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FULL PAPER

Theriogenology

Intrauterine insemination with fresh semen in Amur leopard cat (*Pionailurus bengalensis eutilura*) during non-breeding season

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ABSTRACT. Equine and human chorionic gonadotropins were administered to two female Amur leopard cats to induce estrus and ovulation during non-breeding season. Fresh semen collected from male cats was surgically inseminated into the uterine horn of the females. In one animal, two fetal sacs without heartbeats were observed on abdominal ultrasonography 31 days after insemination, which indicated that embryo death had occurred. In the other animal, fetal heartbeats were detected in two fetal sacs 29 days after insemination, which confirmed as pregnancy. This animal delivered two newborns 68 days after insemination; the one of the kittens was assumed to be stillbirth, and the other grew normally. In this study, we successfully obtained a kitten from an Amur leopard cat by artificial breeding for the first time in Japan.

KEY WORDS: Amur leopard cat, estrus induction, intrauterine insemination, non-breeding season, ovulation induction

The Amur leopard cat (*Pionailurus bengalensis eutilura*) is classified as Least Concern (low danger class) according to the International Union for the Conservation of Nature Red List [16], and it is currently believed that the risk for the extinction of this cat is still low [1]. However, similar to many other wild felids, they may become endangered in the future unless actions are initiated for their conservation. Inokashira Park Zoo has maintained Amur leopard cats since August 2000 and made concerted efforts to promote their reproduction. Reproduction by mating succeeded 13 times until 2005, resulting in many pups being delivered. However, reproduction only succeeded in a limited number of pairs. We attempted to initiate reproduction in another pair, with the aim of conserving genetic diversity, but were unsuccessful. Compatibility between a male and female is most important for successful reproduction in wild felids. Therefore, we consider the active introduction of assisted reproductive technologies (ARTs), particularly artificial insemination (AI), necessary for conserving genetic diversity in these wild felids [2, 21].

Numerous studies have examined AI for domestic cats and endangered wild felids [3, 7, 8, 10, 15, 18, 20, 22, 24, 26]; however, successful AI of Amur leopard cats has not been reported except for a description without detail in the introduction section of one article [11]. Previous findings obtained using domestic cats may be applied to the artificial breeding of Amur leopard cats as they share similarities in their body shapes and size, and they are both small felids. However, their rearing environment and adaptability to humans as well as their reproductive characteristics and abilities including their reproductive cycles are markedly different from domestic cats [5]. Therefore, it may be difficult to directly apply the findings obtained from domestic cats to Amur leopard cats.

Although Amur leopard cats are seasonal breeding post-coital ovulation animals similar to domestic cats, their breeding season (BS) is limited between February and March and the estrous sign is not clear. Moreover, the time to ovulation after the copulatory stimulation has not yet been identified. These characteristics make it difficult to establish the optical timing for AI during the natural BS. On the other hand, the effects of exogenous hormonal agents may vary in the presence of naturally growing follicles in

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the BS in general. Therefore, the induction of estrus and ovulation during non-BS by administration of gonadotropins is necessary for successful AI. If it proves possible to obtain kittens by AI during non-BS, that would be a marked advance in ART for Amur leopard cats. However, induction of estrus and ovulation by a combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) [3, 14, 15, 17–20, 22] has not yet been examined in Amur leopard cats, as it has in other felids. The dose and timing of administration required to induce estrus and ovulation need to be investigated in more detail in these cats. Thus, we considered it is necessary to assess reactivity after the administration of gonadotropins based on fecal hormone dynamics in Amur leopard cats and then determine AI timing [1, 12].

The semen collection method, semen quality and timing and method of AI are important factors that determine whether AI will be successful in general. We collected semen from a male Amur leopard cat using the transrectal electric stimulation method [23] and confirmed that semen quality was favorable before and during the female's BS compared with those after the BS. The mean number of sperm per one ejaculation collected during study period of about four year was 19×10^6 [23]. To achieve a conception rate of >80% by AI with fresh semen in domestic cats, 80×10^6 sperm are required for intravaginal AI, and 8×10^6 sperm are required for unilateral intrauterine AI [24, 26]. Although the number of sperm required for AI in Amur leopard cats has not yet been established, previous findings for domestic cats suggest that intrauterine AI is adequate for obtaining kittens.

In the present study, we administered eCG to female Amur leopard cat during non-BS to induce estrus and administered hCG to induce ovulation. We then determined the timing for AI based on the results of fecal sex steroid hormone measurements. Surgical intrauterine AI using fresh semen collected from the males was performed based on this result, and the potential for achieving conception was investigated.

MATERIALS AND METTHODS

Animals

Two female and two male Amur leopard cats were used in this study. Two females (Nos. 33 and 34) were litter mates and had no previous experience of mating, pregnancy or any hormone administration. At the time of AI, the females were 9 years old, and their body weights were 4.4 and 4.2 kg, respectively. The males were Nos. 15 and 49. The fertility of No. 15 had already been confirmed in May 2004, whereas No. 49 had no experience of mating. At the time of semen collection, No. 15 was 11 years old and weighed 5.7 kg, and No. 49 was six years old and weighed 5.0 kg. Animals were individually maintained at facility without exhibition (5 × 4 × 3 m) and were fed horse and chicken meat as well as chicken heads. Drinking water was available *ad libitum*. Females were maintained and managed in an animal hospital room 10 days before AI until late pregnancy. Thereafter, pregnant female was transferred to a delivery room. This study was conducted in conformity with the animal study guidelines of Japanese Association of Zoos and Aquariums.

General anesthesia

Semen collection [23] and AI were performed under general anesthesia. Using a squeeze cage, anesthesia was induced via intramuscular administration of a mixture of ketamine hydrochloride (Ketalar®50, 1.8–2.0 mg/kg; Sankyo Co., Ltd., Tokyo, Japan) and medetomidine hydrochloride (Domitor®, 60 μg/kg; Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan) and maintained by the inhalation of isoflurane (Escain®; Pfizer Co., Ltd, Tokyo, Japan) after tracheal intubation. Following the completion of these procedures, atipamezole hydrochloride (Antisedan®, 300 μg/kg; Nippon Zenyaku Kogyo Co.) was intramuscularly administered for recovery from anesthesia. For pregnancy diagnosis, the females were accommodated in an anesthetic box; anesthesia was induced and maintained by isoflurane inhalation, and examinations were subsequently performed.

Semen collection and quality test

Semen was collected approximately 2 hr before AI using the previously reported transrectal electric stimulation method [23]. After removing accumulated urine using a 3-Fr. nutrition catheter (Atom Medical Co., Tokyo, Japan), semen was collected. Semen was ejaculated into sterile plastic 1.5-m*l* conical tubes, to which 50 μl semen diluent (egg yolk Tris-fructose citric acid solution [27]) was immediately added to prevent drying. A semen quality test was performed as previously reported [23, 24]. Sperm motility was assessed immediately after semen collection, and the total sperm count, sperm viability and sperm abnormality were examined prior to AI. To reduce the injection volume, semen was centrifuged at 400 × *g* for 5 min, and the supernatant was removed to adjust the liquid volume to 50–150 μl.

Vaginal smear, observation of the ovary and intrauterine AI

A vaginal smear test was performed before AI. Vaginal epithelial cells were collected from the vagina using a swab wetted with saline. The swab was gently rolled onto a glass slide. The slide was stained using Hemacolor® (Merck). Detection of keratinized epithelial cells was considered to indicate follicular development. Intrauterine AI was surgically performed on the females by referring to previous studies on domestic cats [26]. The uterine horn was exposed in the abdominal cavity by an abdominal median incision, and semen was slowly injected using a 1-m*l* syringe with an outer of 24-G indwelling needle. Prior to the semen injection, the ovary was observed to confirm follicular development or ovulation. After surgery, the animal was maintained and managed in an animal hospital room until late pregnancy.

Diagnosis of pregnancy and observation of delivery

Pregnancy was diagnosed approximately 30 days after AI by confirming the presence of a fetal sac and heartbeat using a diagnostic ultrasound imaging system with a 7.5 MHz transducer (HS-2100V, Honda Electronics Co., Ltd., Toyohashi, Japan). Once pregnancy was observed, the animal was transferred to a camera-monitored delivery room 45 days after AI, and delivery was observed. The litter size was determined by observation through the monitor.

Measurement of fecal hormones and serum progesterone

To measure fecal hormones, feces were collected from the female cats at least once a day for 8–10 days before the initiation of eCG administration until 15, 60 and 80 days after the administration of eCG in experiments 1, 2 and 3, respectively. However, there were several days when feces were not able to be collected. Sex steroid hormone metabolites—estrogens (E) and progestagens (P)—were extracted from heat-dried feces with 80% methanol. Fecal concentrations of E and P were determined via enzyme immunoassays using estradiol-17β and progesterone (P_4) antisera, respectively, and their concentration was presented as μg/g unit, as previously reported [1]. The main cross reactivity of estradiol-17β antiserum and P_4 assay were previously reported [1]. Intra- and inter-assay coefficients of variation for estradiol assay were 2.5% and 10.7%, respectively (20–80% binding). Intra- and inter-assay coefficients of variation for P_4 assay were 6.9% and 9.1%, respectively (20–80% binding). At the pregnancy examination, blood was collected from the cephalic vein of the forearm or lateral saphenous vein. Serum was separated by centrifugation (600 × *g*, 10 min) at 4°C and stored at −40°C until P_4 measurement using an automatic immunofluorescence measurement device (SPOTCHEM VIDAS SV-5010, Arklay Inc., Kyoto, Japan).

Experimental design

This study was divided into three experiments: In experiment 1, 200 IU of eCG (ASKA Phamaceutical Co., Ltd., Tokyo, Japan) was intramuscularly administered once to the two female cats during non-BS (Dec 2012), and the presence or absence of the estrous sign (such as calling, rolling or body rubbing against wire netting) was determined. Follicular development without ovulation was confirmed from fecal E and P concentrations, and the timing of hCG (ASKA Phamaceutical Co.) administration to induce ovulation was determined based on the days when E concentration peaked. In experiment 2, 300 IU of eCG was intramuscularly administered once to one female Amur leopard cat (No. 33) in Apr 2013 followed by 200 IU of hCG 5 days after the administration of eCG, and ovulation was confirmed from the fecal P concentration. The time to ovulation after hCG administration was estimated based on the time at which an elevation occurred in P concentration, and the timing of intrauterine AI was determined based on this increase. The timing of AI was set to immediately before ovulation. In experiment 3, 200 IU of eCG was intramuscularly administered once to both the female Amur leopard cats (No. 33 at Nov 2013; and No. 34 at Jan 2014) followed by 200 IU of hCG 5 days later. Surgical intrauterine AI was performed 20–22 hr after the administration of hCG, and vaginal smear examination was performed at the same time, followed by pregnancy and delivery.

RESULTS

Experiment 1

Changes in fecal E and P concentrations in the two female Amur leopard cats after the administration of eCG are shown in Fig. 1. In No. 33, the fecal E concentration increased at 7 days after the administration of eCG, peaked at 9 days and then rapidly decreased to the basal level. In No. 34, the fecal E concentration increased at 5 days after eCG administration, peaked at 7 days and then rapidly decreased to the basal level. Elevations in fecal P concentration did not occur in either animal. No behavioral characteristics for estrus were observed.

Experiment 2

Based on the results of experiment 1, fecal E concentrations were estimated to increase from 5 to 7 days after eCG administration, and follicles developed sufficiently by 5 days after eCG administration taking into consideration the days required for the metabolism and excretion into feces [4]. Thus, hCG was administered at 5 days after eCG administration. As shown in Fig. 2, the fecal P concentration began increasing 3–4 days after the administration of hCG and then increased sharply, which indicated the induction of ovulation and formation of the corpora lutea. No specific estrous sign was noted.

Experiment 3

Based on the results from experiment 2, the ovulation was assumed to occur >24 hr after hCG administration taking into consideration the days required for the metabolism and excretion into feces [4]. Intrauterine AI on No. 33 was performed 20 hr after the administration of hCG using semen collected from male No. 49, whereas AI on No. 34 was performed 22 hr after hCG administration using semen collected from male No. 15. Semen quality immediately after collection is shown in Table 1.

Keratinized epithelial cells were noted in both females based on the vaginal smear test conducted before AI, confirming follicular development and estrus in the animals. Macroscopic observations of the ovary by laparotomy revealed the presence of three follicles with a diameter of 5–6 mm in the left ovary (the right ovary was not examined) in No. 33 and three and four follicles with a diameter of 4–5 mm in the left and right ovaries, respectively, in No. 34 (Fig. 3). The entire semen volume was injected into the left uterine horn in No. 33, whereas semen was divided into two equal aliquots and injected into the bilateral uterine horns in No. 34. In No. 33, the semen volume was adjusted to 150 μl; however, a small volume leaked from the uterine horn when it was

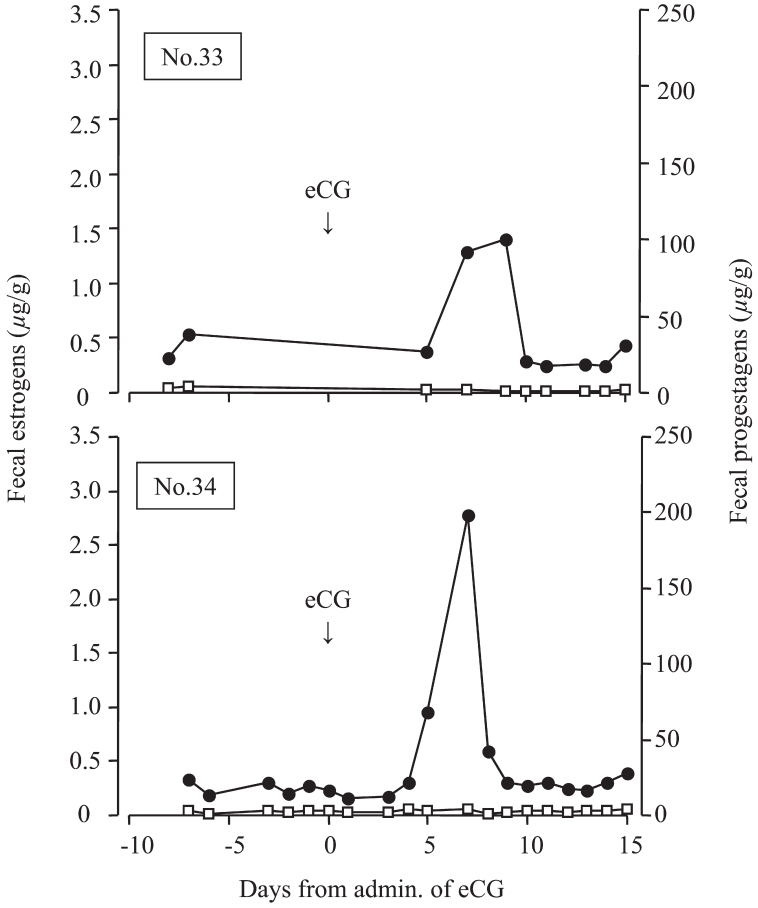

Fig. 1. Changes in fecal concentrations of estrogens (●) and progestagens (□) after the administration of eCG (200 IU) in two female Amur leopard cats (Experiment 1).

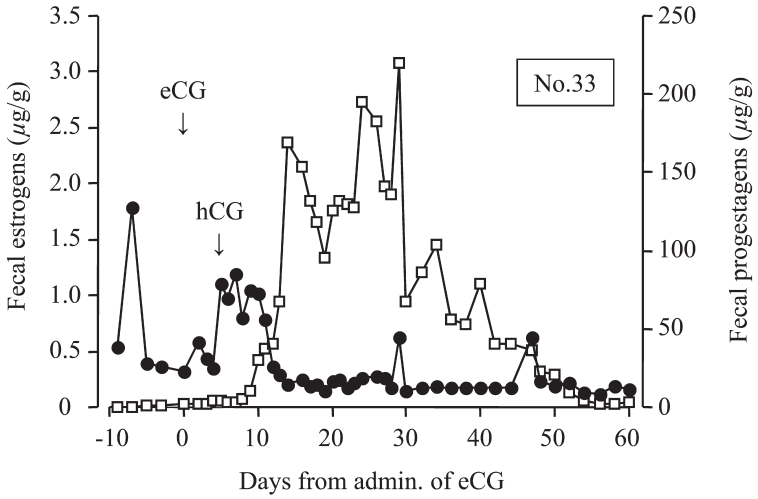

Fig. 2. Changes in fecal concentrations of estrogens (●) and progestagens (□) after the administration of eCG (300 IU), followed by 200 IU of hCG 5 days after the administration of eCG in one female Amur leopard cat (Experiment 2).

injected. In No. 34, the divided volume of semen was separately adjusted to 50 μl and injected without leakage. There were no post-surgical complications.

Pregnancy was confirmed 31 and 29 days after insemination in Nos. 33 and 34, respectively. Pink and slightly enlarged nipples (pinking) were observed in the two cats. When No. 33 was examined using ultrasonography, a fetal sac with a diameter of 2 cm was observed in each uterine horn (Fig. 4). However, their shapes were slightly distorted, and no fetal heartbeats were detected.

Table 1. The quality of semen collected by transrectal electric stimulation method from two male Amur leopard cats

Male No.	Date of collection	Sperm motility (%)	Sperm viability (%)	Sperm abnormality (%)	Total sperm ($\times 10^6$)
No.49	Nov., 2013	70	74.2	- [a)]	9.6
No.15	Jan., 2014	40	71.5	3.2	21.0

a) Not examined.

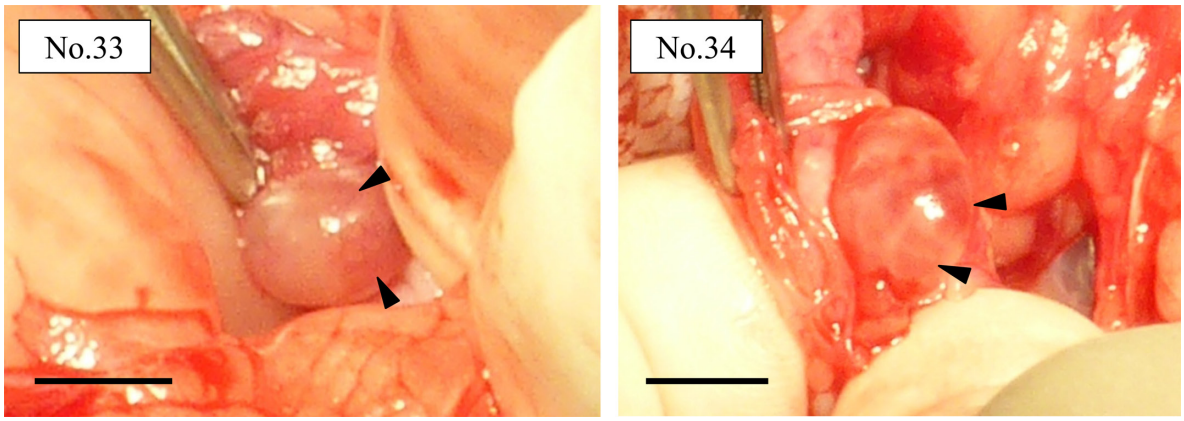

Fig. 3. Appearance of the ovary observed by laparotomy in two female Amur leopard cats at the time of AI. No. 33, left ovary; No. 34, right ovary. The arrows show follicles. The scale bar is 1 cm.

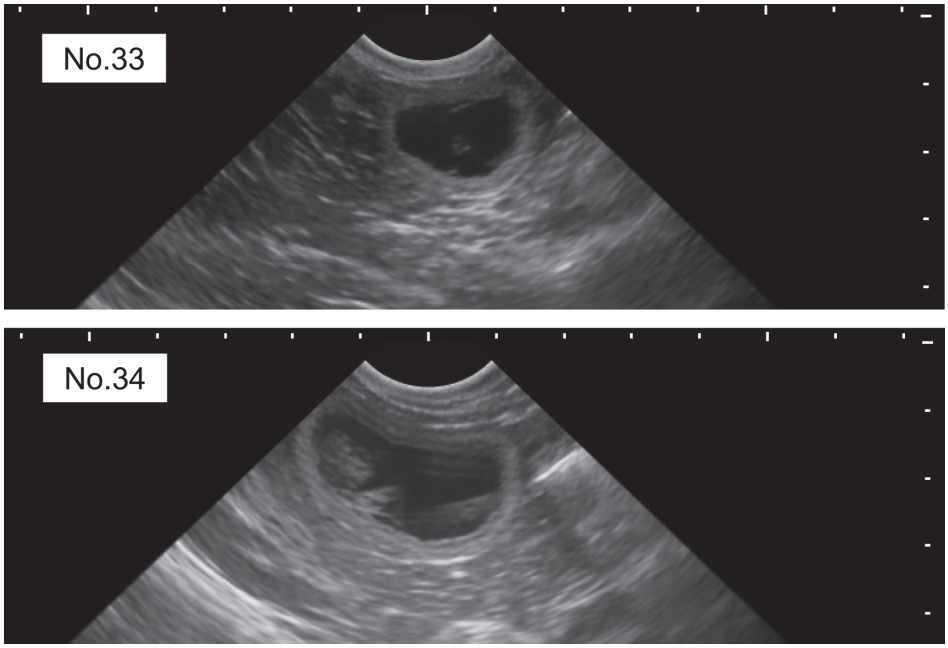

Fig. 4. Ultrasonographic images of fetal sacs in two female Amur leopard cats (No. 33, 31 days after insemination; and No. 34, 29 days after insemination). In No. 33, the shape of the fetal sac was slightly distorted, and no fetal heartbeat was detected. In No. 34, fetal heartbeat in a fetal sac was detected. The distance between the white scales is 1 cm.

Pregnancy diagnosis was performed a week later, and cavities assumed to be fetal sacs were observed, but no change in sac size was noted. At the time of pregnancy diagnosis 60 days after AI, cavities assumed to be fetal sacs had disappeared. In No. 34, a fetal sac with a diameter of 3 cm was observed in each uterine horn on ultrasonography, and fetal heartbeats were detected in both fetal sacs (Fig. 4). Serum P_4 concentration was 50.35 and 9.42 *n*g/m*l* on days 31 and 38 after insemination, respectively, in No. 33, and it was 80 *n*g/m*l* on day 29 after AI in No. 34. After pregnancy was confirmed, spontaneous delivery occurred 68 days after AI in No. 34. The first kitten was delivered in a posterior presentation after 97 min from start of labor pain (confirmed using a camera monitor), but it did not move and was subsequently eaten by the dam. The second newborn was delivered in an anterior

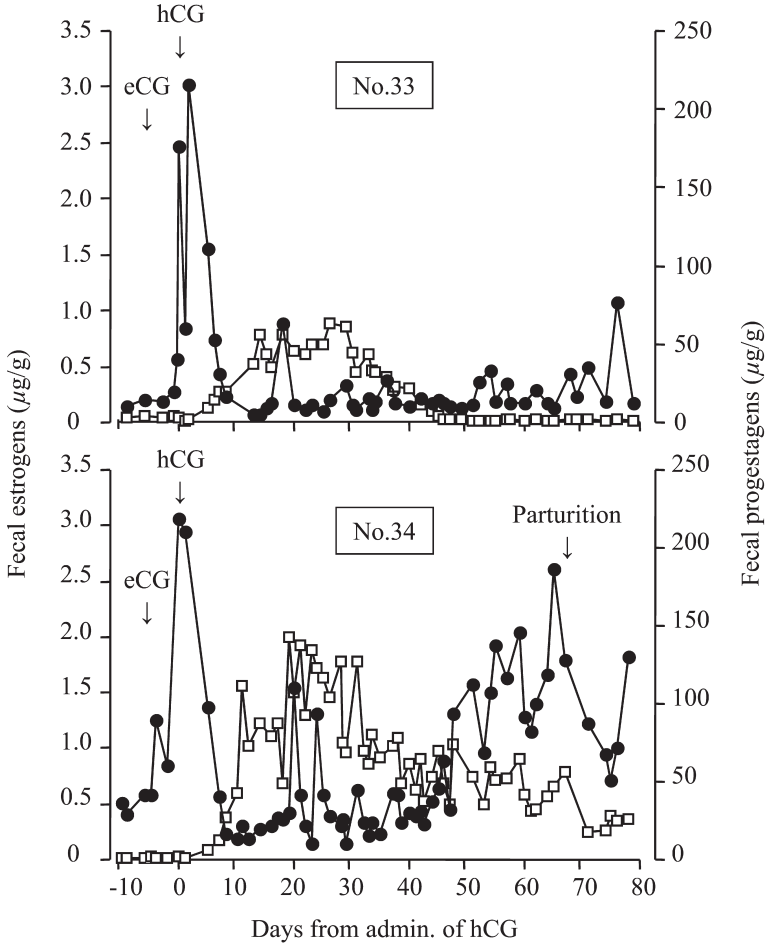

Fig. 5. Changes in fecal concentrations of estrogens (●) and progestagens (□) after the administration of hCG in two female Amur leopard cats (Experiment 3). The dose of eCG was 200 IU. Intrauterine AI was surgically performed 20–22 hr after the administration of hCG.

presentation 13 min later. The dam took care of the kitten, and it grew well thereafter. The kitten was subsequently confirmed to be a male.

The changes observed in fecal E and P concentrations in the two female Amur leopard cats in experiment 3 are shown in Fig. 5. In No. 33, the fecal P concentration was at the basal level approximately 45 days after the administration of hCG. In contrast, No. 34 maintained a level higher than the basal level until delivery. The fecal E concentration slowly increased from approximately 40 days after the administration of hCG and decreased after delivery.

DISCUSSION

The present study is the first to investigate a method to induce ovarian follicular development and ovulation in the Amur leopard cat during non-BS by hormone administration. Although eCG is mainly used to induce estrus (follicular development) in domestic cats and wild felids, the frequency of administration and effective dose vary among species [17, 19]. For example, eCG is administered subcutaneously or intramuscularly at 50–100 IU for 1–3 days to domestic cats [17, 19, 28]. For wild felids, eCG was administered once at 500 IU to ocelot weighing 9–18 kg [22], 100– 400 IU to cheetah weighing 40–65 kg [14] and 200–1,000 IU to tiger weighing 110–160 kg [10, 12]. Amur leopard cats weigh 3–5 kg, which is close to the body weight of domestic cats. However, multiple administrations are technically difficult and stressful for the animals, unlike for domestic cats. Thus, we used a single intramuscular administration of 200 IU, which is slightly higher than the dose used for domestic cats, to ensure estrus induction. As a result, fecal E concentrations increased from 5–7 days after the administration of eCG, suggesting that this dose was sufficient to induce follicular development during non-BS, but no specific change of behavior suggesting estrus was noted after its administration. Thus, the eCG dose was increased to 300 IU in experiment 2, because it was suggested that 200 IU was insufficient to induce change of behavior suggesting estrus in these cats. However, no significant change was noted, and fecal E concentrations increased in a manner similar to that when 200 IU eCG was administered. These results demonstrated that follicular development after eCG administration cannot be determined based on the change of behavior suggesting estrus in Amur leopard cats.

In domestic cats, it is possible to decide the timing for hCG administration based on estrous peak by observing their behavior

[24, 26], and hCG has been administered 5 to 7 days or 80 hr after the administration of eCG when estrus was induced by eCG [17, 28]. In experiment 1, fecal E concentrations peaked at approximately 7 days after eCG administration. Brown [6] previously reported that the time taken to excrete approximately 97% of blood steroid hormones into feces was 1–2 days in felids; therefore, we considered 5 days after eCG administration to be the appropriate time for hCG administration. Although present result was different from the timing of hCG administration, 80–84 hr after eCG administration was reported in many species of wild felids [3, 9, 14, 22, 25], it is similar to report for domestic cat [17, 28], and the Amur leopard cat successfully became pregnant in this study. Thus, the cause of this difference may be specific, or the reported timing for hCG administration to induce ovulation may be too early. As the dose of hCG administered to domestic cats and many wild felids is 100– 200 IU, we set it to 200 IU to ensure the induction of ovulation in wild felid, and it was intramuscularly administered once. Fecal P concentrations increased in hCG-treated animals, confirming the induction of ovulation. The time to ovulation after hCG administration was previously reported to be 25–30 hr in domestic cats [25]. In experiment 2, fecal P concentrations began to increase approximately 72–96 hr after hCG administration. In domestic cats, an increase in plasma P_4 can be detected within 24–48 hr after ovulation [25]. From these findings, the timing of ovulation was supposed to occur >24 hr after hCG administration taking into consideration days required for metabolism and excretion into feces [4]. As ovulation did not occur in the ovaries 20–22 hr later, as confirmed by laparotomy, we assumed that the time at which ovulation occurred after hCG administration was similar to that in domestic cats.

AI was performed 20–22 hr after hCG administration (which was before ovulation) by the surgical intrauterine AI method previously performed on domestic cats [26], and conception was achieved. These results confirmed that the timing for the insemination was appropriate. Ovulation may be inhibited in domestic cats due to the influence of anesthetics in estrus induced by hormone administration [13]. However, in this study, it was demonstrated that ovulation was not inhibited by anesthesia in Amur leopard cats. The targeted sperm count to be inseminated was 8×10^6, as the number required to ≥80% conception rate in domestic cats for unilateral intrauterine AI with fresh semen [26]. Pregnancy was achieved, thereby verifying that the insemination method and sperm count were appropriate. However, in this study, sperm motility and viability of Amur leopard cat semen were lower than that reported for domestic cat semen (mean sperm motility is 84.4%; and mean sperm viability is 89.3%) [26]. Therefore, if semen with high sperm motility and viability was used for AI in Amur leopard cats, it seemed likely that conception could be achieved with a lesser sperm count.

When domestic cats become pregnant in estrus induced by hormone administration during non-BS, serum P_4 levels cannot be maintained throughout the pregnancy period because hormones from the pituitary and hypothalamus do not function and decrease to basal levels 25–30 days after ovulation, resulting in the abortion of some fetuses [28]. It currently remains unclear whether a similar phenomenon occurs in Amur leopard cats. Based on ultrasonography findings during the pregnancy diagnosis, embryo death may have occurred in one (No. 33) of the two cats. Although a decrease in the serum P_4 level was suspected as the cause, it was 50.35 *n*g/m*l* 31 days after AI, which was high at this time of the pregnant period, suggesting that the phenomenon observed in domestic cats does not occur. Thereafter, P_4 gradually decreased and reached basal level approximately 45 days after ovulation. It was suggested that this secretion pattern of P_4 was similar to that of pseudopregnant domestic cats [25]. Accordingly, the cause of embryo death was not due to a decrease in serum P_4 levels; therefore, stress due to changes in the postoperative living environment was suspected. No. 33 was more susceptible to stress than No. 34, which maintained pregnancy; however, the cause of embryo death was not identified. A comparison of the two animals revealed that the timing of pregnancy differed for the two cats. No. 34, which maintained pregnancy, became pregnant nearer the original BS than No. 33, in which embryo death occurred; this may have been one of the reasons for the maintenance of pregnancy in No. 34. To clarify this, more insemination cases between September and November, during which abortion occurred in No. 33, need to be investigated. In addition, fecal E increased in the second half of the pregnancy and decreased after delivery in No. 34, suggesting that estrogen may be secreted from the placenta. A similar phenomenon has been reported in the cheetah, but not in domestic cat, leopard cat and Asian Golden cats [5, 18]. However, because the data in this study were the only set, it will be necessary to address this question by investigating more pregnant Amur leopard cats.

Two newborns were delivered 68 days after AI. However, one was stillborn and was subsequently eaten by the dam. Although exact details are unknown because the kitten was only observed on a monitor screen, it did not move, which indicated that it was stillbirth. Although no differences were observed in size between the two kittens, the gestational time and cause of death remain unclear.

We succeeded in obtaining an Amur leopard cat kitten using surgical intrauterine AI of fresh semen during non-BS. Pregnancy was established in the Amur leopard cat under the following conditions: the eCG dose to induce follicular development was 200 IU, the hCG dose to induce ovulation after 5 days was 200 IU, and the sperm count inseminated 20–22 hr after the administration of hCG was approximately 10×10^6 (unilateral uterine horn). Wild animals that have a BS do not bear kittens during non-BS, but if this artificial breeding technique is applicable, kittens may be obtained during non-BS. We intend to accumulate more cases, clarify unclear points and establish an AI technique with the aim of applying it to Tsushima leopard cats, a rare species of wild felid in danger of extinction.

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