Effects of the addition of a dose of 400 IU of eCG to treatments based on Progesterone and Estadiol on follicular dynamics, ovulation and pregnancy rates in fixed time embryo transfer programs (FTET)

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Introduction

Embryo transfer is the most widespread technique used world-wide to achieve a rapid spread of elite genetics. The embryo transfer industry has undergone major changes in a short period of time (Mapletoft 1981). In its beginnings it was practiced in sophisticated clinics that used surgical techniques for the collection and transfer of embryos. No pharmaceuticals were available that made it possible to act effectively on the estrous cycle of the cow, so that superovulation treatments had to be started towards the end of the cycle, just before natural estrus (Mapletoft, 1981). The variability in estrous cycle length and number of follicles capable of responding to hormonal stimulation at this stage of the estrous cycle made these methods unacceptable. Nor was it possible to freeze embryos and a large number of recipients were necessary so as to have an adequate number of cows in detectable heat on the same day as the donor. The results were extremely unpredictable and therefore costs were very high. As from the 70s, the use of non-surgical techniques made it possible to apply this technology in the field (Mapletoft, 1981; 1986). However, currently less than 1% of bovines in Mercosur countries are in some way included in these schemes (Thibier, 2000).

One of the major problems in embryo transfer programs is management of the recipients (Bo et al., 2002c). These can be selected using a natural heat detection program or a hormonal induced heat program. Whichever method is used, it is important to have a precise heat detection system, since this is a key factor and one of the factors that most affects the maintenance cost of recipients from the moment they enter the program until they are pregnant (Bo et al., 2002c; Beal y Hinshaw, 2001). Therefore, recipient heat synchronization programs must be used to increase efficiency and decrease the number of visits by the embryo transfer technician teams (Bo et al., 1995b; Mapletoft et al., 2000).

Characteristics of Follicular Development during the Estrous Cycle in Cows

The improvement of techniques to monitor hormone concentrations, hormone receptors and ultrasonography to assess dynamic ovarian morphological changes, have made it possible to achieve a greater understanding of follicular development in cows (Bo et al., 1995b). Studies using real time ultrasonography have convincingly documented that antral follicular growth in bovines takes place in waves (Knopf et al., 1989; Savio et al., 1988; Sirois y Fortune, 1988).

Treatments with Progesterone and Estradiol

Progesterones have been on the market for several years and are used in combinations with E_2 and PGF to synchronize ovulation in FTAI programs (Bó et al., 2002). The use of estrogen and progesterone to control follicular development is based

on the potent effect of the combination of these steroids on gonadotrophins (Bó et al., 1995a). The most commonly used protocol, consists in administrating 2mg of intramuscular (im) EB at the time of insertion of an intravaginal device with P_4 (Day 0), to synchronize follicular development (Bó et al., 1996b; Caccia y Bo, 1998). On Day 7 or 8 the intravaginal device is removed and PGF is administered to induce luteolysis and 24 h later 1mg of EB is given to synchronize ovulation. TAI is performed between 52 and 56 h after removal of the device since most animals ovulate at between 66 and 84 h (Cutaia et al., 2001).

Synchronization treatments of ovulation using P_4 and EB devices have resulted in pregnancy rates of between 55-65% in TAI programs (Bó et al., 2002a; Mapletoft et al., 2000). Some studies have attempted to use the knowledge developed in TAI programs to synchronize ovulation of recipients and thus to avoid heat detection when using this biotechnology. In an experiment conducted in Argentina (Tribulo et al., 2000), we compared pregnancy rates in embryo recipients treated with a CIDR-B combined with 2 mg EB and 50 mg im P_4 on Day 0, PGF at the time of device removal (Day 7), 1 mg EB on Day 8 and TET on Day 16, with cows treated with two doses of PGF every 14 d and transferred 7 d after estrous detection. There were no significant differences between the CIDR-B Group and PGF Group every 14 d, either in percentage of cows selected (59 and 60%, respectively) or in final pregnancy rate (pregnant / treated, 37 and 33%, respectively). It was concluded that treatment with intravaginal devices with P_4 combined with injectable EB + P_4,with the injection at the time of placement of the device, and EB 24 h after its removal, can be used to synchronize ovulation, eliminating the need for heat detection in embryo recipient groups. Results

from other experiments confirmed the above, using CIDR-B or DIV-B (1g of P_4, Syntex, Argentina) devices. Moreover comparing 7 and 8 d protocols no significant differences were found between groups in the proportion of cows selected to receive an embryo, or final pregnancy rates (CIDR-B for 7 d: 46.2% and 43.3% vs. CIDR-B for 8 d: 56.3% and 41.7%, respectively, Bó et al., 2000). However, in all these studies, the percentage of cows with a CL detected by rectal palpation at the time of transfer and subsequently transferred was quite low (<60%), and final pregnancy rates (pregnant / treated) did not exceed 40%, which represents a high cost in treatments and unused recipients.

The low rate of recipients selected to receive an embryo in previous studies could be related to high circulating levels of P_4 in recipients during synchronization treatment. The reason for this hypothesis is that in these experiments only cyclic animals (with functional CLs) were used and administration of PGF was at the time of device removal. Therefore, most of the cows would have high levels of P_4 (from CL and from device) during treatment. High levels of P_4 would have decreased the frequency of LH pulses and consequently follicular development (Stock and Fortune, 1993). It has been shown that the growth and persistence of a DF depends mainly on pulsatile LH secretion (Stock and Fortune, 1993). Therefore, if a follicle grows under low P_4 conditions, ovulatory follicle size and the CL which is formed after ovulation could be larger than in cows with high P_4 during treatment. Recently published papers have suggested ovulatory follicle size is related to the size of the resulting CL and subsequent pregnancy rates (Macmillan et al.2003; Vasconcelos et al., 2001). Furthermore, the size

of the DF at the time of treatment with EB may determine the success or failure of ovulation induction (Burke et al., 2001).

Another alternative to increase the size or the number of ovulatory follicles during synchronization treatment and consequently circulating levels of P_4 at the time of embryo transfer, is the inclusion of an injection of equine chorionic gonadotropin (eCG). Fuentes and de la Fuente (1997) reported data from an experiment that used Holstein heifers divided into four groups. The first group consisted of heifers in which natural estrous was observed (Control Group). The second group received a single dose of PGF (PGF Group). The third group (PRID Group) was treated on Day 0 with a PRID (1.55 g of P_4, Sanofi, Sanite Animale, France) and a capsule containing 10 mg of EB and PGF when the device was removed on Day 10. The heifers in the fourth group (PRID Group + eCG) were treated on Day 0 with a PRID together with 5 mg of 17β-estradiol (17β-E) and 100 mg of P_4 im, and on Day 4 with 1000 IU im eCG and PGF on Day 6, and the devices were removed 12 hours later. In this experiment, heat was detected in all groups and heifers were transferred 7 d after detected heat. The number of recipients selected for transfer (transferred / treated) was higher ($P = 0.0001$) in PRID + eCG group heifers (90%) than in the other groups (50% in the control group, 45% in PGF Group and 49% in PRID Group). This also resulted in a greater ($P = 0.008$) Final pregnancy rate (pregnant / treated) in PRID + eCG Group (59%) than in the other groups (29%, 19% and 22%, respectively). These results were supported by another experiment in Brazil, where zebu cross-bred heifers were used that were treated with a CIDR-B for 7 d plus 800 IU of eCG on Day 5 and were transferred at fixed time (Baruselli et al., 2001). No decrease in pregnancy rates due to recipient superovulation

was seen, but on the contrary, the addition of a dose of eCG on Day 5 increased the number of recipients with more than one ovulation, the proportion of recipients used and pregnancy rates, compared to recipients also treated with CIDR-B but without eCG (Baruselli et al., 2001). However, these studies used a very high dose of eCG (800 IU), which greatly increases the cost of treatment. Furthermore, there is also a recent paper (Nogueira et al., 2004) showing that an excessive increase in P_4 can be adverse to the establishment of pregnancy.

Objective

Evaluate the effect of the addition of a dose of 400 IU eCG to treatments based on P_4 and EB on follicular dynamics, ovulation and pregnancy rates in FTET programs.

Materials and Methods.

Experiment A

We used 19 multiparous non-lactating beef cows, with a BCS of 2.5 to 3.5.On Day 0, all cows received an intravaginal DIB (1 g pf P4, Syntex, Argentina) device together with 2 mg EB im and 50 mg P_4 im. On Day 4 the cows were divided into an eCG Group (n = 9) that received 400 IU of eCG im (Novormón, Syntex SA, Argentina) on Day 5 and a Control Group (n = 10) that received an injection of 2 ml of im saline. Additionally, each group was subdivided to receive 500 micrograms of cloprostenol im on Day 4 or Day 5. In all groups DIB devices were removed on Day 8 and 1 mg of EB im was injected on Day 9.

Experiment B

The experiment was performed in three replicates and a total of 312 multiparous cows were used (67, 109 and 136 animals in replicates 1, 2 and 3, respectively). The animals were non-lactating cycling cows of beef breeds (*Bos taurus* and *Bos taurus x Bos indicus*), with a BCS of 2.5 to 3.5. On Day 0, all cows received an intravaginal DIB device together with 2 mg EB im and 50 mg P_4 im. On Day 5, all cows received 500 micrograms of cloprostenol im, and then were divided into two groups to receive either 400 IU of eCG (eCG Group; Novormón, Syntex SA, Argentina)) or an injection of 2 ml of saline im (Control Group). In both groups DIV-B devices were removed on Day 8 and 1 mg of EB im was injected on Day 9. Heat detection was not performed and cows with a CL> 110.0 mm^2 on Day 16, received frozen embryos on Day 17 (Grade 1, n =

206, Grade 2, n = 49 and Grade 3, n = 4 allocated equally to both groups). Embryos were from Fleckvieh-Simmental and red Angus cattle donors superovulated for this purpose and the transfers were performed using non-surgical techniques in the horn ipsilateral to the CL.

Ultrasonography

All animals were examined daily by transrectal ultrasonography (Concept MCV with a 7.5 MHz transducer, Products Group International Inc, Boulder, CO, USA, Figure 1), first to detect ovulation and then from the insertion of the DIV-B to 4 d after the beginning of the new follicular wave. All follicles>4 mm were identified, measured and diagrammed with respect to location in the ovary to assess changes in each of them individually (Knopf et al., 1989). The data evaluated were: diameter of the DF and main subordinate follicles, number of follicles>4 mm present in the ovaries and the day of emergence of the new follicular wave (Ginther et al., 1989a, 1989b)).

The largest follicle present at start of the experiment was defined as the DF of wave 1. Subordinate follicles were defined as the group of follicles originating from the same follicular wave as the DF (Ginther, 1989a). The emergence of a post-treatment follicular wave (wave 2) was defined as the first day that the DF of the new wave was detected retrospectively with 4-5 mm in diameter (Ginther et al., 1989b; Knopf et al., 1989). The time of ovulation was defined by the disappearance of the ovulatory wave DF (Knopft et al., 1989).

Figure 1. Ultrasound used to monitor follicular and luteal dynamics and pregnancy diagnosis in the experiments of this thesis.

Furthermore ultrasonography was performed every 6 h from Day 8, to determine the time of ovulation. On Day 16, ultrasound was performed in all cows to determine CL area (Figure 2), which was calculated according to the formula ½ height * ½ width * π (Kastelic et al., 1990).

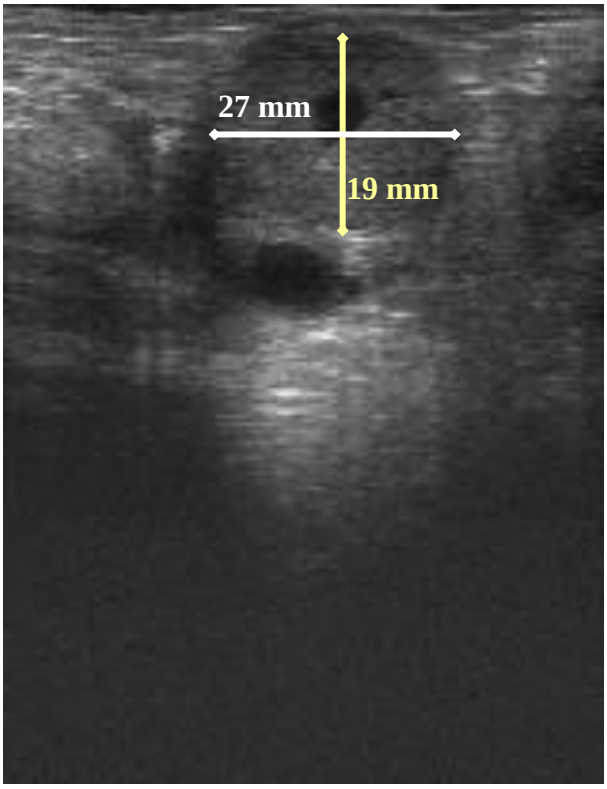


Figure 2. Ultrasonography of an ovary of an embryo recipient on Day 17 (TET Day), with a CL of 410 mm^2.

All animals were examined by transrectal ultrasonography the day before embryo transfer (Day 16) in order to determine CL area and 23 days after transfer to determine pregnancy rate.

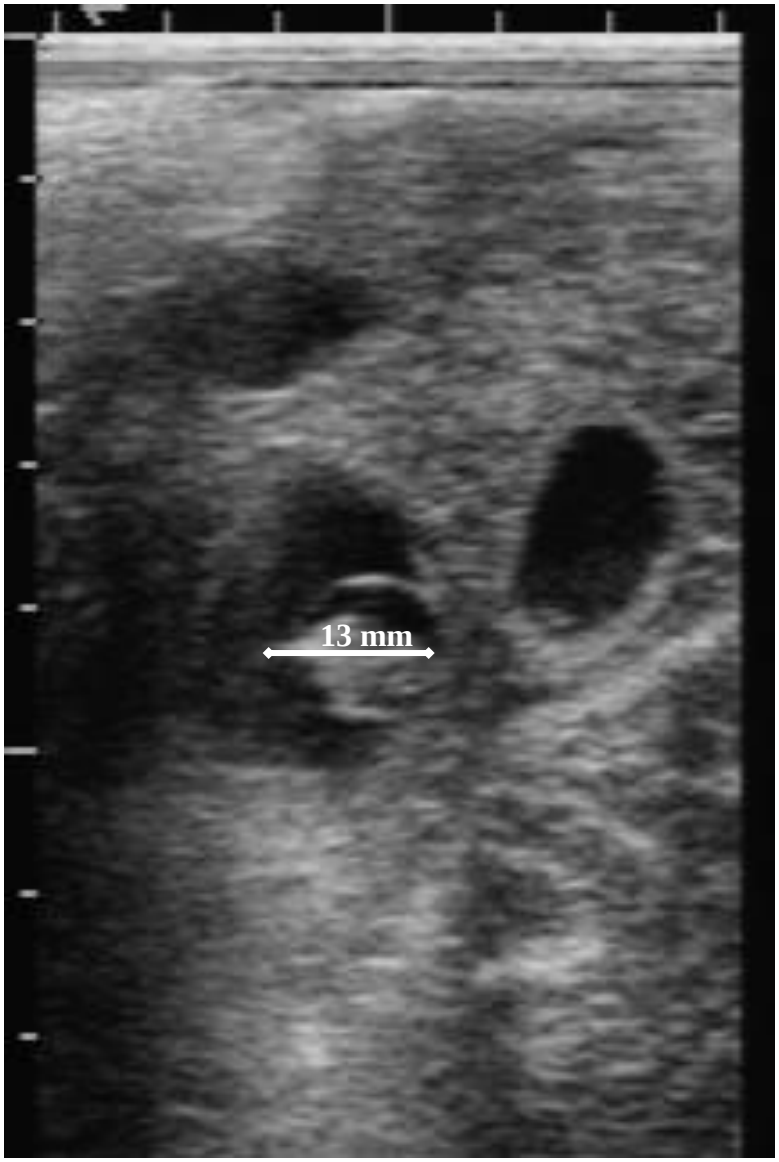


Figure 3. Ultrasonography of a recipient 23 days after TET, with a 13 mm length fetus, of approximately 30 days of age, using a lineal transducer ultrasonograph.

Sample Taking and Hormone Analysis

Serial samples of jugular vein blood were taken from all animals. The samples were collected in heparinized tubes and immediately after collection were centrifuged and the plasma frozen at -20 ° C.

For P_4 analysis an enzyme immunoassay (EIA) previously validated for use in bovine plasma (Aba et al., 2001) was used. Briefly, the "coating" of the plates was performed with progesterone 3 (o-carboxy-methyl) oxine: conjugated BSA and they were blocked with bovine serum albumin. A monoclonal antibody was used as first antibody and as second antibody an anti-mouse antibody produced in goats and conjugated with peroxidase was used. Development was performed with o-Phenylendiamine Dihydrochloride (OPD). The sensitivity of the method was 0.02 ng / mL. Intra-assay variation coefficient was 13% at a concentration of 0.04 ng/mL and 12% at concentrations of up to 5.2 ng/mL. Interassay variation coefficient was 7 and 11% for low control (0.16 ng / mL) and for high control (2.6 ng / mL), respectively.

In Experiment A blood samples were obtained on the day of start of treatment (Day 0), on day of intravaginal device removal (Day 8), on Day 10, and the day before embryo transfer (Day 16). P_4 plasma concentrations were determined by solid phase commercial RIA (DPC, Coat-A-Count Progesterone, Los Angeles, USA). All samples were tested in duplicate. Intra-assay variation coefficients were 9.6, 0.4 and 3.9% for values of 1.6, 5.1 and 25.2 ng/mL, respectively. The sensitivity of the method was

estimated at 0.16 ng/mL.

In Experiment B blood samples were taken from Replicate 1 cows (n = 56), one day before embryo transfer (Day 16) to measure P_4 concentrations.

The samples were collected, processed and evaluated in the same manner as in Experiment A. Intra-assay variation coefficients in this study were 4.9, 1.7 and 3.6% for values of 1.5, 5.2 and 21.6 ng/mL, respectively. Interassay variation coefficients in this study were 6.7, 2.5 and 7.8% for values of 1.6, 5.2 and 21.9 ng/mL, respectively. The sensitivity of the method was estimated at 0.10 ng/mL.

Statistical Analysis

In Experiment A, the pattern of data for follicular growth and hormone levels were analyzed by ANOVA for repeated measurements using a split plot fitted model, and Bonferroni criteria were used for comparisons and post-tests, in situations in which time x treatment interactions were statistically significant. Infostat ® (Infostat, 1999) software was used.

In Experiment B individual quantitative measurement data were analyzed using Student's t test. Ovulation distribution (Experiment A) was compared between groups using Bartlett's test of homogeneity of variance.

In Experiment B the proportions of selected pregnant recipients were analyzed by logistic regression, taking into account factors such as replicate, *Bos taurus* vs. *Bos*

taurus x Bos indicus recipients, treatment, fresh embryos vs. frozen embryos, and technician performing the transfer (Infostat, 1999).

Results

Experiment A

The results of individual comparisons in experiment A are summarized in Table 6. There were no significant difference in the day of emergence of the new follicular wave (P = .49). However, average DF diameter on Day 10 tended to be higher (P = 0.06) in cows treated with eCG than in Control Group cows.

With respect to the time of ovulation, there was a trend (P = 0.08, Table 6) to ovulate earlier in cows treated with eCG on Day 5 than in Control Group cows. Moreover, ovulation in eCG Group cows was less synchronous than in Control Group cows (P = 0.003, Graph 1).

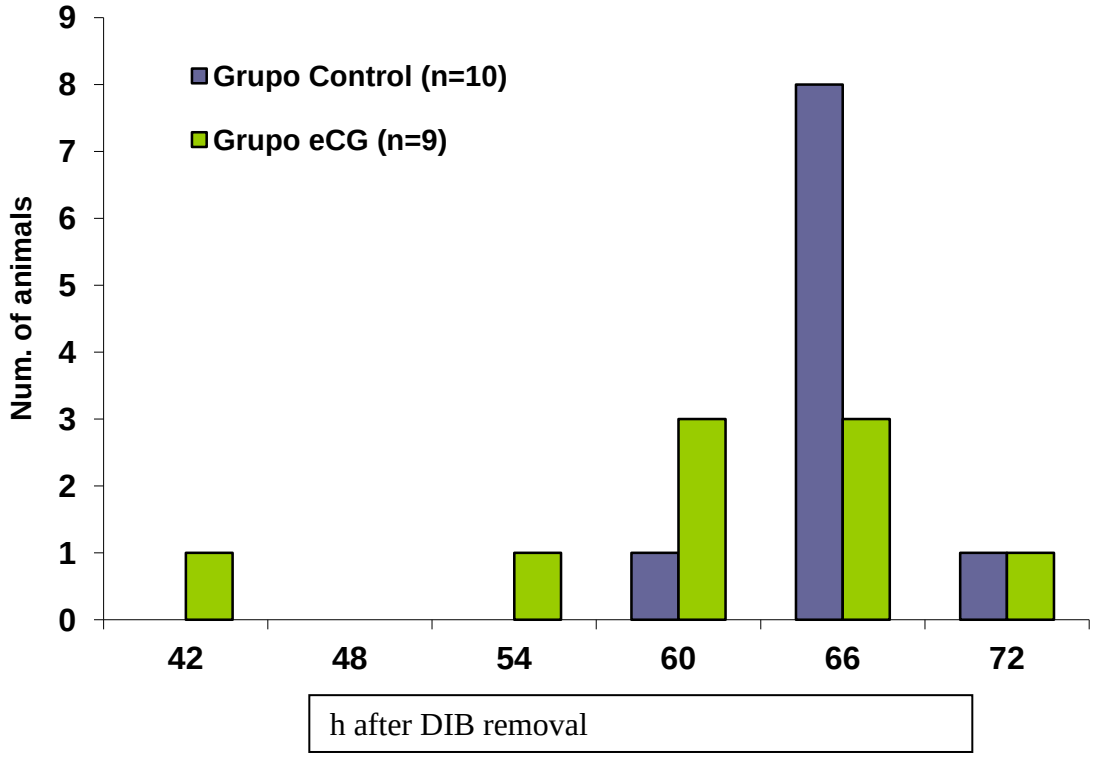
CL area on Day 16 did not differ between groups (P = 0.15, Table 1). However, P_4 plasma concentrations on Day 16 were higher in cows treated with eCG on Day 5 (P = 0.006, Table 6) than in those that did not receive eCG (Control Group).

Results of statistical analysis of follicular development pattern after treatment showed treatment x day interaction (P = 0.0001), due to a higher average DF diameter on Day 10 in cows treated with eCG on Day 5 of treatment, than in Control group cows.

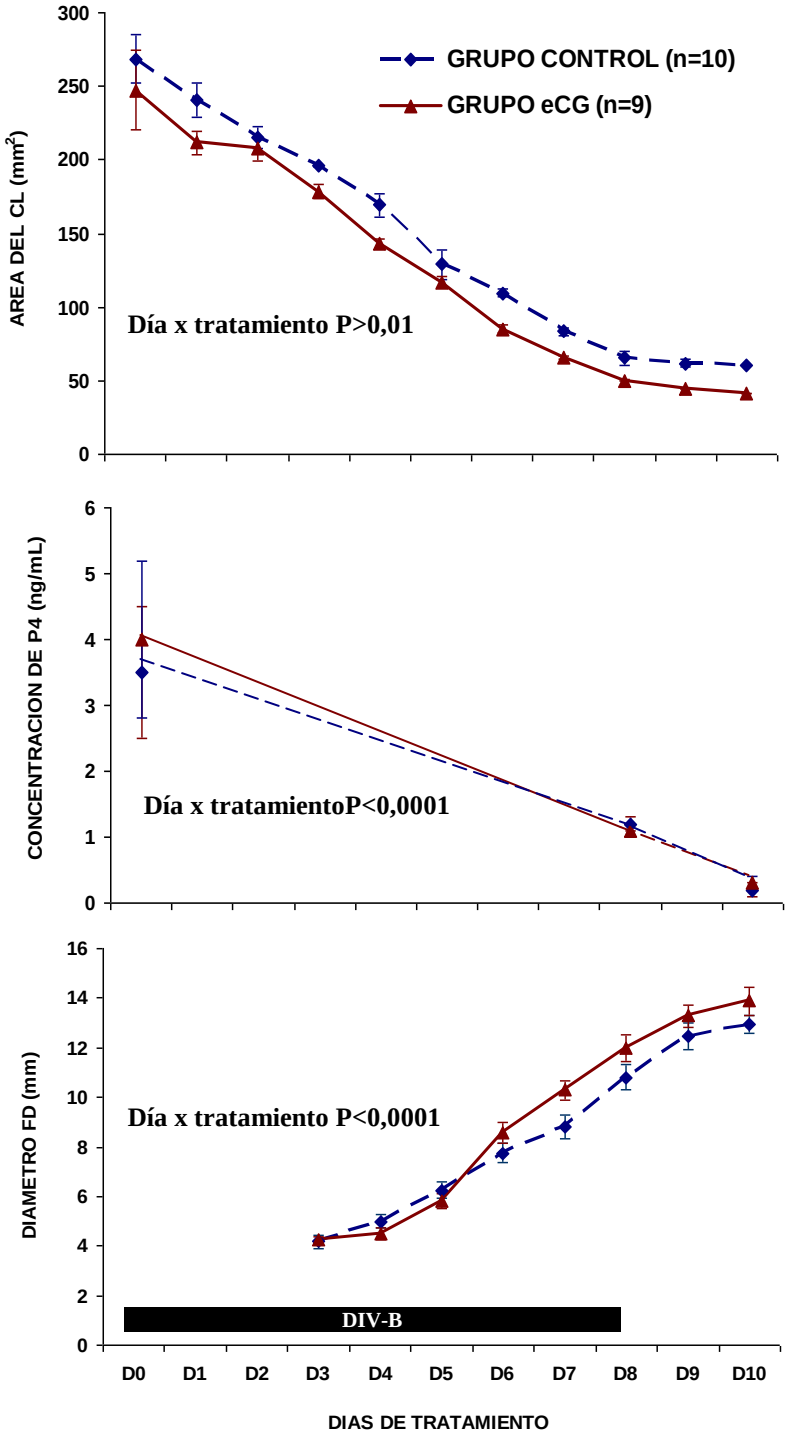
There were no differences between groups in CL development pattern, or P_4 levels (P> 0.5, Graph 2).

Table 1. Beginning of new follicular wave, DF diameter on Day 10, DIV-B device removal to ovulation interval, CL area and P_4 plasma concentration on Day 16, in embryo recipient cows treated with DIV-B devices with P_4 for 8 days, P_4 + EB on Day 0, PGF on Day 5 and EB on Day 9. eCG Group cows also received a dose of 400 IU of eCG on Day 5 of treatment (Means± SD).

	Control Group (n=10)	**eCG Group (n=9)**	***P***
Beginning of new wave (d)	3.5 ± 0.5	3.3 ± 0.5	0.49
DF on Day 10 (mm)	12.7 ± 0.5	14.5 ± 0.8	0.06
Interval DIV-B removal to ovulation (h)	66.0 ± 0.9	60.7 ± 2.9	0.08
CL area on Day 16 (mm^2)	370.0 ± 10.0	450.0 ± 30.0	0.15
P_4 concentration on Day 16 (ng/mL)	4.7 ± 0.5	6.7 ± 0.3	0.006



Graph 1 Distribution of ovulation in embryo recipient cows treated with DIV-B devices with P_4 for 8 days, EB + P_4 on Day 0, PGF on Day 5 and EB on Day 9. Cows in the eCG Group also received a dose of 400 IU of eCG on Day 5 of treatment.



Graph 2 CL area, P_4 concentrations and DF diameter (Mean ± SE) in embryo recipient cows treated with DIV-B devices with P_4 for 8 days, EB + P_4 on Day 0, PGF on Day 5 and EB on Day 9. Cows of the eCG Group also received a dose of 400 IU of eCG on Day 5.

Experiment B

Results of the analysis of CL area measurements, P_4 plasma concentrations on the day prior to embryo transfer (Day 16) in Replicate 1 are shown in Table 2. Because 8 cows treated with eCG had 2 or 3 ovulations, the data for these cows is separated from that of the other cows that had a single ovulation for analysis and comparison with the Control Group. Transferred cows in the eCG Group with 2 or 3 ovulations had a greater CL area ($P = 0.0032$) compared to cows in the eCG Group with a single ovulation and cows in the Control Group.

Measurements of P_4 plasma concentrations in Replicate 1 cows on the day before embryo transfer (Day 16), in cows treated with eCG on Day 5 that had 2 or 3 CLs were greater ($P=0.0028$) than those of cows in the same group that had only one CL. Moreover, this last group had higher concentrations of P_4 ($P=0.0025$) than the cows in the Control Group.

Table 2. CL area and P_4 concentrations on Day 16, in embryo recipient cows treated with DIV-B devices with P_4 for 8 days, P_4 + EB on Day 0, PGF on Day 5 and EB on Day 9. Cows of the eCG Group also received 400 IU of eCG on Day 5 of treatment (Only Replicate 1 animals; Means ± SD).

CORPUS LUTEUM (mm^2)	**Control Group**	**eCG Group (simple CLs)**	**eCG Group (multiple CLs)**	**P**
Transferred Cows	200.0 ± 10.0^{a} (n=29)	230.0 ± 20.0^{a} (n=18)	129.0 ± 22.0^{b} (n=8)	^{ab} P=0.0032
Pregnant Cows	190.0 ± 10.0^{a} (n=15)	250.0 ± 20.0^{a} (n=13)	930.0 ± 150.0^{b} (n=5)	^{ab} P=0.017
Open Cows	200.0 ± 20.0^{a} (n=14)	180.0 ± 20.0^{a} (n=5)	1770.0 ± 330.0^{b} (n=3)	^{ab} P=0.039
PROGESTERONE (ng/mL)				
Transferred Cows	5.7 ± 0.4^{a} (n=29)	7.5 ± 0.7^{b} (n=18)	30.2 ± 8.2^{c} (n=8)	^{ab} P= 0.0028 ^{bc} P= 0.0025
Pregnant Cows	5.5 ± 0.4^{a} (n=15)	7.6 ± 0.8^{b} (n=13)	26.7 ± 15.1^{b} (n=5)	^{ab} P=0.023
Open Cows	5.8 ± 0.6^{a} (n=14)	7.3 ± 1.7^{a} (n=5)	34.6 ± 4.5^{b} (n=3)	^{ab} P= 0.0004

Considering all the animals included in the experiment, no significant differences (P> 0.05) were seen between replicates or between *Bos taurus* or *Bos taurus x Bos indicus*. Data was combined and analyzed for both pregnancy rates and CL area and are shown in Table 3. There were no significant differences between groups in the proportion of transferred recipients (P = 0.45), but a higher percentage was found in the number of pregnant/transferred cows and pregnant/treated cows (P = 0.0082) in the eCG group.

Although no eCG treatment resulted in a high number of multiple ovulations (8/156, 5% of the cows had 2 or 3 CLs), average CL area in transferred cows was greater in eCG-treated cows than in the Control Group (P = 0.0045). Furthermore, higher average CL areas were seen in pregnant eCG treated cows, than in pregnant Control Group cows (P = 0.0018). In open cows there were no significant differences in CL area between groups (P = .37).

Table 3. Pregnancy rates and CL area (Mean ± SD) on Day 16, in embryo recipient cows treated with DIV-B devices with P_4 for 8 days, P_4 + EB on Day 0, PGF on Day 5 and EB on Day 9. Cows in the eCG group also received 400 IU of eCG on Day 5 of treatment (Replicates 1, 2 and 3)

	Control Group (n=156)	**Group eCG (n=156)**	**P**
Transferred / Treated	127/156 (81.4%)	132/156 (85.0%)	0.45
Pregnant / Transferred	53/127 (41.7%)	76/132 (57.6%)	0.011
Pregnant / Treated	53/156 (33.9%)	76/156 (48.7%)	0.0082
CORPUS LUTEUM (mm^2)			
Transferred Cows	240.0 ± 90.0 (n=127)	280.0 ± 110.0 (n=132)	0.0045
Pregnant Cows	220.0 ± 50.0 (n=53)	280.0 ± 140.0 (n=76)	0.0018
Open Cows	250.0 ± 110.0 (n=74)	270.0 ± 20.0 (n=56)	0.37

Discussion:

In Experiments A and B, the effect of a dose of 400 IU eCG on treatments based on P_4 devices combined with EB + P_4 on follicular dynamics, ovulation and pregnancy rates was evaluated. The results obtained in these experiments show that the application of 400 IU eCG on Day 5 of a synchronization of ovulation treatment with P_4 devices and EB, results in increased DF size, and higher levels of P_4 at the time of TET. These results also agree with those seen with eCG treatments in postpartum anestrous cows where treatment increased P_4 levels and pregnancy rates (Baruselli et al., 2004).

Simultaneously, an attempt was made to increase circulating levels of P_4 and pregnancy rates in embryo recipients by inducing multiple ovulations using eCG during synchronization treatments in recipients. Previous work (Baruselli et al.2001; Fuentes and de la Fuente, 1991; Fuentes, 2000) showed that the addition of eCG to recipient synchronization treatments results not only in an increased number of ovulations (number of CLs) and higher concentrations of P_4, but also in a higher proportion of recipients used and higher pregnancy rates (pregnant / treated). However, in recent work (Nogueira et al., 2004) increase in P_4 concentrations induced by injection of eCG resulted in lower pregnancy rates in recipients in TE programs. It should be noted, that in this experiment higher doses of eCG were used on Day 7-12 of the estrous cycle and not at the beginning of a synchronized follicular wave, as in the case of the work done in this thesis and those mentioned above (Baruselli et al.2001; Fuentes, 2000). The authors concluded that the lower pregnancy rates could be caused by an early luteinization of follicles by LH-like action of eCG on theca and granulosa cells and not

by an absolute increase of P_4 on the day of ET . This could have resulted in a premature increase of P_4 concentrations, which in turn caused an early release of PGF and consequently early embryonic death (Stewart et al., 1976). Furthermore, it has been reported that early increase in circulating P_4, achieved by inserting a CIDR-B in the early luteal phase affected CL development and induced short cycles (8 days) with one follicular wave or only two cycle follicular waves with a maximum duration of 18 days (Burke et al., 1994), thereby affecting the ability to maintain pregnancy. Therefore a sudden increase in P_4 levels in the early days of the estrous cycle would be counterproductive for the establishment of pregnancy.

Mann et al., (1999) studied P_4 levels in dairy cows that were pregnant or not, and found that cows that were pregnant had a higher concentration of P_4 from Day 5 of the estrous cycle. Perhaps the main difference between increasing P_4 levels with an accessory CL on Day 5 or 7 and which can occur with the addition of 400 IU eCG, as done in this experiment, is that the increase in P_4 levels is more progressive, probably favoring improvement of the uterine environment for the development of the embryo (Ferguson et al., 2004). High concentrations of plasma P_4 from Day 5 of the estrous cycle were associated with an increase in embryonic development, a greater supply of nutrients to the uterine lumen and the ability of the conceptus to secrete interferon-τ (Mann et al., 1999), causing a decrease in the secretion of PGF2α by uterine endometrium and blocking luteolysis. This phenomenon was associated with an increase in conception rates due to better conditions for maternal recognition of pregnancy (Baruselli et al., 2001, Fuentes, 2000, Marques et al., 2003, Santos et al., 2001) .

Conclusions

Application of 400 IU of eCG on Day 5 of an ovulation synchronization treatment program with P_4 devices and EB increased DF size, P_4 concentrations at the time of FTET and pregnancy rates.

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