Transcervical or Laparoscopic Insemination of Frozen-thawed Semen in Estrus-synchronized Himalayan Tahrs (*Hemitragus jemlahicus*)

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ABSTRACT

Four estrus-induced Himalayan tahrs (*Hemitragus jemlahicus*) were inseminated with frozen-thawed semen by laparoscopic or transcervical insemination techniques with no regard to the site of ovulation in non-breeding season. In June and July, 2009, estrus was synchronized by Eazi-Breed CIDR® (Controlled internal drug release; Pfizer Animal Health, New Zealand) insertion for 16 days and PG 600 (PMSG 400IU, hCG 200 IU; Intervet, Netherlands) injection (IM) a day before removing CIDR®. Forty eight hours later, laparoscopic or transcervical insemination was done to each of two tahrs under anesthetic condition inducted by ketamine (1.5 mg/kg) and medetomidine (0.09 mg/kg). For examination of estradiol and progesterone, blood was collected right before CIDR® insertion, PG 600 injection, CIDR® removal and insemination. Estradiol levels of four tahrs (No. 1, 2, 3, 4) before CIDR® insertion and insemination were 13.3, 8.8, 14.3, 12 pg/ml and 23.5, 25.5, 21.1, 11.5 pg/ml, respectively. Progesterone levels of four tahrs (No. 1, 2, 3, 4) before CIDR® insertion and insemination were 1.8, 0.05, 0.63, 0.61 ng/ml and 1.03, 0.37, 1.48, 2.12 ng/ml. Except for No. 4 tahr, cervices showed cervical mucus and opened enough to penetrate with embryo transfer gun sheet usually used for cows. Therefore, No. 4 was laparoscopically inseminated together with No. 1. In conclusion, none of four Himalayan tahrs was pregnant. However, we proved that estrus could be induced by CIDR and PG 600 injection in non-breeding season, and laparoscopic or transcervical insemination with frozen-thawed semen could be one of assisted reproductive techniques in Himalayan Tahr.

(Key words : Himalayan tahr, estrus synchronization, cryopreservation, laparoscopy, insemination)

INTRODUCTION

Himalayan tahr (*Hemitragus jemlahicus*) is one of three tahr species. This species is currently listed as "Near Threatened (NT)" by International Union for Conservation of Nature (IUCN). Unlike Cervidae and the other Artiodactyla, assisted reproductive technologies established in domestic animals have not well been applied to this species (Asher *et al.*, 1993; Johnston *et al.*, 2000; Garde *et al.*, 2006). Under strict circumstance of international Artiodactyla transaction, enhancing sustainability within domestic animal resources must be considered desperate more than ever. Therefore, we chose Himalayan tahrs as a animal model to apply and establish assisted reproductive technologies with hope of using these technologies at artificially breeding indigenous wild goats or settling down this species in Korea.

In the present study, we tried to get Himalayan tahrs impregnated with estrus synchronization and frozen-thawed semen on the basis of artificial breeding techniques established in domestic goat industry.

MATERIALS AND METHODS

1. Animals

Four female Himalayan tahrs were used in this study. One is nulliparous and the other three are multiparous. They were fed twice a day with hay, carrot, cabbage, letuce and sweet potato with free access to water. Body weight was ranged from 34 to 40 kg. The male tahr that donated semen was 50 kg.

2. Estrus Synchronization

The four female Himalayan tahrs were inserted into vagina with controlled internal drug release (Eazi-Breed CIDR®, Pfizer Animal Health, New Zealand) devices containing 300 mg of progesterone for 16 days (Fig. 1A). Twenty four hours

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before removal of CIDR, PMSG 400IU and hCG 200IU (PG 600®, Intervet, Netherlands) was intramuscularly injected.

3. Semen Collection

In February, 2009, semen was collected by electroejaculation from a male Himalayan tahr using an electrical stimulator (ElectroJac IV, Neogen; USA) under anesthesia (Mejia *et al.*, 2009). The male Himalayan tahr was anesthetized by a blow dart in which 1.2 ml of xylazine (0.48 mg/kg; Xylazine®, Kepro BV, Netherlands) was loaded (Dematteis *et al.*, 2006). After semen collection, the animal was recovered by intramuscular administration of the antidote, 1.2 ml of atipamezol hydrochloride (0.12 mg/kg; Antisedan®, Orion Corp., Espoo, Finland). The use of yohimbine chlorohydrate (0.25 mg/kg; Xyverse®, SF, Ansan, Korea) was not working (not published).

4. Semen Cryopreservation

Collected semen in a plastic tube of 50 ml was liquefied for 30 minutes before diluting it with Triladyl (Minitüb, Germany)-based solution (Triladyl: egg yolk: tri-distilled water, 1:1:3 (v/v), respectively) (Yong *et al.*, 2010). Diluted semen was cooled to 4℃ for 2 hours, loaded into 0.5 ml plastic straws, and exposed to LN_2 vapor 5 cm apart from the surface of LN_2 for 20 minutes before finally plunging into LN_2.

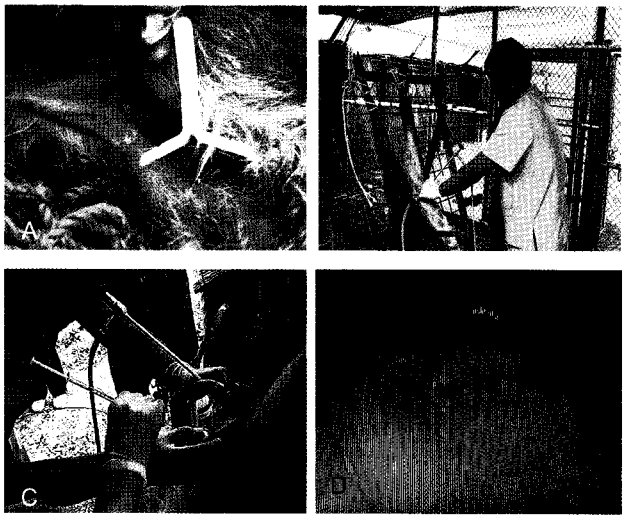

Fig. 1. Procedure of laparoscopic AI in Himalayan tahr. (A) A CIDR device was removed 16 days after insertion. (B) An abandoned, welded wire door was used for restraining Himalayan tahrs and the central part was cut and bent backwards in order to minimize sudden movement during laparoscopic AI. (C) An insemination pipette loading frozen-thawed semen was connected with a 1 ml syringe. Watching uterine horns through telescope, the pipette needle approaches a uterine horn. (D) The 5-mm long needle was injected into a uterine lumen before semen was inseminated.

5. Transcervical Insemination

On July 17, 2009, two Himalayan tahrs (No. 2 and 3) were transcervically inseminated with frozen-thawed semen that was stored in liquid nitrogen tank for five months. The concentration of the semen in straws was 1.65×10^8/ml and inseminated once 48 hours after removal of CIDR. Artificial insemination gun specifically designed for domestic goats was used to Himalayan tahrs because cervical opening is located in the range of less than 15 cm from the entrance of vagina. Xylazine of 1 ml was intramuscularly injected for sedation and the animals werer recovered by injection of the same volume of antidote, atipamezol.

6. Laparoscopic Insemination

Laparoscopic insemination was performed under anesthesia. Two female Himalayan tahrs weighed about 35 kg were anesthetized by a combination of medetomidine hydrochloride (Domitor®, Orion corp., Espoo, Finland) and ketamine hydrochloride (Ketamine®, Yuhan, Chungwon, Korea), 5 mg and 65 mg, respectively. After shaving abdomen area, the female tahrs were upside down restrained onto a welded wire mesh door with its central part cut and bent backwards (Fig. 1B). Application of 2% lidocaine jelly (Xylocaine®, Astorazeneca, Osaka, Japan) onto abdomen skin following skin disinfection with 5% povidone-iodine and alcohol was needed to minimize abdominal pain as two trocars are penetrated into abodomen. Before performing laparoscopic insemination using Laparoscopy (11001 RP, Karl Storz, Tuttlingen, Germany), small incisions on the median line of skin were needed because of tough skin of this species, which were made 4 cm apart from udder and on the right abdominal skin 4 cm apart from the median puncture, two different sizes of trocars were inserted into the abdomen. The 0.5 mL of frozen-thawed semen was loaded into an insemination pipette (No. 005546; I.M.V., Rue Clémenceau, France), which was guided close to proximal part of a uterine horn before being slowly injected into a uterine lumen (Fig. 1C and D). The concentration of frozen-thawed semen was same as by transcervical insemination, 1.65×10^8 /ml. Meanwhile, intraperitoneal infusion of carbon dioxide was continued to secure a clear view. The female Himalayan tahrs were recovered by intramuscular injection of 5 ml of atipamezol hydrochloride.

7. Hormonal Analysis

Right before all treatments, blood was collected from jugular vein and centrifuged at 3,000 rpm for 20 min. Serum samples were transferred to Neodin Veterinary Laboratory (Seoul, Korea) to analyze concentrations of estradiol and progesterone. Estradiol and progesterone were analyzed by ECLIA (electrochemiluminescence immunoassay) and RIA (radioimmunoassay), respectively.

RESULTS

1. Concentration of Estradiol and Progesterone

Difficulty of penetrating cervix was revealed only in No. 4 Himalayan tahr that showed no cervical mucus. As insemination performed in the other three tahrs, discharge of cervical mucus was found, and estradiol concentrations were more than 20 pg/ml (Table 1). Even though these animals were in non-breeding season, estrus synchronization was successfully induced in June and July. Except for No. 1 tahr, three tahrs showed maximal concentration of estradiol at the day of CIDR removal that might mean 16 days of CIDR insertion would be too long to set up optimal estrus synchronization strategy in Himalayan tahrs (Table 1).

2. Pregnancy Diagnosis

Pregnancy was examined by progesterone concentration but no tahrs showed pregnancies on Aug 21 or 24 (Table 1).

DISCUSSION

We showed how to inseminate frozen-thawed semen into uterine lumen of estrus-induced female Himalayan tahrs even though this first trial in the world ended up with failure. Himalayan tahrs chosen in this study are reproductively sound, producing offsprings every year. As that much, there were a lot of accidental deaths between in rutting season and high occurence of inbreeding because they have harem of reproduction pattern and both male and female were raised in the same enclosure. Which male sexually dominates over the other males in ungulates depends on horn size, age, body weight and testosterone concentrations during rutting season (Lovari *et al.*, 2009). As summer moves autumn and winter, the fur color of black changes to brown in Himalayan tahrs. Lighter brown of fur is a primary color that means the male with the lightest color is positioned sexually at the top of rank (Lovari *et al.*, 2009). Aggressive challenges come from subordinate males always resulted in serious, physical injuries, sometimes even to death.

Most of laparoscopic AI was performed in sheep species like Barbary sheep, (Johnston *et al.*, 2000; Lida *et al.*, 2004; Fair *et al.*, 2005; Hiwasa *et al.*, 2009). Anatomical difference of cervical folds between goats and sheep is the reason that laparoscopic insemination in sheep is more popular than in goats (Yong, 2010). In the present study, we failed in producing

Table 1. Changes of estradiol and progesterone from CIDR insertion to pregnancy diagnosis

Treatment	No. 1		No. 2		No. 3		No. 4	
	Hormone	Level	Hormone	Level	Hormone	Level	Hormone	Level
CIDR insertion	E	13.3	E	8.8	E	14.3	E	12.0
	P	1.8	P	0.05	P	0.63	P	0.61
PG600 inj.	E	*	E	20.4	E	21.5	E	23.2
	P	*	P	1.11	P	1.47	P	1.65
CIDR removal	E	15.6	E	28.3	E	22.7	E	23.7
	P	1.56	P	1.31	P	1.01	P	0.53
Laparoscopic/ cervical AI	E	23.5	E	25.5	E	21.1	E	11.5
	P	1.03	P	0.37	P	1.48	P	2.12
Pregnancy check	E	22.1	E	18.4	E	22.8	E	24.3
	P	0.7	P	0.28	P	0.29	P	0.6

E (estradiol) pg/ml, P (progesterone) ng/ml.

No. 1 and 4 were for laparoscopy AI / No. 2 and 3 were for cervical AI.

* Blood sample was contaminated during centrifugation.

offsprings. The estradiol levels at the time of insemination were lower than at the time of CIDR removal (Table 1). It might mean 16 days of CIDR insertion is such a long period even though the duration of CIDR insertion in a Saanen was optimal to induce estrus and produce offsprings (Yong *et al.*, 2010). In addition, the use of luteolytic agent should be considered at the same time of CIDR removal to enhance follicular development and suppress luteal activity arisen by injection PG 600 (Table 1).

To accomplish artificial insemination of Himalayan tahrs, sperm cryopreservation is also important. Fertile, frozen-thawed Himalayan tahr's semen could be made by modifying sperm cryopreservation techniques established in Barbary sheep (*Ammotragus lervia*), Bighorn sheep (*Ovis canadensis*), Mouflon sheep (*Ovis musimon*), Fallow deer (*Dama dama*) and domestic goats (Keskintepe *et al.*, 1998; Purdy, 2006; Mejia *et al.*, 2009).

Egg yolk was used as a cryoprotectant in this study. The semen recovered by electrical stimulation was directly diluted with a diluents including egg yolk (Mejia *et al.*, 2009). Contrary to a situation between bovine seminal plasma and egg yolk, goat seminal plasma and egg yolk interact deleteriously to the sperm (Purdy, 2006). Therefore, ejaculates might be needed to centrifuge and the seminal plasma are removed before resuspending in freezing diluents like semen cryopreservation of blackbuck, *Antilope cervicapra* (Holt *et al.*, 1988). The semen we ejaculated has been preserved in LN_2 tank since April, 2009 which is non-breeding season. The sperm quality was not so excellent that the use of melatonin implants could be considered in the case of ejaculating in non-breeding season (D'Occhio *et al.*, 1984; Zarazaga *et al.*, 2009). To recover two laparoscopically inseminated tahrs, 5 ml of atipamezole was used but actually same volume of medetomidine is enough (unpublished).

In conclusion, we established successful method of estrus synchronization in June and July, non-breeding season for Himalayan tahr of which reproduction is seasonal breeding. Even though this trial was ended up with failure of being impregnated, we showed the possibility of producing offsprings of Himalayan tahrs using transcervical or laparoscopic inseminations for the first time in the world.

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