



Administration of gonadotropin-releasing hormone at sponge insertion increases litter size in ewes subjected to a fixed-time insemination procedure

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ABSTRACT - The effect of a dose of GnRH administration at sponge insertion on pregnancy rate and litter size in ewes was investigated. A total of 162 pluriparous, non-lactating Huiyang ewes (3–4 years old, 40–50 kg body weight, 120–150 days postpartum) were randomly and equally assigned to three groups (n = 54 each): control, G25, and G50. Estrus was synchronized using an intravaginal sponge impregnated with flurogestone acetate for 12 days, followed by an injection of 330 IU of equine chorionic gonadotropin at sponge removal. At sponge insertion, ewes received an intramuscular injection of either 25 µg (G25) or 50 µg (G50) of the GnRH agonist triptorelin, or saline solution (control). Fixed-time artificial insemination was performed at 52 and 60 h after sponge removal. Pregnancy was diagnosed by transabdominal ultrasonography 45 days post-insemination, and reproductive parameters were recorded after lambing. Pregnancy rate was not affected by GnRH administration ($P = 0.3001$). However, the multiple birth rate ($P = 0.0032$) and litter size ($P = 0.0012$) were significantly increased in the G50 group compared with control. In conclusion, administration of 50 µg of GnRH at sponge insertion increased litter size in Huiyang ewes subjected to fixed-time artificial insemination.

Keywords: endocrine manipulation, hormone protocol, ovulation synchronization, reproductive efficiency, triptorelin

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide synthesized in the hypothalamus. GnRH is released in pulses into the hypophyseal portal circulation and reaches the anterior pituitary, where it stimulates the synthesis and release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH is the central initiator of the reproductive hormonal cascade. An extensive body of literature has shown that GnRH can be used to synchronize and induce ovulation in farm animals (Gonzalez-Bulnes et al., 2004; Menchaca and Rubianes, 2004; Reyna et al., 2007; Balara et al., 2016). Although GnRH is already widely used in ovine fixed-time artificial insemination programs, its effects of GnRH on pregnancy rates remain controversial (Menchaca and Rubianes, 2004). Some studies have reported that a single dose of GnRH given 24 to 48 h after progestagen/progesterone pessary removal increased pregnancy rate (Biehl et al., 2017; Kutlu and Dinç, 2021; Pereira et al., 2024), while others have reported no changes (Reyna et al., 2007; Türk et al., 2008; Olivera-Muzante et al., 2011) or even a decreased pregnancy rate (Martemucci and D'Alessandro 2011; Olivera-Muzante et al., 2013; Maggi et al., 2024). In our previous studies, we found that a single dose of GnRH administered at insemination

significantly decreased pregnancy rate but increased litter size in Kazak ewes undergoing a fixed-time artificial insemination procedure during the breeding season (Zhang et al., 2023). Año-Perello et al. (2020) reported that administration of a single dose of GnRH at sponge insertion can enhance the response of ewes, improve follicular turnover, and synchronize a new follicular wave. We hypothesized that a single dose of GnRH administered at sponge insertion could increase pregnancy rate and litter size in estrus-synchronized ewes. Therefore, the objective of the present study was to determine whether a single dose of GnRH administered at sponge insertion increases the pregnancy rate and litter size in non-seasonally prolific Huiyang ewes undergoing a fixed-time insemination procedure.

2. Material and methods

2.1. Animals and ethics

This study was conducted in Zhalantun, Hulun Buir, Inner Mongolia Autonomous Region during the breeding season (November - December). The study area is located at longitude 121.24 degrees east and latitude 47.31 degrees north. The climate is characterized as a mid-temperate continental monsoon type. The average annual rainfall is 480.3 mm, and the annual average temperature is 3.3 °C. A total of 162 clinically healthy Huiyang ewes, free of reproductive disorders, pluriparous and non-lactating (3 - 4 years old with 40 - 50 kg body weight and 120 - 150 days postpartum) and four adult Australian White rams (3 - 4 years old with 80 - 90 kg body weight) were used. During the experimental period, ewes were kept away from the rams to prevent natural mating. Ewes were housed indoors. They were fed a diet of barley and alfalfa hay supplemented with grass. Fresh drinking water and salt licks were provided *ad libitum*. No nutritional flushing was applied to the animals before mating. The Ethics Committee of Hebei Agricultural University approved the use of animals and the procedures included in this study. All procedures in the experiment were conducted in a manner consistent with animal health and welfare guidelines.

2.2. Semen preparation

Semen was collected from rams using an artificial vagina (Muqimuye Sci-Tech Co., Ltd., Shanghai, China) one hour before each insemination. All ejaculates met acceptable parameters for volume (0.7 - 2.0 mL), sperm concentration ($>3 \times 10^9$ sperm/mL), and sperm motility ($>70\%$ progressive motility). Ejaculates were pooled and diluted in ultra-high temperature-treated commercial skimmed milk (YILI™, YILI Group, China) to achieve a final concentration of 400×10^6 spermatozoa cells per mL. Sperm concentration was assessed using a hemocytometer under a light microscope (Zhang et al., 2022). The semen was kept at 30 °C in a water bath until insemination.

2.3. Estrus synchronization and insemination

Each ewe was treated with a polyurethane intravaginal sponge impregnated with 45 mg of flurogestone acetate (Muqimuye Sci-Tech Co., Ltd., Shanghai, China) for 12 days, followed by an intramuscular injection of 330 IU of equine chorionic gonadotropin (Sansheng Biological Technology Co., Ltd., Ningbo, China) at sponge removal. In total, 162 ewes were randomly and equally divided into three groups: the control group (n = 54), the G25 group (n = 54), and G50 group (n = 54). At sponge insertion, ewes in the G25 and G50 groups received intramuscular injections of 25 µg and 50 µg of the GnRH agonist triptorelin (Sansheng Biological Technology Co., Ltd., Ningbo, China), respectively, whereas control ewes received with 1 mL of sterile physiological saline solution. Each ewe was subjected to cervical insemination twice, at 52 h and 60 h after sponge removal using an insemination device (Zhengmu Bio-Tech Co., Ltd, Baoding, China) containing 0.25 mL of diluted semen (approximately 100×10^6 spermatozoa). The cervical opening was located using a speculum and a head light. Each ewe was restrained by elevating the hind limbs against a wooden support. The perineal area was scrubbed with antiseptic soap and warm water thoroughly. A speculum was inserted into the vagina and pushed against the tissue surrounding the cervix to help center the external cervical os with the aid of a head

light. The insemination device was inserted into the cervix and slowly and intermittently pushed down to deposit the semen (Zhang et al., 2022). Semen dilution and insemination were performed by the same experienced technician.

2.4. Ultrasound examination and reproductive performance

Pregnancy was diagnosed by transabdominal ultrasonography using a B-Mode Real-Time ultrasound with a 3.5-MHz probe (Mindray DP-50Vet, Shenzhen, Guangdong, China) 45 days after the sponges removal. The number of ewes carrying at least one live fetus was recorded. The pregnancy rate (number of pregnant ewes/number of inseminated ewes), lambing rate (number of lambing ewes/number of pregnant ewes), multiple birth rate (number of ewes lambing twins or more/total number of lambing ewes), litter size (number of total lambs/number of lambing ewes), survival rate (number of living lambs/number of lambs born), female lamb rate (number of female living lambs/number of total living lambs), and male lamb rate (number of male living lambs/number of total living lambs) were calculated after completion of all deliveries. The gestation length was calculated from the first insemination to delivery.

2.5. Data analysis

The data were statistically analyzed using SAS Version 8.0. The litter size and period of gestation were analyzed using a one-way ANOVA and multiple comparisons. The other reproductive parameters were analyzed using a Chi-squared test for every possible pairwise comparison with Bonferroni correction. It was accepted as significant if the calculated P - values were less than 0.05. Period of gestation are expressed as mean $\pm$ SD.

3. Results

After excluding four ewes due to sponge loss during estrus synchronization, 52, 53, and 53 ewes were inseminated in the control, G25, and G50 groups, respectively. Pregnancy rate did not differ among groups. The multiple birth rate and litter size in the G50 group (100% and 3.05) were significantly higher ($P<0.01$) compared to those in the control group (77.1% and 1.83) (Table 1). The two parameters

Table 1 - Effects of GnRH on reproductive performance of Huyang ewes

Reproductive parameters	Control	G25	G50
Number of ewes	54	54	54
Pregnancy rate (%)	69.2 (36/52)	75.5 (40/53)	79.2 (42/53)
Lambing rate (%)	97.2 (35/36)	95.0 (38/40)	97.6 (41/42)
Multiple birth rate (%)	77.1 (27/35)a	92.1 (35/38)ab	100 (41/41)b
Number of lambs			
Single	8 (22.9%)a	3 (7.9%)ab	0b
Twin	25 (71.4%)a	20 (52.6%)ab	2 (4.9%)b
Triplet	2 (5.7%)a	15 (39.5%)b	35 (85.4%)b
Quadruplet	0	0	4 (9.8%)
Litter size	1.83 (64/35)a	2.32 (88/38)ab	3.05 (125/41)b
Survival rate (%)	96.9 (62/64)	95.5 (84/88)	96.8 (121/125)
Female lamb rate (%)	51.6 (32/62)	46.4 (39/84)	47.1 (57/121)
Male lamb rate (%)	48.4 (30/62)	53.6 (45/84)	52.9 (64/121)
Days of gestation (day)	148.6 $\pm$ 2.63	149.5 $\pm$ 2.51	149.2 $\pm$ 2.74

Ewes in the G25 group, G50 group, and control group were subjected at sponge insertion to intramuscular administration with 25 μ g, 50 μ g of the GnRH agonist triptorelin and 1 mL of sterile physiological saline solution, respectively.

Pregnancy rate = Number of pregnant ewes/Number of inseminated ewes; Lambing rate = Number of lambing ewes/Number of pregnant ewes; Multiple birth rate = Number of ewes lambing twin or more/Total number of lambing ewes; Litter size = Number of total lambs/Number of lambing ewes; Survival rate = Number of living lambs/Number of lambs born; Female lamb rate = Number of female living lambs/Number of total living lambs; Male lamb rate = Number of male living lambs/Number of total living lambs. Period of gestation are expressed as mean $\pm$ SD.

a,b - The difference between values with different letters in the same row is significant at the P-value of <0.05.

in the G25 group (92.1% and 2.32) did not differ significantly ($P>0.05$) compared to those in the control group and the G50 group. The rates of single, twin, triplet, and quadruplet were significantly different ($P<0.01$) among the three groups. These results indicate that GnRH administration at sponge insertion significantly increased the multiple birth rate. Particularly in the G50 group, all ewes had multiple lambs, and four ewes had quadruplets. The lambing rate, survival rate, female lamb rate, male lamb rate, and period of gestation in the three present study groups were not significantly different ($P>0.05$).

4. Discussion

Our data showed that the multiple birth rate and litter size were significantly increased by 50 μg of GnRH. The effects of 50 μg of GnRH on the multiple birth rate and litter size were not significantly different than those of 25 μg of GnRH.

In a previous study, the administration of a single dose of GnRH, concomitant with the insertion of a progesterone-loaded controlled internal drug release device for estrus synchronization, caused regression of gonadotrophin-dependent follicles in all ewes, with 70% of them initiating a new follicular wave and the remaining showing non-dominant follicles (Año-Perello et al., 2020). This is considered, as previously described, the best scenario for applying assisted reproductive techniques and has led to the so-called “Day 0 Protocol,” which significantly improves embryo yield by using the first wave of the estrous cycle (Menchaca et al., 2009). The absence of dominant follicles can induce a higher ovulation rate and better embryo yield in ewes through direct action, independent of systemic pathways (Balara et al., 2016; Gonzalez-Bulnes and Veiga-Lopez, 2008). Therefore, it was speculated that GnRH administration induces the absence of dominant follicles, provides better environment for the development of follicles and multiple ovulations, and thereby increases the litter size of estrus-synchronized ewes subjected to a fixed-time insemination procedure in the present study.

These results were similar to those of a previous study, which showed an additional injection of GnRH prior to estrus induction enhanced the lambing rate, twin pregnancies, and litter size in Karakul ewes using a two-injection protocol of PGF2 α , 10 days apart, for an estrus synchronization protocol (Mirzaei et al., 2017). In our preliminary studies, administration of a single dose of GnRH (50 μg of triptorelin) at sponge insertion significantly increased pregnancy rates and litter size in Kazak ewes, Small-tailed Hanyang ewes, Mongolian ewes, and down-bearing goats in fixed-time insemination procedures during the breeding season (data not shown). In addition, GnRH administration at the beginning of progesterone-based synchronization increased pregnancy per artificial insemination in lactating dairy cows and suckled beef cows (Consentini et al., 2021; Tschopp et al., 2022). Administration of GnRH at sponge insertion offers a time-efficient protocol for improving ovine reproductive performance.

5. Conclusions

A single dose of GnRH administration at sponge insertion increased the litter size in non-seasonally prolific Hanyang ewe after fixed-time artificial insemination. These findings strongly suggest that this protocol should be recommended for sheep reproduction. Further studies are needed to assess the effects of the GnRH administration protocol on pregnancy rate and follicular dynamics in a larger sample of ewes, and across different breeds.

Data availability

Data supporting these findings are available upon request to the corresponding author.

Author contributions

Data curation: Sun, S. **Formal analysis:** Zhang, J. **Funding acquisition:** Liu, Y. **Project administration:** Li, X. **Software:** Yang, N. **Writing – review & editing:** Wu, X. and Li, X.

Conflict of interest

The authors declare no conflict of interest.

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