Successful Transcervical Insemination in Giant Panda at Chiangmai Zoo, Thailand

Boripat Siriaroonrat[1*] Ampika Thongphakdee[1] Wanlaya Tipkantha[1]
Kannikar Nimtragul[1] Kornchai Kornkeawrat[2] Ratchaneewan Khejornwong[1]
Prasertsak Buntragulpoontawee[1] Sumate Kamolnorranath[1]

Giant panda *(Ailuropoda melanoleuca),* a bamboo-eating bear from China, has been globally recognized as a symbol of endangered species conservation due to the loss of bamboo forest habitat and poaching. In wildlife education, giant panda has become worldwide ambassador to promote the need to conserve threatened wildlife, habitats and biodiversity. In China, the Giant Panda Conservation Center has been established to ensure sustainability of the valuable animals as well as their genetic diversity. To date, more than 160 individuals living *ex situ* in China and approximately 30 individuals exhibited in North America, Austria, Mexico, Japan and Thailand. Providing a proper semi-natural environment to encourage natural breeding is the major goal of captive breeding program. However, success from natural mating does not come easy. It was reported that 74% of adults fail to mate naturally, caused mainly by poor mating interest (libido) of sexually matured male pandas. Accordingly, assisted reproductive technologies including artificial insemination (AI) would allow population manager to incorporate genetically valuable male in to gene pools.

In a case of giant panda at Chiangmai Zoo; Chuang Chuang (9-year-old male) and Lin Hui (8-year-old female) exhibited some degree of behavioral incompatibility that resulted in failed natural mating repeatedly from 2006-2009. Moreover, unclear understandings of giant panda reproductive physiology and anatomy, as well as a short window of sexually receptive (2 to 3 days per year), are the challenges that veterinarians and scientists were facing. Attempts have been made to investigate issues related to female fertility to prepare for natural or assisted breeding. Female reproductive cycle has been monitored from the changing patterns of estrus behavior, vaginal cytology and reproductive hormonal profiles i.e. urinary estrogen and progesterone metabolites accessed by enzyme-immunoassay, in order to understand the baseline information of reproductive biology. Laboratory results enabled Thai researchers to pinpoint timing of ovulation, thus the time of insemination can be accurately scheduled. On the male side, fertility assessment had been conducted for Chuang Chuang and the results showed that the Chiangmai male was spermic and no degree of sub-fertility or infertility was observed. However, the first attempted of AI in 2007 resulted no pregnancy. In 2008, it was concluded that the female showed no signs of estrous from both behavioral observation and urinary enzyme-immunoassay data. In the mid-February 2009, estrus signs such as increased vocalization, scent marking and restlessness but decreased appetite, were observed. The high percentage (90%) of vaginal epithelial morphology (anucleated superficial cells) was observed on the day that

[1]**Zoological Park Organization under the Royal Patronage of H.M. the King, Bangkok, Thailand**
[2]**Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand**
[*]**Corresponding author: Email: eldsdeer@yahoo.com**

found peak of urinary estrogen level (111.5 ng/mg creatinine). On the first day of AI (February 17), fresh semen (total volume=4.85 ml) was collected by electroejaculation from the male. Spermatozoa were diluted in TEST egg-yolk cryodiluents. The mixture was divided into 2 parts; the first half was used for immediate AI, and the other half was chilled at 4°C for the second AI 24 h later. Eighteen hours after the peak of estrogen was detected, the female panda was anesthetized and placed in a dorsal recumbence position. Urinary catheter was inserted to empty the bladder and minimize urine contamination to AI procedure. Catheter was inserted into external cervical os. Diluted fresh semen was used for the first AI. The second AI was performed 24 h later with chilled semen. Pregnancy watch plan was prepared to evaluate AI success. Pregnancy was monitored using the combination of progesterone assay, behavior changes and bi-monthly ultrasonography diagnosis. However, during the first 60-100 days, pregnancy is generally indistinguishable from pseudopregnancy by behavioral observation, physical changes of animalís body or urinary hormonal profile. Urinary hormone assay demonstrated significant increased of progesterone level (from baseline at AI day to 440 ng/mg at 82 day after AI). Beating heart- or large arterial-like image was observed by ultrasonography at day 82 but could not confirm pregnancy. Nesting behavior was observed 24 hours before parturition. At day 97 after AI, female panda gave birth to a live healthy female cub (birth weight 235 g, length 17.50 cm) and raised the cub naturally

1. Factors affecting the AI success in giant panda

Ovulation detection

Urinary hormonal profile was monitored throughout the year. In 2009 breeding season, Lin Hui started to show signs of estrous behaviors from January 1. Daily urine samples were collected and analyzed for estrogens and progesterone metabolites to determine timing of ovulation. Durrant et al. (2006) reported that estrogen peak level was recorded (Day -1) and the rapid

decline of estrogen level (Day 0) was considered the day of ovulation. In non-breeding period, average estrogen level of Lin Hui was normally at the baseline (3.37 ± 1.1 ng/mg creatinine; range 1.85-6.9 ng/mg creatinine, n=115). On February 3rd, estrogen level started to rise to 6.05 ± 1.5 ng/mg creatinine (range 4.42-8.05, n=5) and gradually increased until reaching the highest point at 111.46 ng/mg on February 17, and markedly dropped to 38.19 ng/mg creatinine, respectively. Simultaneously, after estrogen decline, the progesterone level was increasing (Figure 1). This switching pattern of the 2 hormones indicated ovulation of the female - Lin Hui. This observation is in accordance with the report by Durrant et al. (2006). The predicable ovulation could be expressed to the estrus behavior as shown in Figure 1.

The relationship between the behavioral and hormonal changes during the estrus cycle has been reported from multiple female pandas. Shuling et al. (1997) reported that 80% of female giant pandas in captivity exhibiting weak estrus signs, or the absence or overt estrus behavior. In case of Lin Hui, estrus signs were expressed in correlation with estrogen level. Increased vocalization of bleating and chirping sounds during the day of estrogen peak was observed, whereas lordosis and tail up or receptive behaviors were closely associated with falling estrogen level.

In accordance to the observation by Durrant and colleagues (2006), prior to the peak of urinary estrogen level, the high percentage of vaginal epithelial morphology (anucleated superficial cells) was observed (Figure 2).

The combination of endocrinology results, behavioral and vaginal cytology data were important information to assist the detection of ovulation in female panda. These parameters were recommended to include in the breeding plan e.g., introduction of male for natural mating or timed AI.

Semen quality

It has been reported that the active spermato-genesis in a male panda can be observed from the period
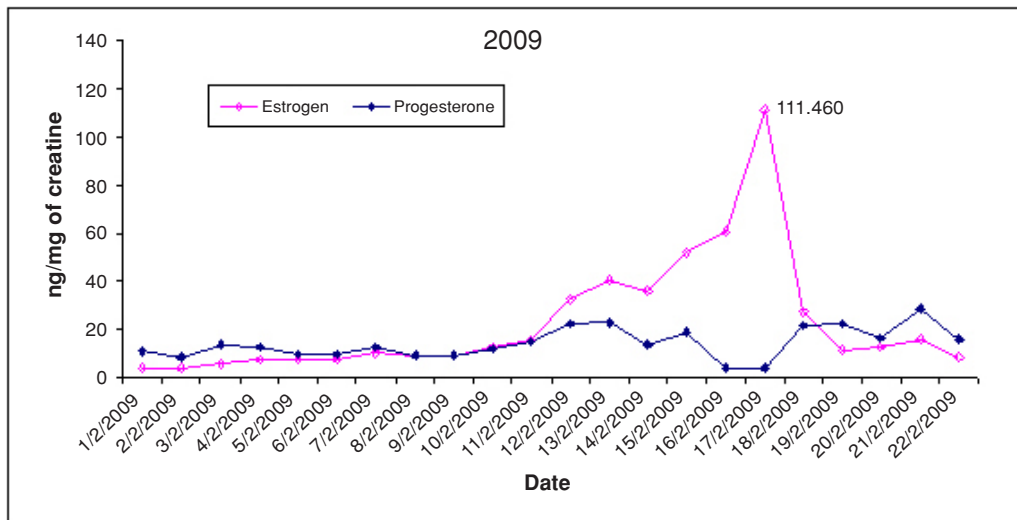
Figure 1. Daily concentration of estrogen and progesterone excretion during 1-22 February 2009 demonstrates the estrogen peak occurred on 17th February 2009.
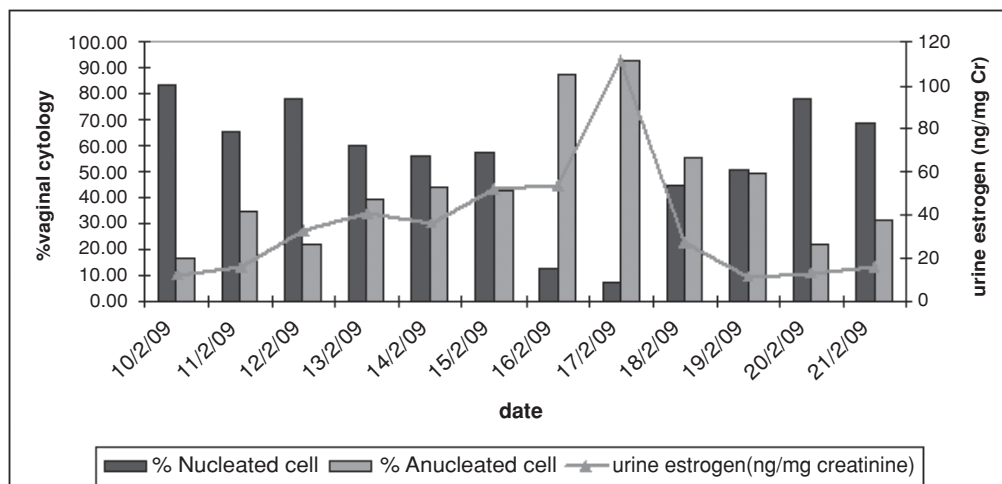

Figure 2. Relationship between urinary estrogen and vaginal cytology demonstrated a high percentage of anucleated cells found on the same day of estrogen peak.

of 3 months before estrus to 2 months after estrus in a presence of female (Tsutsui et al., 2006). Semen collection using electroejaculation was conducted successfully for Chuang Chuang both in non-breeding and breeding seasons (2007-2009). On the day of AI (17 February 2009), semen was collected from the male and milky projectile ejaculate (total volume=4.85 ml; pH 9; Figure 3A) was observed during 4-5 volts electrical induction. Subsequently, semen was diluted (1:1 v/v) in TEST egg-yolk cryodiluents. The mixture was divided into 2 parts; the first half for immediate AI, and the latter

chilled for 24 h at 4°C for the second AI on the following day. Semen analyses revealed excellent quality (95% motility, 4.5 progressive status, and concentration of 640×10^6 spermatozoa/ml). Morphological examination demonstrates 98% normal head and 86% normal tail spermatozoa, respectively (Figure 3B). The abnormalities of spermatozoa were pear and small heads with/without distal and proximal droplets. At 24 hour after refrige-ration, chilled spermatozoa were motile with no apparent abnormalities induced by cold temperature.
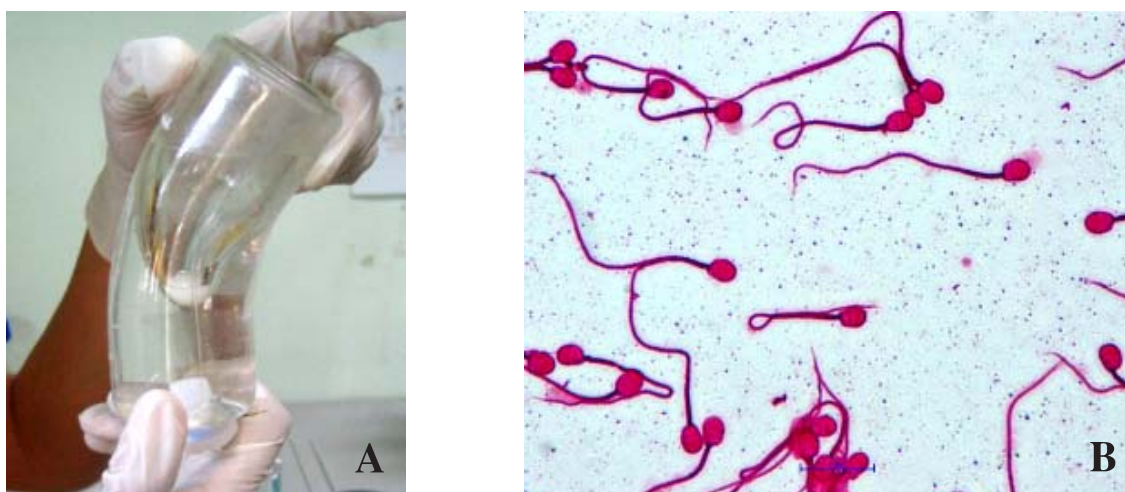
Figure 3. Milky ejaculated semen in a warm water-jacketed glass cup (A), and sperm morphology of giant panda (B) William's staining, x 1000
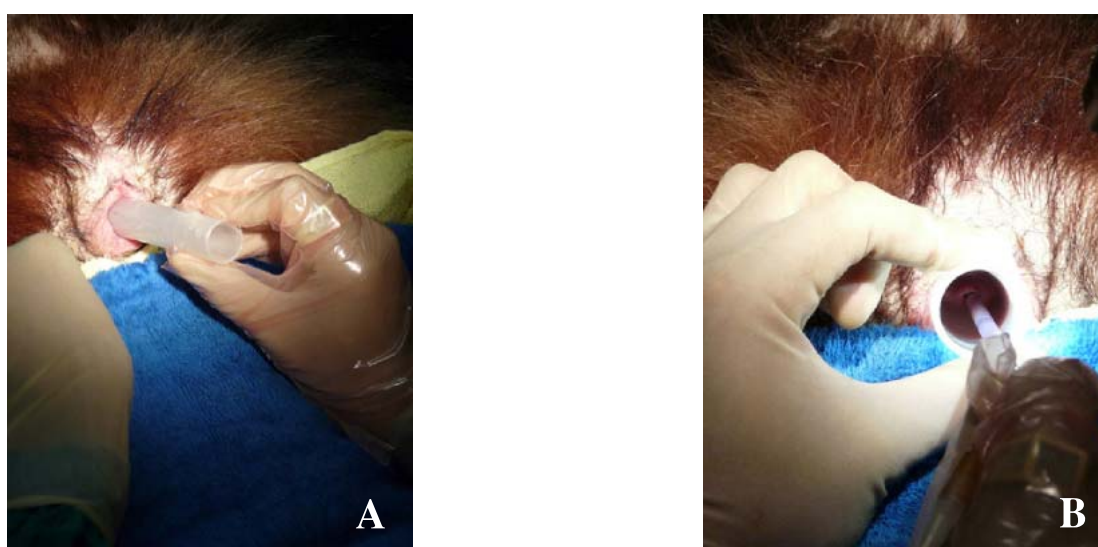

Figure 4. Lubricated vaginal speculum (A) was inserted to visualize the cervical opening. Plastic catheter was transcervical inserted to deposit semen (B).

AI techniques

Eighteen hours after peak of estrogen was detected, the female panda was anesthetized using a combination of 7 mg/kg of ketamine hydrochloride (Ketaleann, Animal Health Inc., Poland) and 0.5 mg/kg of xylazine (Ilium Xylazine-100, Troy Lab. PTY Ltd, Australia) and maintained under isoflurane gas anesthesia. Female was placed in a dorsal recumbence position, and a lubricated vaginal speculum was inserted to visualize the cervical opening (Figure 4A). Urinary catheter was inserted to empty the bladder and minimize urine contamination to AI procedure. Plastic/stainless steel catheter was inserted into external cervical os (approximately 18 cm from vulva, Figure 4B). For the first AI, extended fresh semen (total volume= 5 ml) was deposited in to the uterus. The second AI was performed as described in the first AI with additional 4.7 ml of chilled extended semen 24 h after the first AI. To prevent backflow of inseminated semen, the female rear end was lifted for 5 minutes immediately after insemination. The successful AI procedure

demonstrates that the technique using vaginal speculum and appropriate-sized catheters (5.5 to 6 mm diameter) were suitable for transcervical insemination in this species.

2. Understanding giant panda reproduction

Variation of gestation period caused by delayed implantation

Bears are seasonal breeders. The reproductive biology in most bear species is similar. The bear gestation period is characterized by a long 'embryonic diapause' or ëdelayed implantationí that caused variation in pregnancy lengths. Average pregnancy of giant panda is reported to be 90-160 days (range 80-300 days). The large variation of pregnancy length is mainly dependent on the delayed implantation. This reproductive phenomenon is also known as a discontinuous development of embryos inside the females' uterus. Cell cycle arrest can occur at the G0/G1 or G2 phase, depending on the species (Lopes et al., 2004). In several species that display pre-implantation delay, including some marsupials, rodents, roe deer, nine-banded armadillo, the embryos hatches from its zona pellucida before entering into diapause. This unique biological mechanism is the reason of unpredictable gestation length.

Pregnant or pseudopregnant?

Monitoring of pregnancy using the combination of behavior changes, urinary progesterone assay, and bi-monthly ultrasonography was conducted to maximize the chance to confirm pregnancy. During the first 60-100 days after ovulation, pregnancy is generally indistinguishable from pseudopregnancy both by behavioral observation (increased bamboo consumption and sleep time), physical changes of animalís body (enlarged abdomen, breasts, and milk let down) and hormonal profiles. Only diagnostic nesting behavior was observed 24 h prior to parturition in true pregnancy. Urinary hormone assay demonstrated significant increased of progesterone level from baseline at AI day to 440 ng/mg creatinine at 82 day after AI. However, the observed profile was comparable to pseudopregnancy profile of Lin Huiís 2007 cycle (Figure 5). This similar level of progesterone could also be detected during pseudo-pregnancy illustrating that true pregnancy cannot be distinguished from pseudopregnancy using hormonal assay. Beating heart- or large arterial-like image was observed by ultrasonography at day 82 but could not confirm pregnancy.

3. Conclusion and further researches

The study indicates that 1) ovulation can be detected in female giant panda using behavioral observation and urinary hormone assay thus timed AI can be conducted; and 2) a healthy giant panda cub has been produced successfully by AI with 2 doses of fresh and chilled
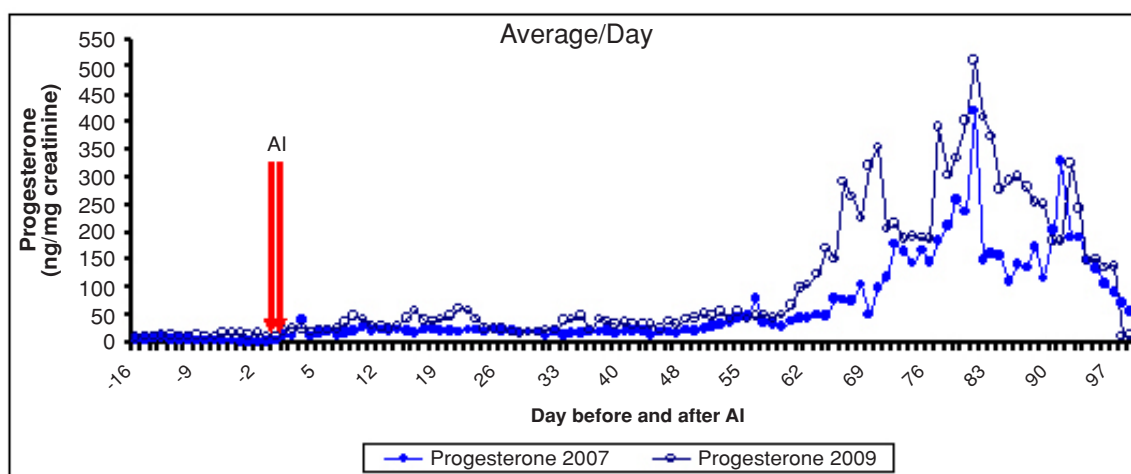

Figure 5. The progesterone profiles of giant panda Lin Hui after AI in 2007 and 2009.

extended semen. Suggested future research and developments are; 1) to investigate the efficiency of frozen sperm for AI; and 2) to improve pregnancy diagnosis by ultrasonography and specific assay to detect embryo-origin signals.

References

Durrant, B.S., Olson, M.A. Anderson, A., Gual-sil, F., Li, D. and Huang, Y. 2006. The value and significance of vaginal cytology. In: Giant Pandas: Biology, Veterinary Medicine and Management. 1st ed. New York: Cambridge University Press. 231-244.

Lopes, F.L., Desmarais J.A. and Murphy, B.D. 2004. Embryonic diapause and its regulation. Reproduction 128: 669-678.

Steinman, K.J., Monfort, S.L., Mcgeehan, L., Kersey, D.C.,Gual-Sil, F., Snyder, R.J., Wang, P., Nakao, T., Czekala, N.M. 2006. Endocrinology of the giant panda and application of hormone technology to species management. In: Giant Pandas: Biology, Veterinary Medicine and Management. 1st ed. New York: Cambridge University Press. 198-230.

Shuling, Z., Zhao, Q., Zhong, X., Wildt, D.E., Seal, U.S., 1997. Report of the giant panda captive management planning workshop, Chengdu, China. In: Shuling, Z., Zhao, Q., Zhong, X., Wildt, D.E., Seal, U.S. (Eds.), IUCN - World Conservation Union/SSC Conservation Breeding Specialist Group. IUCN, Apple Valley, MN, 266.

Tsutsui, T., Hori, T., Nakashige, T., Narushima, E., Hara, T., Akikawa, T., Nose, N., Saito, K., Shichiri, S., Hashizaki, F. and Komiya, T. 2006. Semen quality in a Giant Panda *(Ailuropoda melanoleuca)* in relation to estrus of a nearby resident female panda. Theriogenology. 66(6-7): 1803-1806.