A Novel Subcutaneous Progesterone-Loaded Chitosan Microchip for Estrus Synchronization in Ewes: Comparative Evaluation with Conventional Vaginal Sponges and the Impact of eCG versus Vitamin E/Selenium on Fertility Outcomes

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Abstract

This study's objective Aim: This study aimed to compare the efficacy of two estrus synchronization methods in ewes conventional intravaginal progesterone sponges and a novel progesterone-loaded crosslinked chitosan microchip administered subcutaneously via the ear and to evaluate the effects of subsequent eCG or Vitamin E/Selenium (Vit. E/Se) supplementation on reproductive performance and hormonal profile.

Materials and methods: Thirty clinically healthy adult ewes (2–4 years old) were randomly allocated into two main groups (n = 10 each): Group T1, synchronized using intravaginal progesterone sponges (Chronogest CR) for 14 days, and Group T2, synchronized using subcutaneous progesterone-loaded chitosan microchips. Each group was further divided into three subgroups (n = 5): a single intramuscular injection of 500 IU eCG, a single intramuscular injection of 2 mL Vit. E/Se, or no treatment (control). Blood samples were collected at baseline, at device removal, and 24 hours post-treatment to determine serum progesterone, estrogen, FSH, and LH concentrations by ELISA. Estrus behavior was monitored using teaser rams, and reproductive performance (estrus response, pregnancy, lambing, fertility, and multiple lambing rates) was recorded. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test ($P \leq 0.05$).

Results: Estrus response was 100% across all groups regardless of synchronization method or treatment. Pregnancy, lambing, and fertility rates were lowest in the control group (40%), intermediate in the sponge + eCG group (80%), and highest in the sponge + eCG + Vit. E/Se and microchip + eCG groups (100% each), while the microchip + Vit. E/Se group achieved 60%. The microchip + eCG group also recorded the highest multiple lambing rate (100%). Both synchronization methods induced significant hormonal fluctuations ($P \leq 0.05$), characterized by elevated progesterone during device application, a marked post-removal decline, and a subsequent surge in estrogen, FSH, and LH indicative of renewed follicular activity. The subcutaneous microchip group exhibited a more pronounced post-treatment hormonal rebound compared with the sponge group, suggesting more effective luteal priming.

Conclusion: The progesterone-loaded crosslinked chitosan microchip is an effective and reliable alternative to conventional intravaginal sponges for estrus synchronization in ewes, offering comparable or superior reproductive and hormonal outcomes, particularly when combined with eCG injection. These findings support the microchip's potential as an innovative, minimally invasive tool for reproductive management in small ruminants.

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Introduction

In response, ewes ranchers have embraced innovative artificial reproductive techniques like as estrus synchronization. One interesting way to increase ewes' fertility is to synchronize their estrus cycles. Estrus synchronization boosts reproductive efficiency, and the ability to select when to give birth results in production benefits (Hasani *et al.*, 2018) [10].

In the ewes business, exogenous hormones are frequently utilized for superovulation and estrus synchronization, giving farmers significant control over the timing of flock mating and guaranteeing increased reproductive efficiency (Kusina *et al.*, 2000) [11].

The best way to induce estrus in ewes during the non-breeding season is to administer progesterone, which prolongs the luteal phase in these ewes. They become synchronized as a result, and the ewes enter estrus at the same time when the hormonal treatment is stopped. Ewes frequently use fluorogestone acetate (FGA) sponges to synchronize their estrus (Rowe *et al.*, 2009, Souza *et al.*, 2011, Zohara *et al.*, 2014) [16, 18, 24].

Regardless of the stage of the estrus cycle or the condition of the ovaries at the time of apparatus insertion, FGA or CIDR remain in the vagina for 12–14 days during typical estrus synchronization, imitating the life of a corpus luteum (Menchaca and Rubianes 2004) [13]. Ewes that receive an injection of equine chorionic gonadotropin (eCG) typically enter estrus 24 to 48 hours after the intravaginal device is removed (Wildeus 2000, Swelum *et al.*, 2015) [21, 19].

Micronutrient imbalances and inadequacies, however, are frequently only identified after health problems have already appeared (Masters, 2018) [12]. In comparison to non-ruminants, the rumen environment and the activity of rumen bacteria that transform selenium (Se) into an insoluble form lower the absorption capacity of Se in ruminants (Xun *et al.*, 2012).

Selenium absorption in ruminants is only about 35%, compared with almost 85% in non-ruminants (Galbraith *et al.*, 2016) [9]. Because selenium is thought to maintain the corpus luteum's proper function, it has been associated with increased ovarian structures, ovarian size, and the number of corpora lutea, all of which should be reflected in higher fertility (Cabrera-Mora *et al.*, 2019) [5].

According to Cabrera-Mora *et al.*, (2019) [5] and Wu *et al.*, (2019), vitamin E's strong lipid-soluble antioxidant activity optimizes and strengthens the immune response by preventing lipid peroxidation and removing free radicals, both of which are immunosuppressive and result in cell and tissue damage. It is unclear how supplementing with selenium and vitamin E affects the reproductive performance of sheep given intravaginal sponges.

Materials and methods

Experimental Animals

The study was conducted on 30 clinically healthy adult ewes, aged 2–4 years and with similar body weight. All animals were maintained under uniform management, feeding, and environmental conditions throughout the study period. Clinical examination ensured that all ewes were free from reproductive or systemic disorders before inclusion in the study (Abduljaleel *et al*., 2025; Naeem et al., 2026) [1, 14]

Experimental Design

Ttwo experimental groups (n = 15 per group) were randomly assigned to the animals based on the estrus synchronization technique and subsequent treatment:

Group T1 (Sponge group):

Ewes were synchronized using intravaginal progesterone sponges. After sponge removal, ewes were further divided into two subgroups (n = 5 per subgroup):

T1a: Five ewes injected with 500 IU of equine chorionic gonadotropin (eCG) intramuscularly**.**

T1b: Five ewes injected with 2 mL per ewe of Vitamin E/Selenium (Vit. E/Se) intramuscularly**.**

T1c (Control group): Five ewes received no hormonal or antioxidant supplementation and served as the control group for evaluating natural estrus response.

Intravaginal Sponge Insertion*:*

A sterile applicator was used to introduce synthetic intravaginal sponges (Chronogest CR, an intravaginal sponge loaded with 20 mg of a synthetic progesterone-like hormone) into each ewe's vagina. To reduce the danger of infection, the vulva was cleansed with an antiseptic solution before insertion.

Duration of Treatment

The sponges were left in place for 14 days,after which they were carefully removed**.**

Fig 1: (A) Chronogest® CR, an intravaginal sponge impregnated with 20 mg of the synthetic progestagen cronolone (flugestone acetate). (B) Pregnant mare serum gonadotropin (PMSG), commonly referred to as equine chorionic gonadotropin (eCG), in vials containing 5,000 international units (IU)

Fig 2: (A) Day 0: gentle insertion of the Chronogest® sponge using the applicator.
(B) Day of sponge removal, with intramuscular injection of 500 IU PMSG (Intervet®) or intramuscular injection of 2mL/ewe vitamin E and selenium solution.
(C) Estrus detection and mating, 24–48 hours after sponge removal.
(D) Ultrasonographic pregnancy diagnosis on day 24 post-mating.

Group T2 (Microchip group)
Estrus was synchronized using progesterone-saturated chitosan microchips
injected subcutaneously into the ear, which functioned similarly to vaginal sponges. The microchips were inserted sterilely and left in place for the required synchronization period**.**
T2a (eCG-treated group): Five ewes received a single intramuscular injection of 500 IU equine chorionic gonadotropin (eCG).
T2b (Vitamin E/Selenium-treated group): Five ewes received a single intramuscular injection of 2 mL Vitamin E/Selenium (Vit. E/Se) combination.
T2c (Control group): Five ewes received no hormonal or antioxidant supplementation and served as the control group for evaluating natural estrus response.

Preparation of progesterone-Loaded Crosslinked Chitosan Polymer
One gram of chitosan was dissolved in a 2.0% aqueous acetic acid solution (50 mL), and an appropriate amount of progesterone (3mL) was added at room temperature with continuous stirring for 30 minutes to obtain a pale yellow, viscous chitosan solution. Then, 5 mL of glutaraldehyde solution was included in the answer above. At room temperature, the mixture was swirled for ten minutes, during which it became increasingly viscous and more intensely colored. Subsequently, 5 mL of 10% sodium hydroxide solution was added. The crosslinked polymer containing the drug was then dried at room temperature for a 24 day to eliminate any remaining solvent. Berger, et al.,(2004) [4]; Rinaudo, (2006) [15].

Injection Site Preparation
The external surface of the ear was cleaned with 70% ethyl alcohol to ensure sterile conditions**.**

Injection Procedure
A sterile microchips were used**.**

Post-Injection Care
The injection site was monitored for at least 30 minutes for any local reactions such as swelling or redness**.**No adverse effects were recorded**(0.27 g)**

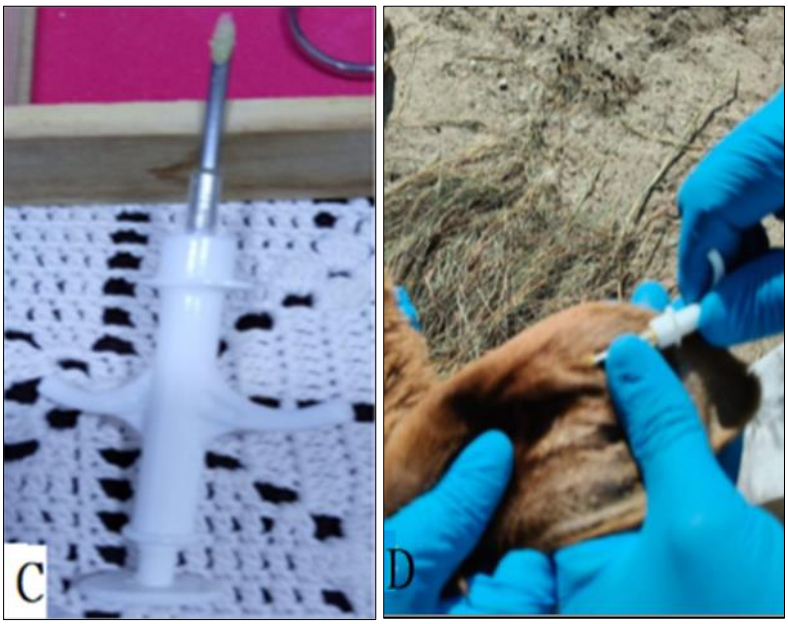

Fig 3: Preparation and subcutaneous administration of progesterone-loaded chitosan microchips in ewes.
A) Chitosan polymer matrix saturated with progesterone during the preparation process..
B) Precision analytical balance used for accurate measurement of the polymer quantity..
C) Progesterone-loaded chitosan microchips prepared for subcutaneous application..
D) Subcutaneous injection of the prepared formulation into the ear region of the ewe.

Blood Drawing and Hormone Testing

The jugular vein was used to draw blood samples (~5 mL). of all ewes at the following time points (Al-Alywi et al.,2025) [2]*:*

- Before estrus synchronization (baseline).
- At sponge or microchip removal.
- After treatment administration at specific intervals (24 hours*).*

After allowing the blood samples to coagulate at room temperature, they were centrifuged for 15 minutes at 3,000 rpm. Until hormonal analysis, the isolated serum was kept at -20 °C.*.*

Commercial ELISA kits were used to measure the following hormones' serum concentrations*:*

Progesterone (ng/ml)

estrogen (pg/ml)

FSH, or follicle-stimulating hormone (mIU/ml)

Hormone Luteinizing (LH, mIU/ml).

Estrus Detection

Ewes were monitored for estrus behavior for 24-48 hours Estrus and mating hours after sponge removal using teaser rams.

Clinical After Sponge Removal + eCG Injection (500 IU) or injected with 2 ml/ewe of Vitamin E/Selenium (Vit.E/Se).

Onset of Estrus

After removing the progesterone sponge and injecting eCG or injected with 2 ml/ewe of Vitamin E/Selenium (Vit.E/Se),Estrus usually appears in 24–48 hours.

Statistical Analysis

The standard error of the mean (SEM) was used to express the data. One-way analysis of variance (one-way ANOVA) was used in statistical studies to compare hormonal measures at various sampling stages as well as reproductive performance between the experimental groups. Tukey's post hoc multiple comparison test was performed to identify differences between means when significant differences were found. At ($P \leq 0.05$)., differences were deemed statistically significant. Statistically significant differences ($P \leq 0.05$). are shown by means with distinct superscript letters within the same row or column.

Result

Table 1. shows the results of ewe reproductive performance after estrus synchronization with various estrus synchronization programs. Estrus response rate was 100% in all experimental groups (control and treated) and this suggests that all the animals responded to the estrus synchronization treatments irrespective of the treatments. As for pregnancy rate, the lowest pregnancy rate was obtained in the control group (40%) and the group treated with vaginal sponge removal followed by eCG injection (80%).

The pregnancy rate for the group treated with sponge removal and eCG injection and Vitamin E/Selenium supplementation was the highest at 100%, which was similar to the Microchip Progesterone removal, eCG injection group (100%). The Microchip Progesterone removal with Vitamin E/Selenium injection group had a 60% in comparison. A similar pattern was observed for lambing rate and fertility rate, both mirroring the pregnancy rate values across all groups (40%, 80%, 100%, 100%, and 60%, respectively).With respect to multiple lambing rate, the control group showed the lowest percentage (20%), followed by the sponge + eCG group (60%). Notably, the Microchip Progesterone with eCG injection group achieved the highest multiple lambing rate at 100%, whereas both sponge + Vitamin E/Selenium and Microchip + Vitamin E/Selenium groups recorded 40%.

Overall, these findings suggest that combining progesterone-based synchronization with eCG injection, particularly using the Microchip Progesterone device, yields superior reproductive outcomes in ewes compared to the control and other treatment protocols.

Table 1: Reproductive performance of ewe following estrus synchronization by different protocols(vaginal sponges and Subcutaneous Microchip Progesterone)

Parameter	After Microchip Progesterone Removal + Vitamin E/Selenium	After Microchip Progesterone Removal + eCG	After Sponge Removal + Vitamin E/Selenium	After Sponge Removal + eCG	Control
Number	5	5	5	5	5
Estrus response (%)	100%	100%	100%	100%	100%
Pregnancy rate (%)	60%	100%	100%	80%	40%
Lambing rate (%)	60%	100%	100%	80%	40%
Fertility (%)	40%	100%	40%	60%	20%
Multiple lambing (%)	—	—	—	—	—

Fertility%=Number of pregnant ÷Number of mated x100

Hormonal profiles in ewes showed statistically significant differences among treatment stages ($P \leq 0.05$), as indicated by superscript letters Table 2 Progesterone concentrations differed significantly between groups and stages ($P \leq 0.05$). Before treatment, progesterone was significantly lower prior to vaginal sponge application (0.144 ± 0.021 ng/ml) compared to before subcutaneous progesterone administration (0.51 ± 0.012 ng/ml). During treatment, progesterone levels increased significantly, reaching 1.96 ± 0.050 ng/ml with vaginal sponges and peaking at 2.60 ± 0.158 ng/ml during subcutaneous progesterone administration. Following treatment, progesterone concentrations decreased significantly after sponge removal and eCG injection (0.43 ± 0.0086 ng/ml), but remained significantly higher after withdrawal of subcutaneous progesterone with eCG administration (1.18 ± 0.58 ng/ml).

The estrogen level was also found to vary significantly among the different treatment stages ($P \leq 0.05$). The subcutaneous progesterone group had significantly higher levels of estrogen (23.00 ± 0.71 pg/ml) than the sponge group (22.00 ± 0.95 pg/ml) before treatment. Levels of estrogen significantly fell to 16.20 ± 0.58 pg/ml with vaginal sponges and 16.00 ± 0.71 pg/ml with subcutaneous progesterone administration during treatment. Estrogen levels were found to be significantly higher post treatment after eCG injection (30.00 ± 0.035 pg/ml) and after subcutaneous withdrawal of progestogen (53.80 ± 1.85 pg/ml).

FSH concentrations exhibited significant differences among stages ($P \leq 0.05$). Before treatment, FSH levels were significantly higher in the sponge group (2.12 ± 0.058 mIU/ml; than in the subcutaneous progesterone group (1.76 ± 0.19 mIU/ml). During treatment, FSH levels remained relatively stable in the sponge group (1.90 ± 0.045 mIU/ml; ^{A}) but decreased significantly during subcutaneous progesterone treatment (0.51 ± 0.178 mIU/ml). After treatment, FSH increased significantly following sponge removal and eCG injection (6.10 ± 0.12 mIU/ml) and then decreased significantly after subcutaneous progesterone withdrawal (3.20 ± 0.11 mIU/ml).

Similar significant trend ($P \leq 0.05$) was observed in the LH concentrations. The sponge group had greater LH levels before treatment (1.54 ± 0.051 mIU/ml) than the subcutaneous progesterone group (0.189 ± 1.54 mIU/ml). LH levels were significantly reduced during treatment in both groups, to 1.22 ± 0.037 mIU/ml (^{A}) in the sponge group and 0.67 ± 0.093 mIU/ml in the subcutaneous progesterone group. LH showed a slight increase following treatment with eCG after sponge removal (1.60 ± 0.071 mIU/ml), but was significantly higher following subcutaneous withdrawal of progesterone with eCG (5.56 ± 0.18 mIU/ml).

Table 2: Hormonal Changes in Ewes Treated with Vaginal Sponges and Subcutaneous Progesterone Injected with 500 IU eCG After Removal Vaginal Sponges and Subcutaneous Progesterone (Mean ± Error)

Parameter	Before Applying Vaginal Sponges	Before Applying Subcutaneous Progesterone	During Applying Vaginal Sponges	During Applying Subcutaneous Progesterone	After Removal of Vaginal Sponges + eCG (24 h)	After Removal of Subcutaneous Progesterone + eCG (24 h)
Progesterone levels (ng/ml)	0.144 ± 0.021 B	0.51 ± 0.012 A	1.96 ± 0.050 B	2.60 ± 0.158 A	0.43 ± 0.0086 B	1.18 ± 0.58 A
Estrogen levels (pg/ml)	22.00 ± 0.95 B	23.00 ± 0.71 A	16.20 ± 0.58 A	16.00 ± 0.71 B	30.00 ± 0.035 B	53.80 ± 1.85 A
FSH levels (mIU/ml)	2.12 ± 0.058 A	0.51 ± 0.178 B	1.90 ± 0.045 A	0.51 ± 0.178 B	6.10 ± 0.12 A	3.20 ± 0.11 B
LH levels (mIU/ml)	1.54 ± 0.051 A	1.54 ± 0.189 B	1.22 ± 0.037 A	0.67 ± 0.093 B	1.60 ± 0.071 B	5.56 ± 0.18 A

* Different capital letters mean significant ($P \leq 0.05$) differences within column

As presented in Table 3 hormonal concentrations in ewes showed significant variations among treatment stages ($P \leq 0.05$), as indicated by different superscript letters .Progesterone levels differed significantly across stages. Before treatment, progesterone was higher prior to vaginal sponge application (0.46 ± 0.031 ng/ml) compared to before subcutaneous progesterone administration (0.19 ± 0.035 ng/ml). During treatment, progesterone increased significantly during vaginal sponge application (2.18 ± 0.026 ng/ml), whereas it was significantly lower during subcutaneous progesterone treatment (1.84 ± 0.187 ng/ml). After removal and Vitamin E/Selenium injection, progesterone decreased significantly following sponge removal (0.378 ± 0.010 ng/ml), but remained significantly higher after withdrawal of subcutaneous progesterone (0.92 ± 0.12 ng/ml) (Table 3.3).

Estrogen levels also showed significant differences ($P \leq 0.05$). Before treatment, estrogen was higher in the subcutaneous progesterone group (22.91 ± 0.39 pg/ml) compared to the sponge group (21.60 ± 0.169 pg/ml). During treatment, estrogen decreased significantly to 12.60 ± 0.40 pg/ml during sponge application, while it was relatively higher during subcutaneous progesterone (14.69 ± 0.34 pg/ml).After treatment, estrogen increased significantly following injection, reaching 29.00 ± 0.254 pg/ml after sponge removal and peaking at 48.18 ± 0.70 pg/ml after subcutaneous progesterone withdrawal (Table 3.3).

FSH levels varied significantly among stages ($P \leq 0.05$), as shown in Table 3.3. Before treatment, FSH was higher in the sponge group (2.56 ± 0.051 mIU/ml) than in the subcutaneous progesterone group (1.64 ± 0.048 mIU/ml). During treatment, FSH remained relatively stable during sponge application (1.54 ± 0.0245 mIU/ml) but decreased significantly during subcutaneous progesterone (0.67 ± 0.014 mIU/ml). After treatment, FSH increased markedly following sponge removal and injection (7.10 ± 0.187 mIU/ml) and then declined significantly after subcutaneous progesterone withdrawal (3.73 ± 0.011 mIU/ml).

A similar pattern was observed for LH levels ($P \leq 0.05$). Initial levels of LH were higher in the sponge group (1.86 ± 0.051 mIU/ml) than in the subcutaneous group receiving PGE2 (1.43 ± 0.0302 mIU/ml).Both groups showed a decrease in LH during treatment, ending at 1.18 ± 0.0734 mIU/ml in the sponge and 0.60 ± 0.010 mIU/ml in the subcutaneous progesterone. Following treatment, LH levels were significantly higher after sponge removal (4.14 ± 0.012 mIU/ml) and after subcutaneous withdrawal of the progesterone (5.12 ± 0.038 mIU/ml) (Table 3).

The changes in the hormones listed in Table 3are the physiological response to the estrus synchronization with progesterone plus antioxidant (Vitamin E/Selenium) stimulation. During administration of the vaginal sponge, there is a significant rise in the level of progesterone, indicating effective exogenous progesterone release which mimics the luteal phase with suppression of ovulation.

Differences in release and absorption efficiency are suggested by the somewhat lower levels observed are suggested by the somewhat lower levels with subcutaneous progesterone therapy. The dramatic drop in progesterone after stopping medication signifies the loss of luteal support, which is necessary to start follicular development and estrus. The subsequent increase in estrogen levels (especially after asubcutaneous progesterone with drawal) indicates a rise in the synthesis of estradiol and follicular activity. Negative feedback action of Progesterone on Hypothalamic-pituitary axis is evident by the suppression of FSH and LH during therapy.

After removal, the notable increase in gonadotropins (FSH and LH) following vitamin E/selenium injection suggests that the ovarian function is reactivated and this leads to expansion of the follicles and ovulation. The subcutaneous progesterone group's larger post-treatment hormonal responses suggest a bigger rebound effect, perhaps as a result of extended hormonal priming prior to withdrawal.

Table 3: Application of Subcutaneous Microchip Progesterone and Vaginal Sponges on Hormonal Levels in Ewes After Subcutaneous Progesterone and Vaginal Sponges Removed and Vitamin E/Selenium Injected (Mean ±SE).

Parameter	Before Applying Vaginal Sponges	Before Applying Subcutaneous Progesterone	During Applying Vaginal Sponges	During Applying Subcutaneous Progesterone	After Removal of Vaginal Sponges + Vitamin E/Selenium (24 h)	After Removal of Subcutaneous Progesterone + Vitamin E/Selenium (24 h)
Progesterone levels (ng/ml)	0.46 ± 0.0311 A	0.19 ± 0.035 B	2.180 ± 0.026 A	1.84 ± 0.187 B	0.378 ± 0.010 B	0.92 ± 0.12 A
Estrogen levels (pg/ml)	21.60 ± 0.1691 B	22.91 ± 0.39 A	12.60 ± 0.40 B	14.69 ± 0.34 A	29.00 ± 0.254 B	48.18 ± 0.70 A
FSH levels (mIU/ml)	2.56 ± 0.051 A	1.64 ± 0.048 B	1.54 ± 0.0245 A	0.67 ± 0.014 B	7.10 ± 0.187 A	3.73 ± 0.011 B
LH levels (mIU/ml)	1.86 ± 0.051 A	1.43 ± 0.0302 B	1.180 ± 0.0734 A	0.60 ± 0.010 B	4.14 ± 0.012 B	5.12 ± 0.038 A

* Significant ($P \leq 0.05$) differences among columns are indicated by different capital letters.

Discussion

Estrus synchronization protocols based on exogenous progestagens remain the cornerstone of controlled breeding programs in sheep, since they permit precise timing of ovulation while allowing the additional manipulation of ovarian and uterine function through hormonal or nutritional adjuncts (Wildeus, 2000; Menchaca and Rubianes, 2004) [21, 13]. The present study compared two delivery routes of exogenous progesterone intravaginal sponges and a subcutaneous progesterone implant each combined with either equine chorionic gonadotropin (eCG) or a vitamin E/selenium (Vit.E/Se) supplement, and evaluated their impact on estrus expression, fertility, peripheral hormonal dynamics, and uterine/ovarian morphometric parameters assessed by transabdominal ultrasonography.

Estrus Response and Fertility Outcomes

All experimental groups, including the untreated control, displayed a 100% estrus response following progesterone withdrawal, confirming that both the conventional intravaginal sponge and the subcutaneous progesterone device were equally capable of synchronizing follicular wave emergence and inducing estrus within the expected 24–48 hour window (Wildeus, 2000; Swelum *et al.*, 2015) [21, 19]. This finding indicates that the route of progesterone delivery did not, by itself, compromise the animals' capacity to express behavioural estrus, and is consistent with the well-established principle that exogenous progestagens mimic luteal-phase progesterone regardless of the carrier device, thereby suppressing the preovulatory LH surge until withdrawal removes the negative feedback (Menchaca and Rubianes, 2004) [13].

A recent comparison of injectable versus intravaginal progesterone protocols in ewes likewise reported equivalent synchronization efficiency between delivery routes at the onset of the breeding season, while noting subtle differences in the subsequent fertility outcome depending on the pharmacokinetic profile of the formulation used (da Silva *et*

al., 2023) [6], a pattern that is mirrored by the present results.
Despite the uniformity of estrus expression, marked differences emerged in pregnancy, lambing, and fertility rates among groups. The untreated control recorded the lowest fertility (40%), reflecting the limited reproductive efficiency that can be expected when synchronization is not reinforced by an ovulation-inducing or antioxidant adjunct. Both gonadotropin-supported groups sponge-eCG and subcutaneous progesterone-eCG، achieved high fertility (80% and 100%, respectively), supporting earlier reports that a bolus of eCG administered at progestagen withdrawal recruits and matures additional follicles, advances the timing of ovulation, and consequently improves conception rates in ewes (Hasani *et al.*, 2018; Kusina *et al.*, 2000) [10, 11].
The superiority of the subcutaneous progesterone-eCG combination over the sponge-eCG combination in this regard may be related to the more stable progesterone profile achieved with the subcutaneous device, which would be expected to provide more consistent priming of the endometrium and hypothalamic-pituitary axis prior to gonadotropin stimulation(Kusina *et al.*, 2000) [11].
The group receiving vaginal sponges together with Vitamin E/Selenium achieved the highest fertility of all groups using this device (100%), corroborating the companion study published by the present authors (Taher and Alywi, 2026) [20], in which Vit.E/Se supplementation after sponge-based synchronization likewise yielded the best reproductive outcome among the protocols tested (100% pregnancy, lambing, and fertility, with a 40% multiple lambing rate).
This The conclusion that selenium and vitamin E have a real, repeatable effect on ovine fertility when given at progestagen withdrawal is strengthened by consistency between two independent experiments, rather than being a coincidental discovery limited to a single small cohort.
In Awassi ewes, three peri-synchronization injections of vitamin E and selenium decreased overall pregnancy loss and quantitatively increased pregnancy rate compared to untreated controls, leading to a generally comparable conclusion (Awawdeh *et al.*, 2019) [3]. also in Mehraban ewes, where the elimination of CIDR did not significantly affect overall fertility, but a pre-breeding vitamin E/selenium regimen increased circulating progesterone and antioxidant capacity (Farahavar *et al.*, 2020) [8].
Additionally, compared to untreated controls, anestrous goats reported that combined selenium/vitamin E supplementation significantly increased estrus induction, ovulatory rate, embryo implantation, and pregnancy rate (Santos-Silva *et al.*, 2025) [17], supporting the cross-species reproducibility of the antioxidant-fertility effect seen in the current study.
The proposed physiological basis for this benefit is twofold: selenium supports normal corpus luteum function and has been linked to increased ovarian structure size and corpora lutea number (Cabrera-Mora *et al.*, 2019) [5], while the combined antioxidant action of vitamin E and selenium limits oxidative damage to oocytes, luteal cells, and the early embryo during the periovulatory period, a stage that is particularly vulnerable to reactive oxygen species generated during follicular rupture and luteinization (Yan *et al.*, 2022). Selenium is incorporated into glutathione peroxidase and other selenoproteins that neutralize peroxides within the ovarian and uterine microenvironment, and adequate selenium status during early gestation has been associated with improved placental antioxidant defense and pregnancy maintenance across livestock species (Dahlen et al., 2022) [7].
Because ruminants absorb dietary selenium relatively poorly compared with non-ruminants ,approximately 35% compared with 85% owing to its conversion to insoluble forms by rumen microorganisms (Xun et al., 2012; Galbraith et al., 2016) [9], parenteral injection at the time of synchronization may be a particularly effective way of bypassing this absorption barrier and ensuring an adequate peri-ovulatory selenium status.
On the other hand, compared to the matching sponge-based Vit. E/Se group, the subcutaneous progesterone with Vitamin E/Selenium group had a relatively low fertility of 60%. This disparity implies that the benefits of vitamin E/Se supplementation may vary depending on the hormonal context in which it is administered; it is plausible that the longer duration of progesterone exposure offered by the subcutaneous implant changed the timing of follicular maturation in comparison to the antioxidant injection (Xun *et al.*, 2012).
With respect to multiple lambing, the subcutaneous progesterone-eCG group achieved the highest rate (100%), exceeding even the sponge-eCG group (60%), which may again reflect the more stable hormonal priming afforded by the implant, increasing the number of ewes ovulating more than one mature follicle in response to eCG. The two Vit.E/Se groups recorded comparable multiple lambing rates (40%), again in close agreement with the 40% multiple lambing rate reported for the Vit.E/Se group in the published companion trial reinforcing the reproducibility of the antioxidant effect on ovulation rate independently of the progestagen delivery route(Taher and Alywi, 2026) [20].

Hormonal (Progesterone, Estrogen, FSH, and LH)

The hormonal assays performed before, during, and after treatment provide a physiological explanation for the fertility differences described above. Progesterone concentrations rose sharply during exposure to both the intra-vaginal sponge and the subcutaneous implant, confirming that each device delivered an effective luteal-phase concentration capable of suppressing the pre-ovulatory gonadotropin surge, consistent with the established mode of action of progestagen-releasing devices (Rowe *et al.*, 2009; Menchaca and Rubianes, 2004) [16, 13].
Notably, the subcutaneous progesterone produced a higher peak concentration (2.60 ng/ml in the eCG sub-trial; 1.84–2.18 ng/ml across measurements) than the sponge (1.96–2.18 ng/ml), and progesterone remained measurably elevated for longer after withdrawal of the subcutaneous (1.18 ng/ml and 0.92 ng/ml in the eCG and Vit.E/Se sub-trials, respectively) compared with the more rapid decline seen after sponge removal (0.43 ng/ml and 0.378 ng/ml). This more gradual decline is compatible with a depot-type subcutaneous release pattern rather than the comparatively abrupt cessation of hormone absorption that follows withdrawal of an intra-vaginal device, and may underlie the more pronounced post-withdrawal gonadotropin and estrogen rebound recorded in the subcutaneous groups.
Progesterone and estrogen, FSH, and LH exhibited the anticipated inverse connection. Due to progesterone's traditional negative feedback on hypothalamic GnRH pulsatility and pituitary gonadotropin release, concentrations of all three hormones were repressed while progesterone was raised (Menchaca and Rubianes, 2004) [13].
Every group experienced a significant increase in estrogen, FSH, and LH after progestagen withdrawal, which was

consistent with the resumption of follicular growth and the approaching pre-ovulatory surge. However, the magnitude of this post-withdrawal rise was consistently higher in the ewes treated with the subcutaneous implant than in those treated with the sponge (estrogen reached 53.80 pg/ml and 48.18 pg/ml after subcutaneous progesterone withdrawal, whereas the eCG and Vit.E/Se sub-trials, respectively.

The hypothesis that more thorough and prolonged luteal-phase priming results in a stronger hypothalamic-pituitary-ovarian response once negative feedback is released is supported by this sharper rebound. This phenomenon may also account for the higher multiple lambing rate seen in the subcutaneous progesterone-eCG group, despite its marginally lower overall pregnancy rate compared to the sponge-Vit.E/Se team.

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