Development of an Enzyme Immunoassay for Urinary Pregnanediol-3-Glucuronide in a Female Giant Panda (Ailuropoda melanoleuca)

Natsuki HAMA1,2*, Hideyasu KANEMITSU2), Michiyo TANIKAWA1), Masami SHIBAYA1), Kensuke SAKAMOTO3), Yuijiro OYAMA1), Tomas J. ACOSTA1), Osamu ISHIKAWA2), Wang PENGYAN3) and Kiyoshi OKUDA1)

1)Graduate School of Natural Science and Technology, Okayama University, 1–1–1 Tushimanaka, Okayama 700–8530, Japan and 2)Kobe Municipal Oji Zoo, 3–1 Oji-cho, Nada-ku, Kobe 657–0838, Japan and 3)China Research and Conservation Center for the Giant Panda, Wolong, Wenchuan, Sichuan, P. R. China

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ABSTRACT. In order to enable monitoring of the reproductive status of the female giant panda after observation of estrus behavior, we developed an enzyme immunoassay (EIA) system for urinary pregnanediol-3-glucuronide (PdG), a progesterone metabolite, using commercial reagents and examined the changes in the urinary concentration of PdG in a female giant panda that showed pseudopregnancy and suspicious pseudopregnancy in 6 consecutive years. The developed EIA system had good reproducibility (intra- and interassay CVs 6.1% and 16.3%, respectively), good parallelism between the standard curve and the dose response curve of serial diluted samples and positive correlation (r=0.836) with the data for PdG in the same samples measured by gas chromatography. Urinary PdG in the female panda showed two phases of increase. The first elevation was observed immediately after estrus with the levels of PdG below 100 ng/Crmg, while the second phase was characterized by a drastic elevation above 100 ng/Crmg until the level began to decrease at the end of pseudopregnancy or suspicious pseudopregnancy. The length of the second phase had wider range than that of the first phase. In the present study, a new EIA assay system for urinary PdG in the female giant panda was developed, and we found that the length of the second phase is unstable in the pseudopregnant and suspicious pseudopregnant giant panda, in contrast with the unstable length of the first phase caused by delayed implantation in the pregnant giant panda.

KEY WORDS: enzyme immunoassay, giant panda, PdG, pseudopregnancy.

The reproductive biology of the giant panda (Ailuropoda melanoleuca) has unique characteristics. The giant panda shows a single estrus associated with ovulation per year followed by a variable period of delayed implantation and pseudopregnancy [16]. The length of pregnancy varies widely (95–178 days [5], 83–181 days [7], 85–185 days [16]). Since the period of time between implantation and delivery is relatively constant (4–6 weeks) [12], the large variation in the length of pregnancy is thought to be mainly due to the variation in the period of delayed implantation.

Pregnanediol-3-glucuronide (PdG) is a metabolite of progesterone present in large amounts in the urine of pregnant animals [8]. Although many researchers have measured urinary PdG to monitor the reproductive status of the giant panda species, there is no study that compares the levels of PdG in pregnant and pseudopregnant giant pandas. However, because monitoring urinary PdG levels after estrus provides information on the timing of delivery and end of pseudopregnancy, a method for monitoring urinary PdG is needed to aid in reproductive management of female giant pandas in captivity.

Enzyme immunoassay (EIA) systems for various steroids including PdG have been developed to assess the reproductive statuses of exotic wild animals [2, 3, 6]. Since the specific antisera used in the above reports are not always available to researchers working in different institutions, development of an EIA system for PdG using commercially available reagents and antisera is desired.

The aim of this study was to establish an EIA system for measuring urinary PdG in a female giant panda using commercial reagents. We also monitored the dynamics of urinary PdG in this panda over a six-year period, in which the panda did not give birth.

MATERIALS AND METHODS

Animal: The female giant panda used in the present study, named ShuangShuang, was born at Wolong Natural Reserve in PR China on September 16, 1995 and was loaned by PR China to Kobe Municipal Oji Zoo on July 16, 2000. A male partner was kept in a neighboring enclosure throughout the study period of July 2000 to April 2006. However, since the first partner, which was kept from July 16, 2000 to December 5, 2002, was found to be a female with a male-like body size and behaviors such that it was mistook for a male, another male mate, named LongLong and born at Wolong Nature Reserve on September 14, 1995, was loaned to the zoo on December 9, 2002.

Management of the animals: ShuangShuang was kept in an enclosure consisting of an indoor space (128.5 m²), including an exhibition room, bedroom and nesting room, and an outdoor space (283.5 m²). The neighboring space was used for her partner, from which she was separated by a stainless steel barrier containing small openings. From...
0730 hr to 1500 hr, she had free access to the indoor and outdoor space; however, from 1500 hr to 0730 hr the next day, she could only utilize the indoor space. She was fed bamboo, carrots, apples and pellets (Mazuri 5MA4, Pmi Nutrition International, LLC) 6 times a day with permanent access to drinking water from an indoor or outdoor pool.

Collection of urine samples: Daily urine samples were collected for ShuangShuang as a pooled sample flowing into a collection cup which was set beneath the drainage hole of the indoor resting room from 1700 hr to 0900 hr the next day between April and August in 2001 and 2002, April and September in 2003, March and July in 2004 and 2005 and March and September in 2006. If the collection cup was empty at 0900 hr, it was left in place during the day until urination. When the urine in the cup was suspected to be contaminated with water, urine samples were immediately re-collected after urination by aspirating with a plastic syringe from the flour of the indoor space during the day. The collected urine samples were centrifuged for 4 min at 650 × g, and the supernatant was stored at −40°C in the zoo laboratory until assay.

Hormone assays: Urinary concentrations of PdG in all samples collected in the periods described above were measured by EIA, and the same samples used for EIA above were also measured by gas chromatography (GC) in a commercial laboratory (Mitsubishi Kagaku Bio-Clinical Laboratory, Inc, Kobe, Japan) between 2001 and 2004 in order to evaluate the correlation coefficient between EIA and GC. The EIA was carried out with a double antibody enzyme immunoassay. A first antibody, anti-5α-pregnane-3α, 20α-diol-3-glucuronide-BSA (Catalog No. FKA334-E), and a HRP-linked antigen, 5β-pregnane-3α, 20α-diol-3-glucuronide-HRP (Catalog No. FKA333), were purchased from Cosmo Bio Co., Ltd., Tokyo, Japan (manufactured by Kanegawa Lab. Tokyo Japan). A standard, 5β-pregnane-3α, 20α-diol-3-glucuronide (Catalog No. P3635) was purchased from Sigma-Aldrich. The cross-reactivity of the 1st antibody is shown in Table 1. The concentrations of standards used in this study were between 0.03125 and 2.0 ng/well. The EIA buffer, coating buffer, wash buffer, substrate buffer and 2nd antibody used in the present study were the same as those used in a previous report [4]. A plate was coated with 2nd antibody and washed with wash buffer 2 times. Then, 20 µl of the standards and control urine sample (dilution rate: × 1, collected from ShuangShuang on April 30, 2003) were added to each well in duplicate. On the same plate, 120 µl and 20 µl of EIA buffer were added to non-specific binding and maximum binding wells in duplicate, respectively, and two wells were prepared as blank wells. One hundred microliters of the 1st antibody (final working dilution: × 700,000) was added to each well except for non-specific binding and blank wells, and 100 µl of HRP binding antigen (final working dilution: × 200,000) was added to each well except for the blank well. After adding all of the standards, samples, control sample and reagents, the plate was incubated in 4–5°C for over 12 hr. After washing with wash buffer 4 times, 150 µl of substrate buffer was added to each well, and then the plate was incubated in a shaking dark incubator at 37°C for 40 min. Fifty microliters of 4 N-H2SO4 was then added to each well to stop the reaction before reading the plate at a wave length of 450 nm with a microplate reader (Bio-Rad Model 550, Bio-Rad Laboratories Inc, Hercules, CA, U.S.A.).

The intra- and interassay coefficients of variation (intra- and interassay CVs, respectively) were evaluated from the concentrations obtained in control samples. The intra-assay CV was calculated from triplicate data within a plate, and the interassay CV was calculated from 26 plates. To evaluate the parallelism between the dose response curve from serial dilutions of giant panda urine and standard curve, the urine collected on July 2, 2006 was used to measure the PdG concentrations at the serial dilutions (×1 – ×32). The methods of measuring the urinary concentration of creatinine and PdG were the same as reported previously [4]. Finally, the concentrations of PdG was calculated as ng/mg of creatinine (Crmg).

Natural mating and artificial insemination (AI): ShuangShuang was not given any opportunities for natural mating or AI in 2001 and 2002 because her partner was female. ShuangShuang and LongLong were kept together in one enclosure for natural mating on May 1 and 2, 2003, March 14, 2005 and April 5 and 6, 2006, and ShuangShuang was received AI on May 3 and 4, 2003, March 22, 2004, March 15, 2005 and on April 7, 2006. These AIs were performed using cryopreserved semen previously collected from LongLong by electroejaculation.

Detection of estrus: To detect estrus in each year, urinary estrone-3-glucuronide (E1G) was measured by EIA as described previously [4]. The week when the urinary concentration of E1G reached a peak value in each year was defined as week 0 to normalize the urinary concentrations of PdG. Weekly averages of the urinary concentration of PdG were calculated by taking the day of the peak as the first day of week-0.

Statistical analysis: The correlation coefficient for the EIA and GC measurements of PdG was analyzed. Regression of both of the standards and diluted samples was

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Cross reaction (%)</th>
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<tbody>
<tr>
<td>5β-pregnane-3α, 20α-diol-3-glucuronide</td>
<td>100</td>
</tr>
<tr>
<td>20α-OH-progesterone</td>
<td>16</td>
</tr>
<tr>
<td>5β-pregnane-3α, 20α-diol</td>
<td>8.9</td>
</tr>
<tr>
<td>Progesterone</td>
<td>2.3</td>
</tr>
<tr>
<td>5β-pregnane-3β, 20α-diol</td>
<td>0.8</td>
</tr>
<tr>
<td>5β-pregnane-3β, 20α-diol-20-one</td>
<td>0.2</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.1</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>0.05</td>
</tr>
<tr>
<td>17α-OH-pregnenolone</td>
<td>0.02</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.01</td>
</tr>
<tr>
<td>Androstenedioid</td>
<td>0.01</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.01</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.01</td>
</tr>
</tbody>
</table>
adapted to linear lines, respectively, and the parallelism among the lines was examined by analysis of covariance. These analyses were performed with SPSS Ver. 10 (SPSS Japan Inc, Tokyo, Japan).

RESULTS

The standard and dose response curves from the serial dilutes samples and their linear regressions are shown in Fig. 1. These two linear lines were parallel (P = 0.082).

The intra- and interassay CVs were 6.1% and 16.3%, respectively.

The PdG concentrations (ng/Crmg) assayed by EIA and GC were not normally distributed (Kolmogorov-Smirnov Z=0.305 for GC and 0.293 for EIA, P<0.05). The Spearman’s rank correlation coefficient for the EIA and GC values was 0.826 (P=9.99×10⁻⁷).

The weekly changes in urinary concentration of PdG over a 6-year period are shown in Fig. 2a-f. Except for 2003 (Fig. 2c), the dynamics of urinary PdG showed 2 phases of increase. Phase 1 showed a slight elevation from week 1 with most values below 100 ng/Crmg, whereas phase 2 showed a second drastic elevation beyond 100 ng/Crmg followed by a decrease to a level lower than that in week 0. Phase 2 was longer and had a wider range than phase 1 (Table 2). The total length of phases 1 and 2 had a wide range, from 14 to 19 weeks.


DISCUSSION

In the present study, the dose response curve of the serial diluted urine sample well-paralleled the standard curve, which suggests that inhibition of the reaction between the antisera and antigen did not affect the quality of the assay, and the effects of other urinary components could be ignored. The reproducibility of the present EIA system was considered to be good based on the intra-assay CV of 6.1% and interassay CV of 16.3%. Furthermore, the PdG concentrations measured by EIA and GC were positively correlated. The above results indicate that the EIA system

![Regressed curves and linear lines for both of standards (a) and serial diluted urine samples (b). Each curve (real line) and linear (dot line) line was expressed using the following formulas.](image-url)

**Fig. 1.** Regressed curves and linear lines for both of standards (a) and serial diluted urine samples (b). Each curve (real line) and linear (dot line) line was expressed using the following formulas. a-1: OD=0.109 C + 0.194 C²– 0.408 C + 0.220. a-2: OD=–0.424C+0.247. b-1: OD=–0.0898 C +0.277 C²–0.718C+0.194. b-2: OD=–0.49C+0.224. OD: Optical Density at 450 nm. C: Log (Concentration of standard in a and dilution rate of sample in b). Each number in parentheses in b indicates the volume (µl) of giant panda urine in a well. The data for the concentrations of PdG in urine samples were calculated by fitting to the standard curve (a-1). Parallelism between the standard and serial diluted sample lines was examined by comparing a-2 with b-2 by ANCOVA.
developed in the present study is practical for measuring urinary PdG concentrations.

Female giant pandas often show pseudopregnant behavior, even if they are not pregnant. However, pseudopregnancy in the giant panda is not well defined because pregnancies resulting in embryo loss or early fetal loss are often mistaken for pseudopregnancies [15]. The latter are referred to as suspicious pseudopregnancies. Pregnant, pseudopregnant and suspicious pseudopregnant females have similar dynamics of urinary progestins showing 2 phases of elevation [15]. The first elevation of urinary progestins signals the occurrence of ovulation [9, 11]. In the present study, changes in the concentrations of urinary PdG, a kind of progestin, also showed 2 phases in all 6 years, indicating that ShuangShuang ovulated in each year. During the first 2 years of the present study, ShuangShuang was apparently pseudopregnant because her partner was female and AI was not performed. In the subsequent 4 years from 2003 to 2006, it could not be determined whether ShuangShuang was pseudopregnant or suspicious pseudopregnant, since AI was performed in all 4 years. In a study of Japanese black bears, the serum progesterone (P4), estradiol-17β, FSH, LH and prolactin (PRL) concentrations were measured in a female that did not mate with a male but could see and touch the male through a fence (group A), 3 females that were...

<table>
<thead>
<tr>
<th>Year</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 1 + Phase 2</th>
</tr>
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<tbody>
<tr>
<td>2001</td>
<td>7</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>2002</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>2003</td>
<td>Unclear</td>
<td>Unclear</td>
<td>18</td>
</tr>
<tr>
<td>2004</td>
<td>8</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>2005</td>
<td>9</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>2006</td>
<td>8</td>
<td>11</td>
<td>19</td>
</tr>
</tbody>
</table>

Mean ± SD 7.8 ± 0.8 8.4 ± 1.7 16.5 ± 1.9
given the chance to mate with several different males and ultimately produced no cubs (group B), 6 females that mated with males and ultimately produced cubs (group C) and 2 females that were maintained together but were completely isolated from other animals (group D) [14]. In the above report, the group A and B females showed 2 phases in the changes of serum P4 as was observed for PdG in the present study. The group C females showed marked increase in serum P4 around 2 months before parturition. One of the group D females did not show elevation of serum P4, whereas the other of group D females did. The frequencies of blood sampling for the group C and D females were lower than for the group A and B females, so it was not known whether the group C females that produced cubs had two phases in their changes of serum P4. However, because the levels of serum PRL in all the groups increased and were positively correlated with elevation of serum P4 in this report, the authors suggested that the elevations of P4 would be supported by secretion of PRL and that once ovulation occurred, the serum P4 concentration might change following the same course regardless of whether or not an embryo existed in the uterus [14]. This reported inferred that the mechanisms of P4 secretion in bears after ovulation may be consistent regardless of whether or not an embryo existed in the uterus and regardless of whether or not implantation occurred. Because the giant panda, which shows delayed implantation and pseudopregnancy like bears, is a very close species to the animals of the family Ursidae, the dynamics of urinary PdG, a metabolite of P4, in giant panda after ovulation seem to be consistent regardless of whether or not an embryo existed in the uterus and regardless of whether or not implantation had occurred.

For ShuangShuang, the length of phase 2 varied more widely than the length of phase 1. Therefore, the variation in the total length of phase 1 and 2 might depend on the wider range of phase 2. Female giant pandas have been demonstrated to have a wide variation in the length of the pregnancy period (95–178 days [3], 83–181 days [5], 85–185 days [15]). Since the period of time between implantation and delivery is relatively constant (4–6 weeks) [10], the large variation in length of the pregnancy period in the giant panda may be the result of wide variation in the period of delayed implantation. Our results suggest that a pseudopregnant or suspicious pseudopregnant giant panda has a large variation in the length of phase 2 and comparatively constant length of phase 1, in contrast with the pregnant giant panda, which has a variable length of phase 1 and constant length of phase 2 [10]. However, the present study only analyzed six years worth of data from one animal, so further study is needed to clarify the reason for the large variation in the lengths of the pseudopregnancy and suspicious pseudopregnancy periods in the giant panda.

A previous study performed at Ueno Zoological Garden, Tokyo, reported the dynamics of urinary PdG in a female giant panda that did not deliver for 10 consecutive years, from 1991 to 2000, despite being artificially inseminated each year [13]. The length of the period from ovulation to the end of pseudopregnancy or suspicious pseudopregnancy, in which the level of urinary PdG was elevated, was calculated as 8.8 ± 2.3 weeks, which is shorter than the period of PdG elevation measured in the present study (16.5 ± 1.9 weeks). There seems to be large individual variation in the length of the period from ovulation to the end of pseudopregnancy or suspicious pseudopregnancy.

In the present study, an EIA system was established to monitor urinary PdG in female giant pandas using commercial reagents. Our data suggest that the period from ovulation to the end of pseudopregnancy or suspicious pseudopregnancy varies widely from year to year and that pseudopregnant or suspicious pseudopregnant giant pandas have a comparatively consistent duration for phase 1 and variable duration for phase 2.

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