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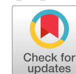

In Vitro, *In Compost*, and *In Vivo* Assessment of Chitosan-Polyethylene Glycol as an Intravaginal Insert for Progesterone Delivery in Sheep

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ABSTRACT

In estrous synchronization, the hormone progesterone is an important element. Various hormone preparations currently available have limitations, especially those related to environmental impact issues. Various alternatives are being studied, and using biodegradable polymeric materials (chitosan-polyethylene glycol combination) to develop new devices is considered one of the solutions. This contribution aims to design and evaluate intravaginal implants that can release progesterone and be degraded in the body and the environment. Implants are made by melting and molding techniques. *In vitro* drug release studies using dyes as drug models. Implant degradation studies tested in compost. Changes in the shape of the implant, while it is in the vagina, are observed by ultrasound. Blood collection was performed three days before and during implantation to obtain a blood progesterone profile. *In vitro* drug release studies using dye as a drug model showed a chitosan-PEG profile that released the drug faster at first, then slowed down. Implant degradation studies in compost and vagina demonstrated a gradual degradation process. The blood progesterone profile increased during implantation, as high as 15 ng/mL on the third day. In conclusion, the chitosan-PEG intravaginal implant formulation designed using the melting and molding technique proved to be degraded in the compost environment. It released the hormone progesterone for four days according to the degradation period of the implant in the vagina.

***Keywords:** biodegradable; chitosan; implant; polyethylene glycol; progesterone*

INTRODUCTION

Estrus synchronization is a technique for causing female herds to go into estrus at a predetermined time. In sheep, estrus synchronization can be done by manipulating hormones (Biehl *et al*., 2019; Arya *et al*., 2023). The administration of progesterone hormone is one of the most relevant steps in synchronization protocols. Different pharmaceutical formulations have been used, including oral, ear implants, intravaginal sponges, and intravaginal devices. These products aim to maintain progesterone levels above 2 ng/mL during the treatment, causing the regression of the dominant follicle and thus preventing estrus (de Graaff & Grimard, 2018). Follicular regression stimulates the secretion of follicle-stimulating hormone (FSH), starting a new follicular wave. After the device removal, progesterone concentration decreases rapidly to subluteal levels (< 1 ng/mL), promoting follicular maturation, estradiol secretion, and a peak of luteinizing hormone (LH) that leads to ovulation (Roche & Ireland, 1981; Silva *et al*., 2023).

Oral progesterone supplementation with melengestrol acetate (MGA) was added to the feed (Arya *et al*., 2023). Feeding duration may vary between protocols, but feeding rates are consistent and critical to success. Animals that fail to consume the required MGA will have a reduced estrus synchronization response (Chaudhari *et al*., 2018). Therefore, the feeding method must be provided so the animals consume the feed simultaneously. Synchro-Mate B ear implants have also been used for the estrous synchronization program (Omontese *et al*., 2016), but these also had to be removed after treatment. Synchro-Mate B has been withdrawn from the market. In addition, intravaginal sponges with different concentrations of medroxyprogesterone acetate (MPA) and fluorogestone acetate (FGA) have been used. However, using an intravaginal sponge is frequently associated with vaginitis and purulent discharge (Martinez-Ros *et al*., 2018).

Progesterone-releasing intravaginal devices are currently the first choice to supplement this hormone. These devices have been developed since the 1970s using silicone rubber as a support material. There are

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several trademarks of intravaginal devices that are commonly used, for example, CIDR® (1.38 g of progesterone, Zoetis), Cue-Mate® (1.56 g of progesterone, Vetoquinol), and PRID-delta® (1.55 g of progesterone, CEVA). However, at the end of therapy, the device must be removed from the animal's body as it is not biodegradable. Compared to sponges, CIDR devices reduce the incidence of vaginitis (Suárez *et al*., 2006), probably due to better design and a CIDR structure that allows the drainage of vaginal secretions (Martinez-Ros *et al*., 2018).

Initially, progesterone is used for an extended period similar to the life of the cyclic corpus luteum in the ovary of 11 to 19 days (Corteel *et al*., 1988), which efficiently synchronizes estrous but with varying degrees of fertility. Long-term use of progesterone is now known to induce subluteal serum progesterone levels at the end of treatment, leading to periods of follicular overgrowth and oocyte senescence *(Gonzalez-Bulnes et al*., 2020). A short synchronization protocol (5–7 days) with an intravaginal device has the advantage of maintaining progesterone concentrations at luteal levels suitable for stimulating follicular renewal and inducing new follicular ovulation while achieving the same level of conception (Gobikrushanth *et al*., 2023). Animals will show estrus 2-3 days after withdrawal of the progesterone device from its site (Silva *et al*., 2015).

Although the intravaginal devices successfully synchronize estrus in sheep, an essential concern about environmental residues and their impact on bio-systems must be considered. Physiology and native animal behavior may be affected by residual progesterone. Agricultural practice must be increased to adopt the three R's of sustainability — reduce, reuse, and recycle — to minimize this problem. In addition, this situation encourages the reuse of CIDR (Bragança *et al*., 2017), and developing new devices is required for successful estrus synchronization in sheep. Also, there is a scope for improved regulations on managing agricultural wastes containing chemical and hormonal residues worldwide (Gonzalez-Bulnes *et al*., 2020).

Several studies have been conducted to address this issue. For example, reducing the thickness of the silicone matrix in CIDR resulted in a decrease in the initial progesterone load from 1.9 to 1.38 g, thus minimizing the residual content after use from 1.31 to 0.72 g (Rathbone *et al*., 2002a). Another example is designing and evaluating prototypes of recyclable intravaginal devices from ethylene vinyl acetate (EVA) copolymer material (Helbling *et al*., 2020). Another alternative that can be done is to replace silicon material with biodegradable polymers such as chitosan and polyethylene glycol (PEG). However, combining chitosan and PEG by intravaginal implant dosage forms to release progesterone has yet to be studied further. Therefore, this study specifically combined chitosan and PEG as an intravaginal implant material expected to release the hormone progesterone, which is absorbed by the body and can be biodegradable in environment.

MATERIALS AND METHODS

All procedures relating to husbandry and research are by animal welfare as approved by the ethics committee of the Animal Ethics Commission, IPB University (Approval no. 19-2016 IPB).

Research Material

The experimental animals used were Garut ewe (*n*= 6), aged 3-4 years with a normal estrous cycle, and had a history of giving birth. Experimental animals were divided into two groups, namely the group that was given a placebo implant (without progesterone) as a control (*n*= 3) and the group that was given a progesterone implant (*n*= 3). Adaptation was carried out for a week before being given treatment in the study. Sheep are maintained by providing forage three times a day (as much as 10% of body weight per day), concentrate in the morning and evening (as much as 2%-4% of body weight per day), and drinking water is given *ad libitum*. Animals are kept in the Reproductive Rehabilitation Unit cages, Division of Reproduction and Obstetrics, Department of Clinics, Reproduction, and Pathology, School of Veterinary Medicine and Biomedical Sciences, IPB University.

Implant Design and Manufacturing

Implants are made by molding the fused material used. The formula for making placebo, color, and progesterone implants can be seen in Table 1. In the manufacture of placebo implants, a mixture of chitosan (Chitosan, PT. Biotech Surindo, Indonesia) and PEG (PEG 4000, Merck, Germany) was melted in an evaporating cup at 70 °C using a hot plate. Once homogeneous, the mixture is poured into the mold and cooled at room temperature until it solidifies and put in the refrigerator at 2-8 °C for one night. The implant is T-shaped, packaged using aluminium foil, and stored at 2-8 °C until the next test is carried out.

In the manufacture of dye implants (for *in vitro* dye release tests) and progesterone implants (for *in vivo* tests), powder dyes (Smelling Good Brand, PD. Sahabat Jaya Cemerlang, Indonesia) or powdered progesterone (Sigma, Sigma-Aldrich Pte Ltd, Singapore) was added to the chitosan-PEG mixture which was already homogeneous at 40 °C, stirred thoroughly using a magnetic stirrer for 15 minutes and then molded as in the manufacture of placebo implants.

Table 1. PEG-chitosan implant formulation in dye release test, biodegradation test in compost, and *in vivo* (vaginal) biodegradation test

Type of test	Implants used		
Dyestuff release test	Color implants		
	Chitosan	15%	
	PEG 4000	80%	
	Dyestuff	5%	
Biodegradation test in compost	Suppository placebo implant		
	Chitosan	15%	
	PEG 4000	85%	
In vivo biodegradation test	T-shaped implant		
		Placebo	Progesterone
	Chitosan	15%	15%
	PEG 4000	85%	80%
	Progesterone		5%

Immersion Test in Simulated Vaginal Fluid (SVF)

One application of vaginal fluid simulation is to evaluate its effect on the performance of vaginal therapy formulations (Fernandes *et al.*, 2023). The SVF solution used was adapted from Owen & Katz (1999). Implants with different weights (1.5, 5, and 8 g) were immersed in 40 mL SVF and stored at 37 °C. This test was conducted to determine the erosion time of the implant and the change in weight of the implant during immersion. Samples were weighed at preset times until the complete dissolution or erosion of the implant. The soluble implant portion was filtered, dried, and weighed daily. The part of the implant that is still intact is immersed again until the entire implant is used up.

Dyestuff Release Test

Prediction of drug release profile can use dye as a drug model (Dodero *et al.*, 2020). Color implant pellets (1 cm in diameter and 1 g in weight) were immersed in 8 mL of physiological NaCl solution (B. Braun, PT. B. Braun Pharmaceutical Indonesia, Indonesia) at 37 °C, pH 7.4. Sample solution (1 mL) was collected every 24 hours for eight days. The control solution is a color solution with a predetermined concentration. The control solution was prepared by diluting the multilevel solution, starting with making a stock solution (100 mg of dye in 1 mL of physiological NaCl). Next, the mother liquor was prepared by taking 100 µL of the stock solution and transferring it to the microplate well no.1 (mother liquor concentration $1x10^{-1}$ mg/µL). The microplate wells no. 2 to no. 12 were filled with 50 µL physiological NaCl. A total of 50 µL of solution from well no.1 was transferred to well no. 2, stirred well, and then take another 50 µL from well no. 2 was transferred to well no. 3, and so on to obtain a control solution with concentration levels from $1×10^{-1}$, $5×10^{-2}$, $2.5×10^{-2}$,...,$4.9×10^{-5}$ mg/µL.

In the second row of microplate wells, wells no. 1 to no. 8 were filled with 100 µL sample solution. The control solution is in the first row of microplate wells, while the sample solution is in the second row. The microplate was then scanned with a scanner (Epson L3110) to obtain a color image of each well. The color intensities of the control and sample solutions were determined using the ImageJ application (NIH, USA). The intensity of the control color and its concentration is made in graphical form, and then the equation formula is obtained. Based on this formula, the concentration of the sample solution can be determined.

Biodegradation Test in Compost

This test aims to evaluate the biodegradation of implants in the environment (Othman *et al.*, 2023) in the hope that the implant components can become substrates for microorganisms in the compost. It is done to deal with implant waste to remain environmentally friendly. The commercial organic compost (GM plus, PT. Sumber Tunas Hijau, Indonesia) was placed in a 60 × 35 × 12 cm container. Eight implants with a length of 4 cm, a diameter of 1 cm, and a weight of 4.7±0.1 g were buried in compost to a depth of 10 cm and stored at room temperature. One implant was removed every 24 hours for eight days. The implants removed from the compost were first cleaned of compost adhering to the surface and then observed for changes in length and weight.

In Vivo Biodegradation Tests

This test was conducted to determine the biodegradation time of implants in the vagina of sheep. Observation of changes in the size of the implants, while they were in the sheep’s vagina, was carried out by measuring the length and the largest diameter once a day using a brightness mode ultrasound device (Aloka SSD500, Hitachi, Japan). Sheep that received intravaginal implants underwent per-rectal ultrasound imaging. The sheep were previously fed and confined in pin cages in a standing position. The feces are then removed from the rectum with fingers so as not to interfere with the imaging process. The rectal probe (frequency 5 MHz) was pre-coated with ultrasound gel to reduce rectal mucosal irritation and to obtain a good ultrasound image. The probe is inserted into the rectum and directed to the vagina. Then the implant’s image is observed, and the size of the implant and the degree of echogenicity of the implant is carried out.

Progesterone Hormone Analysis

All sheep were injected with PGF2α (Lutalyse, Zoetis, Ireland) 2 times at an interval of 11 days. Implants were inserted into the vagina of the sheep on day 11. Blood was taken from the jugular vein to obtain serum to be analyzed for the release of systemic levels of the hormone progesterone. Blood collection was performed once daily from three days before the second PGF2α injection and daily while the implant was in the vagina of the sheep. Implantation protocols can be seen in Figure 1. The volume of whole blood was taken as much as 3 mL using a 21 G syringe and collected in a vacuum serum tube. Whole blood was then left at room temperature until it coagulated for 15-30 minutes. Centrifugation was then carried out at 3000 rpm for 10 minutes to maximize the collection of blood serum obtained. The serum was stored in 2 mL microtube tubes, frozen, and stored at -20 °C until analysis of progesterone levels by ELISA was carried out. Examining the hormone progesterone follows the standard ELISA procedure according to the test manual provided by the biological product company DRG International Inc. Germany.

Data Analysis

The collected data were analyzed statistically using one-way ANOVA (SPSS 22, IBM, USA) in a completely randomized design. The analysis was continued with Duncan’s test when a difference was detected.

RESULTS

Implant Design and Fabrication

The implant consists of two variants, placebo implant (chitosan-PEG) and progesterone implant (chitosan-

PEG-progesterone). Both implant variants are shaped like the letter T and are 4 cm long and 1 cm in diameter (Figure 2). The placebo implants had an average weight of 6.32±0.10 g, while the progesterone implants had an average weight of 6.08±0.10 g. The implant is shaped like the letter T with the aim that the implant does not easily come out of the sheep's vagina during implantation. The color of the two implants looks even, which shows the homogeneity of the two implants.

Immersion Test in SVF

Different implant weights show different erosion periods in SVF. Implants weighing 1.5 g experienced the fastest erosion compared to implants weighing 5 g and 8 g, while the 8 g implant experienced the longest deterioration (Figure 3A). The greater the implant weight, the longer the erosion period (Figure 3B; Equation 1).

$$y = 1.0536x - 6.0536;\ R^2 = 0.9789 \quad (1)$$

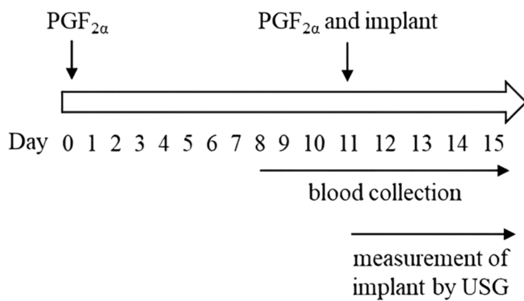

Figure 1. $PGF_{2\alpha}$ treatment protocol, implantation, blood sampling schedule, and ultrasound in the placebo implant and progesterone implant sheep group.

Dyestuff Release Test

The sample solution curve appears linear with the standard curve (control solution) with the equation formula (Figure 4; Equation 2). Based on this formula, the concentration of the sample solution can be determined as in the inset of Figure 4. The release of dyes increased with increasing immersion time (Figure 5). The color concentration of the solution increased from 31.41% to 38.29% on the eighth day. The release rate occurred faster at the beginning, 1.31% in the first 24 hours, then gradually slowed to 0.22%. The color of the control solution was arranged from low to high concentration, while the color of the sample solution was arranged based on the immersion time (first day to the eighth day). The color position of the sample solution is between the control solution of the second and third wells (Figure 6).

$$y = 2\times10^{11}\ln(x) + 1\times10^{10};\ R^2 = 0.9629 \quad (2)$$

Biodegradation Test in Compost

The control implants were stored in a closed dry container at 25 °C while the sample implants were buried in compost at 28 °C. The tremendous weight loss occurred in the early stages of degradation (Figure 7A). Similar to the change in weight, the most remarkable change in implant length also occurred in the early stages of degradation (Figure 7B). The degradation rates of implant weight

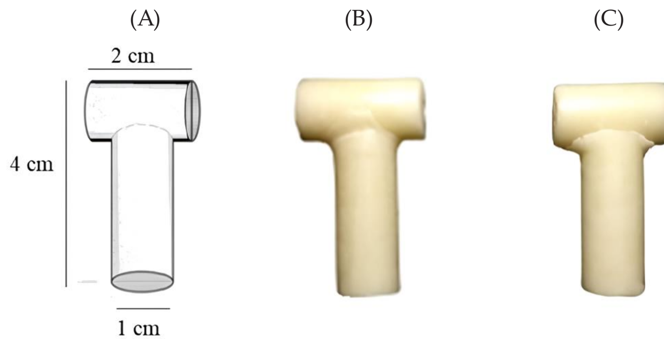

Figure 2. Design drawings and results of PEG-chitosan implant fabrication using the melting method. (A) Implant design, (B) placebo implant fabrication results, and (C) progesterone implant fabrication results.

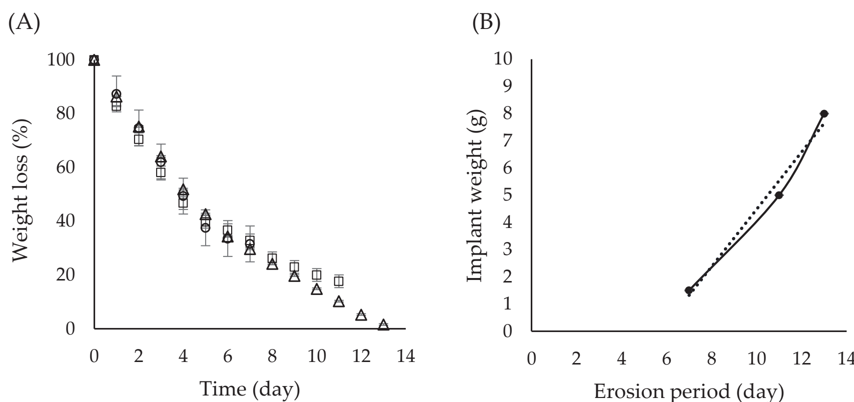

Figure 3. Changes in weight and erosion time of PEG-chitosan implants after immersion in simulated vaginal fluid (SVF). (A) Percentage of implant weight loss (Note: o= 1.50 g; □= 5.00 g; Δ= 8.00 g) and (B) implant erosion period by weight (Note: ─●─ = degradation period; ●●●● = linear line (y= 1.0536x - 6.0536; R^2 = 0.9789).

and length were similar in pattern (Figure 8A). The rate of implant weight and length degradation was fastest in the early stages of degradation and then slowed down. There was no change in ambient temperature in either the control implant environment or the sample implants (Figure 8B). Implants of different weights require different degradation times (Figure 9). Based on the graph, the degradation time of the implant can be predicted with the weight determined by equation 3. The shape of the implant changes over time due to the erosion process with an irregular pattern on the implant's surface (Figures 10).

$$y = 0.2451x + 2.5324;\ R^2 = 0.9132 \quad (3)$$

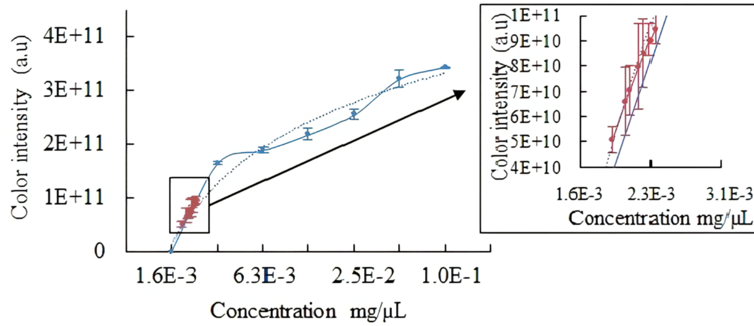

Figure 4. Standard curves of color intensities and color concentrations of control solution and sample, (inset a) color intensity and concentration of sample solution. Note: —●— = control; —●— = sample; ● ● ● ● = log. control ($y = 2\times10^{11}\ln(x) + 1\times10^{10}$; $R^2 = 0.9629$).

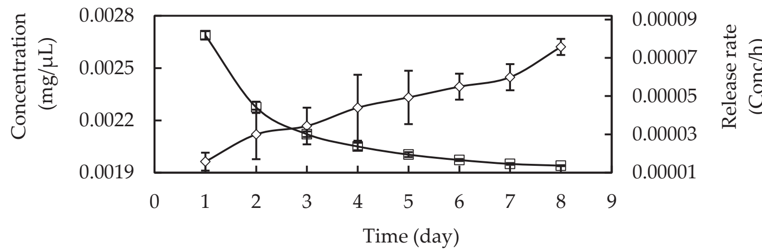

Figure 5. Dyestuff concentrations of sample solution during eight days of PEG-Chitosan implant immersion and the release rate. Note: —◇— = concentration; —□— = release rate.

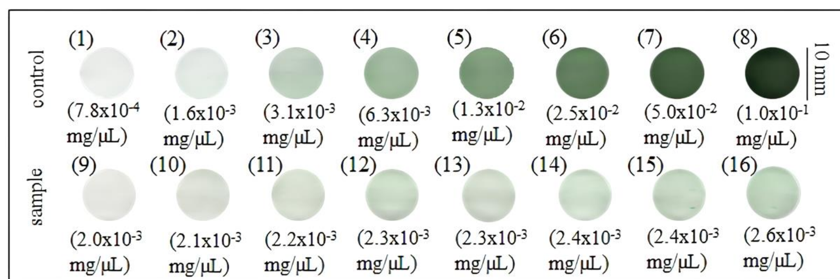

Figure 6. Colors and concentrations of dyestuff solution in microplate wells, (1-8) colors of control solution, (9-16) colors of sample solution during eight days of PEG-Chitosan implant immersion, (9) immersion on day 1, (10) immersion on day 2, (11) immersion on day 3, (12) immersion on day 4, (13) immersion on day 5, (14) immersion on day 6, (15) immersion on day 7, and (16) immersion on day 8.

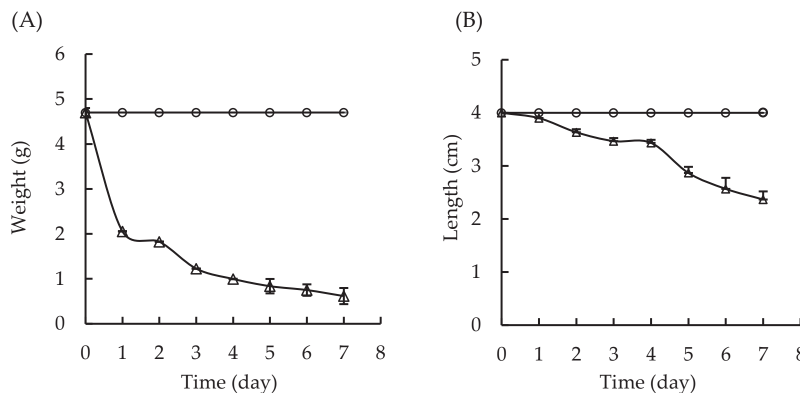

Figure 7. Change in PEG-chitosan implant weight (A) and lengths (B) during burial in compost. Note: —○— = control; —△— = sample.

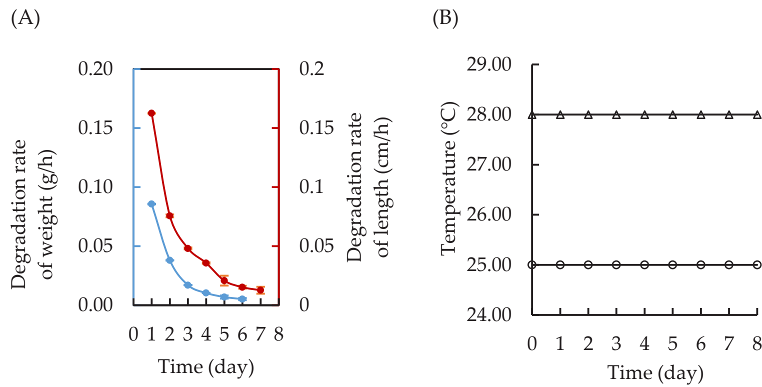

Figure 8. Degradation rates of PEG-chitosan implant weight and length during burial in compost (A) and ambient temperatures of PEG-chitosan implant control (in the room) and sample (in compost). Note: —●— = weight; —●— = length; —○— = control; —△— = sample.

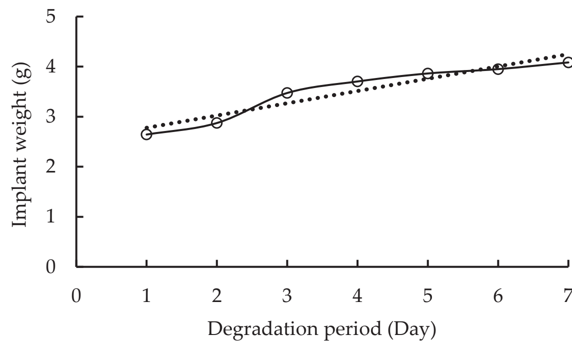

Figure 9. Degradation period of PEG-chitosan implant by weight in compost burial. Note: —○— = degradation period; ●●●● = linear line (y= 0.2451x + 2.5324; R^2 = 0.9132).

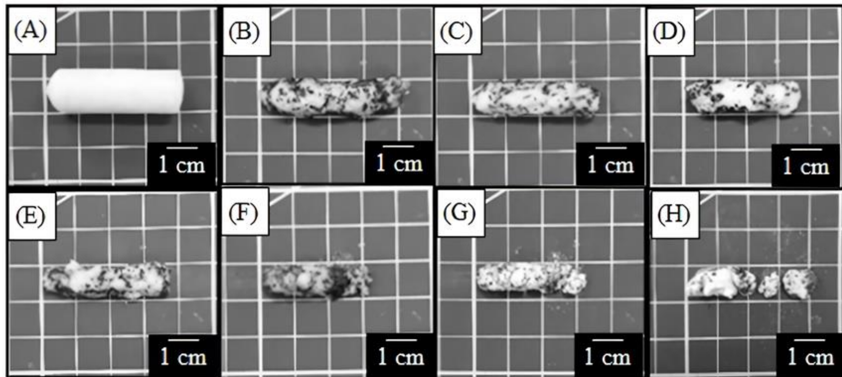

Figure 10. Deformations of PEG-chitosan implants degraded in the compost for eight days, (A) prior to degradation, (B) degradation on day 1, (C) degradation on day 2, (D) degradation on day 3, (E) degradation on day 4, (F) degradation on day 5, (G) degradation on day 6, and (H) degradation on day 7 (divided into several parts).

In Vivo Biodegradation Test

Changes in the length and diameter of the placebo implant and the progesterone implant in the vagina of the sheep followed a similar pattern (p>0.05) (Figures 11A and 11B). The difference in progesterone implant weight results in a different degradation period so that the implant degradation period can be predicted based on the implant weight (Figure 11C, Equation 4). The degradation rates of placebo and progesterone implants in the vagina of sheep also had a similar pattern (Figures 12A and 12B). Similar to the *in vitro* degradation test, the *in vivo* degradation test also showed a rapid degradation rate at first and then slowed down. Retention of the progesterone implant in the vagina reached 100% absorption on day 4 (Figures 13A and 13B). Further, ultrasound images of implant changes in the vagina are shown in Figure 14. The sonogram showed a reduction in the size and shape of the implant while it was in the vagina on days 1 to 3 (Figures 14A-14D), and the implant was no longer visible on day 4 (Figure 14E). Implant echogenicity varies from hypoechoic to hyperechoic.

$$y = 0.6774x2 - 2.0613x + 3.3543; R^2 = 0.9676 \quad (4)$$

Progesterone Hormone Analysis

The results of observing blood progesterone concentrations in the placebo-implanted sheep showed

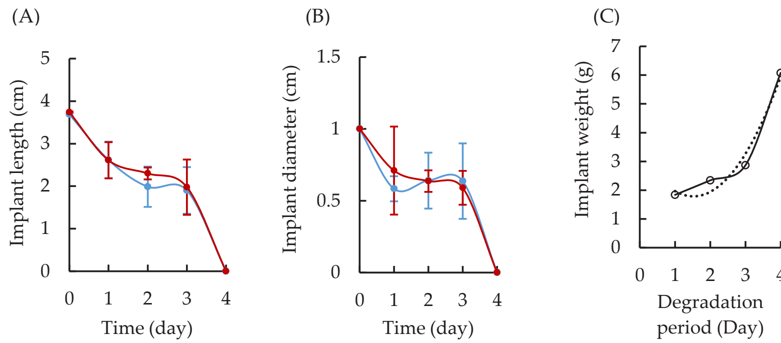

Figure 11. Changes in PEG-chitosan implant length (A), changes in PEG-chitosan implant diameter (B), and degradation period of PEG-chitosan implant by weight in vaginal cavity. Note: = placebo implant; = progesterone implant; = degradation period; ●●●● = linear line (y = 0.6774x2 - 2.0613x + 3.3543; R^2 = 0.9676).

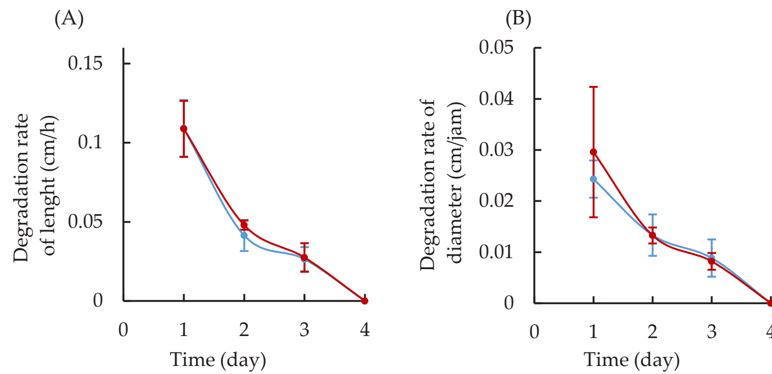

Figure 12. PEG-chitosan implant length degradation rate (A), and PEG-chitosan implant diameter degradation rate (B) during in vaginal cavity. Note: = placebo implant; = progesterone implant.

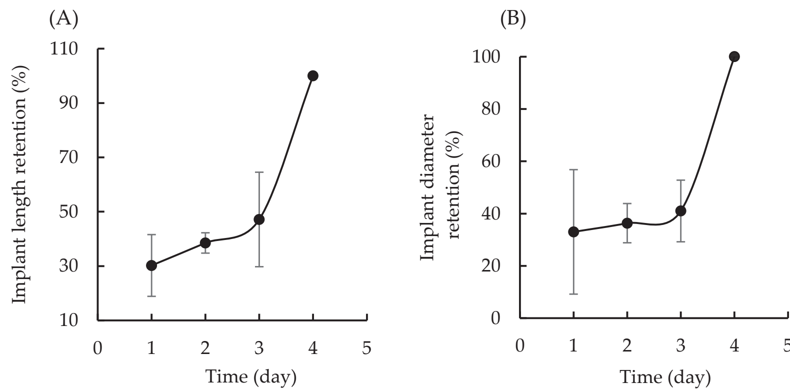

Figure 13. Percentage retention of progesterone implant length (A) and percentage retention of progesterone implant diameter during in vaginal cavity (B).

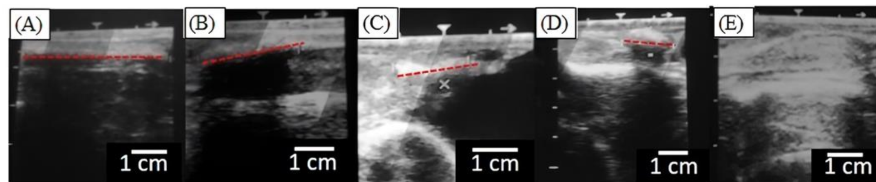

Figure 14. Description of implant biodegradation in the vagina based on ultrasound imaging. (A) ultrasounds taken on day 0, (B) ultrasounds taken on day 1, (C) ultrasounds taken on day 2, (D) ultrasounds taken on day 3, and (E) ultrasounds taken on day 4 (the implant is no longer detected). The red dotted line is the measured portion.

a drastic decrease in blood progesterone levels after the second injection of PGF2α (1.07±0.23 ng/mL). In contrast, the group of sheep given progesterone implants showed increased blood progesterone levels after the second injection of PGF2α (Figure 15A). The highest progesterone concentration was on day 14 (third day of implantation), reaching 15.00±3.70 ng/mL, then decreased drastically on day 15 (fourth day of implantation). The rate of progesterone release in the progesterone-implanted sheep group showed a rapid

rate of progesterone release at first and then slowed down (Figure 15B). It follows the dye release test by the chitosan-PEG combination previously tested (Figure 5).

DISCUSSION

Immersion Test in SVF

The weight of the implant affects the erosion time in the SVF. The erosion process on polymers changes the size and shape of biodegradable polymers. The initial material properties, geometry, and size of the polymer specimen affect the processes of fluid diffusion, hydrolysis, and erosion, and all these factors dictate temporal and spatial changes in the geometry and material properties of the polymer. The processes of fluid diffusion, hydrolysis, and erosion are interrelated, that is, while fluid diffusion is the starting point for hydrolysis and erosion, eroded polymer specimens with changing shapes and sizes also affect the diffusion process of fluid (Shockley & Muliana, 2020).

Dyestuff Release Test

The combination of chitosan-PEG has the potential as a formula for slow-release drug preparations by modelling the hormone release profile using dyes. It can be seen in the pattern of the rate of dye release from implants in physiological NaCl, which occurs faster at first and then slows down to eight days (Figure 5). This release pattern is similar to the release pattern of lovastatin from alginate-chitosan nanoparticles, which were observed for 30 hours in Phosphate Buffered Saline (PBS) pH 7.4 (Thai *et al*., 2020). Similar results also occurred with cellulose nanocrystal/chitosan hydrogel releasing theophylline for nine hours in PBS (pH 7.4) (Xu *et al*., 2019). Despite having the same pattern, the duration of release of the active substance from chitosan-PEG implants in physiological NaCl (0.9%) appears to be faster than alginate-chitosan in PBS (Thai *et al*., 2020) and nanocrystal/chitosan hydrogel in PBS (Xu *et al*., 2019).

The mechanism for rapid drug release at the initial stage occurs due to changes in surface area and particle size in a pH 7.4 solution (Thai *et al*., 2020), which causes dramatic drug dissolution in the chitosan particles. The next stage is the slow and controlled stage, that is the drug mechanism occurs, which is a combination of many processes, including swelling, polymer dissolution, diffusion, drug dissolution, and others.

Biodegradation Test in Compost

Microorganisms in the compost environment, such as *Symbiobacterium*, *Lactobacillus*, *Bacillus*, and *Yaniella* bacteria, play a significant role in the biodegradation process of organic matter (Nakasaki *et al*., 2019). Microbial are chitosanase-producing strains (*B. circulans, B. licheniformis, S. roseolus, S. zaomyceticus, P. islandicum, and P. chrysogenum*), improved the efficiency of the degradation process. Microbial diversity affects the efficiency of chitosan biodegradation in a composting environment (Altun *et al*., 2020).

The degradation rate can increase with increasing humidity. Sample implants placed in a humid compost environment experienced weight and length degradation. In contrast, control implants stored in a dry container did not experience degradation (Figures 7A and 7B). The implant size shortened and the implant surface eroded due to degradation in the compost (Figures 10A-10H). The chitosan-PEG combination implant lost about 87% of weight after being stored in compost for seven days (Figure 7A). These results indicate that the implant degrades more rapidly when compared to intravaginal implants from other biodegradable polymeric materials such as polycaprolactone (approximately 20% weight loss after being stored in compost for approximately one year) (Rathbone *et al*., 2002b). There was no change in temperature in the compost during the observation of the implants made in this study (Figure 8B). However, degradation still occurred because the polymer degradation rate was also influenced by other factors such as humidity, environmental acidity, aerobic and anaerobic conditions, enzymes, polymer structure, and molecular weight (Zeng *et al*., 2016).

In Vivo Biodegradation Test

Biodegradation of chitosan polymers in the body requires hydrolysis or enzymatic processes sensitive to these polymers. *In vivo*, chitosan is degraded mainly by

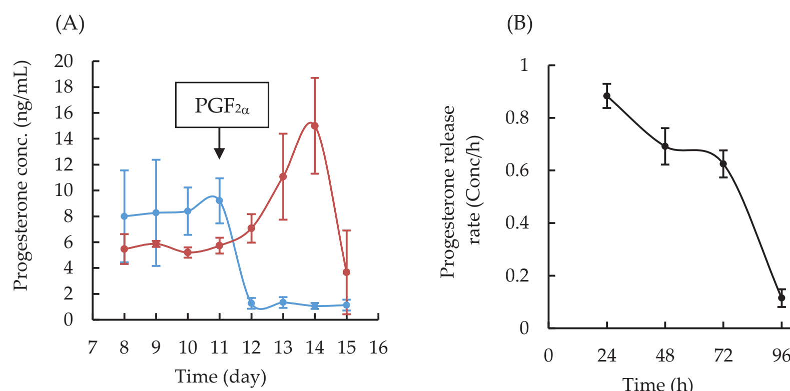

Figure 15. Progesterone profiles in blood serum of sheep treated with placebo implants and progesterone implants. Day 0 was the first PGF2α treatment; day 11 was the second PGF2α treatment and implant insertion. (A) Progesterone concentrations of sheep treated with placebo implants and progesterone implants. (B) Progesterone release rate during progesterone implant administration. Note: —●— = placebo implant; —●— = progesterone implant.

the action of lysozyme (Roman *et al*., 2020). Lysozyme is found in several body fluids, such as serum, tears, saliva, and other body fluids, including mucus in the vagina. The fastest degradation rate occurs in the early stages (Figures 12A and 12B), which can be caused by the role of enzymes in degrading polysaccharide chains. It can be distinguished based on how these enzymes depolymerize polysaccharide chains by cutting glycosidic bonds (Poshina *et al*., 2018). The first type is endo-acting by cutting the interior of the glycosidic bond at random. As a result, polysaccharides break down into smaller forms with random sizes, and the molecular weight of the polymer will decrease rapidly. The second type is exo-action which prefers to cut the glycosidic bond at the end of the chain to produce a monomer form. In general, the molecular weight of the chitosan polymer decreases constantly and slowly in the exo-action type.

Based on the retention percentage of length and diameter of the implant in the vagina, the implant was 100% absorbed in the vagina of the sheep on day 4 (Figures 13A and 13B). Unlike intravaginal implants made from silicone, polycaprolactone, and EVA, implants made from a combination of PEG and chitosan can be absorbed by the body so that the implant does not need to be withdrawn. The ultrasound implant's image in the vagina of the sheep also shows a change in the implant's size and shape while in the vagina (Figures 14A and 14D).

Progesterone Hormone Analysis

Progesterone levels in the follicular phase of the estrous cycle are very low (until < 0.5 ng/mL). After estrus and ovulation, the corpus luteum acquires a functional structure in the luteal phase (diestrus period) and synthesizes > 2 ng/mL progesterone. In this study, two injections of PGF2α at 11-day intervals were aimed at bringing the animals into the luteal phase where there was functional CL. In the group that was given placebo implants (without progesterone), the second PGF2α injection successfully regressed CL. PGF2α causes a vasoconstrictive effect, so blood flow to the CL decreases drastically (Say *et al*., 2016). As a result, the blood progesterone level decreases rapidly to 1.07±0.23 ng/mL. In contrast, in the group that was given the progesterone implant after the second PGF2α injection, the progesterone level increased to 15.00±3.70 ng/mL on the 3rd day of implantation (Figure 15A). The levels of progesterone that were observed to be elevated were from exogenous progesterone inserted via a progesterone implant. It proves that the implanted progesterone can release the hormone intravaginally. The time (days) for the hormone progesterone release in the blood to occur in sheep due to the insertion of progesterone implants corresponds to the length of time (days) the implants last in the vagina of the sheep, which is four days.

The implants in this study could maintain plasma progesterone concentrations above 2 ng/mL for four days (Figure 15A) through a transvaginal application which could avoid cross-metabolism in the gastrointestinal tract and liver, and the active substances could go directly to blood plasma (dos Santos *et al*., 2020). Supplementing progesterone with an intravaginal device during estrous synchronization programs aims to increase plasma progesterone concentrations in the animal. This concentration must be maintained above 2 ng/mL until the device is removed (van Werven *et al*., 2013) to mimic functional CL. At high progesterone levels, progesterone will decrease LH secretion, thereby preventing estrus and ovulation. Treatment using progesterone preparations with intravaginal devices for 7-12 days can induce negative feedback in the hypothalamus and pituitary and inhibit the release of gonadotropin hormones (Macmillan & Burke, 1996). After removal of the device, more significant amounts of gonadotropins will be released to stimulate follicular development, release the hormone estradiol, resume estrus, and synchronize ovulation or for luteinization of the sizeable dominant follicle (Bonacker *et al*., 2020).

Chitosan ability to release hormones is due to its mucoadhesive properties. Adhesive interfacial interactions between mucin and chitosan are complex and depend on several factors, such as solvent type, pH, ionic strength, and temperature of the system in which both "polymers" interact. The interaction between chitosan and mucin occurs mainly due to electrostatic attraction, hydrogen bonding, and hydrophobic interactions. However, the specific domains involved in these interactions have not been completely revealed, even though several works confirmed that the interactions are enhanced with the molecular weight and the degree of acetylation of chitosan (Collado-González *et al*., 2019). The rate of progesterone release in the progesterone-implanted sheep group showed a rapid rate of progesterone release at the beginning (the first 24 hours of implantation), then slowed down. Furthermore, it tends to be stable at 48-72 hours (2-3 days) of implantation. Then it decreases dramatically on the fourth day of implantation (Figure 15B) because the implant in the vagina has reached 0 cm on the fourth day. The pattern of progesterone release rate (Figure 15B) that occurs is similar to the dye release test by the chitosan-PEG combination tested previously (Figure 5).

The rapid rise in the concentration level of serum progesterone after vaginal administration of progesterone implants suggests that the vaginal mucosa is a practical site for hormone absorption and delivery. The vaginal cavity has great potential for systemic delivery due to its large surface area and dense network of blood vessels, which is the key advantage of vaginal drug delivery (Wang *et al*., 2021).

Along with increasing knowledge about the regulation of follicular dynamics, the progesterone treatment program, which was initially 12-14 days, was reduced to 7-9 days (Bilbao *et al*., 2019). Prolonged progesterone priming can induce inappropriate progesterone concentrations at the end of treatment. Because a follicular wave appears every 5-7 days, prolonged progesterone priming is not warranted. Researchers have evaluated various alternatives to short-term progesterone treatment in sheep and goats consisting of 5–7 days of progestogen administration (Martinez-Ros *et al*., 2019; Gore *et al*., 2020; Menchaca *et al*., 2017).

This study successfully explored the potential of the chitosan-PEG combination as a biodegradable intravaginal progesterone insertion device material to minimize adverse environmental impacts. The implant that has entered the vagina cannot be removed from the vagina in

Table 2. PEG-chitosan implant degradation period (days) in simulated vaginal fluid (SVF), compost, and vagina based on differences in implant weight and degradation time equation

Implant weight (g)	Implant degradation period (day) in		
	SVF (Equation 1)	Compost (Equation 3)	Vagina (Equation 4)
6.00	11.44	14.15	4.02
8.00	13.34	22.31	4.55
10.00	15.24	30.47	5.00
12.00	17.14	38.63	5.40
14.00	19.03	46.79	5.77
16.00	20.93	54.95	6.10
18.00	22.83	63.11	6.41
20.00	24.73	71.27	6.71
22.00	26.63	79.43	6.98

its original form because when it is in the vagina, the implant will become soft and sticky, so it will be damaged if it is forcibly pulled to be removed. There is no residual implant or progesterone after the implant is used because the progesterone in the implant will only be released as long as the implant remains in the vagina. Further, because the test results for implant degradation and release of the hormone progesterone *in vivo* are four days and cannot be applied to existing synchronization protocols, adjustments to the implant preparation formula are needed.

After carrying out implant degradation tests on several media, an equation for the implant degradation period in several media was obtained. In simulated vaginal fluid (Equation 1), in compost (Equation 3), and in the vagina (Equation 4). Table 2 shows the prediction of the implant degradation period based on the implant's weight and the test medium according to the equation obtained from the graph of each test medium. If the implant weight is 6.00 g, it is predicted to be degraded for 11.44 days in SVF, 14.15 days in compost, and 4.02 days in the vagina. Therefore, it is necessary to adjust the 10-22 g implant to degrade for 5-7 days in the vagina to allow it to be applied to a short-term synchronization protocol.

CONCLUSION

Based on the study's results, it can be concluded that the chitosan-PEG intravaginal implant formulation design was successfully prepared through a melting and molding process. *In vitro* drug release studies using dye as a drug model showed a chitosan-PEG profile that released the drug faster at first, then slowed down. Implant degradation studies in compost and vagina demonstrated a gradual degradation process. The blood progesterone profile increased during implantation, as high as 15 ng/mL on the third day. The chitosan-PEG intravaginal implant formulation proved to release the hormone progesterone for four days according to the degradation period of the implant in the vagina. Serving weights are suggested to be 10-22 g to obtain a suitable time for short-term synchronization protocols of 5-7 days.

CONFLICT OF INTEREST

There is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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REFERENCES

Altun, E., E. Çelik, & H. Y. Ersan. 2020. Tailoring the microbial community for improving the biodegradation of chitosan films in composting environment. J. Polym. Environ. 28:1548–1559. https://doi.org/10.1007/s10924-020-01711-0

Arya, D., R. Goswami, & M. Sharma. 2023. Estrous synchronization in cattle , sheep and goat. Multidiscip. Rev. 6:2023001. https://doi.org/10.31893/multirev.2023001

Biehl, M. V., M. V. C. de Ferraz Junior, J. P. R. Barroso, I. Susin, E. M. Ferreira, D. M. Polizel, & A. V. Pires. 2019. The reused progesterone device has the same effect on short or long estrus synchronization protocols in tropical sheep. Trop. Anim. Health Prod. 51:1545–1549. https://doi.org/10.1007/s11250-019-01841-1

Bilbao, M. G., L. O. Zapata, H. Romero Harry, S. Perez Wallace, M. F. Farcey, L. Gelid, R. A. Palomares, M. S. Ferrer, & J. A. Bartolome. 2019. Comparison between the 5-day cosynch and 7-day estradiol-based protocols for synchronization of ovulation and timed artificial insemination in suckled BOS taurus BEEF cows. Theriogenology 131:72–78. https://doi.org/10.1016/j.theriogenology.2019.01.027

Bonacker, R. C., K. S. Stoecklein, J. W. C. Locke, J. N. Ketchum, E. R. Knickmeyer, C. M. Spinka, S. E. Poock, & J. M. Thomas. 2020. Treatment with prostaglandin F2α and an intravaginal progesterone insert promotes follicular maturity in advance of gonadotropin-releasing hormone among postpartum beef cows. Theriogenology 157:350–359. https://doi.org/10.1016/j.theriogenology.2020.08.018

Bragança, J. F. M., J. M. Maciel, L. K. Girardini, S. A. Machado, J. F. X. da Rocha, A. A. Tonin, & R. X. da Rocha. 2017. Influence of a device intravaginal to synchronization/induction of estrus and its reuse in sheep vaginal flora. Comp. Clin. Path. 26:1369–1373. https://doi.org/10.1007/s00580-017-2542-z

Chaudhari, A., N. Haque, N. Jamnesha, N. Bhalakiya, G. Patel, M. Madhavatar, D. Patel, & P. Patel. 2018. Synchronization of estrus: A reproductive management tool in veterinary practice. Int. J. Curr. Microbiol. Appl. Sci. 7:1511–1519

Collado-González, M., Y. G. Espinosa, & F. M. Goycoolea. 2019. Interaction between chitosan and mucin: Fundamentals and applications. Biomimetics 4:1–20. https://doi.org/10.3390/biomimetics4020032

Corteel, J. M., B. Leboeuf, & G. Baril. 1988. Artificial breeding of adult goats and kids induced with hormones to ovulate outside the breeding season. Small Rumin. Res. 1:19–35. https://doi.org/10.1016/0921-4488(88)90041-7

de Graaff, W. & B. Grimard. 2018. Progesterone-releasing devices for cattle estrus induction and synchronization: Device optimization to anticipate shorter treatment durations and new device developments. Theriogenology 112:34–43. https://doi.org/10.1016/j.theriogenology.2017.09.025

Dodero, A., M. Alloisio, M. Castellano, & S. Vicini. 2020. Multilayer alginate-polycaprolactone electrospun

membranes as skin wound patches with drug delivery abilities. ACS Appl. Mater. Interfaces. 12:31162–31171. https://doi.org/10.1021/acsami.0c07352

dos Santos, A. M., S. G. Carvalho, V. H. S. Araujo, G. C. Carvalho, M. P. D. Gremião, & M. Chorilli. 2020. Recent advances in hydrogels as strategy for drug delivery intended to vaginal infections. Int. J. Pharm. 590:119867. https://doi.org/10.1016/j.ijpharm.2020.119867

Fernandes, L., R. Costa, M. Henriques, & M. E. Rodrigues. 2023. Simulated vaginal fluid: Candida resistant strains' biofilm characterization and vapor phase of essential oil effect. J. Med. Mycol. 33:101329. https://doi.org/10.1016/j.mycmed.2022.101329

Gobikrushanth, M., R. J. Mapletoft, & M. G. Colazo. 2023. Estrous activity and pregnancy outcomes in Holstein heifers subjected to a progesterone based 5-d CO-Synch protocol with or without administration of initial GnRH. Theriogenology 202:36–41. https://doi.org/10.1016/j.theriogenology.2023.02.024

Gonzalez-Bulnes, A., A. Menchaca, G. B. Martin, & P. Martinez-Ros. 2020. Seventy years of progestagen treatments for management of the sheep oestrous cycle: Where we are and where we should go. Reprod. Fertil. Dev. 32:441–452. https://doi.org/10.1071/RD18477

Gore, D. L. M., J. N. Mburu, T. O. Okeno, & T. K. Muasya. 2020. Short-term oestrous synchronisation protocol following single fixed-time artificial insemination and natural mating as alternative to long-term protocol in dairy goats. Small Rumin. Res. 192:106207. https://doi.org/10.1016/j.smallrumres.2020.106207

Helbling, I. M., F. Karp, A. Cappadoro, & J. A. Luna. 2020. Design and evaluation of a recyclable intravaginal device made of ethylene vinyl acetate copolymer for bovine estrus synchronization. Drug Deliv. Transl. Res. 10:1255–1266. https://doi.org/10.1007/s13346-020-00717-4

Macmillan, K. & C. Burke. 1996. Effects of oestrous cycle control on reproductive efficiency. Anim. Reprod. Sci. 42:307–320. https://doi.org/10.1016/0378-4320(96)01512-6

Martinez-Ros, P., S. Astiz, E. Garcia-Rosello, A. Rios-Abellan, & A. Gonzalez-Bulnes. 2019. Onset of estrus and preovulatory LH surge and ovulatory efficiency in sheep after short-term treatments with progestagen-sponges and progesterone-CIDRs. Reprod. Domest. Anim. 54:408–411. https://doi.org/10.1111/rda.13317

Martinez-Ros, P., M. Lozano, F. Hernandez, A. Tirado, A. Rios-Abellan, M. C. López-Mendoza, & A. Gonzalez-Bulnes. 2018. Intravaginal device-type and treatment-length for ovine estrus synchronization modify vaginal mucus and microbiota and affect fertility. Animals. 8:1–8. https://doi.org/10.3390/ani8120226

Menchaca, A., P. Dos, & S. Neto. 2017. Estrous synchronization treatments in sheep : Brief update. Rev. bras. reprod. anim. supl. 41:340–344.

Nakasaki, K., H. Hirai, H. Mimoto, T. N. M. Quyen, M. Koyama, & K. Takeda. 2019. Succession of microbial community during vigorous organic matter degradation in the primary fermentation stage of food waste composting. Sci. Total Environ. 671:1237–1244. https://doi.org/10.1016/j.scitotenv.2019.03.341

Omontese, B. O., P. I. Rekwot, I. U. Ate, J. O. Ayo, M. U. Kawu, J. S. Rwuaan, A. I. Nwannenna, R. A. Mustapha, & A. A. Bello. 2016. An update on oestrus synchronisation of goats in Nigeria. Asian Pacific J. Reprod. 5:96–101. https://doi.org/10.1016/j.apjr.2016.01.002

Othman, S. H., N. D. A. Ronzi, R. A. Shapi'i, M. Dun, S. H. Ariffin, & M. A. P. Mohammed. 2023. Biodegradability of starch nanocomposite films containing different concentrations of chitosan nanoparticles in compost and planting soils. Coatings. 13:777. https://doi.org/10.3390/coatings13040777

Owen, D. H. & D. F. Katz. 1999. A vaginal fluid simulant. Contraception 59:91–95. https://doi.org/10.1016/S0010-7824(99)00010-4

Poshina, D. N., S. V. Raik, A. N. Poshin, & Y. A. Skorik. 2018. Accessibility of chitin and chitosan in enzymatic hydrolysis: A review. Polym. Degrad. Stab. 156:269–278. https://doi.org/10.1016/j.polymdegradstab.2018.09.005

Rathbone, M. J., C. R. Bunt, C. R. Ogle, S. Burggraaf, K. L. Macmillan, C. R. Burke, & K. L. Pickering. 2002a. Reengineering of a commercially available bovine intravaginal insert (CIDR insert) containing progesterone. J. Control. Release. 85:105–115. https://doi.org/10.1016/S0168-3659(02)00288-2

Rathbone, M. J., C. R. Bunt, C. R. Ogle, S. Burggraaf, K. L. Macmillan, & K. Pickering. 2002b. Development of an injection molded poly(ε-caprolactone) intravaginal insert for the delivery of progesterone to cattle. J. Control. Release. 85:61–71. https://doi.org/10.1016/S0168-3659(02)00272-9

Roche, J. F. & J. J. Ireland. 1981. Effect of exogenous progesterone on time of occurrence of the LH surge in heifers. J. Anim. Sci. 52:580–586. https://doi.org/10.2527/jas1981.523580x

Roman, D. L., V. Ostafe, & A. Isvoran. 2020. Deeper inside the specificity of lysozyme when degrading chitosan. A structural bioinformatics study. J. Mol. Graph. Model. 100:107676. https://doi.org/10.1016/j.jmgm.2020.107676

Say, E., S. Çoban, Y. Nak, D. Nak, U. Kara, S. White, V. Kasimanickam, & R. Kasimanickam. 2016. Fertility of Holstein heifers after two doses of PGF2α in 5-day CO-Synch progesterone-based synchronization protocol. Theriogenology 86:988–993. https://doi.org/10.1016/j.theriogenology.2016.03.026

Shockley, M. F. & A. H. Muliana. 2020. Modeling temporal and spatial changes during hydrolytic degradation and erosion in biodegradable polymers. Polym. Degrad. Stab. 180:109298. https://doi.org/10.1016/j.polymdegradstab.2020.109298

Silva, B. D. M., T. A. S. N. Silva, N. H. Moreira, H. C. A. Teixeira, M. A. Paiva Neto, J. P. Neves, & A. F. Ramos. 2015. Ovulation induction in ewes using GnRH in long and short-term synchronization protocols. Anim. Reprod. 12:312–315

Silva, L. O., N. P. Folchini, R. L. O. R. Alves, G. Madureira, C. E. C. Consentini, J. C. L. Motta, M. C. Wiltbank, & R. Sartori. 2023. Effect of progesterone from corpus luteum, intravaginal implant, or both on luteinizing hormone release, ovulatory response, and subsequent luteal development after gonadotropin-releasing hormone treatment in cows. J. Dairy Sci. 106:4413–4428. https://doi.org/10.3168/jds.2022-22618

Suárez, G., P. Zunino, H. Carol, & R. Ungerfeld. 2006. Changes in the aerobic vaginal bacterial mucous load and assessment of the susceptibility to antibiotics after treatment with intravaginal sponges in anestrous ewes. Small Rumin. Res. 63: 39–43. https://doi.org/10.1016/j.smallrumres.2005.01.011

Thai, H., C. Thuy Nguyen, L. Thi Thach, M. Thi Tran, H. Duc Mai, T. Thi Thu Nguyen, G. Duc Le, M. Van Can, L. Dai Tran, G. Long Bach, K. Ramadass, C. I. Sathish, & Q. Van Le. 2020. Characterization of chitosan/alginate/lovastatin nanoparticles and investigation of their toxic effects in vitro and in vivo. Sci. Rep. 10:1–15. https://doi.org/10.1038/s41598-020-57666-8

Wang, X., S. Liu, Y. Guan, J. Ding, C. Ma, & Z. Xie. 2021. Vaginal drug delivery approaches for localized management of cervical cancer. Adv. Drug Deliv. Rev. 174:114–126. https://doi.org/10.1016/j.addr.2021.04.009

Xu, Q., Y. Ji, Q. Sun, Y. Fu, Y. Xu, & L. Jin. 2019. Fabrication of cellulose nanocrystal/chitosan hydrogel for controlled drug release. Nanomaterials 9:253. https://doi.org/10.3390/nano9020253

Zeng, S. H., P. P. Duan, M. X. Shen, Y. J. Xue, & Z. Y. Wang. 2016. Preparation and degradation mechanisms of biodegradable polymer: A review. IOP Conf. Ser. Mater. Sci. Eng. 137:012003. https://doi.org/10.1088/1757-899X/137/1/012003