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—Original Article—

Intravaginal administration of estradiol benzoate capsule for estrus synchronization in goats

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Abstract. Estrus synchronization requires multiple treatments of hormonal drugs, requiring considerable time and cost. The aim of the present study was to develop an estrus synchronization protocol using intravaginal administration of estradiol benzoate (EB) capsules in goats. Two types of capsules were prepared: an EB capsule that melted immediately after administration and a sustained-release (SR) EB capsule that dissolved slowly and reached a peak after 24 h. Goats with functional corpus lutea were intramuscularly treated with prostaglandin $F_{2\alpha}$ (PG). At 24 h after PG administration, goats were administered 1 mg of EB solution intramuscularly (PG + 24IM; n = 6) or 1 mg of EB capsule intravaginally (PG + 24EB; n = 6). The SR EB capsule was administered intravaginally at the time of PG administration (PG + SR; n = 6). The control group (n = 6) received only PG. All groups showed estrus within 72 h after PG administration. The onset of estrus did not differ significantly between the PG + 24IM and PG + SR groups but was earlier than in the control group. Estradiol concentration in the PG + SR group peaked at 11.5 ± 6.1 h after EB and PG administration. Peak estradiol concentrations were not significantly different between the PG + 24IM and PG + SR groups (78.0 ± 25.8 and 64.0 ± 38.1 pg/ml, respectively), and were higher than the PG + 24EB and control groups (27.3 ± 8.8 and 14.6 ± 6.1 pg/ml, respectively). These results suggest that intravaginal administration of an EB capsule with a sustained-drug release base is applicable for estrus synchronization, as an alternative to intramuscular administration.

Key words: Estradiol benzoate, Estrus synchronization, Intravaginal administration

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As herd sizes increase, visual observation of individual cows is not practical within the available time of herd managers, resulting in unobserved estrus and substantial economic losses [1]. A decline in reproductive efficiency due to weak estrus and ovulation disorders in high-producing dairy cows has also been noted [2, 3]. Therefore, there is an increasing demand for synchronized estrus and ovulation using a hormone preparation to conduct fixed-time artificial insemination (TAI). Current estrus synchronization protocols employ a combination of several hormones, i.e., such as prostaglandin $F_{2\alpha}$ (PG) for inducing luteolysis, an intravaginal progesterone device used to artificially extend the luteal phase, and estradiol sodium or gonadotropin-releasing hormone for the induction of estrus and/or ovulation [4]. However, these protocols involving multiple hormonal treatments require repeated tasks, such as holding and treating cows.

As an alternative to intramuscular administration, the vagina is a potential route for systemic drug delivery. Suppositories, creams, gels, tablets, and vaginal rings are commonly used to administer hormones and antimicrobial agents. There are many advantages to intravaginal administration; this route avoids gastrointestinal absorption and hepatic first-pass metabolism of the drug [5], and direct delivery to the site of action results in reduced systemic side effects [6]. However, the vaginal mucus that coats the vaginal walls can significantly affect penetration, distribution, and residence time of the drug administered via this route [7]. Mucoadhesive polymers are sometimes used in tablet formulations to increase the vaginal residence time of microbicides [8]. Recent studies have shown that the use of mucoadhesive polymers such as polyacrylic acid bases and cellulose derivatives can improve the efficacy of drug release and absorption in the vagina [16, 17]. We reported that intravaginal administration of a progesterone capsule formulated using a mixture of a mucoadhesive polymer and silicone fluid could maintain a plasma progesterone (P_4) concentration similar to that in the natural luteal phase for 9 d in goats [9]. In this way, a reduction in the administration frequency using intravaginal sustained drug release can reduce the time and cost of treatment and reduce pain in animals.

In cattle, estradiol benzoate (EB) is used at the beginning of various estrus synchronization protocols to synchronize the follicular wave or at the end of the protocol to induce estrus and synchronize ovulation for TAI [10–12]. In studies on estrus synchronization using EB, the drug was administered intramuscularly at 24 or 48 h after PG treatment to induce luteolysis [12–16]. The aim of the present study was to develop a new estrus synchronization protocol using the intravaginal route for EB administration in goats. We used goats as an experimental model of cattle because of their similar reproductive characteristics [17, 18]. This study investigated the effect of vaginal sustained-release (SR) EB capsules on the circulating profiles of estradiol and the expression of estrus in goats.

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Materials and Methods

This study was approved by the University Committee for the Use and Care of Animals at the Tokyo University of Agriculture and Technology (no. 31–52).

Preparation of intravaginal estradiol capsules

Two types of intravaginal capsules were prepared. The EB capsule was designed to melt immediately after administration and was formulated using commercially available hard fat (Hosco S-55, Maruishi Pharmaceutical Co., Osaka, Japan) as the suppository base. The SR EB capsule was designed to melt gradually to reach a peak 24 h after administration and was formulated using an SR base developed previously in our laboratory [9].

The EB capsule was prepared by adding 1 mg of EB powder (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 0.8 ml of hard fat to a gelatin capsule (#00, volume of 1.01 ml, Matsuya Co., Osaka, Japan). For the SR capsule, 1 mg of EB powder and hard fat was placed in a small capsule (#2, volume of 0.37 ml, Matsuya Co., Osaka, Japan) and placed in the center of a large capsule (#00) filled with a SR base containing silicone fluid (Q7-9120, Dow Corning, MI, USA) as a dispersing agent and acrylic starch (SANWET®, Sanyo Chemical Co. Ltd., Kyoto, Japan) as a mucoadhesive polymer [9] (Fig. 1). The capsules were then stored at 4°C in a refrigerator until administration.

Dissolution test

One milligram of blue dye (Brilliant Blue FCF CI42090, Fuji Film Wako Pure Chemical Industries, Osaka, Japan) was used instead of EB for *in vitro* dissolution testing of the SR capsule. For this, a capsule was placed in a 50-ml centrifuge tube filled with 0.1% bovine serum albumin phosphate buffer (BSA-PBS). The pH was adjusted to 7.1, which is the average pH of the cervical mucus during the luteal phase [19]. The tube was then incubated for 28 h in a shaking water bath at 37°C. Samples of 1 ml were withdrawn at 4, 8, 20, 24, and 28 h and replaced with the same volume of 0.1% BSA-PBS. Absorbance was measured at 620 nm using an absorbance microplate reader (Multiskan FC, Thermo Fisher Scientific, Tokyo, Japan). Dissolution rates of the SR capsule in the preliminary experiment are shown in Fig. 2.

Animals

Fifteen female Shiba goats (age, 71.4 ± 31.9 [mean ± SD] months; body weight, 29.0 ± 5.8 kg) maintained at the Tokyo University of Agriculture and Technology were used. The goats were housed in outdoor paddocks with sheltered areas and were fed alfalfa hay cubes (350 g) twice a day. Clean water and mineralized salt were provided *ad libitum*. All goats were confirmed to be clinically healthy and in good condition and to have normal estrous cycles before beginning the study from September 2018 to February 2019.

Treatments

All goats were checked for estrus daily. During 7–14 days after estrus, all goats with functional corpus lutea were treated with PG (2 mg of dinoprost, intramuscular; Pfizer, Tokyo, Japan) to induce luteolysis (day 0: the day of PG administration). The control group (n = 6) did not receive any further treatment. However, the PG + 24IM (n = 6) and PG + 24EB (n = 6) groups received 1 mg of EB (Ovahormone®; ASKA Pharmaceutical Co., Tokyo, Japan) intramuscularly or EB

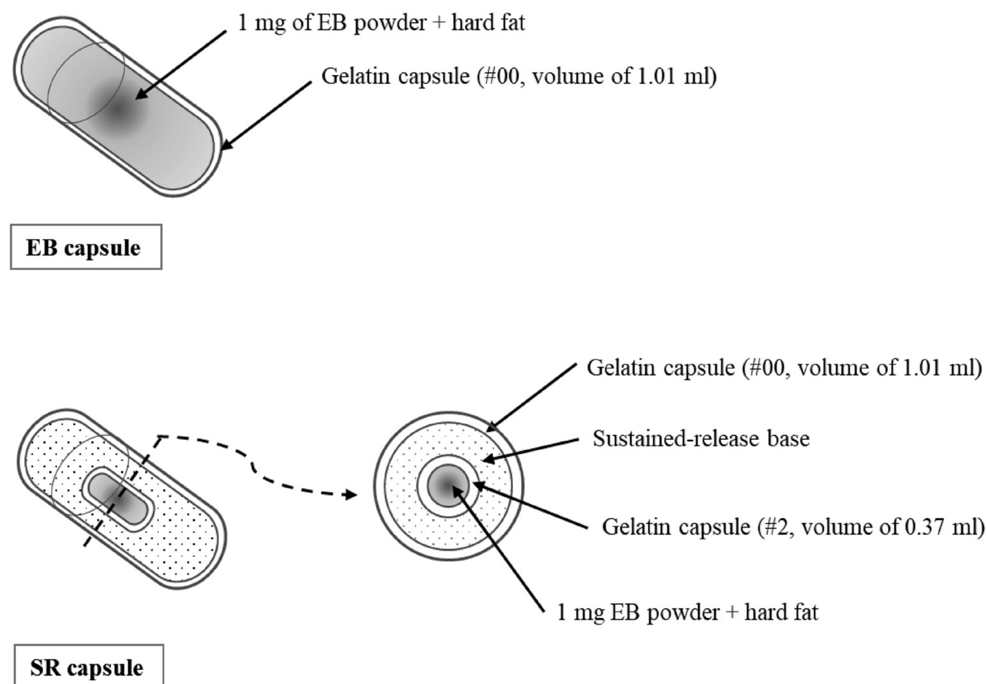

Fig. 1. Schematic diagram of the estradiol benzoate (EB) capsule (upper panel) and sustained-release (SR) EB capsule (lower panel).

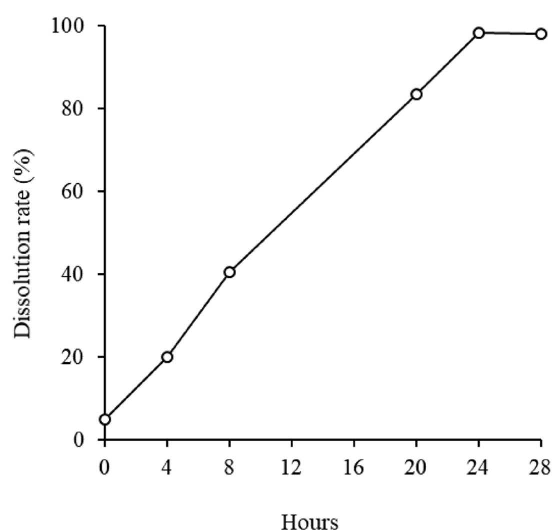

Fig. 2. Dissolution rate of the sustained-release (SR) estradiol benzoate (EB) capsule containing 1 mg of blue dye instead of EB in the preliminary experiment. A capsule was placed in a 50-ml centrifuge tube filled with 0.1% bovine serum albumin-phosphate buffer (BSA-PBS). The tube was incubated for 28 h in a shaking water bath at 37°C. Absorbance was measured at 620 nm using an absorbance microplate reader.

capsule intravaginally at 24 h after PG treatment, respectively. Goats in the PG + SR group (n = 6) were administered an SR capsule at the same time as PG treatment (day 0). The administered intravaginal EB and SR capsules were attached to a Y-shaped silicone device [9] to prevent the capsule from flowing out of the vagina. The device was removed on day 2, and by then, it was confirmed that all capsules had melted in the vagina.

Blood sampling

Blood samples were collected via jugular venipuncture into 5 ml syringes containing heparin. Samples were obtained just before PG administration, and once daily at 24-h intervals for 5 days. In the PG + 24IM and PG + 24EB groups, additional samples were obtained at 9 h after EB administration. In the PG + SR group, additional samples were obtained at 9 and 33 h after SR capsule administration. Goats in the control group were sampled in the same manner as those in the PG + SR group. Blood samples were placed in iced water and then centrifuged immediately at 3000 rpm at 4°C for 20 min. Following separation, the plasma was stored at –20°C until subsequent assays were performed.

Estrus detection

Estrus detection was performed using a male goat at the time of blood collection for 5 days. Standing estrus meant that the female goat stood still and allowed a nearby male goat to mount. The onset of estrus was defined as the first observation time point at which the goat showed standing estrus. The end of estrus was defined as the first observation time point at which the goat showed no standing estrus. The duration of estrus was calculated as the interval between the onset and the end of estrus.

Hormone assays

Plasma concentrations of estradiol and P_4 were measured via an enzyme immunoassay after extraction using dichloromethane and diethyl ether, respectively. Plasma estradiol concentrations were measured using a commercial assay kit (Estradiol ELISA Kit, Cayman Chemical, MI, USA), following the manufacturer's instructions. Plasma P_4 concentrations were measured according to the method reported by Prakash *et al*. [20] with some modifications. The intra- and inter-assay coefficients of variation for E_2 were 33.0% and 9.0%, respectively, with a sensitivity of 0.55 pg/ml. The intra- and inter-assay coefficients of variation for P_4 were 7.6% and 4.1%, respectively, with a sensitivity of 0.78 ng/ml.

Statistical analysis

Data are presented as means ± standard deviations. All data were analyzed using a statistical software (Excel Statistics, Social Information Services, Tokyo, Japan). Comparisons of the estrus characteristics among groups were made using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Continuous variables such as E_2 and P_4 concentrations were compared using two-way repeated-measures ANOVA, followed by Tukey's multiple comparison test. Blood sampling at 9 h after PG administration was performed only in the PG + SR and control groups. Therefore, the data at this time point were analyzed separately using Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Estrus

All goats showed estrous behavior (Table 1). The onset of estrus did not differ significantly between the PG + 24IM and PG + SR groups, but was earlier than that in the control group ($P < 0.05$). The duration of estrus varied widely among the animals (range, 15–96 h for all groups), but no significant differences were found in the duration and end of estrus among groups. These results were calculated from the estrus detection, which was performed once daily at 24-h intervals.

Plasma concentrations of steroid hormones

Plasma P_4 concentrations in all animals declined to less than 1 ng/ml during 24 h after PG administration. There was no significant difference among groups in progesterone concentrations after PG administration during the blood sampling period ($P > 0.1$).

Plasma estradiol concentrations until 96 h after EB administration were compared among the PG + 24IM, PG + 24EB, and PG + SR groups (Fig. 3). There were no significant differences in estradiol concentration at 0 h among the PG + 24IM, PG + 24EB, and PG + SR groups (6.1 ± 1.6, 10.2 ± 5.5, and 5.8 ± 3.8 pg/ml, respectively). The mean estradiol concentration in the PG + 24IM group reached a peak of 74.6 ± 30.0 pg/ml at 24 h after administration. In contrast, the mean estradiol concentrations in PG + 24EB and PG + SR groups peaked at 9 h after administration. The concentration at 9 h after administration in the PG + 24IM and PG + SR groups (57.0 ± 29.1 and 61.0 ± 41.6 pg/ml, respectively) was not significantly different but was significantly higher than that in the PG + 24EB group (24.5 ± 8.3 pg/ml, $P > 0.05$). Estradiol concentrations in the PG + 24IM

Table 1. Estrus detection rate, onset, and end of estrus after PG administration as well as estrus duration in the control, PG + 24IM, PG + 24EB, and PG + SR groups

	Control	PG + 24IM	PG + 24EB	PG + SR
Estrus detection rate (%)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
Onset of estrus (h)	60.0 ± 13.1 [a]	35.5 ± 6.1 [bc]	49.5 ± 12.5 [ab]	30.0 ± 4.6 [c]
Duration of estrus (h)	32.0 ± 12.4	46.0 ± 15.3	26.5 ± 11.1	50.0 ± 27.4
End of estrus (h)	88.0 ± 19.6	80.0 ± 19.6	76.0 ± 23.6	80.0 ± 24.8

All data are presented as means ± standard deviations. Different letters (a, b, c) within a row represent significant differences (P < 0.05). PG, prostaglandin $F_{2\alpha}$; PG + 24IM, 1 mg estradiol benzoate (EB) solution (intramuscular) administered 24 h after PG; PG + 24EB, 1 mg of EB capsule (intravaginal) administered 24 h after PG; PG + SR, sustained-release (SR) EB capsule administered intravaginally at PG administration; control, group receiving only PG.

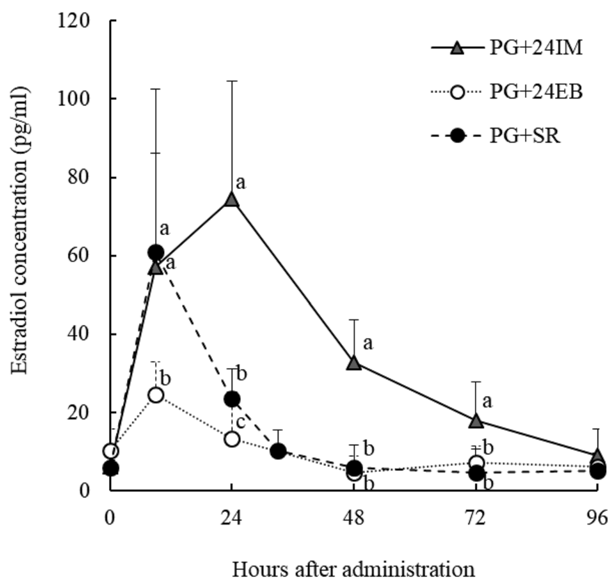

Fig. 3. Plasma estradiol concentrations until 96 h after estradiol benzoate (EB) administration in goats intramuscularly administered 1 mg of EB (PG + 24IM group, n = 6) or intravaginally administered an EB capsule (PG + 24EB group, n = 6) or sustained-release EB capsule (PG + SR group, n = 6). Different letters (a, b, c) represent a significant difference (P < 0.05) at each time point.

group at 24, 48, and 72 h after administration were significantly higher than those in the PG + 24EB and PG + SR groups at respective sampling times.

Plasma estradiol concentrations at different time points after PG administration in all groups are shown in Fig. 4. Goats in the PG + SR group received the SR capsule along with PG. Consequently, the mean estradiol concentration in the PG + SR group reached a peak at 9 h after PG administration, which was earlier than that in the other groups. In the PG + 24IM and PG + 24EB groups, EB was administered at 24 h after PG administration. The mean estradiol concentration in the PG + 24IM group was higher at 33–96 h after PG administration than that in the other three groups. Estradiol concentrations in the PG + 24EB group were significantly higher at 33 and 48 h after PG administration than in the PG + SR and control groups, and they returned to levels similar to those in the PG + SR and control groups at 72 h after PG administration. Peak estradiol concentrations and timing to reach the peak after PG treatment are shown in Table 2. The peak timings were calculated from the sampling times, which were once daily at 24-h intervals. The peak estradiol concentration was not significantly different between the PG + 24IM and SR groups, but the time to reach the peak concentration in the SR group was earlier than that in the other three groups.

Table 2, Figure 3, and Figure 4 show results of analyzing the same estradiol concentration data from different viewpoints.

Discussion

Administration of PG to goats and other ruminants with a functional corpus luteum is the simplest method for inducing estrus. In the present study, administration of PG induced estrus in all goats, regardless of EB treatment. The mean interval from PG administration to the onset of estrus in goats was reported to be 42–47 h [21] or 57 h [22]. In the present study, the interval from PG administration to the onset of estrus in the control group was comparable to that in the aforementioned studies. Estradiol is the primary hormone that induces behavioral estrus. It was proposed that once a threshold of estradiol is achieved, estrus is induced, and additional amounts of estradiol above the threshold do not further enhance the estrous response, duration, or intensity of the estrus [23]. In agreement with this, the results of the present study showed that intramuscular administration of EB at 24 h after PG administration or intravaginal administration of an SR capsule at the same time as PG administration affected the onset of estrus but not the duration or end of estrus. In contrast, intravaginal administration of an EB capsule prepared using a hard-fat suppository base did not advance the onset of estrus unlike that in the control group. These differences could be attributed to the estradiol concentrations after EB administration. The increase in estradiol concentration after 9 h after in the PG + 24EB group was less than half that of the PG + 24IM and PG + 24SR groups. It was considered that this increase was not sufficient to induce estrus immediately after capsule administration, and an additional amount of estradiol secreted from the pre-ovulatory follicles was needed for estrus to commence in some cases.

An estrus synchronization protocol of 1 mg intramuscularly administered EB is generally used in cows, while equine chorionic gonadotropin (eCG) has been used for goats. However, some recent studies indicated the effectiveness of EB as an alternative to eCG

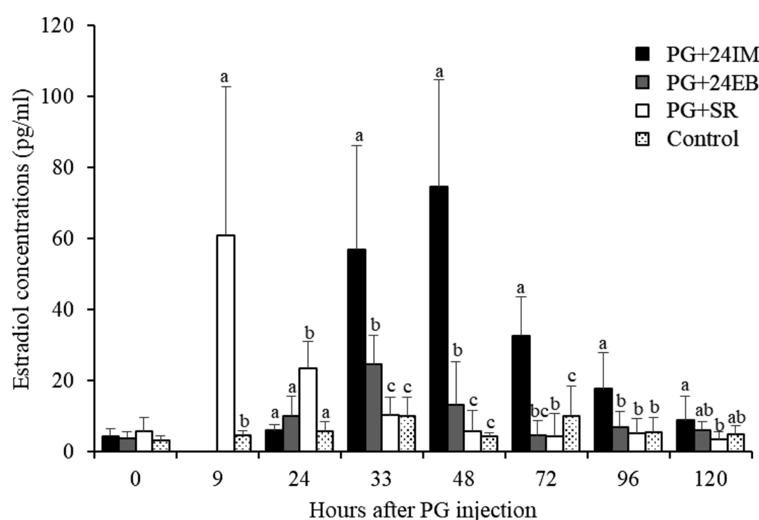

Fig. 4. Plasma estradiol concentrations until 96 h after prostaglandin $F_{2\alpha}$ (PG) administration in goats intramuscularly administered 1 mg of EB (PG + 24IM group, n = 6), intravaginally administered an EB capsule (PG + 24EB group, n = 6), or a sustained-release EB capsule (PG + SR group, n = 6). Different letters (a, b, c) represent a significant difference (P < 0.05) at each time point. Blood sampling at 9 h after PG administration was performed only in the PG + SR and control groups, and the data at this time point were analyzed separately via Student's *t*-test.

Table 2. Peak estradiol concentration and peak timing after PG administration in the control, PG + 24IM, PG + 24EB, and PG + SR groups

	Control	PG + 24IM	PG + 24EB	PG + SR
Peak concentrations (pg/ml)	14.6 ± 6.1 ^{a}	78.0 ± 25.8 ^{b}	27.3 ± 8.8 ^{ac}	64.0 ± 38.1 ^{bc}
Time of peak (h)	59.0 ± 20.1 ^{a}	45.5 ± 6.1 ^{ab}	35.5 ± 6.1 ^{b}	11.5 ± 6.1 ^{c}

All data are presented as means ± standard deviations. Different letters (a, b, c) within a row represent significant differences (P < 0.05). PG, prostaglandin $F_{2\alpha}$; PG + 24IM, 1 mg estradiol benzoate (EB) solution (intramuscular) administered 24 h after PG; PG + 24EB, 1 mg of EB capsule (intravaginal) administered 24 h after PG; PG + SR, sustained-release (SR) EB capsule administered intravaginally at PG administration; control, group receiving only PG.

[24, 25]. In these studies, administration of 0.2 mg EB effectively induced estrus and ovulation, similar to eCG in goats. In the present study, goats were administered 1 mg of EB to determine differences in estradiol profiles caused by changing the method of administration (intramuscular vs. intravaginal) and the suppository base (hard fat *vs.* SR formulation). A previous study examined the effect of EB dose and route on plasma estradiol concentrations in ovariectomized heifers. It showed that intravaginal administration of 10-mg EB powder via a gelatin capsule increased the estradiol concentration to 4.1 pg/ml, which was not significantly different from the concentration after intramuscular administration of 0.5-mg EB [26]. This result implies that an approximately 20-fold intravaginal dose of EB powder was required to attain estradiol profiles similar to those attained by intramuscular administration. Another study reported that, to achieve similar estrus, a gelatin capsule containing four times more EB in powered form than in the intramuscular injection form was required [27]. However, the study did not measure plasma estradiol concentrations. In the present study, when EB was administered intravaginally to goats in a hard-fat suppository base, the increase in plasma estradiol concentration was less than half of that attained following PG administration intramuscularly. In contrast, the concentration was significantly higher in the PG + SR group than in the PG + 24EB group, and the increase at 9 h after administration was close to that in the PG + 24IM group. The efficacy of drug therapy using the vaginal route may be restricted by the short residence time of the drug within the vagina owing to the vaginal fluid that coats the mucosal tissue. The use of a mucoadhesive polymer such as a polyacrylic acid base and cellulose derivatives can promote a prolonged and intimate contact with the vaginal mucus, enhancing the delivery of drugs to the underlying tissue and their sustained release [28–30]. We consider that the use of polyacrylic starch in the SR capsule formulation increased the adhesion and retention of EB on the vaginal wall, preventing EB from leaking out of the vagina.

The plasma estradiol concentration in the PG + SR group was expected to peak at 24 h after administration, based on an *in vitro* test of the SR capsule. This formulation was based on a recently developed estrus and ovulation synchronization protocol for cows, in which intramuscular administration of EB was performed 24 h after PG treatment and TAI was performed 24–28 h after EB administration [13]. This protocol can minimize the cost and time of treatment, while the pregnancy rate after TAI was satisfactory in comparison with conventional protocols. However, in the present

study, the peak estradiol concentration was observed at an average of 11.5 h after SR capsule administration, which was earlier than our expected time. It is possible that the SR capsule disintegrated earlier in the vagina than *in vitro* owing to the pressure and movement of the vaginal wall. Furthermore, physiological factors such as changes in the volume, viscosity, and pH of the vaginal fluid can affect the efficacy of drug delivery systems [28]. The present study was limited to examining two types of intravaginal EB capsules in terms of estrus response and blood estradiol profiles in a goat model. Modification of the size of the surrounding SR suppository base and/or inner EB capsule is one way to extend the drug-release profile and retard the estradiol peak. To validate the effectiveness of intravaginal EB as an estrus synchronization protocol, more *in vivo* clinical data, including the estrus response, blood hormone profiles, follicular development, and ovulation as well as the effect on the pregnancy rate after TAI, will be required in future studies. As intravaginal administration of the SR capsule to goats resulted in a similar estrus onset time and peak estradiol concentration as that following intramuscular administration, estrus synchronization with the SR capsule can be applied to other domestic animals, including cattle.

In conclusion, intravaginal administration of an EB capsule prepared using a mucoadhesive polymer and a sustained-release base at the same time as PG treatment effectively induced estrus in goats. The effect was comparable to that of intramuscular administration of EB at 24 h after PG administration. This novel method of intravaginal EB administration can be applied for estrus synchronization in goats, as an alternative to intramuscular administration.

Conflict of interests: The authors have nothing to declare.

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References

1. **Senger PL.** The estrus detection problem: new concepts, technologies, and possibilities. *J Dairy Sci* 1994; **77**: 2745–2753. [Medline] [CrossRef]
2. **Walsh SW, Williams EJ, Evans ACO.** A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci* 2011; **123**: 127–138. [Medline] [CrossRef]
3. **Dobson H, Smith R, Royal M, Knight C, Sheldon I.** The high-producing dairy cow and its reproductive performance. *Reprod Domest Anim* 2007; **42**(Suppl 2): 17–23. [Medline] [CrossRef]
4. **Pursley JR, Mee MO, Wiltbank MC.** Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. *Theriogenology* 1995; **44**: 915–923. [Medline] [CrossRef]
5. **Roumen FJ, Dieben TO.** Comparison of uterine concentrations of ethinyl estradiol and etonogestrel after use of a contraceptive vaginal ring and an oral contraceptive. *Fertil Steril* 2006; **85**: 57–62. [Medline] [CrossRef]
6. **Major I, McConville C.** Vaginal drug delivery for the localised treatment of cervical cancer. *Drug Deliv Transl Res* 2017; **7**: 817–828. [Medline] [CrossRef]
7. **Ensign LM, Cone R, Hanes J.** Nanoparticle-based drug delivery to the vagina: a review. *J Control Release* 2014; **190**: 500–514. [Medline] [CrossRef]
8. **Ndesendo VMK, Pillay V, Choonara YE, Buchmann E, Bayever DN, Meyer LCR.** A review of current intravaginal drug delivery approaches employed for the prophylaxis of HIV/AIDS and prevention of sexually transmitted infections. *AAPS PharmSciTech* 2008; **9**: 505–520. [Medline] [CrossRef]
9. **Endo N, Rahayu LP, Yamamura T, Tanaka H, Tanaka T.** Intravaginal administration of progesterone using a new technique for sustained drug release in goats. *J Reprod Dev* 2020; **66**: 489–492. [Medline] [CrossRef]
10. **Kasimanickam R, Cornwell JM, Nebel RL.** Fertility following fixed-time AI or insemination at observed estrus in Ovsynch and Heatsynch programs in lactating dairy cows. *Theriogenology* 2005; **63**: 2550–2559. [Medline] [CrossRef]
11. **Dailey RA, James RE, Inskeep EK, Washburn SP.** Synchronization of estrus in dairy heifers with prostaglandin F2 alpha with or without estradiol benzoate. *J Dairy Sci* 1983; **66**: 881–886. [Medline] [CrossRef]
12. **Dailey RA, Price JC, Simmons KR, Meisterling EM, Quinn PA, Washburn SP.** Synchronization of estrus in dairy cows with prostaglandin F2 α and estradiol benzoate. *J Dairy Sci* 1986; **69**: 1110–1114. [Medline] [CrossRef]
13. **Bandai K, Kusaka H, Miura H, Kikuchi M, Sakaguchi M.** A simple and practical short-term timed artificial insemination protocol using estradiol benzoate with prostaglandin F2α in lactating dairy cows. *Theriogenology* 2020; **141**: 197–201. [Medline] [CrossRef]
14. **Melo LF, Monteiro PLJ Jr, Surjus RS, Drum JN, Wiltbank MC, Sartori R.** Progesterone-based fixed-time artificial insemination protocols for dairy cows: Gonadotropin-releasing hormone versus estradiol benzoate at initiation and estradiol cypionate versus estradiol benzoate at the end. *J Dairy Sci* 2016; **99**: 9227–9237. [Medline] [CrossRef]
15. **Ryan DP, Snijders S, Yaakub H, O'Farrell KJ.** An evaluation of estrus synchronization programs in reproductive management of dairy herds. *J Anim Sci* 1995; **73**: 3687–3695. [Medline] [CrossRef]
16. **Miura H, Kotani S, Kohiruimaki M, Ohtsuka H, Kikuchi M, Ohnami Y.** Relationships between the conception rate of estrus synchronization using estradiol benzoate and CIDR (progesterone) and other parameters in holstein lactating dairy cows. *J Reprod Dev* 2008; **54**: 214–216. [Medline] [CrossRef]
17. **Mori Y, Kano Y.** Changes in plasma concentrations of LH, progesterone and oestradiol in relation to the occurrence of luteolysis, oestrus and time of ovulation in the Shiba goat (Capra hircus). *J Reprod Fertil* 1984; **72**: 223–230. [Medline] [CrossRef]
18. **Orita J, Tanaka T, Kamomae H, Kaneda Y.** Ultrasonographic observation of follicular and luteal dynamics during the estrous cycle in Shiba goats. *J Reprod Dev* 2000; **46**: 31–37. [CrossRef]
19. **Mori J, Tomizuka T, Hiroki M, Kariya T.** In vivo pH and electric conductivity of cervical mucus of cows during estrous cycle. *The Japanese Journal of Animal Reproduction* 1979; **25**: 6–11. [CrossRef]
20. **Prakash BS, Meyer HH, Schallenberger E, van de Wiel DF.** Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J Steroid Biochem* 1987; **28**: 623–627. [Medline] [CrossRef]
21. **Bretzlaff KN, Ott RS, Weston PG, Hixon JE.** Doses of prostaglandin F2α effective for induction of estrus in goats. *Theriogenology* 1981; **16**: 587–591. [Medline] [CrossRef]
22. **Romano JE, Alkar A, Amstalden M.** Onset of luteolytic action of exogenous prostaglandinF-2α during estrous cycle in goats. *Theriogenology* 2017; **92**: 45–50. [Medline] [CrossRef]
23. **Allrich RD.** Endocrine and neural control of estrus in dairy cows. *J Dairy Sci* 1994; **77**: 2738–2744. [Medline] [CrossRef]
24. **Menchaca A, Miller V, Salveraglio V, Rubianes E.** Endocrine, luteal and follicular responses after the use of the short-term protocol to synchronize ovulation in goats. *Anim Reprod Sci* 2007; **102**: 76–87. [Medline] [CrossRef]
25. **Menchaca A, Rubianes E.** Pregnancy rate obtained with short-term protocol for timed artificial insemination in goats. *Reprod Domest Anim* 2007; **42**: 590–593. [Medline] [CrossRef]
26. **O'Rourke M, Diskin MG, Sreenan JM, Roche JF.** The effect of dose and route of oestradiol benzoate administration on plasma concentrations of oestradiol and FSH in long-term ovariectomised heifers. *Anim Reprod Sci* 2000; **59**: 1–12. [Medline] [CrossRef]
27. **Smith JF, Mcgowan LT.** Estrogen and the Prid. *Proc New Zeal Soc An* 1982; **42**: 87–89.
28. **Vanić Ž, Škalko-Basnet N.** Nanopharmaceuticals for improved topical vaginal therapy: can they deliver? *Eur J Pharm Sci* 2013; **50**: 29–41. [Medline] [CrossRef]
29. **Khutoryanskiy VV.** Advances in mucoadhesion and mucoadhesive polymers. *Macromol Biosci* 2011; **11**: 748–764. [Medline] [CrossRef]
30. **Hombach J, Bernkop-Schnürch A.** Mucoadhesive drug delivery systems. *Handb Exp Pharmacol* 2010; **197**: 251–266. [Medline] [CrossRef]