Artificial Insemination and Stillbirth in a Giant Panda (*Ailuropoda melanoleuca*)

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(2008年11月30日受領, 2009年5月12日採択)

ジャイアントパンダ (*Ailuropoda melanoleuca*) における人工授精と死産

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ABSTRACT. At the Kobe Municipal Oji Zoo, a female giant panda was artificially inseminated three times during the estrous period of 2007 using fresh and refrigerated semen. Urinary concentrations of estrone-3-glucuronide were monitored to estimate the appropriate time for artificial insemination. Urinary concentrations of pregnanediol-3-glucuronide (PdG) were monitored to determine luteal function and to estimate the timing of delivery or end of pseudopregnancy. During late pregnancy period, the urinary PdG showed large oscillations which was not shown in the previous 6 years. The female occurred stillbirth in 9 days after the rupture of fetal membranes, that is on 137 days after the last artificial insemination.

Key words : Artificial insemination, Giant panda, Stillbirth

Jpn. J. Zoo. Wildl. Med. 14(2) : 119-123, 2009

The female giant panda (*Ailuropoda melanoleuca*) typically exhibits mono-estrus during the breeding season, and ovulates once in each estrous period [1]. This characteristic may result in a comparative low reproductive success rate in both wild and captive populations. Moreover, lack of male libido and aggressive behaviors against females may also contribute towards the low reproductive success in captivity [2]. Therefore, the artificial insemination (AI) is an important technique for the reproduction of giant panda in captivity.

Natural mating and/or AIs were performed in a pair of giant pandas between 2002 and 2006 at the Kobe Municipal Oji Zoo but no offsprings were produced. In 2007, a successful AI was followed by pregnancy and stillbirth for the same giant pandas. In this report, detection of estrus, electroejaculation for semen collection, AI, monitoring of pregnancy and stillbirth in 2007 are described.

A female (ShuangShuang) and a male (LongLong) giant panda which were born at the Wolong Natural Reserve in China on September 16th 1995 and September 14th 1995, respectively, were loaned by China to the Kobe Municipal Oji Zoo on July 16th 2000 and December 9th 2002, respectively.

ShuangShuang was kept in a 128.5 m² indoor enclosure comprising the exhibition, bed and nesting rooms, and was provided access to a 283.5 m² outdoor space. The neighboring enclosure, housing LongLong, was separated from ShuangShuang's enclosure by stainless steel bars. LongLong's 107.0 m² indoor enclosure consisted of an exhibition room and a bedroom. LongLong was also provided access to his own 283.5 m² outdoor space. The two giant pandas were kept separately except when they were mixed for natural mating.

Daily, from 7:30 to 15:00, they could alternatively utilize both the indoor and the outdoor spaces, but, from 15:00 to

next 7:30 they could utilize only the indoor enclosure. Giant pandas were fed bamboo, carrots, apples and food pellets (Mazuri 5MA4, pmi nutrition international, LLC, MO. USA) 6 times a day, with continuous access to drinking water of the indoor or outdoor pool.

To estimate the ovulation timing, urinary estrone-3-glucuronide (E1G) concentrations were measured from March 7th 2007 to March 26th 2007 by enzyme-immunoassay (EIA), as described previously [3]. Pooled urine samples were collected as previously described [3]. In addition, female scent-marking urine samples were also collected when possible. Daily E1G value was indicated by the mean value of all samples collected in a day.

To collect semen by electroejaculation, LongLong was anesthetized with an initial intramuscular injection of ketamine hydrochloride (14.1 mg/kg) and maintained with inhalation of isoflurane (I : O₂ = 1-3 : 3) on March 23rd 2007. A rectal probe used for semen collection was hand-made from a pole of vinyl chloride pipe (diameter: 50 mm, length: 480 mm) with two semicircular electrodes of width of 16.5 mm each. One located at 35 mm and the other located at 70 mm from top of the probe. Stimulation for electroejaculation was accomplished by gradually raising the voltage of a stimulator (Fujihira Kogyo Co. Ltd., Tokyo, Japan) from 1 to 7 V in 1 increments with 3-9 stimulations of 3 second duration at each voltage.

On collection, semen volume, pH, sperm concentration, motility, viability and morphology were evaluated as described previously [4]. Subsequently, semen was diluted 3-fold with a refrigeration medium (TEST Yolk Buffer with gentamicine sulfate and no glycerol, Irvine Scientific, Santa Ana, CA, USA).

To perform intrauterine insemination using a fiberscope (OLYMPUS VQ-8142A, Olympus AVS Co. Ltd., Tokyo, Japan), ShuangShuang was initially anesthetized with an intramuscular injection of medetomidine hydrochloride (28.2-43.5 µg/kg) in combination with ketamine hydrochloride (3.84-5.42 mg/head) and maintained with inhalation of isoflurane at a ration of I : O₂ ratio of 1-3 : 3.

Urinary pregnane-3-diol-glucuronide (PdG) concentrations were measured to monitor the reproductive status before and after estrus from March 16th 2007 to August 26th 2007. And urinary PdG between 2001 and 2006 were also measured retrospectively to compare with 2007. These assays were carried out by EIA as described previously [5].

A total of 4.5 ml semen was obtained from two ejaculates (A and B) at the range from 4 to 6 V on March 23rd 2007. The traits of the ejaculate (A) and (B) were following, volume: 3.0 ml and 1.5 ml, pH: 7.1 and 7.1, concentration: 766×10^6 /ml and 512×10^6 /ml, motility index: 80 and 60, viability: 82.3 % and 76.0 % and normal morphology: 68.2 % and 47.6 %, respectively (Table 1). Both of the ejaculate (A) and (B) were then diluted with refrigeration medium (1:1 v/v), respectively. As the urinary E1G concentration showed a peak value on March 23rd 2007, therefore, 3 serial daily AIs were performed from March 23rd to March 25th 2007 (Fig. 1). A

Fig. 1 Daily urinary E1G levels of female giant panda, ShuangShuang, during the 2007 estrous period. Three arrows indicate AI dates.

half of diluted (A) was inseminated on March 23rd and the remaining portion of diluted (A) and diluted (B) were stored at 4 °C until insemination on March 24th and 25th, respectively. The numbers of spermatozoa which were inseminated at each AI, were $1,149 \times 10^6$, $1,146 \times 10^6$ and 768×10^6 , respectively. Semen qualities immediately before each AI are shown in Table 1.

ShuangShuang's urinary PdG pattern in 2007 showed repeated large oscillations during the late term of pregnancy (Fig. 2), whereas those in the previous 6 years did not show such oscillations (Fig. 3). A small amount of fetal fluid discharge from ShuangShuang suggested the rupture of the fetal membrane in 9 days (August 3rd 2007) before the observed stillbirth. Later, in the morning of August 12th 2007, ShuangShuang was seen cradling a dead neonate. Upon examination, no air was detected in the lung of the dead neonate. The neonate's abdominal skin and internal organs including reproductive organs were absent (Fig. 4). Neonate body measurements are shown in Table 2. Sex could not be determined due to the absent of internal reproductive organs. The body measurements of infant are shown in Table 2.

It has been demonstrated that rising estrogen concentrations are associated with an increase in prospective behaviors, and that peak of receptivity usually occurs after the estrogen concentrations return to the basal level [6-11]. Shuang Shuang's E1G levels on one day prior to the E1G peak in the previous 6 years, ranged from 29 - 41 ng/Crmg [3]; therefore, an E1G concentration of ≥ 30 ng/Crmg was deemed critical for the determination of AI timing. In the present case, an E1G

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Table 1 Characteristics of semen used for AI of female giant panda, ShuangShuang

Status	Immediately after collection		1 day storage	2 days storage
	A	B	Diluted A	Diluted B
Date of insemination	March 23 rd	—	March 24 th	March 25 th
Volume (ml)	3.0	1.5	3.0	3.0
pH	7.1	7.1	ND	ND
Concentration ($\times 10^6/\text{ml}$)	766	512	383	256
Motility index	80	60	60	15
Viability-(%)	82.3	76.0	84.0	89.2
Normal morphology (%)	68.2	47.6	80.8	87.3

ND : not determined

concentration of 34.9 ng/Crmg was measured on March 22nd, and a peak urinary E1G was predicted on March 23rd 2007. Moreover, in 2001-2004, ShuangShuang showed presentation behavior, indicating receptivity, most frequently one day after the urinary E1G peak [3]. Therefore, taking the above previous reports and past findings in ShuangShuang, into account, 3 serial AIs were conducted on March 23rd, 24th and 25th 2007. Czekala et al. [12] reported that 4 successful giant panda AIs were performed 1 or 2 days after the urinary estrogen peak. Masui et al. [6] reported that only one AI performed two

days after the urinary estrogen peak was successful. Based on the above findings, the timing for AIs in the present case was considered to be suitable for successful AI.

Successful AI depends not only on the timing of AI, but also on the semen quality. Howard et al [2] described the semen qualities of non-breeding and breeding male giant pandas: the ejaculate volumes were 3.1 ± 0.4 and 5.5 ± 1.4 ml, ejaculate pH values were 8.2 ± 0.1 and 8.0 ± 0.1 , sperm concentrations were 1370.3 ± 196.4 and $919.7 \pm 289.9 \times 10^6/\text{ml}$, and sperm motilities were 72.1 ± 4.3 and $74.2 \pm 2.7\%$, respectively. In

Fig. 2 Daily urinary PdG levels in female giant panda, ShuangShuang, in 2007. Three small arrows on the left indicate AI dates. Larger dotted and bold arrows indicate dates of fetal membrane rupture and stillbirth, respectively.

Fig. 3 Daily urinary PdG levels in female giant panda, ShuangShuang, in 2001 (a), 2002 (b), 2003 (c), 2004 (d), 2005 (e) and 2006

the present case, ejaculate volumes and pH values were lower, but motilities were similar to those reported in the breeding and non-breeding males. Based on the above findings, it was suggested that LongLong's electroejaculated semen was of a sufficient quality for a successful AI.

Generally in pregnant giant pandas, urinary PdG levels

slightly elevate immediately after the E1G peak in the estrous period, and then maintain elevation until mid-pregnancy, followed by a rapid increase before returning to a basal level [1]. Females who do not delivery neonates, including pseudopregnant females, show similar urinary PdG patterns as pregnant females [1]. In the present case, ShuangShuang showed similar urinary PdG changes as previously observed, however, during the late term of pregnancy in 2007, her urinary PdG showed large oscillations different from the previous 6 years. These oscillations possibly indicated that endocrinologic abnormality occurred in ShuangShuang.

The body weight of dead neonate was 84.4 g, however, abdominal skin and internal organs were missing. Edward

Fig. 4 Stillborn giant panda neonate (abdominal view). Abdominal internal organs and genitalia were absent.

Table 2 Body measurements of stillborn neonate.

	Value
Body weight (g)	84.4
Crown-rump length (cm)	15.8
Tail length (cm)	5.5
Chest girth (cm)	8
Abdominal girth (cm)	9
Neck girth (cm)	4.5

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et al. [13] reported that birth weights of the giant pandas were 152.0 ± 47.4 g (mean $\pm$ SD) ranging from 54.0 to 216.0 g. The weight of the dead neonate in the present case suggested that the fetus had developed adequately in utero. No air was detected in the lungs of the dead neonate. According to this finding, the fetus seemed not to breathe in the air, so we certified that it was dead in utero on some day before parturition. We were unable to evaluate if or how abnormal urinary PdG change affect in utero fetal survival.

We acknowledge Wataru Yoshitake, Akiko Yamada, Eiko Shimokawa, Takashi Kouno and the other staff of the Kobe Municipal Oji Zoo, as well as Yasuyuki Horii, Kodue Kinoshita, Yusuke Aramaki, Haruna Sasaki and Yukiko Yamagami (Students of Kobe University) for their assistance in maintaining these two giant pandas, measuring the hormone levels, and performing AI. We also thank Toshio Fukuoka, the director of the Himeji Municipal Zoo, for allowing us to use the electrostimulator.

要 約

神戸市立王子動物園のジャイアントパンダにおいて 2007 年の発情時に新鮮および冷蔵保存した精液を用いて 3 日間連続で人工授精 (AI) を行った。AI の適期は尿中エストロングルクロニド濃度の測定から推測した。人工授精後は尿中プレグナンジオールグルクロニド (PdG) 濃度の変化を監視した。PdG 濃度は妊娠後期に過去 6 年間と比べると異常な変動を示した。結果的に最終 AI 後 137 日目に破水し、さらにその 9 日後に死産した。

キーワード：死産, ジャイアントパンダ, 人工授精

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