

Five-day protocols of estrus synchronization with new or reused CIDR and different eCG doses in ewes

Susana López-García¹ , María Teresa Sánchez-Torres^{1*} , José Luis Cordero-Mora¹ , Jose Luis Figueroa-Velasco¹ , José Alfredo Martínez-Aispuro¹ , Eulises Díaz-Díaz² 

¹ Colegio de Postgraduados, Programa de Ganadería, Texcoco, Estado de México, México.

² Instituto Nacional de Ciencias Médicas y Nutrición, Laboratorio de Biología de la Reproducción, Tlalpan, Ciudad de México, México.

*Corresponding author:
teresa@colpos.mx

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José Nélio de Sousa Sales
Guilherme Pugliesi

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ABSTRACT - The objective of this study was to compare reproductive performance and progesterone (P4) concentration in serum of synchronized ewes with new and reused (up to five times) CIDR in five-day protocols. Two experiments were conducted in the reproductive season, in two consecutive years. In experiment 1: 125 µg of PGF2α and 200 IU of eCG were applied at removal of the CIDR: CIDR1-200 (new intravaginal devices, control), CIDR2-200, CIDR3-200, CIDR4-200, and CIDR5-200 (groups with CIDR from second to fifth use, respectively). In experiment 2, ewes received the same protocol with a dose of 400 IU eCG: CIDR1-400 (control), CIDR2-400, CIDR3-400, CIDR4-400, and CIDR5-400 (groups with CIDR from second to fifth use, respectively). In the first experiment, estrus rate was 92% in the first and second uses, 100% in the third and fourth uses, and 95% in the fifth use. Pregnancy rates were 85% in first and second uses and 81, 73, and 57% from third to fifth CIDR uses, respectively. In the second experiment, estrus rate was 100, 96, 92, 96, and 95% from the first to fifth uses, respectively. Pregnancy rates were 85, 88, 85, 77, and 62% from the first to fifth uses, respectively. Estrus and pregnancy rates did not differ statistically. Estrus was concentrated between 24 to 48 h in experiment 1 and between 24 to 36 h in experiment 2. Prolificacy index was 1.3 and 1.6 in the first and second experiments, respectively. Progesterone concentration was >2 ng/mL after the insertion of CIDR in all groups. The CIDR (plus PGF2α and eCG) can be used up to four times for estrus synchronization in five-day protocols without affecting reproductive performance.

Keywords: intravaginal devices, equine chorionic gonadotropin, progesterone, short synchronization protocol

1. Introduction

The strategies of estrus synchronization in ewes are based on the insertion of intravaginal devices impregnated with progesterone (P4) or analogues during 12 to 14 days to simulate the *corpus luteum* activity (suppress estrus and ovulation), followed by the injection of equine chorionic gonadotropin (eCG) with both effect of follicle stimulating hormone (FSH) and luteinizing hormone (LH). In the

initial studies, these protocols were developed during the 1950s with daily injections of P4 (Santos-Jimenez et al., 2020); then, in the 1960s with intravaginal sponges, and at the late 1980s with CIDR silicone devices (Gonzalez-Bulnes et al., 2020).

The follicular dynamic studies with ultrasonography at the beginning of the 1990s allowed to determine the number of follicular waves and the time of occurrence of each one (Brogni and Souza, 2021). It was also observed that CIDR insertion induced regression of the large follicles and development of new preovulatory follicles that reach their maximum diameter approximately on day 5 (Año-Perello et al., 2020); moreover, eCG in low doses stimulate LH surge, which therefore induce ovulation, meanwhile in high doses, it also increases ovulatory rate (Hameed et al., 2021). With this in mind, it was possible to shorten the protocols to five (Jackson et al., 2014; Uriol et al., 2019; Altincekic, 2022), six (Martinez-Ros and Gonzalez-Bulnes, 2019; López-García et al., 2023), or seven days (Martinez-Ros et al., 2018). Martinez-Ros et al. (2019) reported the best reproductive results with five-day protocols compared with six, seven, or 14 d. It is important to emphasize that in short protocols, PGF2 α administration is needed, because the insertion of CIDR produces regression of the gonadotropin-dependent follicles and promotes the beginning of a new follicular wave (Año-Perello et al., 2020). However, the devices marketed in México (CIDR 330 SHEEP & GOAT INSERT®), are still recommended for 9-12 d protocols during the breeding or non-breeding season, respectively.

The follicles of the first and second waves grow in an environment with prevalence of P4 that inhibits the high peak of LH and prevents ovulation (Fonseca et al., 2021). The reuse up to the fifth time in five-day protocols implies the maximum advantage of each device, since the use of these protocols is limited because of their cost. We hypothesize that reusing CIDR devices up to five times will not significantly affect the estrus or conception rates, and it would be possible to maintain a short interval to estrus and, at the same time, maintain the prolificacy rate.

Therefore, the objective of this study was to use the CIDR up to the fifth time to compare the reproductive variables in multiparous ewes; in addition, different eCG doses were used to determine if this hormone has benefits in the final maturation of the follicle, ovulation and luteinization.

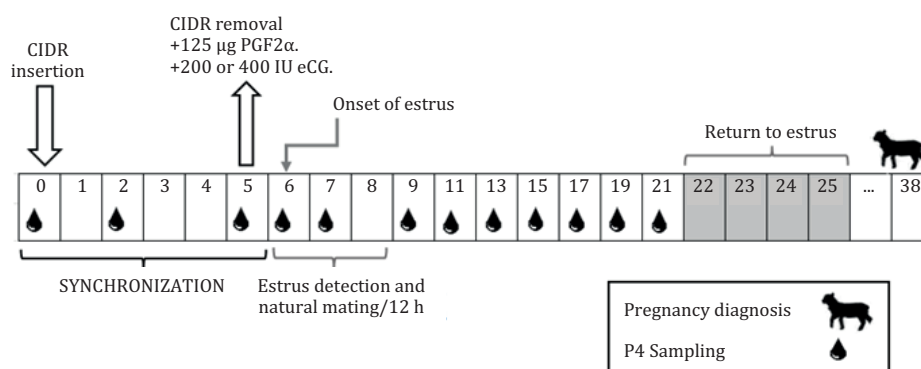
2. Material and methods

All experimental procedures were approved by the institutional committee of animal welfare (COBIAN/010/23) before the beginning of the experiments. The experiments were conducted in Montecillo, Texcoco, Estado de México, México (19°27'52" N, 98°54'44" W, 2220 m above sea level). The climate is C (W0) (W) b (i') g, subhumid temperate with rain in summer and dry season in winter, with little thermal oscillation, an average anual precipitation of 686 mm, and an average mean temperature of 15.9 ° (García, 2004).

2.1. Animal and experimental procedures

We used two hundred and fifty Dorset $\times$ Katahdin multiparous non-pregnant ewes, from two to four years old, 58 $\pm$ 4 kg body weight, and body condition of 3 in a 1-5 scale (Kenyon et al., 2014), fed forage (oat hay and alfalfa) and 400 g of concentrate feed with 14% CP and 2.75 Mcal/kg ME. During the experiment, the ewes were allotted to confinement pens provided with a roof, feeders, and automatic drinkers.

Two experiments were conducted to analyze the estrus synchronization during the reproductive season in a five-day protocol with new and reused intravaginal devices with 0.3 g P4 (CIDR 330 Sheep and Goat Insert®, Zoetis). Immediately after the removal of the CIDR, cloprostenol sodium (Celosil®) and eCG Gonactive® were applied (Figure 1).



In both experiments, 12 blood samples were collected from 10 sheep of each treatment to determine serum P4 concentrations.

Figure 1 - Diagram of the estrus synchronization protocol of the treatments in experiments 1 (200 IU of eCG) and 2 (400 IU of eCG).

2.2. Experiment 1

In the reproductive season (August to October 2021), 125 ewes were randomly allotted to one of five groups ($n = 26$): CIDR1-200, control (new intravaginal CIDR device for five days) and CIDR2-200, CIDR3-200, CIDR4-200, and ($n = 21$) CIDR5-200 (devices of second, third, fourth, and fifth uses, respectively). At time of CIDR removal, the ewes received a dose of 200 IU of eCG and 125 µg of cloprostenol sodium per ewe.

2.3. Experiment 2

In the protocols based on P4, it is common to use eCG; however, the dose varies according to region, breed, body condition, etc. In the temperate zone of México, it is common to use doses from 200 to 400 IU; for this reason, we used the higher dose in the second experiment.

From August to October 2022, we used the same number of ewes randomly allotted, as in the previous experiment, to five groups ($n = 26$): control CIDR1-400 (new intravaginal CIDR device for five days) and CIDR2-400, CIDR3-400, CIDR4-400, and CIDR5-400 ($n = 21$) (devices of second, third, fourth, and fifth use, respectively). Also, in all groups, 400 IU eCG and 125 µg cloprostenol sodium were injected per ewe at the removal of CIDR.

Every time CIDR were removed, they were washed with distilled water, starting from the tail and then the body of the device, removing dirt and mucosa with a sterile gauze, and then were rinsed three times to eliminate impurities and dried at room temperature for 24 h in a laminar flow hood (Telstar Bio II-A®). Once dried, they were placed in their original packaging and refrigerated at 4 °C, removing them from refrigeration 24 h before being used. At each CIDR insertion, florfenicol (0.6 g/100 mL, broad spectrum solution) was applied as a lubricant and antimicrobial.

2.4. Analyzed variables

The analyzed variables were estrus rate, onset (time, h) of estrus, return to estrus, pregnancy rate, prolificacy index, and serum P4 concentration.

Estrus rate and onset of estrus: After 24 h of the removal of CIDR, ewes in estrus were detected four times a day (at 02:00, 08:00, 14:00, and 20:00 h). The ewes had a natural mating 12 h after they exhibited estrous behavior with rams of proved fertility, in a ratio of one ram per five ewes, with a

second mating 12 h after the first one. Estrus rate was determined accordingly with the percentage of ewes that presented estrus between 24 and 72 h after the removal of the CIDR. The onset of estrus was considered as the time between CIDR removal and the first signs of estrous behavior (when the ewe allowed the mating).

Return to estrus: The ewes were exposed to the ram from 14 to 17 d after mating. The ones that allowed mating were classified as ewes that returned to estrus. The data were corroborated accordingly with the embryonic development in the pregnancy diagnosis.

Pregnancy diagnosis and prolificacy index: Pregnancy diagnosis was performed 30 days after the last mating, using ultrasonography, with a CHISON 6 equipment and a transrectal transducer at a 7.5 MHZ frequency. Prolificacy index was calculated with the births data (Number of lambs born/number of ewes lambing).

Serum progesterone analysis: Twelve blood samples were collected from 10 ewes per group using venipuncture in the jugular vein (days 0, 2, and 5 of the synchronization protocol; days 6, 7, and 9 of the mating period; days 11, 13, 15, 17, 19, and 21 of the *corpus luteum* development) to determine serum P4 concentration (Figure 1).

The samples were centrifuged for 20 min at $1500 \times g$ and 5 °C. The serum was transferred to 1.5-mL Eppendorf tubes, where they were maintained in a freezer until analysis. The P4 concentration was determined with ELISA kits (DRG® Progesterone ELISA). The determination was performed with an automatic microplate reader (CHROMATE® 4300); the analytical sensitivity was 0.045 ng/mL.

To control the quality of the measurement, two concentrations of P4 in sheep serum were used as inter-assay control (low control: 0.6-1.7 ng/mL; high control: 7.7-16.9 ng/mL). Internal quality controls were tested in duplicate at the beginning and at the end of the ELISA plates. To determine the precision of the measurement, the results obtained from the four samples of each internal quality control were averaged, verifying that they fell within their corresponding expected ranges; the variation coefficients intra and inter-assays were 7 and 9%, respectively.

2.5. Statistical analysis

In both experiments, completely randomized designs were used (1), with five groups of 26 repetitions from first to fourth uses of CIDR and 21 repetitions in the fifth use. Each sheep was considered as an experimental unit. The proposed statistical model was:

$$\gamma_{ij} = \mu + \tau_i + \varepsilon_{ij} \quad (1)$$

in which γ_{ij} is the observation j in treatment i , μ represents the mean value, τ_i is the fixed effect, and ε_{ij} is their random error.

The response variables estrus rate, return to estrus, and pregnancy rate were analyzed considering a binomial model using logistic regression and a χ^2 test. Shapiro-Wilk and Levene's tests were used to check the normality and homogeneity of variance of onset of estrus and prolificacy; because they did not meet the assumptions, a linear regression test was performed with non-normal data from the robustbase package (lmrob function), and Tukey's test was used to compare treatment means ($P \leq 0.05$) (TukeyHSD).

For the size of the sample used, the power of the statistical test was determined using the pwr package of Rstudio. The input parameters for the function in R were: number of groups (k), size of the sample (n), significance (0.05), effect size ($w = 0.15$), and method (χ^2). Statistical analyses were performed using R software, version 4.2.0.

Progesterone concentration was analyzed with a MIXED model in a completely randomized design with a significance level of $\alpha = 0.05$. Progesterone concentration was measured by sampling 10 sheep from each group (twelve samples per ewe). The main effects of the model were: group, time, and their interaction; the difference among groups was determined by least squares means (LSMeans), using

as covariance structure the Akaike information criteria (AIC) with average covariance structure (CS), unstructured (UN), and autoregressive order 1 (AR1) due to a lower value. The statistical model was:

$$Y_{ijk} = \mu + \tau_i + \delta_{j(i)} + P_k + (\tau P)_{ik} + \varepsilon_{ijk} \quad (2)$$

in which Y_{ijk} = the response variable in observation k , repetition j , and group i ; μ = overall mean effect; τ_i = effect of the i -th group; $\delta_{j(i)}$ = random effect of the j -th animal within the i -th group; P_k = effect of the k -th period; $(\tau P)_{ik}$ = group by period interaction; and ε_{ijk} = random error associated with the k -th repeated measurement within the j -th animal.

Data were analyzed with SAS software (Statistical Analysis System, version 9.4), with $P < 0.05$ as significant value.

3. Results

A hundred percent of the ewes retained CIDR during the five days of treatment, with no incidence of vaginitis observed. The CIDR are made of silicone in a thin, T-shaped design that allows vaginal drainage; unlike sponges, no adhesions were observed, and the short duration of the protocols promotes a healthy vaginal environment.

In experiment 1, for the sample size of 125 ewes and $P = 0.05$, the power of the test was 0.828; and in experiment 2, with the same sample size and $P = 0.05$, the power of the test was 0.958.

3.1. Experiment 1

In experiment 1, 96% of the ewes exhibited estrus with no significant differences among groups ($P > 0.05$). In the ewes of the CIDR1-200 group, estrus began 6 h later compared with all other groups, concentrated from 30 to 48 h; in the CIDR2-200 and CIDR3-200 groups, 80 and 92% of ewes, respectively, exhibited estrus during the first 36 h; while the ewes of the CIDR4-200 group showed a more dispersed estrus (from 24 to 54 h); and in the CIDR5-200 group, estrus was concentrated between 24 and 48 h (Figure 2). The average of groups was in this order: CIDR2-200 (31 ± 10 h), CIDR3-200 (33 ± 7 h), and CIDR5-200 (34 ± 7 h), with lower interval to estrus ($P < 0.05$) compared with CIDR4-200 (38 ± 10 h) and CIDR1-200 (42 ± 10 h).

In the variables estrus rate and prolificacy index, no differences were observed among groups ($P > 0.05$); however, there was a greater rate of return to estrus in CIDR5-200 group ($P < 0.05$) compared

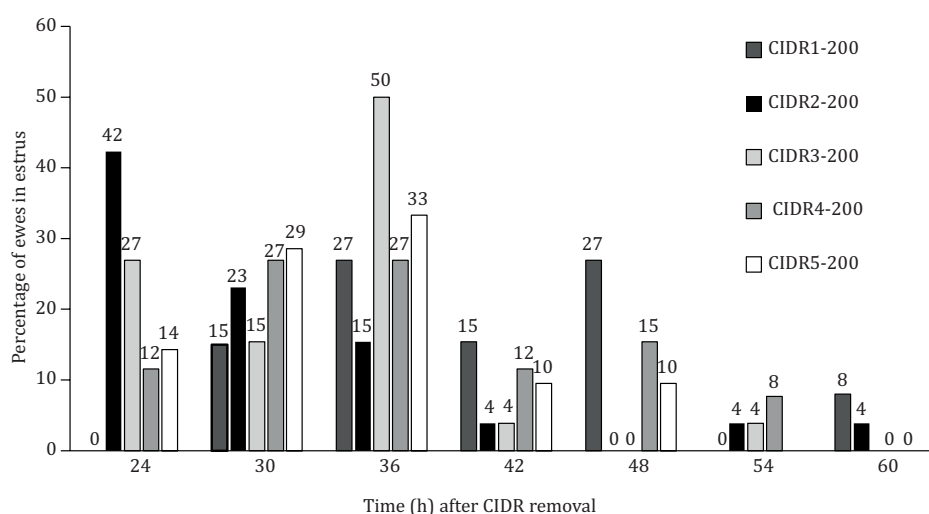


Figure 2 - Distribution of the onset of estrus (%) of ewes treated with new and second, third, fourth and fifth use CIDR with 200 IU of eCG, starting 24 h after CIDR removal.

with CIDR1-200, CIDR2-200, and CIDR3-200 groups, and consequently, a lower percentage in CIDR5-200 ($P<0.05$) with respect to the three groups previously mentioned. In the CIDR4-200 group no difference was observed with respect to the other groups in both variables ($P>0.05$) (Table 1).

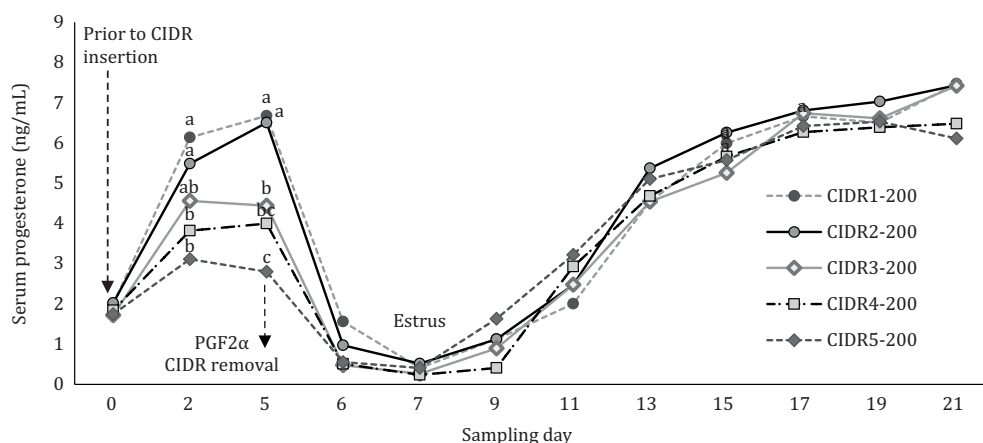
Serum P4 concentrations on day 0, prior to CIDR insertion, averaged 1.9 ± 0.1 ng/mL, without difference among groups ($P>0.05$). Forty-eight hours after CIDR insertion, P4 concentrations increased with an average concentration in CIDR1-200 (6.1 ± 0.9 ng/mL) which was not different from that in CIDR2-200 (5.5 ± 0.8 ng/mL), but both were different ($P<0.05$) from those in CIDR3-200, CIDR4-200, and CIDR5-200 (4.5 ± 0.3 , 3.8 ± 0.7 , and 3.1 ± 0.8 ng/mL, respectively); this increase in P4 across all groups demonstrates that the devices provided exogenous P4 up to their fifth use. A significant difference ($P<0.05$) was observed on day 5, corresponding to the samples obtained immediately before CIDR removal, with 6.7 ± 0.5 and 6.5 ± 0.4 ng/mL in the groups with new and second use CIDR, compared with the third and fourth uses (4.4 ± 0.6 and 4.0 ± 0.2 ng/mL) that were higher ($P<0.05$) than the concentration observed with the fifth use (2.8 ± 0.5 ng/mL). This reduction of P4 concentration from day 2 and 5 may be due to the lowered P4 content with the reutilization of the CIDR. From day 6 to 21, there was no difference ($P>0.05$) among groups (Figure 3).

Table 1 - Reproductive variables ($\pm$ SEM) of sheep treated with new and second, third, fourth, or fifth use CIDR and 200 IU of eCG (experiment 1)

Variable	Group ¹				
	CIDR1-200	CIDR2-200	CIDR3-200	CIDR4-200	CIDR5-200
Estrus rate (%)	92.31 $\pm$ 5.31a (24/26)	92.31 $\pm$ 5.31a (24/26)	100.00 $\pm$ 0.00a (26/26)	100.00 $\pm$ 0.00a (26/26)	95.24 $\pm$ 4.75a (20/21)
Onset of estrus (h)	41.52 $\pm$ 10.09b	31.00 $\pm$ 9.64a	32.77 $\pm$ 7.04ab	38.31 $\pm$ 9.91ab	34.2 $\pm$ 7.05ab
Return to estrus (%)	7.69 $\pm$ 6.27a (2/26)	7.69 $\pm$ 6.27a (2/26)	19.23 $\pm$ 7.73a (5/26)	26.92 $\pm$ 8.71ab (7/26)	38.09 $\pm$ 10.62b (8/21)
Pregnancy rate (%)	84.62 $\pm$ 7.07a (22/26)	84.62 $\pm$ 7.07a (22/26)	80.77 $\pm$ 7.73a (21/26)	73.08 $\pm$ 8.71a (19/26)	57.14 $\pm$ 10.80b (12/21)
Prolificacy index	1.3a	1.2a	1.4a	1.4a	1.3a

¹ CIDR1-200 - new intravaginal device, control; CIDR2-200, CIDR3-200, CIDR4-200, and CIDR5-200 - groups with CIDR from second to fifth use, respectively.

a-b - Values with different letters in a row indicate significant difference ($P<0.05$).



a-b - Different letters indicate differences among treatments ($P<0.05$).

Figure 3 - Serum P4 concentration during the synchronization protocol, estrus, and development of the *corpus luteum* in treatments with new and second, third, fourth and fifth use CIDR with 200 IU of eCG (experiment 1).

3.2. Experiment 2

In all groups, estrus was concentrated between 24 and 36 h after the CIDR removal: 92% with CIDR1-400, 89% with CIDR2-400, 85% with CIDR3-400 and CIDR4-400, and 77% with CIDR5-400. The other ewes exhibited estrous behavior the following 12 h. In the CIDR4-400 group, the ewes showed estrus later (39 ± 10 h; $P < 0.05$) than ewes from CIDR1-400 (33 ± 6 h), CIDR5-400 (32 ± 7 h), CIDR2-400 (31 ± 7 h), and CIDR3-400 (31 ± 7 h) (Figure 4).

On the other hand, no differences were observed among groups ($P > 0.05$) in the variables estrus rate and return to estrus. There was a difference in pregnancy rate in CIDR5-400 ($P < 0.05$) with respect to CIDR1-400, CIDR2-400, and CIDR3-400; CIDR4-400 was not different from the groups mentioned above; there was a high percentage of return to estrus (from 8 to 33%). From the 22 pregnant ewes in the CIDR1-400 group, one of them had embryonic loss; therefore, only 21 gave birth. The prolificacy index was similar ($P > 0.05$) in all groups, varying between 1.4 and 1.7 (Table 2).

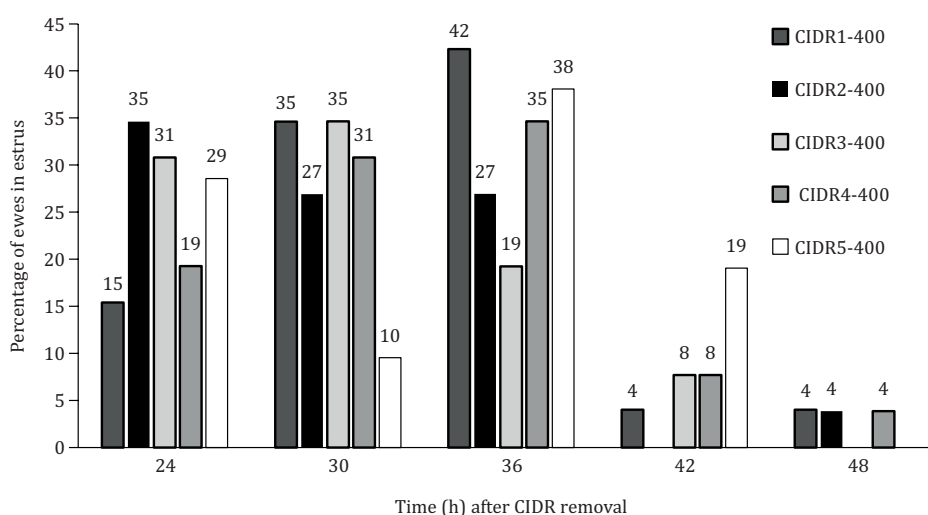


Figure 4 - Distribution of the onset of estrus (%) of ewes treated with new and second, third, fourth and fifth use CIDR with 400 IU of eCG, starting 24 h after CIDR removal.

Table 2 - Reproductive variables ($\pm$ SEM) of sheep treated with new and second, third, fourth, or fifth use CIDR with 400 IU of eCG (experiment 2)

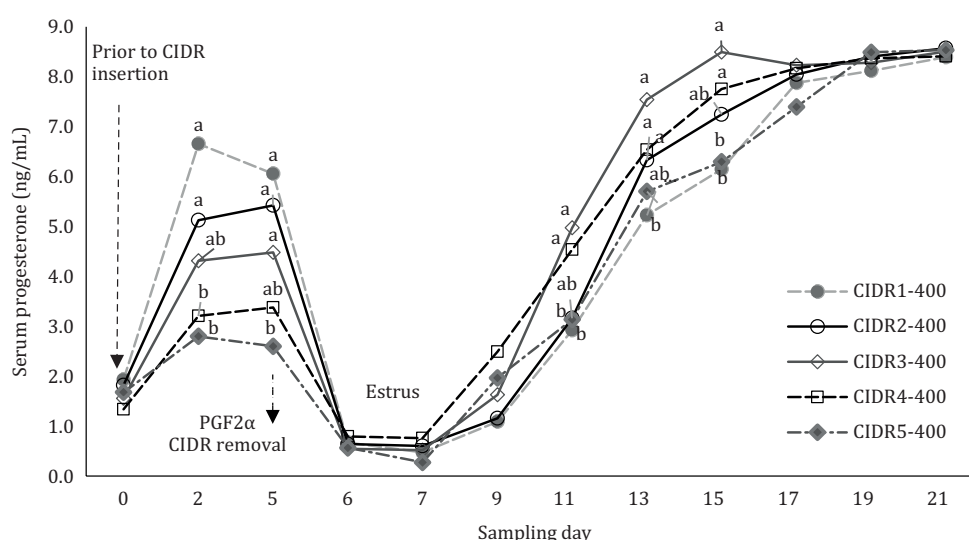
Variable	Group ¹				
	CIDR1-400	CIDR2-400	CIDR3-400	CIDR4-400	CIDR5-400
Estrus rate (%)	100.00 $\pm$ 0.00a (26/26)	96.15 $\pm$ 3.77a (25/26)	92.31 $\pm$ 5.22a (24/26)	96.15 $\pm$ 3.77a (25/26)	95.24 $\pm$ 4.65a (24/26)
Onset of estrus (h)	33.00 $\pm$ 6.18ab	30.96 $\pm$ 6.86a	31.30 $\pm$ 6.76a	38.88 $\pm$ 9.97b	31.80 $\pm$ 6.77a
Return to estrus (%)	15.38 $\pm$ 7.07a (4/26)	7.69 $\pm$ 5.22a (2/26)	15.38 $\pm$ 7.07a (4/26)	19.23 $\pm$ 7.73a (5/26)	33.33 $\pm$ 10.3a (6/21)
Pregnancy rate (%)	84.62 $\pm$ 7.07a (22/26)	88.46 $\pm$ 6.26a (23/26)	84.62 $\pm$ 7.07a (22/26)	76.92 $\pm$ 8.25ab (20/26)	61.90 $\pm$ 10.8b (13/21)
Prolificacy index	1.7a	1.6a	1.5a	1.6a	1.4a

¹ CIDR1-400 - new intravaginal device, control; CIDR2-400, CIDR3-400, CIDR4-400, and CIDR5-400 - groups with CIDR from second to fifth use, respectively.

a-b - Values with different letters in a row indicate significant difference ($P < 0.05$).

The overall average of P4 concentration before the insertion of CIDR was 1.7 ± 0.2 ng/mL. The ewes had an increase in P4 concentration 48 h after CIDR insertion, with 6.7 ± 0.3 and 5.1 ± 0.6 ng/mL in the new and second-use CIDR, different ($P < 0.05$) than the fourth- and fifth-use groups (3.2 ± 0.3 and 2.8 ± 0.7 ng/mL respectively), while the third-use group (4.3 ± 0.5 ng/mL) was not different from the other four groups ($P > 0.05$).

On day 5, corresponding to the removal of the CIDR, the groups CIDR1-400, CIDR2-400, and CIDR3-400 had higher P4 concentration (6.1 ± 0.3 , 5.4 ± 0.1 , and 4.5 ± 0.02 ng/mL, respectively; $P < 0.05$) compared to the fifth-use group (3.1 ± 0.2 ng/mL); however, the CIDR4-400 was not different from the other groups (3.4 ± 0.6 ng/mL). On day 6, the first estrous behavior began in all ewes, and the P4 concentration had an average of 0.8 ± 0.4 ng/mL; the lower P4 values were observed on day 7 (0.4 ± 0.1 ng/mL). On the ninth day, P4 concentrations increased and were similar from days 17 to 21 without difference among groups ($P > 0.05$): 8.1 ± 0.3 ng/mL in CIDR1-400, 8.3 ± 0.3 ng/mL in CIDR2-400, 8.3 ± 0.1 ng/mL in CIDR3-400 and CIDR4-400, and 8.1 ± 0.6 ng/mL in CIDR5-400 group (Figure 5).



a-b - Different letters indicate differences among treatments ($P < 0.05$).

Figure 5 - Serum P4 concentration during the synchronization protocol, estrus period, and development of the *corpus luteum* in treatments with new and second, third, fourth and fifth use CIDR with 400 IU of eCG (experiment 2).

4. Discussion

The use of short protocols is an opportunity to reuse CIDR after an appropriate washing and disinfection (Cox et al., 2012; Pinna et al., 2012; Martínez-Ros et al., 2019; López-García et al., 2023) in the same herd free of diseases to reduce the time of exposure to P4 in ewes, and, at the same time, to lower costs.

Although actual CIDR device has evolved to synchronize estrus and decrease the reported cases of vaginitis compared with the use of sponges (Martínez-Ros et al., 2018), additional improvements are required in the development of protocols to synchronize estrus at lower cost and in the clearance of the hormonal content from devices after use, when they are discarded to minimize environmental impact.

Some studies have concluded that the use of CIDR for a five-day period is enough to obtain a successful estrus synchronization in ewes, and there is no change in reproductive results compared with conventional 12-14-d protocols (Jackson et al., 2014; Martinez-Ros and Gonzalez-Bulnes, 2019; Altincekic, 2022). According to Skliarov et al. (2021), using CIDR, either in short or long protocols, injecting or not eCG, the percentage of onset of estrus were close to 100%, which is similar to the values observed in this research, where in both experiments (200 or 400 IU) 96% of ewes showed estrus. Although the initial dose of P4 (0.3 g) in the CIDR was gradually reduced with each reuse, the estrus percentages were maintained up to their fifth use. It is important to emphasize that in those studies, the utilization of the CIDR was in reproductive season; however, other research conducted both in reproductive season as well as in anestrus season indicated that the estrus percentage was not affected when the CIDR were used up to three times in 5-7-d protocols with or without eCG (Cox et al., 2012; Pinna et al., 2012; Biehl et al., 2019; Uriol et al., 2019; López-García et al., 2023).

The average onset of estrus in both experiments were similar (35.4 ± 9 h and 33.2 ± 7 h) when using 200 or 400 IU eCG, respectively. In this regard, Atalla (2018) did not find differences in onset of estrus in Assaf ewes after the removal of intravaginal sponges impregnated with 60 mg of medroxyprogesterone acetate and 300 or 600 IU eCG (60.7 ± 20 h and 51.3 ± 25 h, respectively), with more delayed estrus than that observed in our study, which may be due to the use of progestogen with prolonged duration (14 d).

In experiment 1 (200 IU of eCG), ewes exhibited estrus between 24 to 60 h after CIDR removal (mainly concentrated between 24 and 48 h). In fact, in the CIDR1 200 group, the first ewes showing estrous behavior were observed until 30 h after removal of the CIDR, corroborating the reports of Biehl et al. (2019) also with new CIDR in seven-day protocols. They concluded that a higher concentration of P4 in new CIDR during the follicle development may induce a smaller ovulatory follicle due to the reduction of LH pulsatility.

Despite the results with CIDR1-200 group, there was no delay on the onset of estrus with CIDR1-400. The 400 IU eCG application at removal of new CIDR concentrated the estrus between 24 and 36 h, a shorter interval than the one reported by Santos-Jimenez et al. (2020), who used CIDR for five days plus PGF2 α (5 mg Dinolytic) and 400 IU eCG and observed 80% ewes in estrus between 36 and 48 h after the CIDR removal.

The reduction of interval between the CIDR removal and the onset of estrus in the 400 IU eCG group is important because it is the time that the preovulatory follicle needs to reach its maximum estradiol secretion and induction of estrous behavior (Martinez-Ros et al., 2019). It would be useful to complete these results with a measure of the intervals of LH preovulatory surge and ovulation in protocols of synchronization for fixed-time artificial insemination.

In Experiment 1, the return to estrus rate increased until the fifth use of CIDR ($P < 0.05$). In Experiment 2, there were no differences among treatments ($P > 0.05$), although CIDR5-400 resulted in a 33% return. This percentage indicates a reduction in fertility. Pregnancy rate decreased ($P < 0.05$) in the fifth use in both experiments (57% with CIDR5-200 and 62% with CIDR5-400).

In both experiments, good pregnancy rates were achieved from the first to fourth use of CIDR. The P4 released by CIDR was sufficient to inhibit LH secretion; the decrease in serum P4 was more dramatic upon CIDR removal, and the injection of eCG during this process aided LH release. Without eCG, the reuse of CIDR could negatively affect fertility, because this hormone is known to have FSH and LH function (Yoshimura et al., 2021). The reduction of pregnancy rate in CIDR5-200 and CIDR5-400 was expected, because the CIDR decreases P4 content with each reutilization; at the fifth reuse, the P4 level probably was enough to prevent estrus while the CIDR was intravaginally maintained, but not enough to completely suppress LH secretion in the ewes. The consequence was an abnormal follicular development that induced a persistent growth of the dominant follicle (Gonzalez-Bulnes et al., 2020); therefore, fertility was deficient. In this regard, Menchaca et al. (2017) reported that the percentage of ewes showing estrus and ovulation was not affected by the CIDR used one, two, or three times. However, pregnancy rate was markedly reduced with CIDR used three times compared with the new ones.

The prolificacy index (lambs born/ pregnant ewes) was similar ($P>0.05$) from the new to the fifth use of CIDR in both experiments. However, the average of the five groups in experiment 1 (200 IU eCG) was 1.3, while with 400 IU eCG in experiment 2, it was 1.6. The eCG is related with this variable; however, during seasonal anestrus, Atalla (2018) did not find differences in prolificacy index using either 300 or 600 IU (1.30 and 1.37, respectively).

In our study, progesterone released by new CIDR maintained P4 concentrations higher than 6 ng/mL in ewes during the five days of treatment, in both experiments. These concentrations decreased as the number of reutilization of CIDR increased, until they reached an average of 3.0 ± 0.9 and 2.7 ± 0.4 ng/mL (in experiment 1 and 2, respectively) during the fifth use. However, serum P4 concentration was not only supplied by the CIDR, either new or reused, it was also influenced by endogenous secretion from *corpus luteum* in the ovary of the ewes during the experiment.

On day 6 (24 h after CIDR removal plus the injection of 125 µg PGF2α), P4 concentration decreased in all groups. However, the CIDR1-200 still showed a value of 1.6 ng/mL, which explains the six-hour delay in the onset of estrus compared with the other groups. Estrus occurs until P4 concentration decreased to approximately 1 ng/mL or less (D'Avila et al., 2022). In experiment 2, P4 concentrations were lower than 1 ng/mL in all groups. The dose of 400 IU eCG reduced the interval between CIDR removal and the onset of estrus, reversing the inhibitory effects of P4 on follicle growth. Even 15% of ewes treated with new CIDR exhibited estrus behavior at 24 h later.

Four days after the CIDR removal (day 9) P4 concentrations were 1.0 ± 0.4 ng/mL in experiment 1 and 1.7 ± 0.5 ng/mL in experiment 2, indicating the supply of P4 at least by a growing *corpus luteum*. On days 11, 13, and 15, the highest serum P4 concentration was observed on CIDR3-400 and CIDR4-400 groups ($P<0.05$). Although it was not possible to make a statement, it is likely that the high concentrations of P4 in ewes with the new and second-use CIDR caused a reduction in LH pulsatility, which affected the growth of the dominant follicle and caused a delay in ovulation. As for the CIDR5-400 group, although P4 concentration during estrus synchronization was not high, the percentage of pregnant ewes was lower and may have been due to subnormal *corpus luteum* development.

In the last sampling, 15 d after the onset of estrus, the P4 concentrations increased to 7 ± 0.57 ng/mL in experiment 1; however, in CIDR5-200 group, a slight decrease in the average of P4 concentration was observed between 19 and 21 d, because three ewes in this group showed P4 concentration < 2 ng/mL, which could be produced by different causes: early embryonic resorption, failure in fertilization, or persistent *corpus luteum* (Younis and Hatif, 2023). In experiment 2, the highest P4 concentration was observed on day 15 (8.5 ± 0.7 ng/mL) in all groups. The serum P4 concentration significantly increased in pregnant ewes.

It is worth mentioning that factors such as breed, age, environment, and temperature can influence the percentages of estrus, pregnancy, and lambing. Nevertheless, this research was performed in two consecutive years, during the reproductive season (August to October) with ewes adapted to the region. So, it is considered that the climate and environmental conditions did not have a negative effect on reproductive behavior.

5. Conclusions

The 5d protocols combined with PGF2α at the CIDR removal allows to use them up to five times without affecting percentage of estrus, pregnancy rate, or prolificacy index. Serum P4 concentration is reduced according to the number of reutilization of CIDR. Pregnancy rate is reduced up to the fifth use; consequently, the return to estrus is increased. However, there is still a high percentage of estrus up to the fifth use in ewes. These findings represent the opportunity to get the most benefits from the CIDR, to reduce, with each reuse, the final cost of the protocols without a major detriment to their efficiency and to reduce the hormonal content of the devices to a minimum when they are discarded, with the ecological benefit that this implies.

Data availability

The contents underlying the research are included in the article.

Author contributions

Conceptualization: López-García, S.; Sánchez-Torres, M. T.; Cordero-Mora, J. L.; Figueroa-Velasco, J. L. and Martínez-Aispuro, J. A. **Data curation:** Sánchez-Torres, M. T. **Formal analysis:** López-García, S.; Martínez-Aispuro, J. A. and Díaz-Díaz, E. **Funding acquisition:** Sánchez-Torres, M. T. **Investigation:** López-García, S.; Sánchez-Torres, M. T.; Cordero-Mora, J. L.; Figueroa-Velasco, J. L.; Martínez-Aispuro, J. A. and Díaz-Díaz, E. **Methodology:** López-García, S.; Sánchez-Torres, M. T.; Cordero-Mora, J. L. and Martínez-Aispuro, J. A. **Project administration:** Cordero-Mora, J. L. **Software:** López-García, S. and Martínez-Aispuro, J. A. **Supervision:** Cordero-Mora, J. L. **Visualization:** Sánchez-Torres, M. T. and Figueroa-Velasco, J. L. **Writing – original draft:** López-García, S.; Sánchez-Torres, M. T. and Figueroa-Velasco, J. L. **Writing – review & editing:** López-García, S.; Sánchez-Torres, M. T.; Figueroa-Velasco, J. L. and Martínez-Aispuro, J. A.

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