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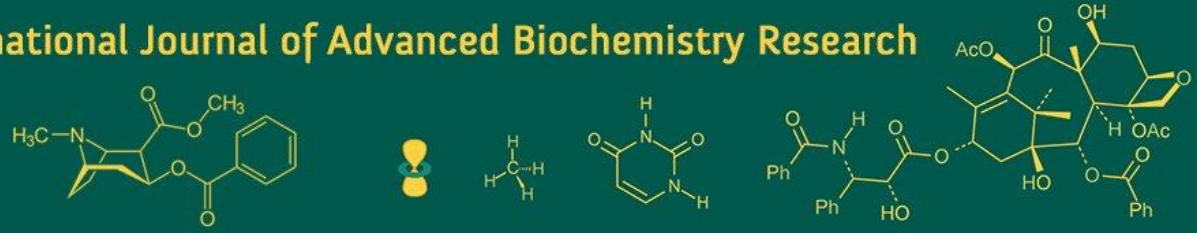

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Naveen Gowda PJ
M.V.Sc. Student, Department of Animal Reproduction, Gynaecology and Obstetrics, Veterinary College, Hassan, Karnataka, India

AJ Shankare Gowda
Associate Professor, Department of Animal Reproduction, Gynaecology and Obstetrics, Veterinary College, Bengaluru, Karnataka, India

Ravikumar BP
Professor & Head, Department of Animal Reproduction, Gynaecology and Obstetrics, Veterinary College, Gadag, Karnataka, India

P Mohan
Professor & Head, Department of Animal Reproduction, Gynaecology and Obstetrics, Veterinary College, Hassan, Karnataka, India

MC Shivakumar
The Dean, Veterinary College, Koila, Puttur, Karnataka, India

Manjunath DR
Associate Professor, Department of Veterinary Clinical Complex, Veterinary College, Hassan, Karnataka, India

Corresponding Author:
Naveen Gowda PJ
M.V.Sc. Student, Department of Animal Reproduction, Gynaecology and Obstetrics, Veterinary College, Hassan, Karnataka, India

Efficacy of different doses of intravaginal letrozole sponges for synchronization of estrus in ewes

Naveen Gowda PJ, AJ Shankare Gowda, Ravikumar BP, P Mohan, MC Shivakumar and Manjunath DR

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Abstract
This study assessed the efficacy of varying doses of letrozole-impregnated intravaginal sponges for estrus synchronization in ewes, focusing on sponge retention, estrus response, interval to estrus, duration of estrus, and conception rate. Thirty-six healthy, cyclic, non-pregnant ewes were randomly assigned to six groups (n=6). Four groups received polyurethane sponges containing 2.5, 5, 7.5, or 10 mg letrozole; the placebo and positive control groups received blank sponges and Avikesil-S® sponges, respectively. On Day 7, all ewes received 125 µg $PGF_2\alpha$ intramuscularly at sponge removal and were observed for estrus expression over 72 h. Natural mating followed, and conception was confirmed by ultrasonography 30 days post-mating. Sponge retention was 100 per cent across all groups, indicating excellent device stability. Estrus response was highest (100%) in the 7.5 mg letrozole and Avikesil-S® groups, followed by 5 mg (83.33%), 2.5 mg (66.67%), and 50 per cent in both 10 mg and placebo groups. The interval from sponge removal to estrus increased significantly ($p<0.05$) with letrozole dose, from 49.00±1.29 h (2.5 mg) to 81.33±0.67 h (10 mg), compared with 37.00±0.86 h (Avikesil-S®) and 38.33±1.20 h (placebo). Estrus duration also extended with dose, from 27.00±1.29 h (2.5 mg) to 52.00±1.15 h (10 mg), compared with 32.00±1.15 h (Avikesil-S®) and 28.67±1.76 h (placebo). Conception rates were highest in the 7.5 mg letrozole and Avikesil-S® groups (83.33%), followed by 5 mg (80.00%), 2.5 mg (75.00%), and 66.67 per cent (10 mg and placebo), with no significant differences ($p>0.05$). These findings indicate that a 7.5 mg intravaginal letrozole sponge provides optimal estrus synchronization and fertility outcomes, comparable to a conventional progestogen-based protocol, offering a promising non-steroidal alternative for reproductive management in ewes.

Keywords: Ewes, estrus synchronization, letrozole, Avikesil-S®, conception rate

Introduction
Estrus synchronization is an effective hormonal approach for enhancing reproductive performance in small ruminants (Kusina *et al.*, 2000) [18], as it enables controlled breeding and lambing schedules while optimizing the benefits of seasonal changes in forage supply, photoperiod, labor availability, and market demands. In sheep, synchronization is typically accomplished by manipulating the luteal phase of the estrous cycle either shortening or prolonging it through the administration of prostaglandin $F_2\alpha$ or its analogues, or by using exogenous natural or synthetic progestagens (Jainudeen *et al.*, 2000; Kusina *et al.*, 2000) [11, 18]. In most traditional protocols rely on steroid hormones such as progesterone and estrogen, but public health concerns over hormonal residues have prompted restrictions in several countries (Daxenberger *et al.*, 2001; Johnson & Hanrahan, 2010) [6, 12]. As a result, interest in non-steroidal alternatives has been increasing.

Letrozole, a third-generation non-steroidal competitive aromatase inhibitor, functions by binding to the heme moiety of the cytochrome P-450 subunit of the aromatase enzyme, thereby blocking the final and rate-limiting conversion of androstenedione and testosterone into estrogens (Goss & Strasser, 2001) [9]. In cattle, its use has recently been explored for ovulation synchronization, where it was shown to extend the growth phase and lifespan of the dominant follicle without disrupting progesterone secretion (Yapura *et al.*, 2018) [37]. Its application in estrus synchronization for sheep has also been documented (Abdel Dayem *et al.*, 2020) [1]. Beyond its follicular effects, letrozole exerts a luteotropic influence, which has been associated with improved conception rates and reduced embryonic losses in high-yielding dairy cows (Yapura *et al.*, 2018) [37]. Concerns regarding the potential health risks of steroidal hormones such as estradiol and progesterone have led to their prohibition in food-producing livestock across several European nations. In light of these restrictions, letrozole

has gained attention as a promising non-steroidal alternative for estrus synchronization and inducing fertile ovulations in sheep.

However, the optimal dose for ewes remains uncertain, as underdosing may be ineffective while overdosing risks excessive estrogen suppression and disrupted estrous behavior. This study aimed to evaluate the efficacy of different doses of intravaginal letrozole sponges for estrus synchronization and conception rate in ewes, using blank and progesterone-loaded sponges as controls.

Materials and Methods

The present study was carried out on 36 non-pregnant, healthy, local cyclic ewes maintained at Livestock Farm Complex, Veterinary College, Hassan. A total of 36 healthy, cyclic, non-pregnant ewes were randomly allocated into six equal groups (n = 6). Polyurethane intravaginal sponges (30 × 20 × 20 mm) with 15 cm cotton retrieval threads were prepared in-house, sterilized by autoclaving (121 °C, 15 psi, 15 min) then in hot-air oven (60 °C, 48 h), and stored. Under aseptic laminar airflow conditions, commercially available letrozole tablets (2.5 mg) were powdered, dissolved in 1 mL solvent (30% ethanol, 70% PEG 400, with a small amount of distilled water), and impregnated into sponges to achieve doses of 2.5, 5, 7.5, or 10 mg. Sponges were air-dried (48 h), coated with antibiotic powder (procaine penicillin G, sodium penicillin G, streptomycin), packed, labeled, and stored. Blank sponges were similarly treated without drug incorporation to improve antibiotic adhesion before drying, coating, and sterile packaging. Permission from the Institutional animal ethical committee was obtained vide No. HVC/IAEC/06/2025 for handling of the animals and carrying out the research.

Groups I-IV received in-house fabricated polyurethane intravaginal sponges impregnated with 2.5 mg, 5 mg, 7.5 mg, or 10 mg letrozole (Fempro®, Cipla Ltd., Goa), respectively; Group V (placebo control) received blank sponges, while Group VI (positive control) was treated with Avikesil-S® sponges containing 350 mg natural progesterone (ICAR-CSWRI, Avikanagar, Rajasthan) for 7 days. On Day 7, all sponges were removed and each ewe was administered 125 µg cloprostenol sodium ($PGF_2\alpha$; Estrumate®, MSD Animal Health) intramuscularly. From Day 8 to Day 10, ewes were observed twice daily for behavioral signs of estrus such as vaginal hyperaemia, vulvar oedema, frequent urination, and a positive "stand-to-be-mounted" response-using sexually active rams. Ewes exhibiting estrus were naturally mated with proven fertile rams at a 1:10 ram-to-ewe ratio. The interval from sponge removal to estrus onset and the duration of estrus were recorded. Pregnancy was confirmed on Day 30 post-mating via transrectal ultrasonography using a real-time B-mode scanner equipped with a 5 MHz linear-array probe. Parameters like sponge retention rate, estrus response rate, estrus duration and conception rate was recorded and analyzed using IBM SPSS Statistics Version 31.0.0.0 (Build 117), with a significance level of $p < 0.05$. Categorical variables (sponge retention, estrus response, conception rates) were expressed as percentages and compared using the Chi-square (χ^2) test. Continuous parameters (interval to estrus, duration of estrus) were reported as Mean±SE, analyzed via one-way ANOVA, and where significant, Duncan's multiple range test was applied for pairwise comparisons.

Results

Vaginal sponge retention rate

All treatment groups, including ewes administered letrozole sponges (2.5-10 mg), placebo, and Avikesil-S®, demonstrated 100 per cent sponge retention throughout the treatment period (Table 1). This finding highlights the suitability of polyurethane-based intravaginal sponges for sustained drug delivery, consistent with earlier reports using both steroidal and non-steroidal devices (Karaca *et al.*, 2009; Martemucci & D'Alessandro, 2011; Abdel Dayem *et al.*, 2020; Surappa *et al.*, 2023; Gavimath *et al.*, 2023) [13, 22, 1, 30, 8]. Comparable results have also been documented with Avikesil-S® in different ewe breeds (Mahendra, 2016; Kumar *et al.*, 2016; Yadav *et al.*, 2020; Suhas *et al.*, 2021) [20, 17, 34, 29].

A whitish, non-offensive vaginal discharge was observed across groups, which likely reflected local mucosal irritation from sponge contact rather than infection (Al Hamedawi *et al.*, 2003; Manes *et al.*, 2015) [2, 21]. Such discharge has been previously reported following both short-and long-term sponge use (Simonetti *et al.*, 2000; Martinez-Ros *et al.*, 2018) [28, 23]. The prophylactic use of streptopenicillin in this study may have helped prevent purulent vaginitis, which is often associated with opportunistic pathogens such as *Staphylococcus aureus* or *E. coli* (Vasconcelos *et al.*, 2016; Braganca *et al.*, 2017) [33, 5]. Importantly, the discharge did not interfere with estrus expression or conception, suggesting minimal clinical impact.

Estrus response rate

Estrus response varied among treatment groups, with the 7.5 mg letrozole dose achieving 100% induction, comparable to Avikesil-S®, while lower responses were observed at 5 mg (83.3%) and 2.5 mg (66.7%). Interestingly, the 10 mg dose produced only 50 per cent response, similar to placebo (Table1 and Figure 1). This suggests a dose-dependent effect, where insufficient aromatase inhibition at lower doses reduces synchronization efficiency, whereas excessive estrogen suppression at higher doses may disrupt hypothalamic-pituitary-ovarian axis function, delaying the LH surge and impairing estrus expression (Yapura *et al.*, 2018; Surappa *et al.*, 2023) [37, 30].

These findings are consistent with earlier studies where letrozole demonstrated effective estrus induction in small ruminants (Abdel Dayem *et al.*, 2020; Gavimath *et al.*, 2023) [1, 8]. In contrast, Avikesil-S® and other progestagen-based protocols have consistently achieved near-complete estrus response (Simonetti *et al.*, 2000; Mahendra, 2016; Yadav *et al.*, 2020) [28, 20, 34]. Similar high induction rates have also been documented with MAP and FGA sponges in sheep (Lombardo *et al.*, 2020; Swelum *et al.*, 2015) [19, 31].

Although statistical differences were not significant ($p > 0.05$), the biological trend highlights 7.5 mg letrozole as the most effective dose, offering synchronization comparable to conventional steroid-based methods. Moreover, the PEG 400 vehicle likely supported uniform intravaginal release and absorption of letrozole, contributing to its effectiveness (Nunes *et al.*, 2018) [25].

Interval to estrus

In this study, the mean interval from sponge withdrawal to the onset of estrus differed significantly among groups. The shortest intervals were recorded in the Avikesil-S® (37.00±0.86 h) and placebo (38.33±1.20 h) groups, aligning with conventional progestagen-based synchronization protocols, where estrus typically occurs within 36-45 h (Mahendra, 2016; Martemucci & D'Alessandro, 2011;

Suhas *et al.*, 2021) [20, 22, 29]. Conversely, letrozole-treated ewes exhibited a clear dose-dependent delay: 49.00±1.29 h (2.5 mg), 59.20±1.02 h (5 mg), 72.00±0.73 h (7.5 mg), and 81.33±0.67 h (10 mg) (Table 2 and Figure 2). This progressive extension is attributable to letrozole's pharmacodynamic action as a potent aromatase inhibitor, which suppresses estradiol synthesis in granulosa cells and delays the hormonal cascade required for estrus expression (Yapura *et al.*, 2013; Zwiefelhofer, 2020) [36, 39].

Comparable delays have been reported in small ruminants; Abdel Dayem *et al.* (2020) [1] recorded 54 h with 7.5 mg letrozole (5-day protocol), while Surappa *et al.* (2023) [30] and Gavimath *et al.* (2023) [8] reported intervals exceeding 85 h under 7-day regimens. Prolonged exposure to letrozole via PEG 400 sponges may enhance systemic absorption, thereby extending the time to estrus onset (Yapura *et al.*, 2013) [36]. Although the 10 mg dose produced the longest interval, potentially leading to estrus-ovulation desynchrony, the 7.5 mg dose provided a favorable balance between synchronization efficacy and timing.

Duration of estrus

Estrus duration differed significantly among groups, showing a clear dose-dependent effect of letrozole. The mean duration increased from 27.00±1.29 h (2.5 mg) to 34.00±1.41 h (5 mg), 40.00±1.15 h (7.5 mg), and 52.00±1.15 h (10 mg), compared to 32.00±1.15 h in the Avikesil-S® group and 28.67±1.76 h in the placebo group (Table 2 and Figure 3). These results indicate that higher letrozole doses prolong estrus expression beyond the range observed with conventional progestagen-based protocols.

Letrozole, a third-generation aromatase inhibitor, suppresses estradiol synthesis, thereby altering both the timing and magnitude of the preovulatory estradiol rise and LH surge (Mitwally & Casper, 2001) [24]. This explains the dose-related extension of estrus seen in this study. The 7.5 mg dose provided a balanced outcome (40 h), aligning closely with durations reported for Avikesil-S® and other progestagen regimens (Ozyurtlu *et al.*, 2011; Lombardo *et al.*, 2020; Mahendra, 2016) [26, 19, 20]. In contrast, the 10 mg dose produced a markedly longer estrus (52 h), which may reflect excessive estrogen suppression and has also been associated with delayed ovulation in cattle (Yapura *et al.*, 2013) [36].

Comparable findings have been documented in ewes and does, where moderate doses of letrozole prolonged estrus duration without disrupting synchronization (Abdel Dayem *et al.*, 2020; Surappa *et al.*, 2023; Gavimath *et al.*, 2023) [1, 30, 8]. Conversely, shorter estrus durations have been reported with some MAP and FGA sponge protocols, particularly when combined with gonadotropins (Kulaksiz *et al.*, 2013; Khalilavi *et al.*, 2016; Yadav *et al.*, 2020) [16, 15, 34]. Such variation across studies may be influenced by breed, nutrition, hormonal supplements, and management practices (Greyling *et al.*, 1997; Zeleke *et al.*, 2005) [10, 38].

Overall, the present results demonstrate that letrozole significantly extends estrus duration in a dose-dependent manner. The 7.5 mg dose appears optimal**,** producing synchronization comparable to Avikesil-S® without excessive prolongation, whereas the 10 mg dose may disrupt physiological timing. These findings underscore the importance of dose optimization when developing letrozole-based synchronization protocols in ewes.

Conception rate

Conception outcomes varied among treatment groups. The highest rates (83.3%) were observed in ewes treated with 7.5 mg letrozole and Avikesil-S®, while conception was slightly lower in the 2.5 mg (75%) and 5 mg (80%) groups. Both the 10 mg letrozole and placebo treatments resulted in 66.7 per cent (Table 1 and Figure 4). Although these differences were not statistically significant ($p > 0.05$), the biological trend indicates that 7.5 mg letrozole achieves fertility results comparable to conventional progestagen-based protocols.

Comparable findings have been reported previously: Surappa *et al.* (2023) observed conception rates of 70 per cent with letrozole and 50 per cent with Avikesil-S®, while Gavimath *et al.* (2023) [8] recorded equal rates (71.4%) in does treated with either protocol. Letrozole's efficacy may be linked to its luteotropic effect, supporting corpus luteum function and implantation (Yapura *et al.*, 2011; Yapura *et al.*, 2018) [35, 37]. Similar mechanisms have been described in women, where reduced estradiol prior to implantation improved endometrial receptivity and embryo development (Bayar *et al.*, 2006; Simón *et al.*, 1995) [4, 27].

Lower conception at 2.5 mg and 5 mg may reflect insufficient ovarian stimulation, while the reduced success at 10 mg could result from excessive estrogen suppression, delaying ovulation and impairing luteal activity (Yapura *et al.*, 2013) [36]. The placebo group's 66.70 per cent rate reflects natural seasonal fertility but emphasizes the value of synchronization for improving reproductive efficiency.

Previous studies using Avikesil-S® have reported conception rates ranging widely from 66 to over 80 per cent (Mahendra, 2016; Kumar *et al.*, 2016; Yadav *et al.*, 2020; Suhas *et al.*, 2021) [20, 17, 34, 29], with lower outcomes also noted under certain field conditions (Gangadharaiah, 2017) [7]. Variability across studies likely reflects differences in breed, body condition, ram effect, hormonal supplementation, and seasonal factors (Karagiannidis *et al.*, 2001; Ataman *et al.*, 2006; Ungerfeld *et al.*, 2008) [14, 32].

Table 1: Effect of synchronization protocols on non-parametric reproductive traits in **ewes.**

Treatment groups	Parameters		
	Sponge Retention Rate (%)	Estrus Response Rate (%)	Conception Rate (%)
Letrozole 2.5 mg	100	66.67	75
Letrozole 5 mg	100	83.33	80
Letrozole 7.5 mg	100	100	83.33
Letrozole 10 mg	100	50	66.67
Placebo control	100	50	66.67
Positive (Avikesil-S®) control	100	100	83.33
p-value		0.133	0.984

Table 2: Effect of synchronization protocols on non-parametric reproductive traits in ewes.

Treatment groups	Parameters (Mean±SE).	
	Estrus Interval (h)	Estrus Duration (h)
Letrozole 2.5 mg	$49.00^{\pm}1.29^{b}$	$27.00^{\pm}1.29^{a}$
Letrozole 5 mg	$59.20^{\pm}1.02^{c}$	$34.00^{\pm}1.41^{c}$
Letrozole 7.5 mg	$72.00^{\pm}0.73^{d}$	$40.00^{\pm}1.15^{d}$
Letrozole 10 mg	$81.33^{\pm}0.67^{e}$	$52.00^{\pm}1.15^{e}$
Placebo control	$38.33^{\pm}1.20^{a}$	$28.67^{\pm}1.76^{ab}$
Positive (Avikesil-S®) control	37.00 ± 0.86^{a}	32.00 ± 1.15^{bc}
p-value	<0.001	<0.001

Note: Mean±SE values with different superscripts a, b, c, d, e vary significantly ($p<0.05$)

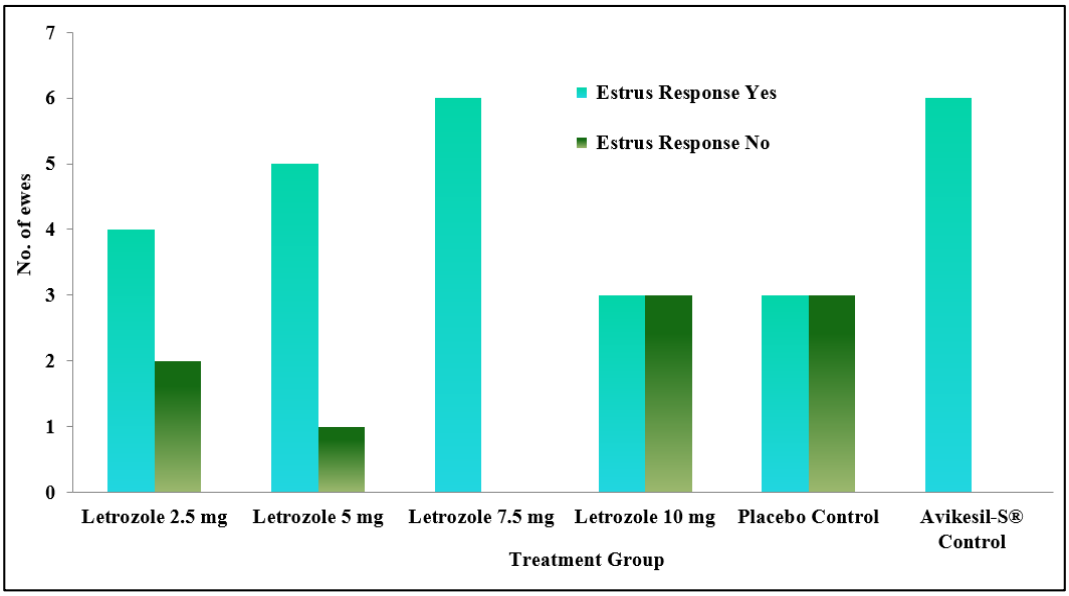

Fig 1: Estrus response in ewes treated with varying doses of intravaginal Letrozole sponges, Placebo and Avikesil-S® control.

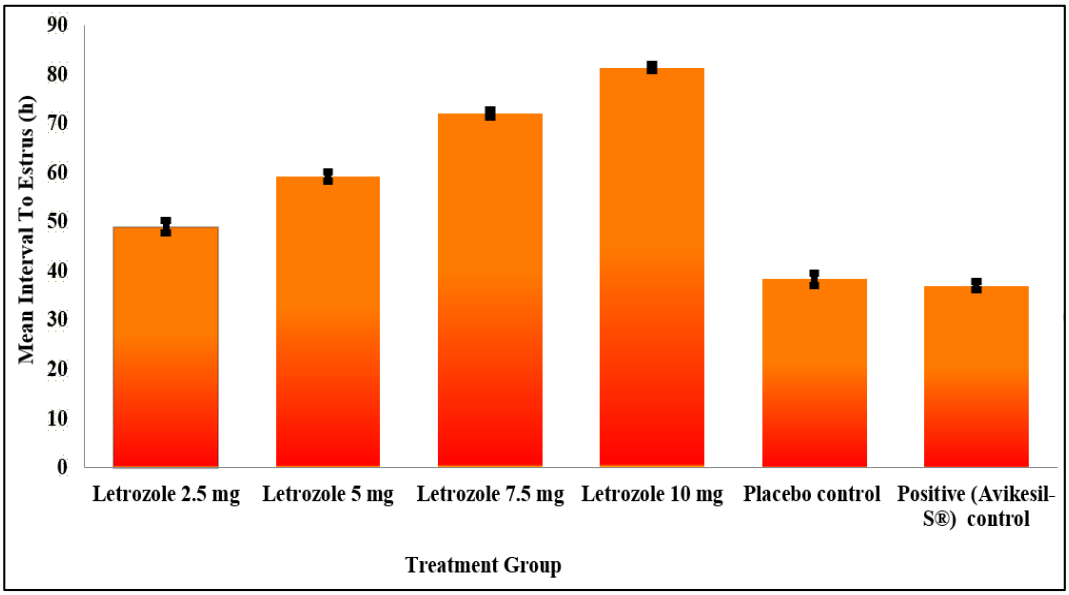

Fig 2: Mean interval to estrus in ewes treated with varying doses of intravaginal Letrozole sponges, Placebo and Avikesil-S® control.

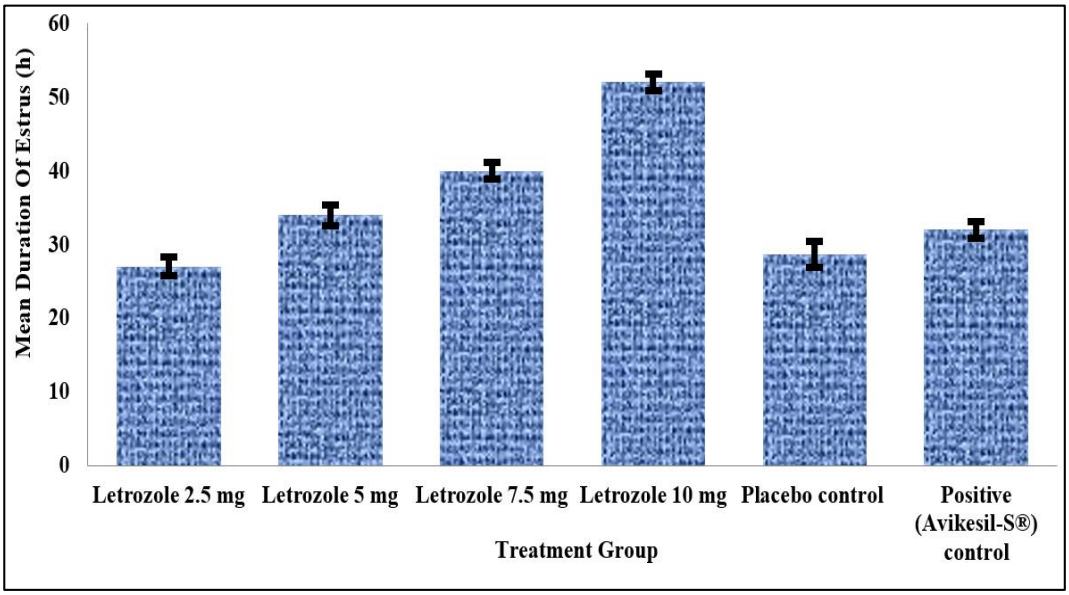

Fig 3: Mean duration of estrus in ewes treated with varying doses of intravaginal Letrozole sponges with Placebo and Avikesil-S® control.

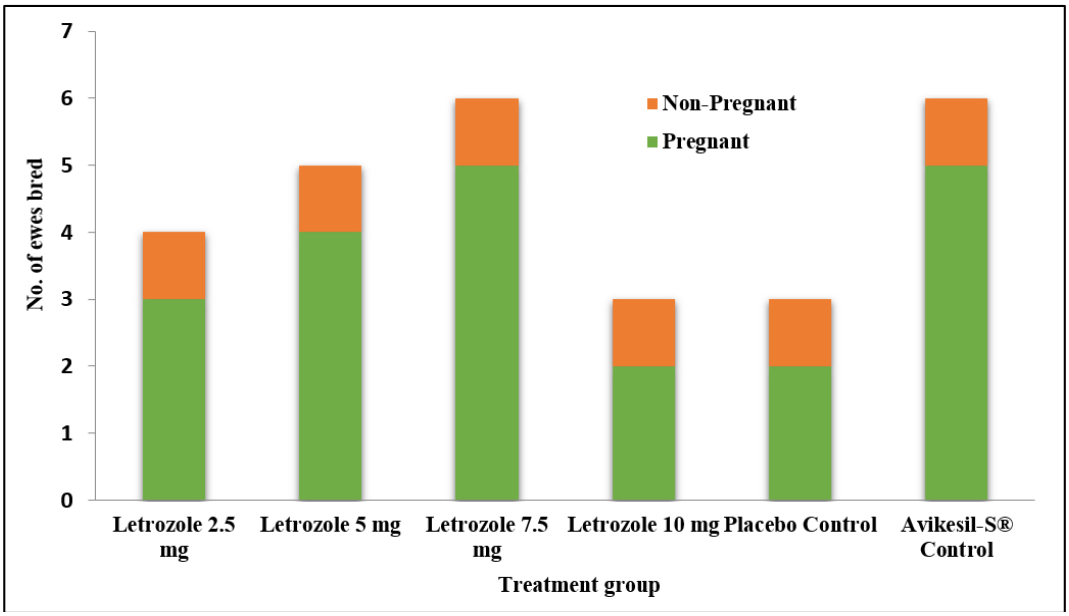

Fig 4: Conception in ewes treated with varying doses of intravaginal Letrozole sponges, Placebo and Avikesil-S® control.

Conclusion

In this study, intravaginal administration of letrozole using PEG 400 as a carrier proved effective for estrus synchronization in ewes, with 100 per cent sponge retention recorded across all groups. Among the tested doses, 7.5 mg emerged as the most suitable, producing a 100 per cent estrus response, an optimal interval to estrus (72 h), a prolonged but physiologically acceptable estrus duration (40 h), and the highest conception rate (83.3%), comparable to the conventional Avikesil-S® protocol. Lower doses (2.5-5 mg) achieved only moderate responses, while the highest dose (10 mg) excessively delayed estrus and reduced conception, likely due to excessive estrogen suppression. These findings highlight the dose-dependent effects of letrozole and suggest that a 7.5 mg intravaginal sponge offers a reliable, non-steroidal alternative to progestagen-based methods for estrus synchronization, with the potential to enhance reproductive management in ewes. Further large-scale field trials with hormonal profiling are warranted to refine dosing strategies, confirm reproductive efficiency, and establish letrozole as a viable alternative for sustainable small ruminant reproductive management.

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