Theriogenology 251 (2026) 117751

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

# A novel therapeutic approach: The effect of letrozole-assisted synchronization on reproductive performance in suckling ewes during the non-breeding season

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ARTICLE INFO

*Keywords:*
Ewes
Fertility
Letrozole
Non-breeding season
Suckling

ABSTRACT

The aim of the present study was to investigate the effect of letrozole treatment on the success of a progesterone-based synchronization protocol and fertility parameters in suckling Awassi ewes during the anoestrus period.

One hundred and five multiparous, suckling Awassi ewes were used. Ewes ($n = 105$) were randomly divided into three groups as Control ($n = 35$), LET-4 ($n = 35$) and LET-0 ($n = 35$). Four days before the synchronization protocol (D-4), letrozole at a dose of 1 mg/kg was administered subcutaneously (s.c.) to the LET-4 group, while the LET-0 group received it on the day of protocol initiation (D0). The control group received 1 mL of benzyl alcohol s.c. at both time points as a placebo. Subsequently, intravaginal sponges containing 60 mg medroxyprogesterone acetate were inserted into all ewes (D0). The sponges were kept in the vagina for 7 days and 500 IU eCG and 75 μg d-cloprostenol were administered intramuscularly on the day the sponges were removed. Blood samples were taken for measurement of serum $E_2$, FSH and $P_4$ before sponge insertion (D-4 and D-2), on the day of sponge insertion (D0), after sponge insertion (D2 and D4), at mating (DM) and post-mating days (DPM-7, 14, 21, 35 and 50). Pregnancy was diagnosed by transrectal ultrasonography at DPM-21, 35, and 50.

The overall mean serum FSH concentrations, calculated across all sampling days, were significantly higher in the LET-4 group (27.68 ± 1.56 mIU/mL) than in LET-0 (21.85 ± 0.87 mIU/mL) and Control (18.54 ± 0.91 mIU/mL) ($P < 0.001$). Subsequent to the FSH elevation, serum $E_2$ levels at the time of mating were also significantly higher in LET-4 (22.24 ± 1.20 pg/mL) than in LET-0 (14.47 ± 0.92 pg/mL) and Control (10.77 ± 0.69 pg/mL) ($P < 0.001$). Post-mating $P_4$ concentrations were likewise significantly elevated in the LET-4 group (3.79 ± 0.24 ng/mL) compared to LET-0 (3.29 ± 0.24 ng/mL) and Control (2.78 ± 0.24 ng/mL) groups ($P < 0.001$). Estrus rate was highest in the LET-4 group (90.62 %) compared to LET-0 (68.57 %) and Control (57.57 %) ($P = 0.010$). Pregnancy rate on day 50 was significantly greater in LET-4 (59.37 %) than in LET-0 (37.14 %) and Control (27.27 %) ($P = 0.027$). Fecundity rate was also highest in LET-4 (87.50 %) compared to LET-0 (57.14 %) and Control (33.33 %) ($P < 0.001$). No significant differences were observed among groups in litter size, twinning or pregnancy losses rates ($P > 0.05$).

In conclusion, letrozole administration four days before the synchronization protocol significantly enhanced reproductive performance in suckling ewes during the non-breeding season. These results suggest that this approach could be a promising alternative strategy to improve protocol success without resorting to early weaning.

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https://doi.org/10.1016/j.theriogenology.2025.117751
Received 30 September 2025; Received in revised form 4 November 2025; Accepted 10 November 2025
Available online 10 November 2025
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## 1. Introduction

The overall efficiency of sheep production systems is predominantly determined by reproductive performance and subsequent lamb output [1]. Therefore, shortening breeding intervals and increasing the number of lambs per ewe are among the main goals of increasing flock productivity [2]. Ensuring lambing twice a year by applying estrus synchronization during the anoestrus period is considered an important strategy to achieve this goal [3–5]. However, the response to synchronization protocols applied during anoestrus is significantly lower compared to the breeding season [6–8]. There are several primary reasons why synchronization protocols implemented outside the breeding season result in lower pregnancy rates. The first is that endogenous gonadotropin levels are low during anoestrus, resulting from the negative feedback estradiol ($E_2$) exerts on gonadotropin-releasing hormone (GnRH) [9–11]. This condition leads to an inadequate follicular response to hormonal treatments compared with the breeding season, resulting in reduced estrus rates, decreased ovulation rates, and diminished pregnancy success [4,12]. On the other hand, the presence of lactation and suckling during the anestrous period makes the situation even more complex. During suckling, elevated prolactin levels and increased endogenous opioid peptides suppress the activity of kisspeptin neurons in the hypothalamus, thereby further reducing the secretion of GnRH and consequently follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [13–16]. This condition further limits follicular development, deepening seasonal anoestrus [17,18]. Therefore, in response to conventional synchronization protocols applied to lactating ewes outside the breeding season, estrus rates and ovulation frequency remain low, the functional capacity of the formed corpus luteum (CL) is limited, and premature luteolysis and early embryonic losses increase [19–21].

The most common strategy suggested to overcome these disadvantages is either early weaning of lambs or limiting suckling [13]. Indeed, many reports have shown that inhibition of suckling significantly increases the success rate of synchronization protocols [17,21–24]. Nevertheless, although this strategy may provide potential reproductive advantages, it compromises lamb growth potential and milk yield, leading to considerable drawbacks from both economic and animal welfare perspectives [25]. These concerns have prompted the search for pharmacological or hormonal adjuvant treatments that could enhance synchronization success without resorting to early weaning [26,27]. However, to the best of our knowledge, no effective strategy or treatment has so far been documented. Hence, the demand for adjuvant interventions capable of enhancing reproductive performance under suckling conditions persists.

In this regard, it is reported that aromatase inhibitors, which have been used for a long time in ovulation induction in humans and have also found potential application in farm animals in recent years, may be an important alternative in increasing the effectiveness of synchronization protocols [28,29]. Aromatase enzyme (cytochrome P450) is the enzyme that catalyses three sequential hydroxylations that convert 19-carbon androgens to 18-carbon aromatic estrogens [30]. Aromatase inhibitors are classified into two categories, type I (steroidal) and type II (non-steroidal), according to their chemical structure and mechanism of action. Type I inhibitors, which are derivatives of androstenedione, irreversibly inhibit aromatase activity by covalently binding to the active site of the cytochrome P450 aromatase enzyme [31]. Type II aromatase enzyme inhibitors, on the other hand, contain a triazole group with nitrogen in their structure and suppress enzyme activity by reversibly binding to the heme moiety of the cytochrome P450 aromatase enzyme. Although this group includes several compounds such as anastrozole and fadrozole, letrozole stands out as the most widely used inhibitor due to its high efficacy [29,32].

Letrozole is a third-generation, nonsteroidal aromatase inhibitor that blocks the conversion of androgens to estrogens by inhibiting the cytochrome P450 aromatase enzyme [33]. Letrozole attenuates the negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis by suppressing $E_2$ synthesis, resulting in increased endogenous FSH secretion [11]. This increase in FSH promotes the emergence and development of a new follicular cohort [11,29,32]. Moreover, studies in cattle have reported that letrozole prolongs the follicular growth phase, resulting in larger preovulatory follicles and increased luteal progesterone production [29,34,35]. Therefore, aromatase inhibition may potentially exert a luteotrophic effect that enhances CL function. Indeed, in high-yielding dairy cows, such effects have been associated with improved pregnancy rates and reduced embryonic loss [29,36]. Studies in ewes, similar to the findings in cattle, have demonstrated that letrozole stimulates follicular development, induces a new follicular wave, and supports CL function [11,37–40].

Based on this background, it is predicted that letrozole administered before the synchronization protocol in suckling ewes outside the breeding season will increase endogenous FSH secretion by eliminating the inhibitory effect of $E_2$ on the HPG axis. We hypothesized that increased FSH secretion would stimulate follicular growth by supporting the development of a new follicular cohort before synchronization, strengthening the functional capacity of the CL and ultimately improving both the success of the synchronization protocol and overall reproductive performance. Accordingly, the present study aimed to evaluate the effects of integrating letrozole into a conventional synchronization protocol on synchronization success and fertility parameters in suckling ewes during the non-breeding season. The overall objective was to increase lambing frequency without resorting to early weaning.

## 2. Materials and methods

This study was carried out with the approval of Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee, Hatay, Turkey (2025/02–03, 2025). The experiment was conducted out during the non-breeding season (March–September 2025) in Reyhanlı District of Hatay Province, located in the Southeastern Anatolia Region (36° 1842.4″ N, 36° 2617.4″ E, and altitude: 160 m).

### 2.1. Animals and management

A total of 105 multiparous, suckling Awassi ewes, clinically healthy, 2–5 years old, mean B.W. 41.18 ± 0.28 kg, average body condition score (BCS) 2.65 ± 0.37 (BCS ranging from 1 = emaciated to 5 = obese) [41], about 60 days postpartum, were used. The ewes were grazed on natural pastures for 12 h (h). During grazing breaks, the ewes were brought to semi-open pens and allowed to suckle lambs first and then hand-milked once a day. An average of approximately 20 kg of milk per day was obtained from all ewes ($n = 105$). A total of 15 experienced Awassi rams that actively mated and produced offspring from matings in the previous season were used. To eliminate the male effect, when the rams had to be separated from the flock due to the experimental procedure, they were kept in a separate pen where the ewes could see and smell them. Ewes and rams were not given any extra concentrated feed or roughage except for pasture, but they were provided ad libitum access to water for 24 h. Nutritional flushing was not applied to the animals before mating.

### 2.2. Experimental design

Letrozole (Purity ≥99.95 %, MedChemExpress, USA, Cat. No: HY-14248), produced for research purposes, was dissolved in benzyl alcohol (Purity ≥99.50 %, KimyaLab, Türkiye, Cat. No: V30968.500G) to prepare an injectable solution at a concentration of 50 mg/mL (175.254 mM). The application dose was set at subcutaneously (s.c.) 1 mg/kg body weight (B.W.) as reported by Kıvrak et al. [11].

The ewes included in the study ($n = 105$) were randomly divided into three groups: Control ($n = 35$), LET-4 ($n = 35$), and LET-0 ($n = 35$). Four days before the synchronization protocol (D-4), the LET-4 group

received a s.c. injection of 1 mg/kg B W. letrozole, whereas the Control and LET-0 groups received 1 mL of benzyl alcohol (vehicle). On the day the synchronization protocol began (D0), the LET-0 group received 1 mg/kg B W. letrozole, and the Control and LET-4 groups received 1 mL of benzyl alcohol. Subsequently, commercial sponges (Esponjavet®, Hipra, Spain) impregnated with 60 mg medroxyprogesterone acetate (MAP) were applied intravaginally to all ewes ($n = 105$) for 7 days (D0). On the day of sponge removal (D7), 500 IU eCG (Oviser®, Hipra, Spain) and 75 μg d-cloprostenol (Senkrodin®, Vetaş, Türkiye) were administered intramuscularly (i.m.) (Fig. 1).

On the day of sponge removal, it was observed that intravaginal sponges had fallen out in a total of five ewes (two from the Control and three from the LET-4 group). These ewes were marked and excluded from the study. From the 24th to the 72nd h after sponge removal, fertile rams (ewe/ram ratio 7:1) were introduced to the flock twice daily (07:00–09:00 and 19:00–21:00) for 2 h. During this period, matings were monitored, and ewes that agreed to mate were admitted during estrus. Mated ewes were marked, and their mating times were recorded; these animals were then immediately separated from the flock and placed in a separate pen. After the mating monitoring was completed at the 72nd h, the rams were completely separated from the ewes and were not returned to the flock for the duration of the study.

### 2.3. Collection of blood samples and hormone analyses

To monitor the effects of treatments administered during the study on serum hormone levels ($E_2$, FSH, and $P_4$), approximately 8 mL of blood was collected from the jugular vein via puncture into dry tubes containing clotting activator gel (Vacusera®, Disera, Türkiye). Blood samples were collected before the synchronization protocol (D-4 and D-2), at the start of synchronization (D0), after synchronization (D2 and D4), on the mating day (DM), and on post-mating days (DPM-14, 21, 35, and 50) (Fig. 1). Collected blood samples were centrifuged at 3000 rpm for 10 min, and the resulting serum was stored at −20 °C until analysed.

Determination of serum $E_2$ and $P_4$ concentrations was performed using a chemiluminescence immunoassay analyser (CLIA) (MAGLUMI-X8, Snibe Diagnostic, China), while FSH levels were measured with a commercial sheep-specific enzyme-linked immunosorbent assay (ELISA) kit (Sheep FSH ELISA Kit, FineTest, China, Cat. No: ESH0028). Serum FSH assays had a sensitivity of ≤1.875 mIU/mL, with intra- and inter-assay coefficients of variation (CVs) of 4.82–5.92 % and 4.68–6.32 %, respectively (measuring range: 3.125–200 mIU/mL). Serum $E_2$ assays demonstrated a sensitivity of ≤5.0 pg/mL, with intra- and inter-assay CVs of 1.50–6.84 % and 1.87–5.05 %, respectively (measuring range: 5.0–6000 pg/mL). Serum $P_4$ assays had a sensitivity of ≤0.1 ng/mL, with intra- and inter-assay CVs of 2.02–4.33 % and 1.83–7.21 %, respectively (measuring range: 0.1–80 ng/mL).

### 2.4. Ultrasonographic pregnancy examination

Pregnancy diagnosis was performed by transrectal ultrasonography (USG) on days 21, 35, and 50 after mating (Fig. 1). A portable, real-time B-mode ultrasonography device (SonoSite M-Turbo, Fujifilm, USA) equipped with a 5–10 MHz linear rectal probe was used for transrectal examinations. Pregnancy diagnosis was based on ultrasonographic visualization of the CL, embryonic vesicle, amniotic fluid, embryo, and heartbeat [42]. In addition, CL vascularization was also evaluated along with pregnancy findings to increase the reliability of pregnancy diagnosis on the 21st day after mating. Ovaries were first examined using B-mode ultrasonography to identify CL, followed by color Doppler imaging for vascularization. The degree of luteal vascularization was scored from 1 to 4 as 1 (0–25 %), 2 (26–50 %), 3 (51–75 %), and 4 (76–100 %) according to the proportion of the CL area showing colored Doppler signals, as described by Arashiro et al. [43]. Ewes were classified as pregnant when both an embryo (or embryonic structures) and at least one CL with a vascularization score of ≥2 were identified, whereas the absence of both an embryo and a CL was considered non-pregnant. All ultrasonographic and Doppler evaluations were performed by a single experienced operator to ensure consistency. Late embryonic loss was considered for ewes identified as pregnant on day 21 after mating

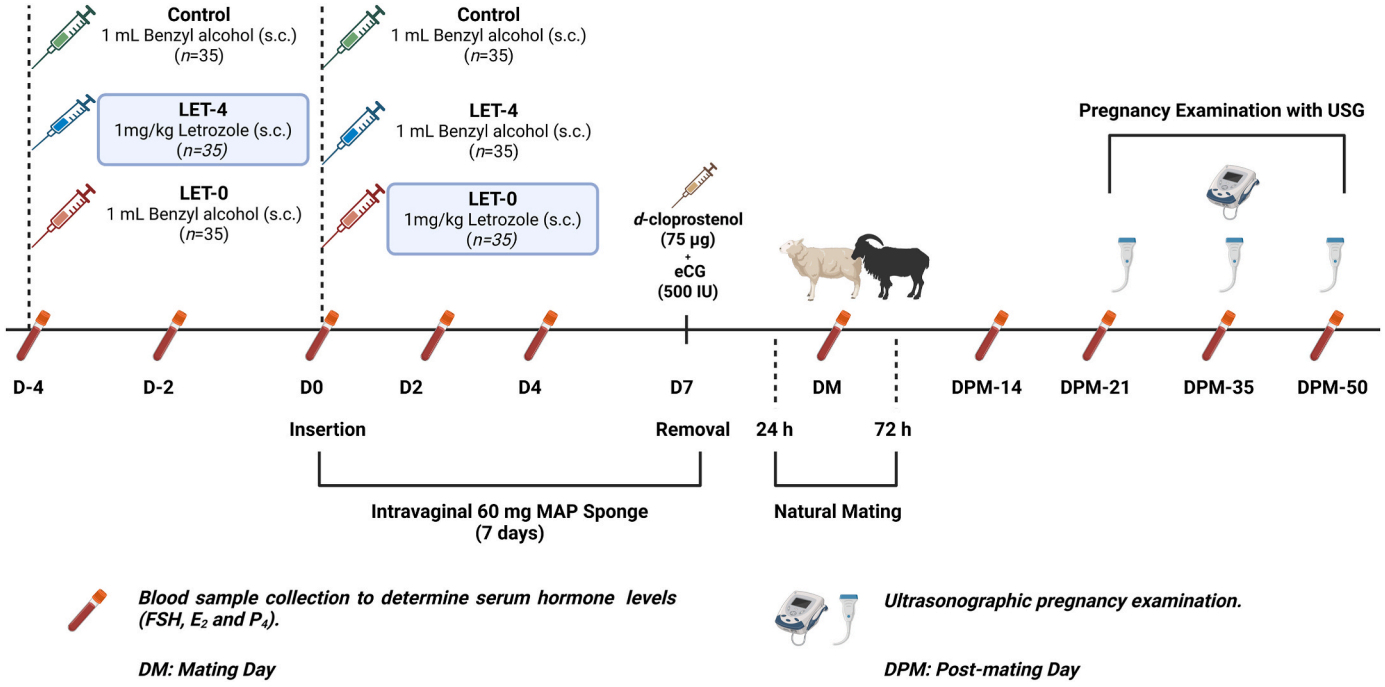



**Fig. 1.** Schematic representation of the experimental design used to evaluate the effects of letrozole treatment in ewes. Ewes ($n = 105$) were randomly assigned into three groups: LET-4 (1 mg/kg letrozole D-4), LET-0 (1 mg/kg letrozole D0) and Control (1 mL benzyl alcohol D-4 and DO). For estrus synchronization: medroxyprogesterone acetate (MAP) for 7 days and 500 IU eCG + 75 μg d-cloprostenol ($PGF_2\alpha$) during the removal of MAP (D7). Natural mating was allowed within 24–72 h after sponge removal (DM: mating day). Blood samples were collected at the indicated time points to determine serum hormone concentrations (FSH, $E_2$, and $P_4$). Pregnancy diagnosis was performed by transrectal ultrasonography at days 21, 35, and 50 post-mating (DPM: post-mating day).

but without signs of pregnancy (gestational sac, embryo, heartbeat, or CL) on day 35. Early fetal loss was considered for ewes identified as pregnant on day 35 but with no signs of pregnancy on day 50. Although the number of offspring was determined during pregnancy examinations, lambing was followed to precisely determine litter size and twinning rates.

### 2.5. Assessment of fertility parameters

Fertility parameters of ewes were calculated using the formulas given below [44].

Estrus rate (%) = Number of mated ewes/Total number of ewes in the group × 100.

21st day pregnancy rate (%) = Number of pregnant ewes on day 21/Total number of ewes in the group × 100.

35th day pregnancy rate (%) = Number of pregnant ewes on day 35/Total number of ewes in the group × 100.

50th day pregnancy rate (%) = Number of pregnant ewes on day 50/Total number of ewes in the group × 100.

Late embryonic loss rate (from day 21–35) (%) = Number of non-pregnant ewes on day 35/Number of pregnant ewes on day 21 × 100.

Early fetal loss rate (from day 35–50) (%) = Number of non-pregnant ewes on day 50/Number of pregnant ewes on day 35 × 100.

Total pregnancy loss rate (from day 21–50) (%) = Number of non-pregnant ewes on day 50/Number of pregnant ewes on day 21 × 100.

Twinning rate (%) = Number of ewes with twin lambing/Number of ewes with lambing × 100.

Litter size = Number of lambs born/Number of lambing ewes.

Fecundity rate (%) = Total number of lambs/Total number of ewes × 100.

### 2.6. Statistical analysis

G*Power software version 3.1 was used for the sample size calculation. It resulted that the minimum number of ewes was 101, considering a medium effect size of 0.31, an alpha value of 0.05, and a power of 0.80.

All the obtained quantitative variables (serum FSH, $P_4$, $E_2$ concentrations and litter size) were evaluated in terms of parametric test assumptions before the significance tests. Quantitative variables (serum FSH, $P_4$, $E_2$ concentrations and litter size) were evaluated according to D'Agostino-Pearson test for normality and Levene test for homogeneity of variances. A chi-square test for categorical variables was used to compare the groups in terms of fertility parameters (except litter size). The differences of litter size and serum $E_2$ concentration between groups was determined using ANOVA because of providing parametric test assumptions.

To evaluate the differences in serum FSH concentration in the groups at different sampling times, linear mixed model for repeated measures was used according to the model $Y_{ijk} = \mu + G_i + SD_j + (G\ x\ SD)_k + e_{ijk}$ , where $Y_{ijk}$ is the single observation, $\mu$ is the overall mean, $G_i$ is the effect of treatment (i = control, LET-4 and LET-0), $SD_j$ is the effect of sampling day (j = D-4, D-2, D0, D2 and D4), $(G\ x\ SD)_k$ is the interaction term and $e_{ijk}$ is the random error.

In addition, to evaluate the differences in serum $P_4$ concentration in the groups at different sampling times, linear mixed model for repeated measures was used also according to the model $Y_{ijk} = \mu + G_i + SD_j + (G\ x\ SD)_k + e_{ijk}$ , where $Y_{ijk}$ is the single observation, $\mu$ is the overall mean, $G_i$ is the effect of treatment (i = control, LET-4 and LET-0), $SD_j$ is the effect of sampling day (j = DM, DPM-14, DPM-21, DPM-35 and DPM-50), $(G\ x\ SD)_k$ is the interaction term and $e_{ijk}$ is the random error.

In cases where differences were found, simple effects analysis with Bonferroni correction was applied as a multiple comparison test. Descriptive statistics of quantitative variables were shown as "Least Square Mean ± Standard Error" and descriptive statistics of categorical variables were shown as " Percentage-Frequency". In all analyses, it was accepted as significant if the calculated *P*-values were less than 0.05, and as a tendency if $0.05 \leq P \leq 0.10$ and were performed using Stata version 15.1.

## 3. Results

### 3.1. Safety

No local or systemic side effects were observed following s.c. administration of 1 mg/kg B W. of letrozole or 1 mL/ewe of benzyl alcohol to ewes. Additionally, no adverse findings were observed in ewes during pregnancy (e.g., abortion) or in lambs after lambing (e.g., congenital anomalies).

### 3.2. Serum hormone ($E_2$, FSH and $P_4$) concentrations

Serum FSH concentrations (mIU/mL) showed statistically significant differences by group, day, and their interaction ($P < 0.001$) (Table 1). When comparing group means, the LET-4 group (27.68 ± 1.56 mIU/mL) had significantly higher FSH levels than the control group (18.54 ± 0.91 mIU/mL) ($P < 0.001$). In contrast, the LET-0 group (21.85 ± 0.87 mIU/mL) did not differ significantly from either the control or the LET-4 group ($P > 0.05$). Regarding changes according to days, FSH concentrations in the control group remained stable throughout the sampling days without significant fluctuations. In contrast, the LET-4 group exhibited a marked increase in FSH levels on D-2 (37.40 ± 4.02 mIU/mL) and D0 (34.19 ± 3.53 mIU/mL) compared to the treatment day (D-4; 18.65 ± 1.03 mIU/mL) and other groups. In the LET-0 group, a significant increase was observed on D2 (27.65 ± 0.82 mIU/mL) compared to both the treatment day (D0; 18.54 ± 1.30 mIU/mL) and the control group. However, the rise in FSH levels in the LET-0 group was clearly lower in magnitude and shorter in duration compared to the LET-4 group (Table 1).

Serum $E_2$ levels (pg/mL) on the day of mating (DM) showed statistically significant differences between the groups ($P < 0.001$). The LET-4 group had the highest $E_2$ level (22.24 ± 1.20 pg/mL), which was significantly higher than both the LET-0 (14.47 ± 0.92 pg/mL) and control groups (10.77 ± 0.69 pg/mL). Furthermore, the LET-0 group also had significantly higher $E_2$ levels than the control group but was significantly behind the LET-4 group.

Finally, serum $P_4$ levels were statistically significantly affected by both group and day factors ($P < 0.001$), but the interaction of group and day was not significant ($P = 0.603$). When mean serum $P_4$ levels of the groups were compared, the LET-4 group (3.79 ± 0.24 ng/mL) had a significantly higher $P_4$ concentration than the LET-0 (3.29 ± 0.24 ng/mL) and control (2.78 ± 0.24 ng/mL) groups. Due to the lack of a significant group and day interaction, intergroup comparisons by specific sampling day were not statistically evaluated. Nevertheless, on all post-mating sampling days (DPM-14, DPM-21, DPM-35, DPM-50), the LET-4 group had numerically higher serum $P_4$ levels than both the LET-0 and control groups (Table 2). It is also noteworthy that at the beginning of the study (D-4), all ewes ($n = 105$) exhibited serum $P_4$ concentrations (ng/mL) below 0.5 ng/mL (0.224 ± 0.008 ng/mL), indicating that all animals were in a state of deep anoestrus [44].

### 3.3. Fertility parameters

When the estrus results were evaluated, estrus induction rates were significantly different among the groups ($P = 0.010$). The LET-4 group exhibited the highest estrus response (90.62 %), which was significantly greater than both the control (57.57 %) and LET-0 (68.57 %) groups. No significant difference was observed between control and LET-0 (Table 3). Analysis of estrus onset timing showed that the majority of ewes expressed estrus between 36 and 48 h, with the highest proportion in the LET-4 group (75.86 %) compared to control (47.3 6 %) and LET-0 (58.33 %), although these differences were not statistically

**Table 1**
Serum follicle-stimulating hormone (FSH) concentrations (mIU/mL) by group and sampling day. Data are least-squares means ± standard error of the mean (SEM) for Control, LET-4, and LET-0 (D0: sponge insertion day).

| Parameter | Group | Day | | | | | *Overall Mean (Group)* | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | D-4 | D-2 | D0 | D2 | D4 | | G | D | G × D |
| **FSH (mIU/mL)** | **Control** | 16.77 ± 1.66 | 18.25 ± 1.86 $^{B}$ | 19.09 ± 1.97 $^{B}$ | 18.36 ± 2.19 $^{B}$ | 20.22 ± 2.51 | **18.54 ± 0.91** $^{B}$ | **<0.001** | **<0.001** | **<0.001** |
| | **LET-4** | 18.65 ± 1.03 $^{b}$ | 37.40 ± 4.02 $^{a, A}$ | 34.19 ± 3.53 $^{a, A}$ | 25.05 ± 3.08 $^{b, AB}$ | 23.10 ± 2.92 $^{b}$ | **27.68 ± 1.56** $^{A}$ | | | |
| | **LET-0** | 17.50 ± 1.59 $^{b}$ | 19.35 ± 1.49 $^{ab, B}$ | 18.54 ± 1.30 $^{ab, B}$ | 27.65 ± 0.82 $^{a, A}$ | 26.22 ± 2.65 $^{ab}$ | **21.85 ± 0.87** $^{B}$ | | | |
| ***Overall Mean (Day)*** | | **17.64 ± 0.83** $^{b}$ | **25.00 ± 1.99** $^{a}$ | **23.94 ± 1.74** $^{a}$ | **23.69 ± 1.38** $^{a}$ | **23.18 ± 1.57** $^{a}$ | | | | |

*Different lowercase superscripts (a, b, c) within the same row indicate statistically significant differences among the sampling days in the "Overall Mean (Days)" column ($P < 0.05$).
*Different uppercase superscripts (A, B) in the "Overall Mean (Group)" column indicate statistically significant differences among the treatment groups ($P < 0.05$). (G: Group; D: Day; G × D: Group × Day interaction).

**Table 2**
Serum progesterone ($P_4$) concentrations (ng/mL) by group and sampling day. Data are least-squares means ± standard error of the mean (SEM) for Control, LET-4, and LET-0 (DM: mating day; DPM: post-mating day).

| Parameter | Group | Day | | | | | *Overall Mean (Group)* | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | DM | DPM-14 | DPM-21 | DPM-35 | DPM-50 | | G | D | G × D |
| **$P_4$ (ng/mL)** | **Control** | 0.26 ± 0.04 | 3.58 ± 0.37 | 3.95 ± 0.45 | 3.01 ± 0.25 | 3.22 ± 0.26 | **2.78 ± 0.24** $^{B}$ | **<0.001** | **<0.001** | **0.603** |
| | **LET-4** | 0.28 ± 0.03 | 4.97 ± 0.35 | 5.29 ± 0.45 | 4.18 ± 0.24 | 4.20 ± 0.42 | **3.79 ± 0.24** $^{A}$ | | | |
| | **LET-0** | 0.23 ± 0.04 | 3.90 ± 0.29 | 4.59 ± 0.41 | 3.85 ± 0.42 | 3.88 ± 0.29 | **3.29 ± 0.24** $^{B}$ | | | |
| ***Overall Mean (Day)*** | | **0.26 ± 0.02** $^{c}$ | **4.33 ± 0.22** $^{ab}$ | **4.80 ± 0.27** $^{a}$ | **3.82 ± 0.19** $^{b}$ | **3.88 ± 0.23** $^{b}$ | | | | |

*Different lowercase superscripts (a, b, c) within the same row indicate statistically significant differences among the sampling days in the "Overall Mean (Days)" column ($P < 0.05$).
*Different uppercase superscripts (A, B) in the "Overall Mean (Group)" column indicate statistically significant differences among the treatment groups ($P < 0.05$). (G: Group; D: Day; G × D: Group × Day interaction).

**Table 3**
Fertility parameters of ewes treated with letrozole (1 mg/kg) on the day of sponge insertion (LET-0) or four days before (LET-4) compared to Control.

| Fertility parameters | Groups | | | *P*- value |
|---|---|---|---|---|
| | Control ($n$ = 33) | LET-4 ($n$ = 32) | LET-0 ($n$ = 35) | |
| **Estrus (%)** | **57.57 (19/33)** $^{a}$ | **90.62 (29/32)** $^{b}$ | **68.57 (24/35)** $^{a}$ | **0.010*** |
| **21st day pregnancy (%)** | **36.36 (12/33)** $^{a}$ | **68.75 (22/32)** $^{b}$ | **42.85 (15/35)** $^{a}$ | **0.022*** |
| **35th day pregnancy (%)** | **30.30 (10/33)** $^{e}$ | **59.37 (19/32)** $^{d}$ | **40.00 (14/35)** $^{ed}$ | **0.055**** |
| **50th day pregnancy (%)** | **27.27 (9/33)** $^{a}$ | **59.37 (19/32)** $^{b}$ | **37.14 (13/35)** $^{a}$ | **0.027*** |
| **Late embryonic loss (%)** | 16.66 (2/12) | 13.63 (3/22) | 6.66 (1/15) | 0.707 |
| **Early fetal loss (%)** | 10.00 (1/10) | 0.00 (0/19) | 7.14 (1/14) | 0.413 |
| **Total pregnancy loss (%)** | 25.00 (3/12) | 13.63 (3/22) | 13.33 (2/15) | 0.645 |
| **Twinning (%)** | 22.22 (2/9) | 47.36 (9/19) | 53.84 (7/13) | 0.312 |
| **Litter size** | 1.22 | 1.47 | 1.53 | 0.321 |
| **Fecundity (%)** | **33.33 (11/33)** $^{a}$ | **87.50 (28/32)** $^{b}$ | **57.14 (20/35)** $^{c}$ | **<0.001*** |

*Different superscripts (a, b, c) in the same row indicate significant differences among the groups ($P < 0.05$).
**Different superscripts (d, e) in the same row indicate tendency differences ($0.05 \leq P \leq 0.1$) among the groups.

**Table 4**
The percentage (%) of ewes showing estrus at different time intervals (h) following sponge removal in groups treated with letrozole (1 mg/kg) on the day of sponge insertion (LET-0) or four days before (LET-4) compared to Control.

| Time periods | Groups | | | *P*- value |
|---|---|---|---|---|
| | Control ($n$ = 19) | LET-4 ($n$ = 29) | LET-0 ($n$ = 24) | |
| **24–36 h** | 0.0 (0/19) | 0.0 (0/29) | 0.0 (0/24) | – |
| **36–48 h** | 47.36 (9/19) | 75.86 (22/29) | 58.33 (14/24) | 0.120 |
| **48–60 h** | 31.57 (6/19) | 17.24 (5/29) | 25.00 (6/24) | 0.335 |
| **60–72 h** | 21.05 (4/19) | 6.89 (2/29) | 16.66 (4/24) | 0.340 |

significant ($P$ = 0.120). No estrus activity was observed in any group within the first 36 h after sponge removal (Table 4).

Considering pregnancy rates, the LET-4 group (68.75 %) had a significantly higher rate than the control (36.36 %) and LET-0 (42.85 %) groups at day 21 ($P$ = 0.022). However, pregnancy rates at day 35 tended to differ between groups ($P$ = 0.055); the LET-4 group (59.37 %) was higher than the control group (30.30 %), while the LET-0 group (40.00 %) was similar to both groups. At day 50, this difference reached statistical significance again ($P$ = 0.027); the LET-4 group (59.37 %) was significantly higher than the control (27.27 %) and LET-0 (37.14 %) groups (Table 3). On the other hand, no significant difference was found in late embryonic loss, early fetal loss, or total pregnancy loss between the groups ($P > 0.05$).

Finally, when offspring productivity was evaluated, there was no statistically significant difference between the groups in terms of twinning and litter size ($P > 0.05$). In contrast, fecundity rates differed significantly between the groups ($P < 0.001$). LET-4 achieved the highest fecundity (87.50 %), followed by LET-0 (57.14 %), both of which were significantly higher than the control group (33.33 %) (Table 3).

## 4. Discussion

The present study is, to the best of our knowledge, the first clinical investigation evaluating the effects of letrozole administration either four days before (D-4) or on the initiation day (D0) of a short-term $P_4$ based synchronization protocol on protocol success and reproductive

performance in suckling ewes during the non-breeding season. Overall, the findings support our hypothesis by showing that letrozole treatment prior to synchronization significantly improves both protocol success and reproductive outcomes, and that the extent of this improvement is markedly influenced by the timing of administration.

When evaluating FSH concentrations, which constitute the basis of the study's main hypothesis, both treatment groups (LET-4 and LET-0) exhibited varying degrees of post-treatment increases in FSH compared to the control group (Table 1). These findings are consistent with previous reports indicating that letrozole treatment elevates FSH levels in humans [45], cattle [46], and ewes [11]. Aromatase inhibition selectively suppresses circulating $E_2$ concentrations [28,31]. In anoestrous ewes, the negative feedback exerted by $E_2$ on the HPG axis suppresses endogenous gonadotropin release [10]. Therefore, letrozole treatment in anoestrous ewes may attenuate the inhibitory action of $E_2$ on the HPG axis, thereby promoting increased gonadotropin secretion [11]. Moreover, letrozole has been reported to elevate intra-follicular androgen concentrations, which can upregulate FSH receptor expression in granulosa cells and enhance follicular responsiveness to FSH [32,47]. In line with our findings, Kıvrak et al. [11] demonstrated that a single s.c. injection of letrozole at 1 mg/kg B W. in anoestrous ewes significantly increased circulating FSH concentrations, with a reported bioavailability of 75.34 % and a half-life of 41.07 h. Although FSH levels in the LET-0 group were higher than in the control group, the increase was substantially lower than that observed in the LET-4 group. While this discrepancy requires further investigation, it may be partly explained by the timing of letrozole administration in the LET-0 group, coinciding with the insertion day (D0) of the intravaginal MAP sponge. It is well established that high $P_4$ concentrations exert an inhibitory effect on hypothalamic GnRH pulse frequency [48]. Although this effect primarily suppresses LH secretion, $P_4$ has also been reported to reduce FSH peak amplitude in anoestrous ewes [49]. Therefore, in the LET-0 group, the elevated $P_4$ environment resulting from intravaginal MAP insertion may have limited or suppressed the stimulatory effect of letrozole on FSH secretion.

In line with the FSH increase observed prior to synchronization, serum $E_2$ concentration at the time of mating was significantly higher in the LET-4 group compared to the other groups ($P < 0.001$). Although ovarian ultrasonography would be required to definitively determine the source of elevated $E_2$, this increase likely originated from a greater number and/or size of developing follicles. Indeed, previous studies have demonstrated a strong correlation between serum $E_2$ concentrations and both follicle number and diameter [23,50,51]. Supporting our findings, Abdeldaiem et al. [50] reported that intravaginal administration of letrozole for five days during the breeding season significantly increased the number and size of follicles in ewes compared to the conventional GPG protocol, which was associated with higher serum $E_2$ levels at estrus. Similarly, in our study, the increased FSH concentrations observed following letrozole administration may have supported the development of a new follicular cohort in anestrous ewes prior to synchronization, thereby contributing to the formation of a more advanced follicular population during the subsequent synchronized estrus.

The most prominent support for this assumption is the significantly elevated serum $P_4$ concentrations observed throughout the luteal phase in the LET-4 group (Table 2). It is well established that the diameter and maturity of follicles developing after synchronization directly affect the morphological and functional capacity of the CL formed following ovulation [52,53]. Indeed, several studies in ewes and cattle have reported a positive correlation between dominant follicle diameter, CL size, and serum $P_4$ concentrations [40,51,54]. In addition, the number of dominant follicles developing after synchronization has also been proposed to contribute to luteal $P_4$ output. The stimulation of follicular development may result in an increased number of follicles, leading to the formation of multiple CLs after ovulation and thereby enhancing $P_4$ production [40,55]. Although this could not be directly confirmed via ovarian ultrasonography in the present study, our findings suggest that the enhanced follicular response observed during the synchronization protocol may have increased the number and/or size of ovulating dominant follicles, which in turn may have contributed to elevated luteal $P_4$ concentrations. In line with our findings, it has been reported that letrozole treatment increased the number and diameter of preovulatory follicles in ewes [50] and cattle [29], which subsequently elevated the number and area of CLs, resulting in higher luteal-phase $P_4$ concentrations and indicating luteotropic effects. Therefore, the high $P_4$ concentrations observed in the LET-4 group of our study support the previously reported luteotropic effects of letrozole and provide additional evidence that it may enhance follicular development.

The most critical indicator of synchronization protocol efficacy during the non-breeding season is the estrus response rate [56]. In this context, the LET-4 group in our study demonstrated a significantly higher estrus response rate (90.62 %) compared to the control (57.57 %) and LET-0 (68.57 %) groups, which was consistent with the elevated $E_2$ concentrations observed in this group ($P = 0.010$) (Table 3). In anoestrous ewes, suppressed endogenous gonadotropin levels limit post-synchronization follicular development, ovulation success, and estrus expression [4,9,10]. In our study, the letrozole-induced increase in FSH secretion appears to have partially overcome this limitation and contributed to improved protocol performance. This finding aligns with previous reports in ewes and cattle indicating that letrozole treatment enhances ovarian response and follicular dynamics [11,34–36,40,46,50,57]. Accordingly, our findings provide promising evidence that letrozole administration prior to synchronization may exert a priming effect that supports follicular development and improves synchronization success.

The extent to which the increased estrus rates translate into improved pregnancy outcomes is crucial for evaluating the overall effectiveness of synchronization protocols. Synchronization during the non-breeding season is often associated with limited success, and pregnancy rates remain substantially lower than those observed during the breeding season [5,7,12]. Moreover, the lactational and suckling status of ewes strengthens the negative feedback mechanisms on the HPG axis, further compromising fertility [4,21,23,44,58]. This is reflected in the low pregnancy rate observed in the control group (27.27 %) (Table 3). Conversely, the hormonal improvements and enhanced estrus response induced by letrozole led to significantly higher pregnancy rates, especially in the LET-4 group, which outperformed both the LET-0 and control groups on all examination days (Table 3). Since there is no previous study directly investigating the use of letrozole in suckling ewes during anoestrus, it is difficult to conduct a direct comparison. Nevertheless, our results are in line with findings in ewes and women, where letrozole has shown potential to improve pregnancy outcomes [38,50,59] The improved pregnancy rates observed in our study may be attributed to the stimulatory effects of letrozole on folliculogenesis, which enhanced estrus expression, increased luteal-phase $P_4$ concentrations, and likely established a more favourable hormonal environment for embryo implantation. Similarly, Abdeldaiem et al. [50] reported that intravaginal letrozole administered for five days improved pregnancy rates in ewes compared to the GPG protocol (60.0 % vs. 50.0 %), with this numerical increase considered a notable trend despite lacking statistical significance ($P > 0.05$). On the other hand, the improvement observed with letrozole was comparable to outcomes achieved through early weaning strategies [19,22–24], underscoring its high potential. Indeed, Ronquillo et al. [21] showed that early weaning significantly improved estrus (92 % vs. 52 %) and pregnancy (76 % vs. 28 %) rates ($P < 0.001$). In this context, letrozole yielded similarly high pregnancy success despite the suppressive effects of suckling, highlights its considerable clinical promise.

Finally, when lambing performance was evaluated, both litter size and twinning rate were numerically higher in the treatment groups compared to the control; however, these differences did not reach statistical significance (Table 3). Nevertheless, the fecundity rate was found to be significantly higher in the LET-4 (87.50 %) and LET-0 (57.14 %) groups compared to the control group (33.33 %) ($P < 0.001$), indicating

a potential trend toward improved lamb production that extends beyond merely increased pregnancy rates. The notably high fecundity observed in the LET-4 group supports our hypothesis that letrozole may enhance reproductive performance by promoting folliculogenesis and exerting a luteotropic effect. Fecundity, as a critical production outcome, reflects not only pregnancy success but also the number of lambs produced per animal [60]. Consistent with our results, previous studies have suggested that letrozole may stimulate folliculogenesis, increase follicle numbers, and induce multiple ovulations, thereby laying the groundwork for improved prolificacy [29,32,38,50]. Indeed, Abdeldaiem et al. [50] reported that letrozole treatment significantly increased not only pregnancy rates but also twinning rate and total lamb yield. In our study, the significant improvement in fecundity, despite the lack of statistical difference in twinning and overall lamb yield, may be attributed to the limited sample size. Therefore, further studies involving larger experimental groups and including detailed assessment of follicle number are needed to clarify the potential effects of pre-synchronization letrozole treatment on multiple pregnancies and total lamb production.

## 5. Conclusion

The overall findings of the study supported our hypothesis that administration of letrozole prior to short-term $P_4$ based synchronization protocols could significantly improve reproductive outcomes in suckling ewes in the non-breeding season. The results demonstrated that early administration significantly positively impacted gonadotropic activity, $E_2$ secretion, luteal function, and ultimately fertility performance, highlighting the critical importance of treatment timing. Specifically, they provide promising evidence that a single dose of letrozole (1 mg/kg, s.c.) administered four days before the synchronization protocol can effectively prime the follicular environment, overcome the neuroendocrine suppression associated with suckling and seasonal anoestrus, and promote more synchronized and efficient ovulatory responses. However, to validate this potential, further studies with larger sample sizes are needed to assess the reproducibility of the findings under different farm conditions, optimize the timing of administration, and clarify the effects of the treatment at the ovarian level in greater detail.

## CRediT authorship contribution statement

**Gökhan Uyanık:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Data curation, Conceptualization. **Murat Abay:** Writing – review & editing, Resources, Project administration, Methodology. **Ahmet Gözer:** Writing – review & editing, Investigation. **Murat Yüksel:** Writing – review & editing, Investigation. **Ebru Arslanhan:** Investigation. **Filiz Kara:** Investigation. **Ufuk Kaya:** Writing – review & editing, Validation, Formal analysis.

## Funding

This research was supported by the Erciyes University Scientific Research Projects Coordination Unit, Türkiye, project number THD-2025-14805.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Murat Abay reports financial support was provided by Erciyes University Scientific Research Projects Coordination Unit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We would like to thank the students of the Faculty of Veterinary Medicine at Hatay Mustafa Kemal University for their generous assistance during the fieldwork.

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