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—Original Article—

A Three-day $PGF_{2\alpha}$ Plus eCG-based Fixed-time AI Protocol Improves Fertility Compared with Spontaneous Estrus in Dairy Cows with Silent Ovulation

Irina GARCIA-ISPIERTO[1)] and Fernando LÓPEZ-GATIUS[1)]

[1)]Department of Animal Production, University of Lleida, 25198 Lleida, Spain

Abstract. This study was designed to test the treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) plus equine chorionic gonadotrophin (eCG) and GnRH 48 h later (PEG protocol) followed by fixed-time AI (FTAI) in dairy cows with silent ovulation (cows with a mature corpus luteum and no signs of estrus detected in the preceding 21 days). In Experiment I, ovulation following the PEG protocol monitored in 24 cows with silent ovulation was recorded in 83% of the cows 36 h after GnRH treatment. In Experiment II, control cows were artificially inseminated during spontaneous estrus (4615 AI), while cows in the PEG group (1266 AI) were subjected to FTAI 24 h after GnRH treatment. Binary logistic regression revealed a significant effect of the interactions of treatment by season, by parity or by repeat breeding syndrome (>3 AI) on the conception rate. The conception rate was negatively affected by the warm season and age in controls but not in treated cows, whereas repeat breeder cows in the control and PEG groups were less (by a factor of 0.7) or more (by a factor of 1.5) likely to become pregnant, respectively, than the remaining animals. Moreover, the likelihood of twin pregnancy was lower in multiparous PEG (by a factor of 0.4) cows than in the remaining cows. This protocol, besides overcoming the negative effects of heat stress and age on the conception rate, increased fertility in repeat breeder cows compared with spontaneous estrus. Moreover, this treatment regimen reduced the twin pregnancy rate in multiparous cows.

Key words: Bovine, Fertility, Hormone, Twin pregnancy

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The worldwide decline in fertility is a major concern for the dairy industry [1, 2]. For good reproductive performance postpartum, a cow has to resume ovarian cyclicity and undergo normal uterine involution, and estrus needs to be detected in the animal. Although many advanced technological procedures exist to detect estrus in high-producing dairy cows such as pedometers or video recording, silent ovulation is a significant reproductive performance dysfunction that causes sub-estrus [3].

Silent ovulation and sub-estrus are the terms used when a cow with a corpus luteum (CL) estimated to be at least 15 mm in diameter (mean maximum and minimum measurements) shows no visual or pedometer-detected signs of estrus in the previous 21 days [4]. In well-managed herds, the rate of sub-estrus can be as high as 40% [4, 5]. The reasons proposed for this lack of estrus signs have been the farmer [3], reduced estradiol production [6] or the high metabolic clearance of estradiol related to high milk production [7]. Moreover, in our geographical area of study, this dysfunction is further aggravated by heat stress [5, 8].

Among our cows suffering silent ovulation detected in a weekly reproductive visit and receiving a prostaglandin F2α ($PGF_{2\alpha}$) dose, only 64% of animals (571/889) were finally inseminated [5]. This means that despite this specific treatment for sub-estrus, 36% of animals did not show estrus again. It is therefore likely that sub-estrous cows need a protocol so that AI can be conducted with fixed-time AI (FTAI) to avoid prolonging the problem. This protocol needs to be short and affordable for dairy herds. In a recent work, equine chorionic gonadotrophin (eCG) added to a progesterone-based AI protocol (upon progesterone device removal) was found to improve the reproductive performance of anestrous animals [9]. ECG has both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) effects [10]. The objective of the present study was thus to determine the effects of $PGF_{2\alpha}$ and eCG given at the time of sub-estrus diagnosis (presence of a mature corpus luteum in cows not detected to be in estrus in the preceding 21 days) in a fixed-timed AI protocol on the subsequent reproductive performance of high-producing dairy cows. Two consecutive experiments were performed. Experiment I was designed to establish the best time for FTAI in Experiment II.

Materials and Methods

Cattle and herd management

The data used were obtained from a reproductive control program conducted at the University of Lleida on four well-managed, high-producing Holstein-Friesian dairy herds in northeastern Spain. Briefly, herd management included the following common features: the use of pedometers, housing in free stalls with concrete slatted floors and cubicles, the use of fans and water sprinklers in the warm season (May-September), rigorous postpartum checks, a similar reproductive health program, confirmation of estrus at artificial insemination (AI) by palpation per rectum, and most AIs (over 90%)

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Correspondence: F López-Gatius (e-mail: flopez@prodan.udl.cat)

performed by veterinarians.

The mean annual culling rate was 30%. Mean annual milk production for the four herds over the study period was 11,250 kg. The cows were grouped according to age (primiparous versus multiparous), milked three times daily and fed complete rations. Feeds consisted of cotton seed hulls, barley, corn, soybean, bran and roughage, primarily corn, barley or alfalfa silage and alfalfa hay. Rations were in line with National Research Council recommendations [11]. Dry cows were kept in a separate group and transferred, depending on their body condition score and age, 7–25 days prior to parturition to a ''parturition group.'' An early postpartum, or "fresh cows," group was established for postpartum nutrition and controls, and 7–20-day postpartum primiparous and multiparous lactating cows were transferred to separate groups. All cows were artificially inseminated. Although estrus detection started on day 14 postpartum, the voluntary waiting period for the herds was 50 days.

Only cows showing estrus signs were inseminated. Estrus was detected using a pedometer system (AfiFarm System; S.A.E. AFIKIM, Kibbutz Afikim, Israel). Walking activity values were recorded at the milking parlor (three times daily) and analyzed automatically using the herd management computer program. A walking activity greater than 80% of the mean activity recorded in the previous 2 days was taken as the lower limit for a cow to be considered in estrus. Since these herds have been observed to show a very significant positive relationship between increased walking activity and fertility provided this increase is 80% to 993% [8], values lower than 80% were not considered as estrus signs. Prior individual information concerning estrus detection was also taken into account. For example, if a cow normally showed a 400% increase in activity, yet during its two last estrus periods, the increase noted was around 120%, the cow was explored for possible incidences other than estrus such as acute lameness or change of location. The cows were ultimately inseminated after estrus was confirmed by examination of the genital tract and vaginal fluid [12, 13].

Reproductive health management

In postpartum checks (daily), the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e., vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (fetal membranes retained longer than 12 h after parturition), or puerperal metritis (diagnosed during the first or second week postpartum in cows not suffering placental retention).

The herds were maintained on a weekly reproductive health program. This involved examining the reproductive tract of each animal by ultrasound with a 7.5 MHz transducer (Easi-Scan, BCF™ Technology, Scotland, UK) from 30 to 36 days postpartum to check for normal uterine involution and ovarian structures. Reproductive disorders diagnosed at this time such as endometritis or ovarian cysts were treated until resolved. Detectable cloudy intrauterine fluid was interpreted as endometritis [14]. An ovarian cyst was diagnosed when a follicular structure larger than 20 mm in diameter (external diameter including the wall) was detected in either or both ovaries in the absence of a CL and uterine tone [15].

Cows with a retained placenta or primary metritis were always treated by introducing oxytetracycline boluses into the uterus. Prostaglandin F2α or a synthetic analogue was given at the end of treatment for a retained placenta and primary metritis, and was also used to treat endometritis and ovarian cysts. In the latter case, treatment was subsequent to manual rupture of the cystic structure per rectum. All postpartum reproductive disorders were resolved before 60 days in milk.

Study population

Cows more than 60 days in milk and not detected to be in estrus in the preceding 21 days were examined weekly by ultrasound until estrus following treatment or until AI was performed during a natural estrus. At the weekly visit, ovarian structures and uterine status or contents were recorded. The types of anestrus and treatments have been described previously [5]. Only healthy cows (with no signs of mastitis, lameness or digestive or uterine disorders) showing the presence of a mature CL (with a of at least 15 mm for the maximum and minimum measurements) detected by ultrasonography and no estrous signs in the preceding 21 days were recorded as cows with silent ovulation and included in the study.

Experiment I. Timing of ovulation following treatment

This experiment was designed to establish the best time for an FTAI schedule following GnRH treatment in Experiment II. Ovulation was monitored in 24 cows with silent ovulation given prostaglandin $F_{2\alpha}$ (25 mg Dinoprost i.m.; Enzaprost, CEVA Santé Animale, Libourne, France) and eCG (500 IU i.m.; Syncostim, CEVA Santé Animale) at the time of silent ovulation diagnosis, and GnRH (100 µg i.m.; Cystoreline, CEVA Santé Animale) 48 h later (PEG treatment). Ovarian follicle checks were performed by both ultrasonography and palpation per rectum the moment of GnRH treatment and every 12 h until ovulation (disappearance of the largest follicle). Palpation per rectum was used to evaluate the sequence the largest ovarian follicle goes through at palpation during the periovulatory period, from a firm/soft follicle (young preovulatory follicle) to a very soft follicle (mature preovulatory follicle) and then to an evacuated/disappeared follicle (follicle associated with ovulation) [16].

Experiment II. Conception rates of fixed-time inseminated cows following treatment compared with cows inseminated following spontaneous estrus

The data used were derived from 5881 inseminations performed over the period of September 2010 to April 2012. Control cows were artificially inseminated following spontaneous estrus (n=4615). Cows with a CL and no estrus signs in the previous 21 days were assigned to the PEG group (n=1266), and received the PEG treatment as cows in Experiment I. FTAI was performed in these cows 24 h after GnRH treatment. After the CL was identified, two cross-sectional images covering the largest areas of the CL were recorded. Only cows with a CL with a mean of at least 15 mm for the maximum and minimum measurements were included in the study.

In both the control and PEG groups, if a cow returned to estrus, their status was confirmed by examination per rectum, and they were recorded as nonpregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post insemination. Cows diagnosed as nonpregnant were included in the weekly reproductive program. Pregnancy was confirmed by palpation per rectum 60–66 days post insemination.

The following data were recorded for each animal: herd, parity (primiparous versus multiparous), treatment (control versus PEG), season of insemination (cool, October to April, versus warm, May to September [17, 18]), milk production (mean production in the three days before treatment) and days in milk at treatment, previous AI number, AI date, semen providing bull, AI technician, pregnancy diagnosis (PD) 28–34 days subsequent to treatment and twin pregnancy (single versus twin/multiple). A cow subjected to four or more AIs was described as a repeat breeder.

Two binary logistic regression analyses were performed. The dependent variables entered in these two analyses, respectively, were positive PD and twin pregnancy.

Regression analyses were conducted according to the method of Hosmer and Lemeshow [19] using the logistic procedure of SPSS version 18.0 (SPSS, Chicago, IL, USA). Significance was set at P<0.05.

Results

Experiment I. Timing of ovulation following treatment

In all cows at the time of GnRH treatment, the disappearance of the CL and presence of a young preovulatory follicle (the largest ovarian follicle could be felt by palpation per rectum as a firm/soft follicle [16]) was confirmed. A very soft follicle distinguishable from the remainder of the ovary (mature preovulatory follicle) was recorded in all cows: in 4 (17%) and 20 (83%) cows at 12 and 24 h after GnRH treatment, respectively. The time of ovulation corresponding to the absence of the mature preovulatory follicle previously registered was determined at 24 h (4 cows) and 36 h (20 cows) after GnRH treatment, respectively. Thus, since most of the cows had a preovulatory follicle ready for service 24 h after GnRH treatment, 24 h was the time selected for FTAI in Experiment II.

Experiment II. Conception rates of fixed-time inseminated cows following treatment compared with cows inseminated following spontaneous estrus

Mean values for milk production, lactation number and previous insemination number for the study period were 39.3 ± 5 kg, 2.3 ± 1.4 lactations and 3 ± 2 AIs, respectively (mean ± SD). The mean conception rate was 35% with a twin pregnancy rate of 14.1% for the study period.

Binary logistic regression analysis revealed a significant effect of the interactions of treatment × season, treatment × parity and treatment × repeat breeding syndrome on conception rate (Table 1). This meant that control animals inseminated during the warm season were less likely to become pregnant (by a factor of 0.7) compared with control animals inseminated during the cool season (no significant differences shown by PEG animals and the reference). Also, control multiparous animals were less likely to become pregnant (by a factor of 0.8) compared with control primiparous cows (no significant differences between PEG animals and the reference). Finally, cows with repeat breeding syndrome in the control and PEG groups were less (by a factor of 0.7) and more (by a factor of 1.5) likely to become pregnant, respectively, compared with control animals not suffering this syndrome.

Based on the odds ratio, the likelihood of twin pregnancy was lower in herd 2 (0.3-fold), compared with the other herds. The interaction of treatment × parity was also found to be significant. This meant that multiparous cows in the control and PEG groups were more (by a factor of 1.5) or less (by a factor of 0.4) likely to carry twins, respectively, compared with all other animals (Table 2).

Discussion

To the best of our knowledge, this is the first synchronization protocol for FTAI of cyclic animals found to improve fertility in repeat breeders compared with cows inseminated at spontaneous

Table 1. Odds ratios of the variables included in the final logistic regression model for factors affecting conception rate after treatment in high-producing dairy cows

Factor	Class	n	% CR[a]	Odds ratio	95% Confidence interval	P
Treatment* × season	Control × cool	1119/3975	36.4	Reference		
	Control × warm	441/1540	28.6	0.7	0.6–0.8	<0.001
	PEG × cool	331/847	39.1	1.1	0.9–1.3	0.15
	PEG × warm	170/419	40.6	1.2	0.9–1.4	0.09
Treatment × parity	Control × primiparous	562/1550	36.3	Reference		
	Control × multiparous	998/3065	32.6	0.8	0.7–0.9	0.006
	PEG × primiparous	161/412	39.1	1.1	0.8–1.3	0.4
	PEG × multiparous	340/854	39.8	1.1	0.9–1.3	0.1
Treatment × repeat breeding syndrome[b]	Control × 0	1130/3123	36.2	Reference		
	Control × 1	429/1489	28.8	0.7	0.6–0.8	<0.001
	PEG × 0	335/912	36.7	1.0	0.8–1.1	0.9
	PEG × 1	166/354	46.9	1.5	1.2–1.9	<0.001

P=0.0001. R^2 Nagelkerke=0.15. *Treatment: Control = natural estrus; PEG = prostaglandin $F_{2\alpha}$ and eCG given on the day of sub-estrous diagnosis (presence of a corpus luteum), GnRH given 48 h later and FTAI conducted 24 h after GnRH treatment. [a] Conception rate. [b] Repeat breeding syndrome: 0 ≤ 3 AI; 1> 3 AI.

Table 2. Odds ratios of the variables included in the final logistic regression model for factors affecting the twin pregnancy rate in high-producing dairy cows

Factor	Class	n	Twinning rate %	Odds ratio	95% Confidence interval	P
Herd	1	94/636	14.8	Reference		
	2	19/332	5.7	0.3	0.2–0.5	<0.001
	3	24/168	14.3	1.1	0.7–1.7	0.5
	4	153/922	16.6	1.0	0.8–1.4	0.5
Treatment* × parity	Control × primiparous	68/561	12.1	Reference		
	Control × multiparous	184/997	18.5	1.5	1.4–2.0	0.005
	PEG × primiparous	17/161	10.6	0.7	0.4–1.3	0.4
	PEG × multiparous	21/339	6.2	0.4	0.2–0.7	0.001

P=0.0001. R^2 Nagelkerke=0.11. *Treatment: control = natural estrus; PEG = prostaglandin $F_{2\alpha}$ and eCG given on the day of sub-estrous diagnosis (presence of a corpus luteum), GnRH given 48 h later and FTAI conducted 24 h after GnRH treatment.

estrus and to overcome heat stress and parity effects. Moreover, this short protocol seems to considerably reduce the twin pregnancy rate in multiparous animals compared with primiparous control cows.

Repeat breeder syndrome causes huge economic losses in dairy farms worldwide [20]. Treatments for this syndrome have been extensively reviewed [21], but with the exception of GnRH treatment at the time of AI, their effectiveness has been poor [22]. Repeat breeders can be classified into those with a reproductive tract disease and clinically normal animals [23]. Consequently, it is feasible that this latter group of cyclic animals can be somehow manipulated to achieve normal fertility.

Recently, Khoramian *et al.* [24] showed in a small number of animals that treatment with human chorionic gonadotrophin or intravaginal progesterone devices during the very early stages of pregnancy improved fertility in repeat breeders compared with control cows, in agreement with prior reports [25]. Such treatments are thought to rescue cows with poor luteal activity [26]. In practice, post-AI treatments can be used in repeat breeder cows, but not in cows suffering silent ovulation in the cycle subsequent to the AI, and these animals are logically not inseminated. Used in cyclic animals, the short protocol presented here promoted pregnancy in repeat breeder cows suffering sub-estrus, probably by inducing ovulation.

Poor luteal activity alone does not serve to explain repeat breeding syndrome, and evidence exists for a delayed LH peak around estrus [27, 28], a prolonged lifespan of the preovulatory follicle [29] and ovulation failure [30] in repeat breeder cows compared even with non-repeat breeders. Our results suggest that the positive effects of treatment on repeat breeders were related to enhanced ovulation and better synchronization of ovulation. Although this study does not identify the nature of $PGF_{2\alpha}$/eCG effects on ovulation, it does prompt several questions. Thus, the point is made that $PGF_{2\alpha}$ plus eCG treatment aids ovulation and significantly improves the conception rate in repeat breeder cows compared with non-repeat breeders. The question that arises is why the conception rate in non-repeat breeders receiving treatment was similar to that observed in control non-repeat breeder cows. Could it be attributable to delayed insemination? Or could there be sufficient endogenous gonadotropin secretion masking the positive effects of eCG?

Endocrine patterns at the farm level are difficult to assess, but an approach addressing the time of ovulation could shed some light on these questions. Despite the results obtained in Experiment I, in which most cows ovulated 36 h after GnRH treatment, in future work, it needs to be determined if ovulation occurs earlier in non-repeat breeder syndrome cows following treatment. In such a case, insemination could be performed 12 h post-GnRH treatment in the cows.

Combined with intravaginal progesterone devices, eCG has proved effective in anestrous buffalo [31], beef [32] and dairy cows [9]. Herein, the use of this hormone alone with a luteolytic agent followed by a GnRH dose 48 h later was designed to achieve the rapid recruitment of a single dominant follicle. In previous studies [33, 34], *Bos indicus* cows receiving eCG showed as much as doubled follicular growth compared with controls. Thus, eCG acts by providing final gonadotropic support leading to a greater percentage of cows undergoing ovulation [35, 36]. However, our results also raise the question of why this protocol decreases the twin pregnancy rate only in multiparous cows.

Multiple pregnancies are undesirable in dairy herds because there is a greater risk of pregnancy loss [37, 38], peripartum diseases and culling [39–41]. In a recent study including cyclic and anestrous cows, treatment with 500 IU or 750 IU of eCG at the time of PRID removal was linked to an increased twinning rate [42]. However, the mechanisms whereby eCG exerts its effects remain to be determined. Thus, it seems that the PEG protocol eliminates the negative effect of this hormone via the addition of GnRH only 48 h after the eCG dose but is able to preserve the beneficial effects of enhancing follicular growth patterns and corpus luteal function [32, 43].

Heat stress and parity have been strongly related to low conception rates in dairy cows [1, 18, 44, 45]. The PEG protocol proposed here was able to overcome the negative effects of these factors, producing a probability of becoming pregnant equivalent to that under more ideal conditions (cool season and first lactation).

In conclusion, the combination $PGF_{2\alpha}$, eCG and GnRH used in a FTAI protocol proved to be effective in cows with silent ovulation. Besides offsetting the negative effects of heat stress and parity on the conception rate, this AI protocol increased fertility in repeat breeder cows compared with spontaneous estrus. Moreover, when used in multiparous animals, the twin pregnancy rate was reduced.

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