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Synchronization of estrus using progesterone injections followed by human menopausal gonadotropin in ewes

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Abstract

Serial progesterone injections followed by human menopausal gonadotropin (hMG), instead of equine chorionic gonadotropin (eCG), were used to synchronize estrus in ewes. Shal ewes (n = 189) were assigned into five groups and each group was divided into two sub-groups to receive gonadotropins including eCG (300 IU; intra-muscular) or hMG (one ampoule; subcutaneously, SC). All ewes received prostaglandin (PG) $F_2\alpha$ six days after introducing ram (day 0). Ewes received 0 (control), one, two, three or four injections of progesterone (50.00 mg; SC), 72 hr apart. The first progesterone was injected at the time of PG injection. Ewes in treatment groups received gonadotropins 48 hr after the last progesterone injection. Control group ewes received gonadotropins, at the time of PG injection. Mating was recorded after introducing fertile rams. Data were analyzed using GLM and GENMOD procedures in SAS. The incidence of estrus was less in control and ewes received a single progesterone (34.20%) compared to ewes received two (64.10%), three (81.10%) and four injections (68.40%) of progesterone. Time to estrus was earlier in control (45.70 ± 4.41 hr) than progesterone-treated groups (63.60 ± 1.79 hr). Fertility (51.30%) and fecundity (78.40%) of ewes received three progesterone injections were significantly greater than other progesterone-treated groups. There was no significant difference in reproductive indices between eCG and hMG sub-groups. In conclusion, during the non-breeding season, three injections of progesterone, three days apart, starting six days after ram exposure, in association with hMG, 48 hr after the last progesterone injection, could provide a sound reproductive performance in Shal ewes.

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Introduction

Three lambing in two years and enhanced twining rates are two common strategies to enhance productive performance of sheep flock.[1] Therefore, management of reproduction during the non-breeding season becomes prominent. During the non-breeding season, ovarian follicle growth occurs without estrous expression.[2,3] Therefore, progesterone priming was used to enhance estrous expression and ovulation.[4,5] Although progestogen priming for 9 to 14 days was commonly used,[6] there is no study to elaborate the least number of progesterone injections required to induce fertile estrus in ewe. It might be thought that intra-vaginal devices could be a sound way to deliver progesterone for long term; however, such devices not only involved cost and technician for hygienic insertion of device, but also could develop problems such as a reduced fertility, vaginitis, sponge's retention, purulent secretion and foul-smelling fluids, which in turn, could affect the animal health and welfare.[7-9]

Earlier studies used progesterone injections to induce estrus and ovulation in ewes during the non-breeding season.[4,10,11] In all earlier studies using progesterone injection, they failed to provide fertility results and the number of animals was not sufficient to make valid conclusion. Moreover, the serum concentration of progesterone following serial injections was not elaborated. The main question of this study was to investigate the least number of progesterone injections necessary to induce fertile estrus in ewes during the non-breeding season.

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The most common approach to enhance twining rates in sheep is to inject follicle-stimulating hormone (FSH) or FSH-like hormones at the end of progestogen priming.[12] Equine chorionic gonadotropin (eCG; previously named as pregnant mare serum gonadotropin) is commonly used in sheep practice.[13,14] It has the half-life of two - five days,[15] both FSH- and luteinizing hormone (LH)-like activities[16] and the ability to bind to both FSH and LH receptors, located at the granulosa and theca cells.[17] However, there are some reports indicating that application of eCG might induce anti-eCG antibodies, rendering the female refractory to the treatment.[18,19] Moreover, the price and availability of eCG could be a problem. Substitution of cheap and more available product not causing refractoriness could be useful for sheep industry.

Human menopausal gonadotropin (hMG) is known to induce super-ovulation in cattle,[20] camel,[21] goat[22] and sheep.[23] There is no report of using hMG for inducing estrus in sheep. We hypothesized that hMG could replace eCG to enhance fertility in ewes. Present study was designed to elaborate the least number of progesterone injections followed by hMG administration to induce fertile estrus during the non-breeding season in ewes.

Materials and Methods

Experimental location and animals. This study was conducted at the Veterinary Research Institute, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran (latitude: 35° 39' 8" N, longitude: 51° 26' 38" E and altitude: 1,029 m) during the non-breeding season (May - June 2019). Healthy, non-pregnant Shal ewes (Iranian native breed; n = 189), 41.10 ± 1.79 months of age and body condition score (BCS) of 2.90 ± 0.05, in the scale of one - five, and fertile adult Shal rams (n = 14; 47.90 ± 4.91 months of age) were used in this study. They received a ration according to National Research Council recommendation.[24] In brief, they received corn silage (400 g), wheat straw (450 g), alfalfa hay (700 g) and concentrate (400 g) as a mixed ration. Ewes received 300 g extra concentrate as a supplementary feeding. Present study was approved by the Animal Ethics Committee of the Faculty of the Veterinary Medicine, University of Tehran, Tehran, Iran (LAT940/20.11.2018).

Experimental design. Following two months isolation of rams and ewes, teaser Shal rams equipped with aprons were introduced to ewes (1:14; day 0 of experiment; Fig. 1). Ewes received a supplementary feeding three weeks before introducing teaser ram (from 09.5.2019). Ewes received prostaglandin (PG) $F_2\alpha$ analog (75.00 µg D-cloprostenol, Aburaihan Pharmaceutical Company, Tehran, Iran) on day six and were randomly assigned into five main groups, considering their age and BCS (Fig. 1). Ewes in control (n = 38) did not receive any progesterone injection. Group 1 (n = 38), group 2 (n = 38), group 3 (n = 37) and group 4 (n = 38) received one, two, three or four subcutaneous (SC) injections of progesterone acetate in oil (50.00 mg; Aburaihan Pharmaceutical Company, Tehran, Iran), three days apart, respectively. Each main group was divided equally into two sub-groups and received intra-muscular (IM) gonadotropins including 300 IU eCG (Gonaser; Hipra, Girona, Spain) or hMG (one ampule including 75.00 IU FSH and 75.00 IU LH, SC; Karma Pharmatech GmbH, Marburg, Germany) concurrent with PG (in control group) or 48 hr after the last progesterone injection in progesterone-treated groups (Fig. 1). Supplementary feeding ceased at the time of gonadotropin injection. Fertile rams (1:10) were introduced 24 hr after gonadotropin injection (Fig. 1). Estrus detection was performed for three consecutive days, twice a day, morning (6.00 – 11.00 am) and evening (18.00 - 23.00 PM), and during the night time using teaser ram equipped with harness and crayon. For ewes expressed estrus during night time and drafted in the morning, the time of estrous expression was considered as 2:30 AM. During each period of observation, fertile rams were replaced every 1 hr. Pregnancy was confirmed on day 35 after mating using a real-time linear array, B-mode ultra-sound scanner (HS-1500; Honda, Toyohashi, Japan) equipped with a 7.50 MHz rectal probe and rectal holder.

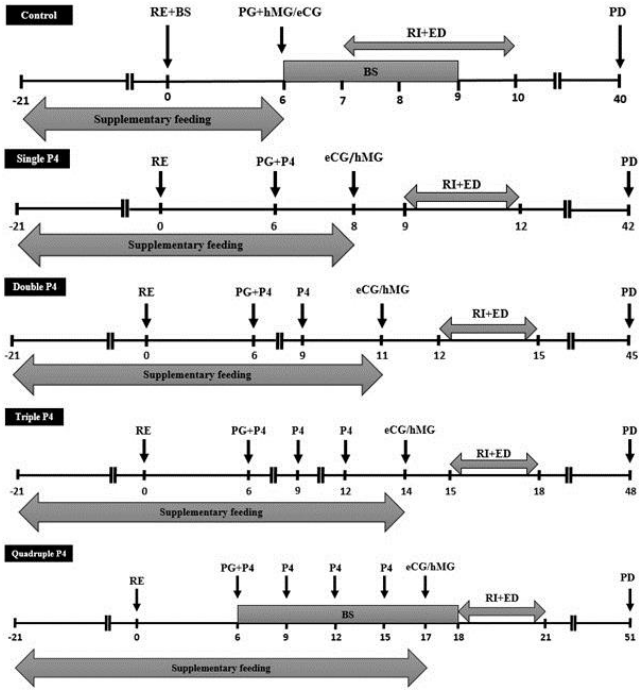

Fig. 1. Experimental design for control and progesterone-treated Shal ewes receiving gonadotropin (eCG or hMG) during the non-breeding season. Ewes in progesterone-treated groups received 1, 2, 3 or 4 injections of progesterone (P4; 50.00 mg, subcutaneously), 3 days apart. RE: ram exposure; BS: blood sampling; PG: prostaglandin F2α; P4: progesterone; eCG: equine chorionic gonadotropin; hMG: human menopausal gonadotropin; RI: ram introduction; ED: estrous detection; PD: pregnancy diagnosis.

Rams' preparation and semen analysis. Rams received supplementary feeding three weeks before beginning of the experiment. Prior to the ram introduction, semen was collected by an electro-ejaculator and the quality of semen was evaluated according to the methods described by Evans and Maxwell.[25] Rams with mass motility of ≥ 3 (scale 0 to 5) and individual motility of ≥ 70.00% were used in this study.

Blood sampling and progesterone assay. Blood samples of ewes were collected via the jugular vein into plain vacutainers tubes. In a subset of ewes (n = 77), two blood samplings, nine days apart, were performed prior to nutritional supplementation to determine cyclicity based on progesterone concentrations ≥ 1.00 ng mL^{-1}. Blood samplings were also taken on days 0 and 6 to 9 in 10 ewes belonged to the control group. Daily blood samples were collected from 10 ewes received four progesterone injections (representative of all progesterone-treated groups) for 15 days starting from the day of the first progesterone injection (Fig. 1). Blood samples were kept at 4.00 ˚C until centrifugation. Serum samples were separated by centrifugation at 2,000 *g* for 20 min and stored at – 21.00 ˚C until the progesterone assay. Progesterone was measured by enzyme-linked immunosorbent assay using commercial kit (Monobind, Lake Forest, USA), validated for sheep serum. The sensitivity, intra- and inter-assay coefficients of variation were 0.10 ng mL^{-1}, 3.80% and 7.50%, respectively.

Statistical analysis. Data with discrete nature including estrus response (number of ewes on heat/total number of ewes × 100), fertility (ewes lambed/ewes exposed to the ram × 100), prolificacy (lambs born/ewes lambed × 100) and fecundity (lambs born/ewes exposed to the ram × 100) were analyzed using GENMOD procedure including logistic regression as Link Function and Binomial (for fertility analysis) or Poisson (for prolificacy and fecundity analyses) statements as a type of distribution in the model. The percentages of events were calculated using FREQ procedure. Data with continuous nature including time to estrus were analyzed using GLM procedure after testing for normality (Shapiro-Wilk) using univariate procedure. Tukey's Studentized range test was used for pair-wise comparisons. The pattern of progesterone concentrations over time was analyzed using GLM procedure by either univariate or multivariate analyses with repeated measures analysis included in the model. Multivariate analyses were used where variance and covariance structures over time did not conform to the analysis of variance assumptions, using sphericity test. Data were presented as mean ± SEM and percentage. All analyses were conducted in SAS Software (version 9.20; SAS Institute, Cary, USA).

Results

Progesterone concentration. Prior to the initiation of the experiment, less than half of the ewes (33/77; 42.85%) had progesterone concentrations of ≥ 1.00 ng mL^{-1}. Progesterone concentration profiles in ewes belonged to the control group and those received four injections of progesterone (group 4), are depicted in Figure 2. Following PG injection, progesterone declined in control group (Fig. 2A; $p < 0.05$). Twenty-four hr after each progesterone injection, the concentration of progesterone elevated (2.53 ± 0.11; $p < 0.05$) followed by the gradual decline until the next progesterone injection (1.03 ± 0.07 ng mL^{-1}) in group 4 (Fig. 2B; $p < 0.05$).

Reproductive performance. There were no interactions between the number of progesterone injections and gonadotropin. Thus, the main effects were analyzed and presented. The incidence of estrus in control and group 1 (34.20%) was lower than group 2 (64.10%), group 3 (81.10%) and group 4 (68.40%; Table 1; $p < 0.05$). Time to estrus in control group was earlier than that in other groups (Table 1; $p < 0.05$). The time to estrus was not different between treatment groups ($p > 0.05$). Fertility in control (2.60%) and group 1 (7.90%) was less than group 2 (26.30%), 3 (51.30%), and 4 (31.60%; $p < 0.05$). Fertility of ewes in group 3 was greater than other progesterone-treated groups (Table 1; $p < 0.05$). Prolificacy was similar among groups ($p > 0.05$). Fecundity in control (5.30%) and group 1 (10.50%) was similar ($p > 0.05$) and less than that in other treatment groups (Table 1; $p < 0.05$). Fertility and fecundity were not different between groups 2 and 4 ($p > 0.05$). Ewes in group 3 had greater fecundity (78.40%) compared to other treatment groups ($p < 0.05$). The incidence of estrus and the time to estrus between eCG (54.20%; 55.71 ± 2.19 hr) and hMG (58.10%; 66.9 ± 2.51) sub-groups were not different (Table 2; $p > 0.05$). Moreover, fertility, prolificacy and fecundity were similar between eCG- and hMG-treated groups (Table 2; $p > 0.05$).

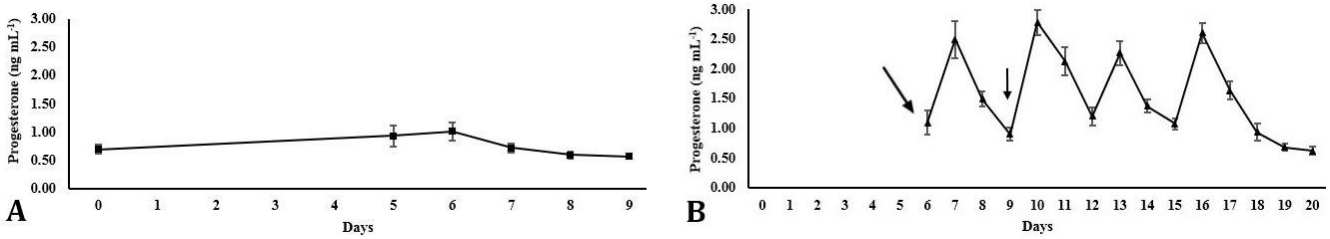

Fig. 2. Progesterone (P4) concentrations in **A)** control group which did not receive progesterone, and **B)** treatment group, 4 ewes which received four injections of progesterone (50.00 mg; subcutaneously), three days apart.

Table 1. Estrous response (number of ewes exhibited estrus within 72 hr and time from gonadotropin treatment to estrus), fertility (ewes lambed/total ewes), prolificacy (lambs/ewes lambed), fecundity (lambs/total ewes) in Shal ewes that received different number of progesterone injections, during the non-breeding season. Data were presented as mean ± SEM and percentage.

Number of injections	Number of ewes	Estrus (%)	Time to estrus (hr)	Fertility (%)	Lamb		Total Lamb	Prolificacy (%)	Fecundity (%)
					Single	Twin			
0 (control)	38	13 (34.20)[a]	45.70 ± 4.41[a]	1 (2.63)[a]	0	1	2	200	5.30[a]
1	38	13 (34.20)[a]	64.80 ± 5.87[b]	3 (7. 90)[a]	2	1	4	133.30[a]	10.50[a]
2	38	24 (63.10)[b]	62.80 ± 3.09[b]	10 (26.30)[b]	5	5	15	150	39.50[b]
3	37	30 (81.10)[b]	64.40 ± 3.66[b]	19 (51.30)[c]	9	10	29	152.60[a]	78.40[c]
4	38	26 (68.40)[b]	62.70 ± 2.76[b]	12 (31.60)[b]	9	3	15	125[a]	39.50[b]

[abc] Values within column with different superscripts differ significantly ($p < 0.05$).

Table 2. Estrous response (number of ewes exhibited estrus within 72 hr and time from gonadotropin treatment to estrus), fertility (ewes lambed/total ewes), prolificacy (lambs/ewes lambed) and fecundity (lambs/total ewes) in Shal ewes treated with different gonadotropins (eCG or hMG) during the non-breeding season. Data were presented as mean ± SEM and percentage.

Gonadotropin	Number of ewes	Estrus (%)	Time to estrus (hr)	Fertility (%)	Lamb		Total Lamb	Prolificacy (%)	Fecundity (%)
					Single	Twins			
eCG	96	52 (54.20)	55.71 ± 2.19	20 (20.80)	11	9	29	145	30.20
hMG	93	54 (58.10)	66.90 ± 2.51	25 (26.90)	14	11	36	144	38.70

eCG: Equine chorionic gonadotropin; hMG: Human menopausal gonadotropin.

Discussion

This study was designed to elaborate reproductive performance of Shal ewes during the non-breeding season using varying progesterone injections followed by hMG or eCG administrations. Progesterone injections (50.00 mg; SC), 3 days apart, provided progesterone levels above 1.00 ng mL^{-1} throughout the injection period. Using this experimental design, we were able to examine the effect of the length of progesterone exposure on reproductive performance of Shal ewes during the non-breeding season. Earlier studies have suggested 14 daily progesterone injections during the breeding season[10] or six injections of progesterone two days apart, during the non-breeding season[26] to control and synchronize ovine estrous cycle. However, several injections over a long period are not practically feasible in sheep flock. Therefore, other routes of progesterone administration were used including intra-vaginal devices,[8,27-29] ear implant[28,29] and oral progestogens.[30] The cost-effectiveness of each protocol depends on the cost of materials and labor used, number of interventions that might be associated with stress and/or infections and finally the reproductive indices. There is a chance of losing intra-vaginal sponge (6.70%) and controlled internal drug release (CIDR; 13.50%) and foul-smelling mucus discharge following sponge (80.00%) and CIDR (12.50%) withdrawal.[7] Similarly, Godfrey *et al.* have found that 18.50% of ewes (5/27) lose their CIDR prior to the ram introduction.[8] Swelum *et al.* have reported that the retention, vaginal discharge and drawstring breakage rates after sponge removal are 94.00, 98.58 and 9.22, respectively.[31] Sponge-treated ewes seem to have low fertility (45.00%) compared to the CIDR-treated ones (70.00-75.00%).[32] The functionality and viability of ram sperm could be negatively affected by the cervical mucus of ewes pre-treated with progestagen sponge.[33]

The frequency of estrus expression (34.20%) and fertility (7.90%) was decreased significantly following single injection of progesterone. This clearly indicated that a single injection of progesterone was not sufficient to prime ewes to show fertile estrus as indicated previously.[34] However, estrous expression pattern following three progesterone injections, every third day (81.10%), was similar to those studies used six injections, every second day (80.00%),[26] and five injections, every third day (88.80%).[11] The concentration and length of progesterone exposure could be important factors to induce fertile estrus during the non-breeding season in ewe. A minimum of three days exposure to progesterone seemed to be necessary to induce estrus during the non-breeding season in ewe.[5] Insertion of vaginal sponge for three days was as effective as three or towel days to induce fertile estrus in ewes during the non-breeding season; whereas, one- or two-days treatments were not sufficient.[35]

Reproductive performances including fertility (51.30%) and fecundity (78.40%) of ewes received three consecutive injections of progesterone were significantly greater than those received one, two or four progesterone injections. Therefore, short or long durations of progesterone exposure could have negative impacts on reproductive indices. In any protocol, based on intra-vaginal progestogen devices, two interventions are necessary, one for device insertion and another one for withdrawal. Therefore, the protocol used in the present study using three injections of progesterone could be cost-effective without necessity to manipulate reproductive tract. In more recent study, we compared the reproductive performance of Shal ewes after inducing estrus using intra-vaginal sponges and progesterone injection followed by eCG administration, during the non-breeding season. In this particular study, we did not use ram effect; therefore, we used four progesterone injections. The ultimate result

(fecundity) was not different between progesterone injection (61.60 %) and intra-vaginal sponge (55.60%).[35]

In the present study, ram effect was used to enhance cyclicity of ewes during the non-breeding season. Sudden introduction of ram (ram effect) was also associated with ovulation in some ewes during the non-breeding season,[36,37] and those in the early breeding season.[38] However, the ovulation following ram exposure was associated with silent estrus and short-lived corpus luteum (CL).[36,39] We suggested that the combination of ram effect to induce short-lived CL followed by three progesterone injections could provide sufficient and optimal progesterone priming for follicle growth, estrus expression and ovulation and enhance reproductive performance during the non-breeding season. This strategy could reduce the cost and avoid the consequences of progestogen device insertion.

The second objective of this study was to substitute eCG with hMG at the end of estrus induction program in ewe. There was no significant difference in the incidence of estrus, time to estrus and reproductive performance between eCG- and hMG-treated ewes. Therefore, eCG can be successfully substituted with hMG at the end of the estrus induction program. The eCG can stimulate follicular development and enhance reproductive performance of ewes during the non-breeding season.[40,41] The dose of eCG varies from 250 to 750 IU,[13] depending on age (250 - 300 IU in ewe lambs and 350 - 500 IU in adult ewes), season (400 - 500 IU during the non-breeding season and 300 - 350 IU during the breeding season) and breed (low doses for prolific breeds).[42] The eCG can be injected from two days before to 0 hr of progestogen cessation.[6] There is no report of using hMG for estrus induction in sheep.

The interval from gonadotropin treatment to estrus expression for ewes in control group received PG injection was significantly shorter (45.70 ± 4.41 hr) than those received progesterone injections (63.60 ± 1.79 hr). Similarly, Hashemi *et al*. have reported that the interval from the cessation of progesterone treatment to the onset of estrus during the non-breeding season was significantly longer in ewes received IM progesterone injection (51.40 ± 10.00) compared to CIDR (30.10 ± 7.60) and sponge groups (29.60 ± 5.60 hr).[26]

In the present study, 42.85% (33/77) of the ewes displayed cyclicity according to progesterone concentrations (≥ 1.00 ng mL^{-1}). In control group received PG and gonadotropin at the same time, only 13/38 (34.20%) expressed estrus following PG injection. Whereas, during the breeding season in cycling ewes, 66.00% responded to PG and displayed estrus.[43]

Beside of low estrus response, the fertility of ewes in control group was substantially low (1/38; 2.63%). Pregnancy rate is generally low in ewes bred at a synchronized estrus with PG compared to untreated[44] and progestogen-treated ewes.[45] Moreover, low fertilization was reported when ewes were synchronized with PG (7.00%) compared to progestogens (69.00%).[46] Low pregnancy rate obtained after PG administration could be due to alterations in myometrial contractions,[47] in which a decreased number of uterine contractions toward the oviduct resulted in fewer sperm reaching the fertilization site.[48] Other authors have suggested that the alterations of the vaginal mucus impair sperm transport from the cervix to the uterus following PG injection.[49] Immobilized and dead sperms were present in the anterior third of the cervix and uterine body in PG-treated ewes, probably as a result of the presence of spermicidal factors, or because of the absence of substances protecting the semen.[50]

Based on the protocol used in the present study in which ewes received nutritional supplementation and ram effect, three injections of progesterone (50.00 mg; SC; three days apart) and hMG, 48 hr after the last progesterone injection, could be used for inducing fertile estrus in ewes during the non-breeding season.

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Conflict of interest

The authors have no conflicts of scientific interest with respect to the manuscript.

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