Studies on assisted reproductive technology in Amur leopard cats

Summary of Doctoral Thesis

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Small wild cats of the Felidae family indigenous to Japan, Tsushima leopard cats and Iriomote wild cats, are on the verge of extinction, as are many wild cats of the Felidae family. To protect these wild animal species, the introduction of various artificial reproductive technologies (ART) as ex-situ conservation is necessary, in addition to in-situ conservation. However, ARTs have not yet been established for small wild cats of the Felidae family including Tsushima leopard cats.

Thus, using a subspecies of leopard cats belonging to the same species, Amur leopard cats, to which Tsushima leopard cats belong, as a model of Tsushima leopard cats, we performed a series of studies to establish ART techniques.

This report is comprised of 8 chapters: Chapter 1 is the Introduction, Chapters 2 to 7 report experimental results, and Chapter 8 overviews the report. The experimental results are summarized below:

1. Semen collection from an Amur leopard cat employing the transrectal electric stimulation method (Chapter 2)

Aiming at establishing a method for collecting semen with favorable characteristics, semen was collected from a male Amur leopard cat under generalized anesthesia at a frequency of once about every 2 months for about 4 years employing the transrectal electric stimulation method and the electric stimulation conditions and seasonal and time-course changes in the semen characteristics, body weight, testicular volume, and serum testosterone (T) level were investigated. For analysis of seasonal changes, the data was divided into subgroups by the semen collection period into the breeding season (BS) group (January-April), post-BS group (May-August), and pre-BS group (September-December).

The body weight in the pre-BS group was significantly higher than those in the other groups ($p<0.05$ and $p<0.01$). The testicular volume in the post-BS group was significantly smaller than that in the BS group ($p<0.05$). In addition, the serum T level was significantly higher in the pre- than post-BS group ($p<0.05$).

It was clarified that the optimum conditions minimizing contamination with urine and collecting many sperm were the use of a rectal probe assembled with electrodes into a single bundle on the ventral side parallel with the rectum, insertion of this at about 6.5 cm from the anus, and application of stimulation at 1-4 V.

Regarding the semen characteristics, the total sperm count, sperm motility, sperm viability, and sperm abnormality in the post-BS group were inferior to those in the other groups, but the differences were not significant. The mean total sperm count was about 15 million.

Based on the above findings, semen collection from the Amur leopard cat using the transrectal electric stimulation method under the conditions was favorable, clarifying that September-April is appropriate for the semen collection period.

2. Semen collection from Amur leopard cats by urethral catheterization method (Chapter 3)

Using 3 male Amur leopard cats, semen was collected from each animal 2-3 times by urethral catheterization method (CT group) before transrectal electric stimulation method (EE group), the usefulness of urethral catheterization was investigated based on the characteristics of collected semen, and at the same time, the influence of individual differences in the semen characteristics was investigated.

The sperm motility and sperm viability immediately after collection were higher in the EE than CT group, and the sperm abnormality was slightly lower in the EE than CT group. The mean total sperm count was slightly higher in the EE than CT group. On analysis regarding that in the BS as a standard, the total sperm count was higher in the BS than post-BS group. The individual difference in the total sperm count was large, but no individual difference was noted in any of the other test items.

Based on the above, the transrectal electric stimulation method may be more appropriate than the urethral catheterization method as a semen collection method for Amur leopard cats, but further investigation of the urethral catheterization method, such as modification of the anesthesia method, may be necessary.

3. Cryopreservation and low-temperature storage of semen of Amur leopard cats (Chapter 4)

Aiming at establishing a semen storage technique, using semen collected in Chapter 2, frozen semen was prepared using the same method as that for domestic cats and its usefulness was investigated with regard to the semen characteristics after thawing as Experiment 1. As Experiment 2, semen collected by 3 times of ejaculation from a male Amur leopard cat using the transrectal electric stimulation method (EE group) and urethral catheterization method (CT group) was subjected to low-temperature storage to investigate its usefulness.

In Experiment 1, the mean sperm motility after freezing and thawing was 27.0±5.6%, being high, but the mean sperm abnormality was 15.8±3.3%, being slightly high. The sperm motility before freezing and after thawing was higher in the BS group than in the other groups, whereas in the post-BS group, the sperm motility markedly declined after thawing compared with that before freezing.

In Experiment 2, the sperm motility and sperm viability sharply decreased after low-temperature storage for 2 days and thereafter in the EE group compared with those in the CT group and differences between the 2 groups increased over time. The sperm abnormality became high after storage for 3 days in the CT group. In storage at 8℃, the sperm motility sharply decreased after storage for 1 day and the sperm viability was kept at a slightly low level after storage for 2 days and thereafter compared with that stored at 4℃.

Based on the above, it was clarified that favorable frozen semen of Amur

leopard cats can be prepared using the same method as that used for domestic cats. In addition, it was clarified that semen collected by urethral catheterization method can be more favorably maintained in low-temperature storage for several days compared with that collected using the transrectal electric stimulation method. It was suggested that 4℃ is more appropriate than 8℃ for the storage temperature.

4. Characteristics and cryopreservation of cauda epididymal sperm of Amur leopard cat (Chapter 5)

Aiming at establishing a cryopreservation technique of sperm collected from the epididymis, the characteristics of sperm collected from the cauda epididymis of 3 dead male Amur leopard cats were compared with those of 5 male domestic cats. The cauda epididymal sperm was cryopreserved and its usefulness was investigated based on the characteristics after thawing.

The characteristics of cauda epididymal sperm immediately after collection from the Amur leopard cats were favorable and no difference from those of domestic cats was noted. The mean total counts of sperm collected were about 80, 55, and 100 million, respectively, being slightly smaller than that of domestic cats, but the differences were not significant. The characteristics of cauda epididymal sperm after freezing and thawing were favorable.

Based on the above, although the total count of sperm in collected semen was smaller, that collected from the cauda epididymis of the Amur leopard cats was almost the same as the count of domestic cats, clarifying that sperm can be collected. The sperm characteristics after cryopreservation were also favorable, suggesting that it is sufficiently applicable for AI.

5. Induction of estrus and ovulation in Amur leopard cat in the non-breeding season (Chapter 6)

Since Amur leopard cats show no marked estrus sign in captive breeding and identification of the optimum time of mating is difficult, induction of estrus and ovulation was considered necessary to perform AI. Thus, using 2 female Amur leopard cats in the non-BS, the usefulness of the estrus induction method by administration of eCG preparation and method of subsequent ovulation induction by administration of hCG preparation was investigated. The states of follicular growth and ovulation were confirmed by measuring metabolites of fecal estradiol-17β (E2) and progesterone (P4).

The fecal E2 level increased after administration of eCG 200 IU for 5 days and peaked after administration for 7-9 days, confirming follicular growth. Since the fecal P4 level did not increase, it was judged that there was no spontaneous ovulation. Considering the number of days required for metabolism of steroid hormones into feces based on the above, 200 IU of hCG was administered on day 5 of eCG administration at which the serum E2 level was estimated to reach the peak. The fecal P4 level rose from day 3 of hCG administration, being judged that ovulation was induced.

Based on the above, it was clarified that this method is capable of inducing follicular growth and ovulation in Amur leopard cats in the non-BS.

6. Intrauterine artificial insemination of fresh semen in Amur leopard cats (Chapter 7)

Considering that intrauterine AI is necessary to achieve a high conception rate using the number of sperm collectable from Amur leopard cats, follicular growth and ovulation were induced by eCG and hCG administration in 2 female Amur leopard cats in the non-BS and they received surgical intrauterine AI of fresh semen collected from 2 male Amur leopard cats to investigate whether a newborn could be acquired.

Semen (sperm count: 9.60 million, sperm motility: 70%, sperm viability: 74.2%) was injected into the left uterine horn 20 hours after hCG administration in a cat. The presence of 2 fetal sacs with a 2-cm diameter was confirmed on ultrasonography 30 days after AI, but no fetal heart beat was confirmed, being judged that early embryonic death occurred. In the other cat, semen containing 10 million sperm (sperm motility: 40%, sperm viability: 71.5%) was injected into the bilateral uterine horns 22 hours after hCG administration. Ultrasonography was performed 28 days after AI and the presence of 2 fatal sacs with a 3-cm diameter and fetal heart beats was confirmed. Natural delivery occurred 68 days after AI and one fetus was stillborn, but the other was successfully delivered as a normal newborn.

Based on the above, it was clarified that it is possible to acquire a normal newborn by surgical intrauterine AI with fresh semen collected from Amur leopard cats treated with induction of follicular growth and ovulation in the non-BS.

The conditions to collect sufficient semen for AI from male Amur leopard cats employing the transrectal electric stimulation method were clarified. In addition, the conditions of induction of follicular growth and ovulation in female Amur leopard cats in the non-BS were clarified. Using fresh semen, surgical intrauterine AI was performed under these conditions and acquisition of a normal newborn succeeded. The birth of a newborn by artificial reproduction in this animal species was the first in the world. In addition, the conditions of cryopreservation and low-temperature storage of semen were investigated and the usefulness of the stored semen was clarified. Furthermore, the characteristics of sperm collected from the cauda epididymis and those of the collected sperm and those after freezing and thawing were favorable, clarifying that these sperm may be used for AI.

These techniques may be useful for reproduction of not only Amur leopard cats but also small wild cats of the Felidae family on the verge of extinction and to help increase the currently reduced population.