as benefits of a parenteral application, fast, precise and with a high assurance of absorption by the animal, eliminating cases of losses of devices. Second, there are hygienic-sanitary advantages avoiding vaginitis and/or vulvovaginitis cases frequently observed in the use of intravaginal device, especially devices are reused. Third, there is less labor mainly because there is a greater facility in the parenteral application and it does not involve clearing as observed in intravaginal devices. Fourth, it involves no cost in silicon devices. Finally, there is no problems related to the devices in the environment.

In conclusion, cows that underwent an ovulation synchronization protocol using injectable P4 showed lower rates for ovulation and pregnancy when compared to animals treated with intravaginal device of P4. However, the use of injectable P4 on a single day can largely facilitate the management of animals due practical aspects.

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