

The effects of a single or double GnRH dose on pregnancy survival in high producing dairy cows carrying singletons or twins

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Abstract. Pregnancy loss during the late embryonic and early fetal periods influences dairy herd economy. The objectives of this study were to (1) investigate the effects of a single or double GnRH dose administered at the time of pregnancy diagnosis (28–34 days post-AI) on the pregnancy survival of cows in their third lactation or further carrying live singletons or unilateral twins, and (2) examine the impacts of GnRH treatment on subsequent twin reduction in twin pregnancies. Cows carrying singletons ($n = 1,054$) or unilateral twins ($n = 379$) were assigned at the time of pregnancy diagnosis to the following groups: control (no treatment), GnRH (100 μg GnRH), and 2GnRH (200 μg GnRH). Pregnancy loss was recorded in 180 of the 1,433 cows (12.6%) at 58–64 days post-AI. Based on the odds ratios, there was a significant ($P < 0.0001$) interaction between the treatment group and twin pregnancy. This interaction implies that control cows carrying twins were 3.2 times more likely to suffer pregnancy loss than the other cows, whereas the GnRH and 2GnRH treatment groups cows carrying singletons or twins had pregnancy loss rates similar to the control cows carrying singletons. Twin reduction was observed in 35 twin pregnancies (9.2%). Cows in the GnRH and 2 GnRH groups were seven times more likely to show twin reduction than control cows. Our results indicate that GnRH administered at the time of pregnancy diagnosis had no beneficial effects in cows carrying singletons. In contrast, for twin pregnancies, the treatment increased the rate of pregnancy survival and was accompanied by an increase in the twin reduction rate.

Key words: Cattle, Pregnancy loss, Twin pregnancy, Twin reduction

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Pregnancy loss during the late embryonic and early fetal period is an important factor for dairy herd economy [1, 2]. Parity (third lactation or more) has been related to pregnancy loss in dairy cows [3, 4]; however, the main non-infectious factor that compromises pregnancy maintenance during the first trimester of gestation is carrying twins [5, 6]. In some dairy herds, twin pregnancy rates can be 18% or higher, and twins are much more common in older cows [7]. Despite the reports on spontaneous embryo reduction rates from 11.2 to 28.4% occurring around 28–40 days of gestation [8–10], twin pregnancy losses can exceed 50%, especially during the warm period of the year [11, 12]. Thus, the risk of pregnancy loss for cows carrying twins may be three to seven times higher than that for cows carrying singletons. In addition, losses are five to nine times higher for unilateral twins than for bilateral twins, whereas spontaneous twin reduction mostly occurs in bilateral twins [6, 8].

Studies have shown that GnRH or hCG treatment applied at the time of pregnancy diagnosis (i.e., 28–40 days) has no apparent effect on the subsequent pregnancy loss rate in cows carrying singletons [13] or other pregnancy types [14, 15]. However, it has been estab-

lished that GnRH treatment in cows carrying twins favors pregnancy maintenance [6, 16]. In a previous study, it was found that GnRH treatment promoted twin reduction compared to progesterone-based treatment, but this study did not include untreated control cows [13]. It remains unclear why GnRH administration at the time of pregnancy diagnosis does not reduce the losses in single pregnancies but does so in twin pregnancies. Increased GnRH doses enhance pituitary LH release [17]; therefore, the first hypothesis tested in this study was whether a double dose treatment of GnRH would reduce pregnancy loss rates in both single and twin pregnancies. Our second hypothesis was that twin reduction in unilateral twin pregnancies may be related to improved pregnancy maintenance. Cows in their third lactation or further carrying unilateral twins or singletons are a good model to assess hormone treatments that could help sustain pregnancy. Accordingly, the objectives of this study were to (1) to examine the effects of the recommended dose (100 μg) or a double dose (200 μg) of GnRH (gonadorelin) administered at the time of pregnancy diagnosis (28–34 days post-AI) on pregnancy survival in cows in their third lactation or further and were carrying singletons or unilateral twins, and (2) examine the possible relationship between GnRH treatment and subsequent twin reduction in twin pregnancies.

Materials and Methods

Cattle, herd management, and pregnancy diagnosis

The study population was a commercial dairy herd of 2,120 Holstein-Friesian lactating dairy cows in northeastern Spain. During

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the study period (November 2014 to April 2016), the mean number of lactating cows in the herd was 2,120, and the mean annual milk production was 12,190 kg per cow. An early postpartum, or “fresh cow”, group was established for postpartum daily checks and nutrition controls from 3 to 25 days postpartum. During the postpartum daily checks, the following puerperal diseases were treated until they were resolved or until cows were culled: signs of injury to the genital area (i.e., vaginal or recto-vulvar lacerations), metabolic diseases, such as hypocalcemia and ketosis (for the latter, the diagnosis was during the first or second week postpartum), retained placenta (fetal membranes that were retained longer than 12 h after parturition), or puerperal metritis (diagnosed during the first or second week postpartum in cows without placenta retention). Cows were milked three times daily and fed complete rations. Only healthy cows in their third lactation or further with no signs of mastitis, lameness, or digestive disorders (28–34 to 58–64 days post-AI) were included in the study. This strict protocol was designed to minimize the variation in the general health state of the animals so that pregnancy loss could be attributed to the factors not associated with these clinical conditions. Although estrus detection, using a pedometer system, started on day 14 postpartum, the voluntary waiting period for the herd was 50 days. All cows were artificially inseminated, and the herd was maintained on a weekly reproductive health program, as described in a previous study [18]. At the end of the voluntary waiting period (40–46 days postpartum), the reproductive tract of each animal was examined by ultrasound to check for normal uterine involution and ovarian structures. Reproductive disorders diagnosed during time, such as endometritis or ovarian cysts, were treated until resolved. During the weekly reproductive visit, open cows that were more than 60 days in milk, that had no estrous signs for at least 21 days, and in absence of a corpus luteum received a five-day progesterone-based synchronization protocol for fixed-time artificial insemination (FTAI). These cows were treated with a controlled intravaginal progesterone-releasing device (CIDR; Zoetis Spain SL, Alcobendas, Madrid, Spain) that contained 1.38 g of progesterone and GnRH (100 µg gonadorelin diacetate tetrahydrate IM; Cystoreline, CEVA Salud Animal, Barcelona, Spain) upon the insertion of the CIDR. The CIDR was left in place for 5 days, and these animals were also administered cloprostenol (500 µg cloprostenol IM; Cyclix bovino, Virbac España S.A., Barcelona, Spain) when the CIDR was removed. Twenty-four and 36 h later, the cows received a second cloprostenol dose and a second GnRH dose, respectively, and were inseminated 50–56 h after the removal of the CIDR [18]. If a cow had a corpus luteum that was estimated to be at least 15 mm in diameter (the mean of the maximum and minimum measurements), the cow was treated with cloprostenol. The cows that failed to show signs of estrus during the 5 days following treatment received the FTAI protocol.

Pregnancy was diagnosed by transrectal ultrasonography from 28 to 34 days post-AI using a portable B-mode ultrasound scanner that was equipped with a 5–10-MHz transducer (E.I. Medical IBEX LITE; E.I. Medical Imaging, Loveland CO, USA). Each ovary was scanned in several planes by moving the transducer along its surface to identify luteal structures, and the number and location of the corpora lutea were recorded. Scanning was performed along the dorso/lateral surface of each uterine horn. The presence of twins was determined by an observation of two embryos in different posi-

tions within one uterine horn using the two screen scan, when there were two embryos simultaneously present on the screen (unilateral twin pregnancy), or when one embryo was in each uterine horn (bilateral twin pregnancy). The viability of an embryo/fetus was confirmed by the observation of heartbeat during all examinations. Only cows carrying single or unilateral twins with live embryos at the time of pregnancy diagnosis were included in the study. Animals were excluded from the experiment if the following four conditions existed: First, cows with bilateral twin pregnancies ($n = 245$) with twin reduction induced by amnion rupture [6] were excluded, and this was routinely performed in all bilateral pregnancies for the herd. Second, cows where the number of corpora lutea exceeded the number of embryos (422 cows carrying singletons and 9 cows carrying unilateral twins) were excluded, because an additional corpus luteum is as a strong factor that promotes pregnancy maintenance [19, 20]. Third, cows with a dead co-twin ($n = 64$) were excluded, because the presence of a dead co-twin at the time of pregnancy diagnosis is highly related to pregnancy loss [6]. Fourth, cows that had a previous pregnancy loss during the same lactation period ($n = 128$) were excluded. Cows were included only once in the experiment.

The final study population comprised of 1,433 cows carrying live singletons ($n = 1,054$) or unilateral twins ($n = 379$) during the experimental period. All embryos were located ipsilateral to their corresponding corpora lutea.

The number of fetuses was revised by ultrasound from 58 to 64 days post-AI. Pregnancy loss was recorded when this exam proved negative. Twin reduction was recorded when the remnants of a dead co-twin were observed next to a live fetus in twin pregnancies. All gynecological exams and pregnancy diagnoses were performed by the last author (FLG).

Experimental design

All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida (license numbers CEEA.06-01/12 and CEEA.09-01/13).

Cows carrying live singletons (S) or unilateral twins (T) at the time of pregnancy diagnosis were alternately assigned on a weekly rotational basis according to the chronological order of the gynecologic visits to the groups. The three groups were the control with no treatment (S: $n = 349$; T: $n = 122$), GnRH with a treatment of one GnRH dose (S: $n = 349$; T: $n = 133$), and 2GnRH with a treatment of a double GnRH dose (S: $n = 356$; T: $n = 124$). Cows in the GnRH group were treated with 100 µg gonadorelin diacetate tetrahydrate IM (Cystoreline, CEVA Salud Animal, Barcelona, Spain). Cows in the 2GnRH group received 200 µg gonadorelin diacetate tetrahydrate IM.

Data collection and statistical analysis

The following data were recorded for each animal: parturition and AI dates, lactation number, insemination number, treatment (control vs. GnRH and 2GnRH), milk production at the time of treatment (mean production during the three days before the positive pregnancy diagnosis) (low producers < 40 kg vs. high producers ≥ 40 kg), days in milk at AI (DIM; < 90 days postpartum vs. ≥ 90 days postpartum), semen-providing bull, conception between 28 to 34 days post-AI, presence of twins, and pregnancy loss or twin reduction between 58 to 64 days post-AI. The pregnancy loss rate was defined as the

Table 1. Independent variables recorded at the time of treatment, and the effects of the different treatments on pregnancy loss (n = 1,433: 1,054 single pregnancies and 379 unilateral twin pregnancies)

	Control (n = 471)	Single GnRH dose (n = 482)	Double GnRH dose (n = 480)
Independent variables *			
Lactation number (mean ± SD)	3.5 ± 0.7	3.6 ± 0.8	3.5 ± 0.7
AI number (mean ± SD)	2.8 ± 1.8	2.7 ± 1.7	2.7 ± 1.7
Milk production (≥ 40 kg)	291 (61.8%)	292 (60.6%)	295 (61.5%)
Days in milk (> 90 days)	271 (57.4%)	292 (60.6%)	290 (60.4%)
Pregnancies during the warm period	264 (56.1%)	254 (52.7%)	270 (56.3%)
Dependent variable **			
Pregnancy loss	81 (17.2%) ^a	60 (12.4%) ^b	59 (12.3%) ^b

* No significant differences were detected by the Student's *t*-test (means ± SD) or the chi-square test (percentages). ** Values with different superscripts within rows denote significant differences detected by the chi-square test ($P < 0.001$).

Table 2. Odds ratios for pregnancy loss of the variables included in the final logistic regression model (n = 1,433: 1,054 single pregnancies and 379 unilateral twin pregnancies)

Factor	Class	n	% losses	Odds ratio	95% confidence interval	P
Period of AI	Cool	69/645	10.7	Reference		
	Warm	131/788	16.6	1.6	1.2–2.2	0.001
Twin pregnancy × treatment	S × C	43/349	12.3	Reference		
	S × GnRH	45/349	12.9	1.2	0.6–2.0	0.5
	S × 2GnRH	44/356	12.4	1.1	0.6–2.1	0.8
	T × C	38/122	31.1	3.2	1.6–5.3	< 0.0001
	T × GnRH	15/133	11.3	0.9	0.5–2.4	0.4
	T × 2GnRH	15/124	12.0	0.9	0.4–2.3	0.5

S: single pregnancies; T: twin pregnancies; C: control cows; GnRH: cows that received a single GnRH dose; 2GnRH: cows that received a double GnRH dose. Hosmer and Lemeshow goodness-of-fit = 4.8; $P = 0.91$. R^2 Nagelkerke = 0.12.

number of cows with a pregnancy loss as a percentage of the total number of cows in each group. The twin reduction rate was defined as the number of cows carrying twins that had twin reduction as a percentage of the total number of twin pregnancies in each group. The insemination dates were used to assess the effects of time of year (warm vs. cool period) on subsequent reproductive performance of cows. It should be noted that in our geographical region, there are only two clearly differentiated weather periods, which are the warm period from May to September and the cool period from October to April [3, 21].

Overall the reproductive performance of the three treatment groups was evaluated using a chi-square test (percentages) or a Student's *t*-test (means ± SD). The effect of the treatment group on pregnancy loss and the twin reduction rate were analyzed using a logistic regression (logistic procedure of PASW Statistics for Windows Version 18.0, SPSS Inc., Chicago, IL, USA) adjusting for lactation, days in milk, milk production, insemination number, and time of year. The estimates and Wald 95% limits were used to calculate the odds ratios and 95% confidence intervals (CI). The explanatory variables and the interaction were evaluated using a backward elimination procedure, and the variables that significantly affected pregnancy or ovulation rate remained in the model [22].

The level of significance was set at $P < 0.05$. Values are expressed as the mean ± the standard deviation (SD).

Results

The mean milk production at the time of treatment, days in milk at AI, number of lactations, and the number of inseminations were 47.7 ± 7.5 kg (25–87 kg), 128.7 ± 42.4 days (51–291 days), 3.5 ± 0.7 (3–8 lactations), and 2.7 ± 1.7 (1–9 inseminations), respectively (mean ± SD, range in parentheses). Cows were inseminated using semen from 16 bulls. Pregnancy loss was recorded in 180 cows (12.6%) 58–64 days post-AI, whereas twin reduction was observed in 35 twin pregnancies (9.2%). The independent variables recorded for each of the three treatment groups and the effects of the different treatments on pregnancy loss are shown in Table 1. Pregnancy loss rate showed a significant increase ($P < 0.001$) in the control group (17.2%) compared to that in the GnRH (12.4%) and 2GnRH (12.3%) groups.

Considering the presence of single or twin pregnancy, Table 2 summarizes the pregnancy loss rate, odds ratios, and 95% CI. The final model included the effect of the warm period of the year and the interaction between type of pregnancy and treatment. Lactation, milk

Table 3. Odds ratios for twin reduction of the variables included in the final logistic regression model for the twin pregnancies (n = 379)

Factor	Class	n	% twin reduction	Odds ratio	95% confidence interval	P
Treatment	C	2/122	1.6	Reference		
	GnRH	15/133	11.3	7.0	1.5–25.0	0.007
	2GnRH	18/124	14.5	7.5	1.8–28.2	0.002

C: control cows; GnRH: cows that received a single GnRH dose; 2GnRH: cows that received a double GnRH dose. Hosmer and Lemeshow goodness-of-fit = 4.2; P = 0.92. R² Nagelkerke = 0.10.

production, days in milk, semen-providing bull, and repeat breeder syndrome were not significant and were not included in the final model. The likelihood of pregnancy loss was 1.6 times higher (P = 0.001) during the warm period of the year than that of the cool period of the year. There was a significant (P < 0.0001) interaction between the treatment group and the type of pregnancy on the probability of pregnancy loss. This interaction implies that a lack of treatment may increase the pregnancy loss rate in cows with twin pregnancies by 3.2 times, where the cows in the GnRH and 2GnRH treatment groups that carried singletons or twins had similar pregnancy loss rates to that of the control cows that carried singletons.

Twin reduction was observed in 35 of the 379 (9.2%) cows that carried unilateral twins. There were no significant effects detected on the twin reduction rate with lactation, milk production, days in milk, time of year, semen-providing bull, or repeat breeder syndrome, and these variables were not included in the final model. Based on the odds ratios (Table 3), cows in the GnRH and 2GnRH groups were 7 (P = 0.007) and 7.5 times (P = 0.002) more likely to show twin reduction than the control cows, respectively. No interactions were found. In the binary logistic regression analysis that excluded the control cows, no differences were detected between the two treatment groups.

Discussion

Our study population was comprised of old cows (in their third lactation or further) carrying singletons or unilateral twins to examine the possible dose-dependent effects of GnRH treatment. These animals showed a higher risk of pregnancy loss compared to younger cows carrying singletons or cows carrying bilateral twins [3, 6]. Furthermore, we excluded cows with an additional corpus luteum, since this promotes pregnancy maintenance, and cows with a dead co-twin at the time of pregnancy diagnosis, since this has a detrimental effect on pregnancy survival [19, 20]. Under these conditions, however, there were similar impacts observed from the single or double GnRH dose for the single and twin pregnancies. Hence, our starting hypothesis that a double GnRH dose would reduce the pregnancy loss rate was not supported. The GnRH treatment was unable to mitigate the losses in cows with single pregnancies, which is in agreement with prior findings [13, 14], but, irrespective of the dose, the pregnancy loss rate was drastically reduced in unilateral twin pregnancies, which also reinforces previous results [6]. It is likely that the amount of gonadotropins released in response to a single GnRH dose reaches an arbitrary level, or a threshold, that triggers a sequence of events

that favors pregnancy for cows that are carrying twins. To this end, a double GnRH dose has similar consequences once the threshold concentration of gonadotropins has been reached.

The findings of this study support our second hypothesis where there would be an expected higher rate of pregnancy survival for treated twin pregnancies that were accompanied by an increase in twin reduction rate. We were able to effectively link the GnRH treatment to twin reduction in cows bearing unilateral twins, such that treated cows were 7 (GnRH) and 7.5 times (2GnRH) more likely to show twin reduction than control cows. This link likely explains a similar percentage of pregnancy loss in the GnRH-treated cows with unilateral twin pregnancies (11.3–12%) and the untreated control cows carrying singletons (12.3%) when compared to high losses in the control twin pregnancies (31.1%). In other words, most losses in the unilateral twin pregnancies may be related to a process of twin reduction in the control cows. Thus, a GnRH treatment does not induce twin reduction but favors the maintenance of pregnancy when one of the twins dies. The question that arises is how the GnRH treatment is able to sustain pregnancy in cows that undergo twin reduction. The reduction of twins involves the gradual dissolution of a single conceptus without compromising the viability of its co-twin [8, 10]. This event is likely accompanied by a local release of cytokines and prostaglandins from the uterine tissue, and the GnRH, presumably, has an indirect antiluteolytic effect. Depending on the follicular status at the time of pregnancy diagnosis, GnRH will advance follicular atresia, luteinization, or ovulation followed by luteinization that results in reduced estradiol-17 β production [23]; this could disrupt the estradiol control of the uterus leading to PGF_{2 α} release [23, 24]. Thus, a reduction in estradiol at this time might inhibit luteolytic mechanisms, which may allow some pregnancies with embryo reduction to continue. A previous study that reinforces this idea showed that an anti-prostaglandin treatment with flunixin meglumine and GnRH administered immediately after induced twin reduction favors pregnancy maintenance [6]. Moreover, the presence of two corpora lutea should further benefit pregnancy maintenance of a single embryo with its corresponding corpus luteum. More studies that include information about follicle activity, number of corpora lutea, plasma concentrations of progesterone, estradiol, and pregnancy associated glycoproteins might help identify the mechanism that inhibits luteolysis.

Most cases of single embryo mortality in twins occurs around 28–40 days of gestation and rarely after day 60 [8–10]. This has important practical implications for the fate of a pregnancy with live twins on day 60, in which there will be twin delivery or abortion.

Accordingly, the assessment of live twins or twin reduction at this time point in GnRH-treated cows or in cows subjected to induced twin reduction should be a main component of herd management policy. Thus, for non-aborting cows carrying twins, the drying period can be advanced by several days, and additional care at parturition can help increase the risk of survival of the twin calves. In contrast, cows showing twin reduction will remain pregnant with a singleton. Thus, increasing the twin reduction rate through a GnRH treatment will reduce pregnancy losses in twin pregnancies and will offer a way to lower twin birth rates in dairy herds.

In conclusion, our first hypothesis that a double GnRH dose would reduce the pregnancy loss rate was denied. A double GnRH dose administered at the time of pregnancy diagnosis did not have better effects than a single GnRH dose on the subsequent twin reduction rate or the pregnancy maintenance rate. Our findings indicate that treatment with GnRH at the time of pregnancy diagnosis has no impact on the course of the pregnancy in cows carrying singletons. In contrast, in twin pregnancies this treatment increased the rate of twin reduction and is linked to a substantially reduced likelihood of pregnancy loss.

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