

Effects of human chorionic gonadotropin on reproductive outcomes in estrus-synchronized ewes subjected to two different insemination methods

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Abstract

This study compared the effects of a dose of human chorionic gonadotropin (hCG) administration at the time of insemination on the reproductive outcome of estrus-synchronized Mongolian ewes subjected to two artificial insemination methods during the breeding season. All females were treated with a polyurethane intravaginal sponge impregnated with 45.00 mg of flurogestone acetate for 12 days, followed by an intramuscular injection of 330 IU of equine chorionic gonadotropin at sponge removal. In Experiment 1, 150 ewes were inseminated using a laparoscopic intrauterine artificial insemination method 48 hr after sponge removal. The ewes were randomly assigned to the hCG group which received 500 IU of hCG at the time of insemination, and the control group which received 1.00 mL of sterile saline solution. In Experiment 2, 85 ewes were inseminated twice at 48 and 60 hr after sponge removal using a cervical artificial insemination method. The ewes were randomly assigned to the hCG group, which received 500IU of hCG at the time of the first insemination and the control group which received 1.00 mL of sterile saline solution. The pregnancy rate in the control group was not significantly different compared to the hCG group in Experiment 1 and the pregnancy rate in the control group was significantly higher compared to the hCG group in Experiment 2. In conclusion, the administration of hCG at the time of insemination could not be recommended in ewes when implementing a fixed time artificial insemination protocol during the breeding season.

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Introduction

The combination of artificial insemination and estrus synchronization, commonly called fixed time artificial insemination (FTAI), is well diffused in sheep reproduction. The FTAI procedure can significantly increase reproductive efficiency, improve flock management and simplify the international trade of male genetic material and the conservation of genetic resources.^{1,2} Moreover, it preserves animal health by limiting the spread of several infectious diseases.³ In the FTAI procedure, the most commonly used protocol for estrus synchronization in ewes relies mainly on the use of progesterone combined with the administration of a single dose of equine chorionic gonadotropin (eCG) at the end of the progesterone treatment. This facilitates the synchronization of estrus by management of the dynamics

of follicular waves.⁴ To improve reproductive outcomes, a single dose of gonadotropin-releasing hormone was administered at the time of insemination.^{2,5} Although gonadotropin-releasing hormone administration increased litter size, the pregnancy rate was decreased.^{2,6,7} Therefore, there is a need to find an alternative hormone to gonadotropin-releasing hormone to increase the pregnancy rate and litter size after estrus synchronization treatments in ewes.

Human chorionic gonadotropin (hCG) can interact with luteinizing hormone receptor, making it a favored choice for inducing oocyte maturation and improving ovulation rate when applied in protocols for estrus synchronization in ewes. The hormone has been used to improve the reproductive efficiency of ewes, goats and heifers.^{8,9} Administered in the presence of functional ovulatory corpora lutea (CL), hCG induces the formation of an

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accessory CL and enhances the total number of born lambs and the fecundity rate in small ruminants.¹⁰ Human chorionic gonadotropin can also be used at the time of mating or artificial insemination to increase crown-rump length, amniotic sac width, the number of placentomes and the number of lambs born.^{11,12}

In sheep artificial insemination procedures, semen is released at the external cervical os through the vaginal canal (cervical artificial insemination, CAI) or it is directly released into the uterine lumen by laparoscopy (laparoscopic intrauterine artificial insemination, LAI). This study originally aimed to investigate the effects of a single dose of hCG administration at the time of insemination on the reproductive outcome of estrus-synchronized ewes during the breeding season. Insemination methods were not considered in the original experiment design and then two different insemination methods (LAI and CAI) were employed in the two experiments with different sample size. Later, however, it was inadvertently discovered that different insemination methods influence the effects of a single dose of hCG administration at the time of insemination. This interesting discovery was presented in this study. To the best of our knowledge, it is the first time, in the literature, that the effects of hCG on reproductive outcomes in estrus-synchronized ewes subjected to two different insemination methods were investigated.

Materials and Methods

Location and animals. This study was carried out on a farm near Tongliao, in Northeast China, during the breeding season. It was conducted in accordance with the Institutional Animal Care and Use Committee of Hebei Agricultural University (Approval No. 20220910-03). The authors declare that all procedures in the experiment were conducted in a manner consistent with animal welfare guidelines. This area is located at longitude 123.312 degrees east and latitude 44.126 degrees north. The climate is characterized by a mid-temperate continental monsoon climate. The average annual precipitation is 411.50 mm, and the annual average temperature is 5.90 °C. A total number of 235 clinically healthy, free of reproductive disorders, multiparous and non-lactating Mongolian ewes (3 - 4 years old with 40.00 - 50.00 kg body weight and 120 - 150 days postpartum) and three adult Australian White rams (3-4 years old with 70.00 - 80.00 kg body weight) were used. All animals were kept indoors. They were fed on a diet of corn straw and alfalfa hay, supplemented with grass, and had free access to water and salt bars.

Semen preparation. Semen was collected using an artificial vagina (Muqimuye Sci-Tech Co., Ltd., Shanghai, China) from the rams. All ejaculates were within acceptable parameters for volume (0.70 - 2.00 mL), sperm

concentration ($> 3.00 \times 10^9$ sperm *per* mL), and sperm motility ($> 70.00\%$ progressive motility). The ejaculates were pooled and diluted in ultra-high temperature-treated commercial skimmed milk (YILI™; YILI Group, Hohhot, China) to reach a concentration of 4.00×10^8 spermatozoa *per* mL. Sperm motility was assessed using a hemocytometer slide under a light microscope.² The semen was kept at 30.00 °C in a water bath until insemination.

Experimental procedure. Two experiments were carried out to evaluate the effects of administering of hCG (Sansheng Biological Technology Co. Ltd., Ningbo, China) intramuscularly at the time of LAI (Experiment 1) and CAI (Experiment 2) on the reproductive outcome in cyclic Mongolian ewes. Based on previous pilot experiments, 500 IU of hCG was employed. All females were treated with a polyurethane intravaginal sponge impregnated with 45.00 mg of flurogestone acetate (Muqimuye Sci-Tech Co. Ltd) for 12 days followed by an intramuscular injection of 330 IU of eCG (Sansheng Biological Technology Co. Ltd) at sponge removal. In Experiment 1, 150 ewes were randomly and equally assigned to two groups: The control group (n = 75) and the hCG group (n = 75). Animals in the control and the hCG groups were inseminated using an LAI method 48 hr after sponge removal. In the hCG group, the ewes received 500 IU of hCG at the time of insemination and the control group received 1.00 mL of sterile physiological saline solution. The LAI method was conducted as previously described.¹³ In brief, the ewes were fasted for at least 24 hr prior to LAI. Each ewe was sedated with a xylazine hydrochloride intramuscular injection (0.10 mg kg⁻¹; Huamu Animal Pharmaceutical Co., Ltd, Yushu, China) and restrained in a head-down position in dorsal recumbency in a cradle. The belly wool was clipped and scrubbed with antiseptic soap and warm water thoroughly. It was then dried using paper towels. Two small incisions were made on either side of the abdominal midline, approximately 8.00 - 10.00 cm anterior to the mammary glands, for insertion of the laparoscope and insemination pipette via cannulas. For each ewe, the semen (0.20 mL) was loaded into a pipette (IMV, Shanghai, China) with half of the insemination dose released into the lumen at the middle of each uterine horn. After the procedure, all animals were removed from the cradles and allowed to recover in a nearby pen. In Experiment 2, 85 ewes were randomly assigned to two groups: The control group (n = 42) and the hCG group (n = 43). Animals in the control and hCG groups were inseminated twice at 48 and 60 hr after sponge removal using a CAI method. In the hCG group, the ewes received 500 IU of hCG at the time of the first insemination and the control group received 1.00 mL of sterile physiological saline solution. The CAI method was conducted using an insemination device (Zhengmu Bio-Tech Co. Ltd, Baoding, China) containing 0.25 mL of diluted semen (approximately 1.00×10^8 spermatozoa). The ovine

cervical opening was located using a speculum and a head light. Each ewe was restrained by raising the hind limbs against a thick stick. 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 release the semen.² Semen dilution and two insemination methods were performed by the same experienced technician.

Ultrasound examination and reproductive outcome. Pregnancy diagnosis was performed by trans-abdominal ultrasonography using a B-Mod Real-Time ultrasound with a 3.50 MHz probe (DP-50Vet; Mindray, Shenzhen, China) 45 days after the removal of sponges. The number of ewes carrying at least one live fetus was recorded. The pregnancy rate (number of pregnant ewes *per* number of inseminated ewes), lambing rate (number of lambing ewes *per* number of pregnant ewes), ratio of males to females (number of male living lambs *per* number of females living lambs) and litter size (number of total lambs *per* number of lambing ewes) were calculated after completion of all deliveries.

Statistical analysis. The data were statistically analyzed using SAS Software (version 8.0; SAS Institute, Cary, USA). The pregnancy rate, lambing rate and litter size were submitted to the *t*-test. It was accepted as significant if the calculated *p* values were less than 0.05.

Results

The effects of treatment with hCG at the time of insemination including LAI and CAI on the reproductive outcome in two experiments are shown in Table 1. Three ewes were eliminated due to sponge loss during estrus synchronization. In Experiment 1, the pregnancy rate, the lambing rate and the litter size in the control group were not significantly different compared to the hCG group (81.33 vs 78.08%, *p* = 0.62; 96.72 vs 98.24%, *p* = 0.60; 1.15 vs 1.13, *p* = 0.67, respectively). In Experiment 2, the lambing rate and litter size in the control group were not

significantly different compared to the hCG group (95.65 vs 92.30%, *p* = 0.67; 1.18 vs 1.17, *p* = 0.9128, respectively). However, the pregnancy rate in the control group was significantly higher compared to the hCG group (54.76 vs 30.95%, *p* = 0.03). The ratio of males to females was not significantly different between the hCG group and the control group in the two experiments.

Discussion

According to our data, the administration of hCG at the time of the CAI insemination significantly decreased the pregnancy rate, while the administration of hCG at the time of the LAI insemination did not decrease the pregnancy rate in ewes undergoing the FTAI procedure. The lambing rate, the ratio of males to females and the litter size were not significantly different between the hCG group and the control group in the two experiments.

An extensive body of literature has shown that a single dose of hCG administration in the early luteal phase after insemination/mating in estrus-synchronized or super-ovulated ewes can induce the formation of an accessory CL.¹⁰ However, the effects of a single dose of hCG given before or at the time of insemination/mating in estrus-synchronized ewes during the breeding/non-breeding season are controversial as pregnancy rates are commonly decreased. Dias *et al.* reported that the administration of 500 IU of hCG 24 hr after progesterone device removal in estrus-synchronized Santa Ines ewes inseminated using a CAI insemination method between 48 - 49 hr and 60 - 61 hr after the removal of the device, respectively, improves ovulatory synchronization.¹⁴ However, it causes a significant decrease in the pregnancy rate by 22.80% (56.70 vs 33.90) during the breeding season. This is consistent with our results. In a previous research performed during the reproductive season, a total number of 26 ewes were divided into two equal groups, which were treated with one intravaginal progesterone device for five days plus one intramuscular dose of 5.00 mg of prostaglandin F2 α at progesterone withdrawal. Half of the ewes were treated with 400 IU of eCG at progesterone removal whilst the remaining received 500 IU of hCG at

Table 1. Effects of hCG on reproductive outcome in estrus-synchronized ewes subjected to two different insemination methods. Ewes in the hCG group and the control group were subjected to intramuscular administration of 500 IU of hCG and 1.00 mL of sterile physiological saline solution, respectively, at the time of insemination.

Reproductive outcome	Experiment 1: LAI		Experiment 2: CAI	
	Control	hCG	Control	hCG
Number of ewes with sponge insertion	75	75	42	43
Number of ewes inseminated	75	73	42	42
Pregnancy rate (%)	81.33 (61/75)	78.08 (57/73)	54.76 (23/42) ^a	30.95 (13/42) ^b
Lambing rate (%)	96.72 (59/61)	98.24 (56/57)	95.65 (22/23)	92.30 (12/13)
Number of lambs (Male/Female)	68 (36/32)	63 (30/33)	27 (12/15)	15 (9/6)
Litter size	1.15 (68/59)	1.13 (63/56)	1.18 (26/22)	1.17 (14/12)

LAI: Laparoscopic intrauterine artificial insemination, CAI: Cervical artificial insemination, and hCG: Human chorionic gonadotropin.

^{ab} Difference between values with different letters in the same row is significant at *p* < 0.05.

24 hr after progesterone removal. The estrus-synchronized ewes were subjected to natural mating and pregnancy diagnosis was performed at 35 days after progesterone removal using an ultrasound scanning. The administration of 500 IU of hCG significantly decreased the pregnancy rate by 30.70% (69.20 vs 38.50) compared to eCG.⁹ Dias *et al.* reported that the administration of 200 IU of hCG at the time of progesterone device removal significantly decreased the pregnancy rate by 16.00% (34.60 vs 18.60) in estrus-synchronized Polwarth ewe lambs subjected to natural mating during the non-breeding season.¹⁵ By comparing pregnancy rates in estrus-synchronized multiparous Dorper ewes treated with 300 IU of hCG at the time of removal of a progesterone device during the non-breeding season, the results observed in the hCG-treated ewes showed a lower pregnancy rate (30.70%) compared to the eCG-treated ewes (72.70%) after natural mating.⁴ This was in agreement with previous studies.^{9,15} Santos *et al.* reported that a dose of 200 IU of hCG given at the time of sponge withdrawal in estrus-synchronized Texel ewes subjected to a CAI insemination method decreased the pregnancy rate by 30.00% during the non-breeding season.¹⁶ Furthermore, this hCG administration decreased the pregnancy rate by 37.50% in Polypay ewes subjected to natural mating during the breeding season.¹⁷ Estrus was synchronized in fat-tailed Ghezel ewes with a 2-injection protocol of prostaglandin F₂ α analogue 8-days apart during the breeding season. Twenty-four hours after the second injection of prostaglandin F₂ α , the ewes were intramuscularly injected with 500 IU of hCG. All ewes were then exposed to five fertile rams. The results showed that the fertility rate (number of ewes lambing/number of ewes mated) was significantly decreased by 28.60%.¹⁸ In summary, an extensive body of literature has shown that the administration of hCG at the time of the CAI insemination or natural mating significantly decreased the pregnancy rate. Little documents reported the administration of hCG at the time of the LAI insemination in ewes.

It has been reported that the low pregnancy rates observed in protocols that involve the administration of hCG to induce ovulation during estrus synchronization in sheep may be attributed to a high incidence of abnormal follicular growth patterns, disruptions and delays in ovulation as well as the concurrent development of follicular cysts in treated females.^{9,15} This was not in agreement with previous studies in ewes,¹⁹ in which repeated administration of hCG at sponge removal supported CL formation, increased serum progesterone concentration and prevented premature luteal regression in eCG-superovulated Sanjabi ewes. In addition, in our previous study, two groups of 20 Black Suffolk ewes were synchronized and superovulated, and had ovulation induced with hCG or physiological saline. This was

followed by an LAI insemination and surgical embryo collection. The ovulatory response and yield of transferable embryos were modest with no significant differences between the two groups (data not shown). Hence, in view of the previous data, the insemination method may be related to the effects of hCG given at the time of insemination on the pregnancy rate in ewes undergoing FTAI procedures. In this present study, the administration of hCG at the time of CAI insemination significantly decreased the pregnancy rate (54.76 vs 30.95%), while the administration of hCG at the time of LAI insemination did not decrease the pregnancy rate (81.33 vs 78.08%) in Mongolian ewes. The phenomenon was also observed in Huiyang ewes, and Huiyang \times Australian White hybrid ewes (data not shown). These results were consistent with a previous study in which a single intramuscular injection of 500 IU of hCG at the time of LAI insemination did not decrease the pregnancy rate in estrus-synchronized Manchega ewes with progestagen sponges and eCG treatment.²⁰ In a CAI insemination method, semen is released at the external cervical os. Administration of hCG may prevent the entry of sperm into the uterine cavity by altering cervical physiological characteristics and intracervical sperm transport in ewes, thus, decreasing the pregnancy rate in ewes.²¹ However, no any experimental evidences (e.g., uterine physiological or histological analysis) support this hypothesis. It is presented as speculation. More studies will be conducted to assess the effects of hCG administration on the profile of serum progesterone concentration, follicular growth patterns, and uterine parameters.

In conclusion, a single dose of hCG administration at the time of CAI insemination significantly decreased the pregnancy rate of estrus-synchronized Mongolian ewes during the breeding season in this present study. These findings strongly suggested that hCG administration at the time of insemination/mating could not be recommended in ewes when implementing an FTAI protocol. Future research will be conducted to detect the effects of lower doses of hCG or alternative administration timings on sheep reproduction.

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Conflict of interest

The authors declare no conflict of interest.

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