

Monitoring the Reproductive Status of Dairy Cows by Urinary Pregnanediol Glucuronide

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ABSTRACT : This study was undertaken with the aim to establish a reliable radioimmunoassay (RIA) system for urinary pregnanediol glucuronide (PdG) and to employ it for monitoring the reproductive status of dairy cows. Urine and blood samples were collected from the Holstein cows both pregnant and non-pregnant. The samples were then investigated for evaluating the relationship between progesterone (P₄) in blood and PdG in urine adjusted with or without urinary creatinine basis. Biweekly urine collection was employed for three cows in estrous and those artificially inseminated, while urine from pregnant cows was collected on a monthly basis. P₄ and PdG levels were measured by enzymeimmunoassay (ELA) and RIA techniques, respectively. Our results indicated the sensitivity of PdG for RIA being 35 pg/tube and the recovery rate of 100%. Urinary creatinine concentrations also fluctuated within a day, but change at midday was not noteworthy. Regardless of the time of urination the change in concentrations of PdG was relatively smaller and did not vary significantly. The urinary PdG concentration showed periodic changes as that with serum P₄ levels during the cow's estrus cycle. The correlation coefficient rose when creatinine level in urine was adjusted but the change was also not significant. The concentrations of PdG during the luteal phase were detected between 8.2 and 17.4 ng/ml, three to five times higher than that in the follicular phase. The concentration of PdG from pregnant cows (21 days after conception) was three to four times higher than in the non-pregnant cows. Our finding suggests that the determination of urinary PdG could be reliably employed for early pregnancy detection. The urinary PdG level continued to raise until 30 days pre-partum while the concentration reached its peak at 30 ng/ml, after which it started to fall 18 to 30 days before parturition and finally fell to its nadir value one week after parturition. As the correlation coefficient between the urinary PdG and serum P₄ was higher than that corrected by urinary creatinine it can be suggested that the adjustment is not needed. The concentrations of urinary PdG could be maintained stably for 2 days in urine samples stored at room temperature and extended to 8 days when the samples were pretreated by boiling for 30 minutes. In conclusion urinary PdG concentration even without the need for creatinine basis adjustment can be used directly for monitoring the reproductive status of dairy cows. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 4 : 460-466)

Key Words : Dairy Cow, Progesterone, Pregnanediol Glucuronide, Urine

INTRODUCTION

The complex physiology of reproduction in mammalian species still remains a mystery, delicately controlled by neuroendocrine and other associated physiological systems. Sex hormones play a major role in those systems and its assessment has been a crucial tool to monitor the reproductive status both in humans and animals. The serum sex hormone assessment especially progesterone (P₄) have evolved as a major tool for understanding reproductive status of animals mostly the females, which carry high economic impact. But need for a non-invasive technique has been greatly felt and urine as a biological fluid presented an ideal opportunity. Their importance and application in modern day animal husbandry and wildlife

conservation efforts have generated valuable information. Existing serum hormone analysis is constrained by shortcoming like disruption of clear reflection by medicaments like anaesthetic drugs (Johnson and Gay, 1981; Clarke and Doughton, 1983). Not to mention the need for skilled hands and pain inflicted to the animal in collection of blood sample.

As a result of added advantage over traditional serum hormone assay, non-invasive techniques of detecting hormone or its metabolite status in the animals is becoming popular. It narrows the misinterpretation of findings from blood hormone level. The fluctuating levels of hormones and its metabolites in the sampled blood may not be genuine reflection of its actual level in circulation. On the other hand, urinary sample represents excreted hormonal metabolites over a period of hours (Schwarzenberger et al., 1996). The higher concentration of hormone metabolites in the urine by two to four folds than that of parent sex steroid in blood provides another advantage (Lasley and Kirpatrick, 1991). Relative ease in collection of the sample over a long term makes assessment of physiological status more precise. Long term storage possibility and greater volume of samples make it ideal to undertake better investigation.

Pregnanediol glucuronide (PdG) is a common urinary

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metabolite of progesterone in wide range of species, but not perissodactyla (Loskutoff et al., 1983) and Old World monkeys (Liskowski and Wolfe, 1972). Rhinoceroses also showed species difference in urinary metabolite of P_4 (Ramsay et al., 1987; Hindle and Hodges, 1990; Hindle et al., 1992), while in cow it is considered a major metabolite of the parent steroid P_4 (Klyne and Wright, 1959). As P_4 level determination in cow urine is not reliable (Yang et al., 1998), alternatively employing its metabolite assessment was a challenge for lack of reliable assay system. Realizing the importance of such assay protocol in dairy industry we undertook this study to establish a radioimmunoassays (RIA) of urinary pregnanediol glucuronide (PdG) and then to characterize long-term urinary PdG excretion during estrous cycle and pregnancy in the dairy cows.

MATERIALS AND METHODS

RIA for urinary pregnanediol glucuronide (PdG)

Reagents : Pregnanediol glucuronide (PdG) used as standards assay was purchased from Sigma. (P3635 Germany). The antiserum was obtained from Biodesign International, USA and it was raised in rabbits to generate pregnanediol glucuronide antibody for PdG assay. As per the product specifications it had cross reactivity of 1 mg/ml estriol, testosterone, progesterone with 0 mg/ml PdG < 3 µg/ml PdG < 10 µg/ml PdG respectively. The [6,7- 3 H] pregnanediol-3 α -glucuronide (sp. act. 30 ci/mol) was obtained from Radio Chemical Center, Amersham, UK. The assay buffer used was phosphate buffer (10.86 g of NaH_2PO_4 and 5.38 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 2 liter distilled water) containing 17.5 g NaCl, 2 g gelatin and 0.2 g thimerosal and made up in deionized water to pH 7.0. Dextran/charcoal suspension, consisting of 625 mg norit A charcoal and 62.5 mg dextran in 100 ml-assay buffer was used, the suspension was continuously stirred on ice water during dispensation. The scintillation fluid was purchased from BDH, England (Scinutra, 14509).

Procedure : The assay for urinary PdG was modified from the RIA system of milk progesterone and urinary estrone sulfate developed in our lab and clearly explained in our earlier works (Lin et al., 1988; Yang et al., 2003). A standard curve of counts bound was plotted against the logarithm of the concentration of PdG in the standard. The PdG concentrations in urinary samples were calculated from this standard curve by interpolation.

Sensitivity : Five replicates of 0, 5, 10, 25, 50, 100, 250, 500 and 1,000 pg/0.5 ml PdG were measured through RIA, the binding percentages were expressed as logit value, and calculated as the mean \pm SD. The sensitivity of this system was calculated from the average binding percentages of the blank minus 2 SD, and then the concentration was obtained by the equation of standard curve.

Precision : 5 ng PdG/ml of urinary sample was measured for precision test, the intra-assay variation was determined by the simultaneous assay of 10 replicates of the added PdG samples. The inter-assay variation was obtained following the determination of the added PdG in 5 separate assays. From these results, the coefficients of variation were calculated by the method of Wilson and Miles (1978).

Accuracy : Parallelism test was determined by the measurement of known amounts of PdG (25, 50, 100 and 250 pg/tube) added to native sample of urine with various dilution (1:2, 1:5, 1:10).

Recovery : Five different amounts of PdG were added to steroid free urinary sample at the concentration of 0.5, 1, 2.5, 5 and 10 ng/ml urine respectively, the level of PdG was determined by the RIA of all five samples in five replicates, and then compared with the added amount for the recovery.

Specificity : The PdG antiserum was obtained from Biodesign International, USA, the product specification is as mentioned above. For the double check, PdG, Pd, cholesterol (C), cortisol (F), corticosterone (B), estrone (E_1), estradiol (E_2), estriol (E_3), progesterone (P_4), pregnenolone (P_5), 17 α -OH progesterone and testosterone (T) with the serial concentrations of 0, 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 pg/500 µl cross reacted with PdG standard solution under the concentrations of 0, 5, 10, 25, 50, 100, 250, 500 and 1,000 pg/500 µl and PdG antiserum by RIA and then the specificity was determined by CR 50% (Abraham, 1969; Spieler et al., 1972).

Creatinine assay of the urine

The creatinine content in each of the cow urine sample was measured using a modification of the method described by Yeh (1973), the detail procedure of which is mentioned in our earlier work (Yang et al., 2003).

The enzyme immunoassay (EIA) for progesterone

We employed competitive enzyme immunoassay for progesterone that was established in our laboratory using monoclonal antibody G7 (Wu et al., 1997). The details of which are also mentioned in our earlier work (Yang et al., 2003).

Sampling

Samples collection for 24 h : The urine and blood samples were collected in series within a day from three pregnant Holstein cows (#8523, #8532 at 3 yrs old and #8626 at 2 yrs old) housed in the dairy cattle farm of National Taiwan University. The samples were used for evaluating the relationship between P_4 in blood and PdG in urine with or without the base of urine creatinine. The cows were housed in a paddock during the time of waiting for urination, with fresh water provided *ad libitum*. Blood samples (2 ml) were obtained from the tail vein using no.

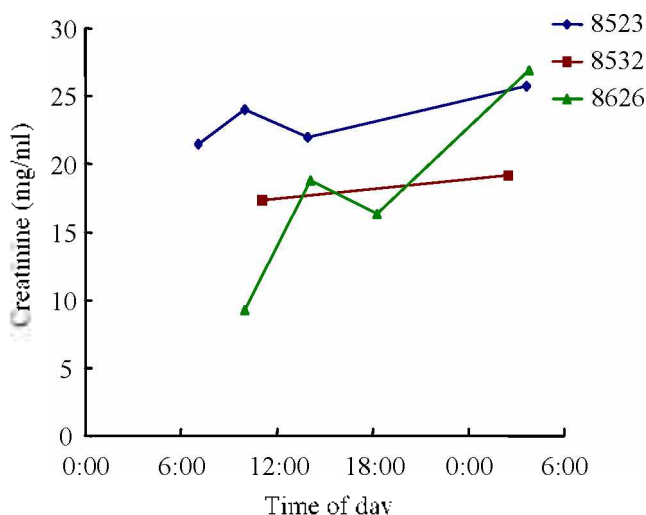


Figure 1. The diurnal shift of urinary creatinine concentration in pregnant Holstein dairy cows.

21 gauge needles immediately after urine collection.

Sample collection during estrus cycle and the pregnant period : The urine and blood samples were collected biweekly for cows on estrus cycle (#8402 at 4 yr. old, #8524 and #8528 at 3 yr old) and those artificially inseminated, collections from pregnant cows (#8329 and #8303 at 5 yr, #8015 at 8 yr old) were made on a monthly basis respectively. The sampling procedure employed was the same as that mentioned above for 24 h.

Samples treatment : The urine samples were obtained after centrifugation (4°C, 800×g for 15 minutes) and the clotted blood samples were centrifuged (4°C, 1,000×g for 10 minutes). The supernatants were stored at -20°C until EIA and RIA were used for measuring the levels of P₄ and PdG in the samples, respectively.

Urine preservation study

To determine the ability of preservatives to maintain urinary PdG immunoactivity in unfrozen samples, urine was collected from each cow (n=3) in the second half of pregnancy and duplicate 10 ml aliquots were stored at room temperature (-25°C) in glass vials (2.5×6 mm) containing no-treatment, boiling in water bath for 30 minutes and autoclave (121°C, 20 min, 1.2 kg/cm²) treatments respectively. These samples were then analyzed for any change in the concentration of PdG after 0, 1, 2, 4 and 8 days of storage in a dark room.

Statistical analysis

The PdG concentration of urine sample was adjusted with creatinine concentration, and were expresses as ng/mg Cr. The data obtained from the experiment with or without creatinine basis were tested with serum P₄ for statistical

Table 1. The diurnal shift of serum progesterone and urinary pregnanediol glucuronide concentrations in pregnant Holstein cows

Steroids (ng/ml)	Cow*		
	No.8523 (n=4)	No.8532 (n=2)	No.8626 (n=4)
Serum progesterone	8.72±0.49	10.78	10.77±0.99
Urinary pregnanediol glucuronide	17.72±4.11	17.87	13.52±2.14

significance by using Spearman's correlation coefficient and Z* test (Steel and Torrie, 1981). A value of p<0.05 was considered a statistically significant difference.

RESULTS

A system established for PdG RIA

Sensitivity : The limit of urinary PdG detection significantly different from zero concentration was 35 pg/tube corresponding to 70 pg/ml, which was sensitive enough for the monitoring of urinary PdG RIA.

Precision : The precision of the PdG RIA was analyzed by using the method of Wilson and Miles (1978). The intra and the inter-assay precision were 6.82 and 9.62%, respectively. CV, less than 10% at the two-assay system were within the acceptable range.

Accuracy : A linear regression was obtained in each dilution for estimated PdG with 25, 50, 100 and 250 pg/tube. The dilutions were not significantly different from the standard (p>0.05) and showed parallelism with the standard linear. It appears that there is no interference in the urine dilution and no matrix effect was found correlating to earlier findings (Shah et al., 1988). The recovery for various concentrations of PdG ranging from 94 to 118% and the average at 100%, is within the acceptable range of 80 to 120% mentioned in earlier works (Abraham, 1975).

Specificity : The specificity of PdG antiserum has immuno-cross reactivity of 100% with PdG, 6.8% with Pd, 0.08% with corticosterone and progesterone and <0.01% with the rest of tested steroids.

The number of urination, daily change of the urinary creatinine, steroids in the blood and urine

The daily urinary creatinine concentrations of cows are as shown in Figure 1. The number of urination of the three cows within 24 h varied from two to six times. The variation of urine creatinine changed slightly during the midday; Cow #8626 showed great variation in creatinine concentrations in three samples of urine, it appeared that urinary creatinine varied individually. The diurnal shift of serum P₄ and urinary PdG is shown in Table 1 and did not vary with the time of urination.

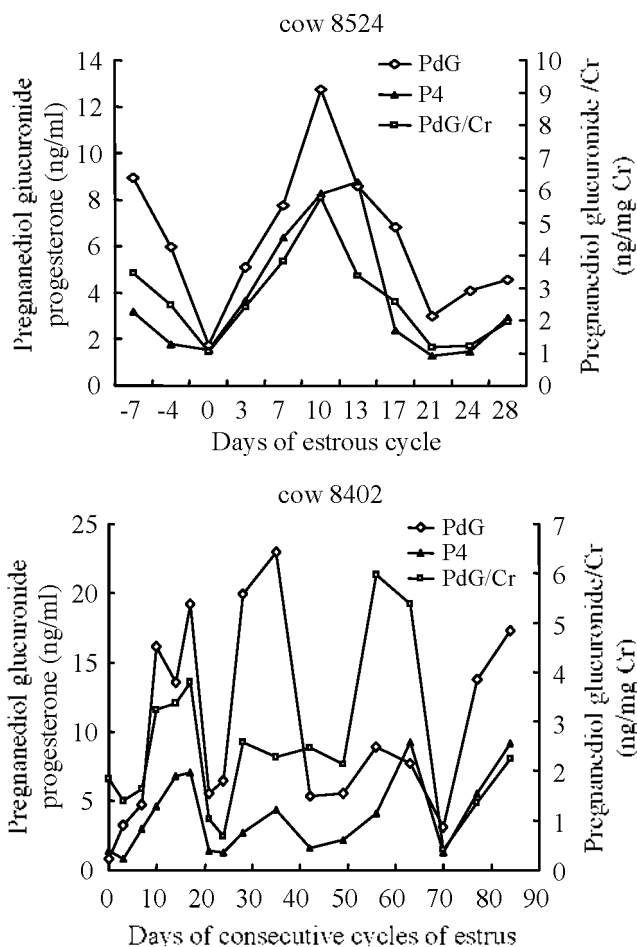


Figure 2. Changes in serum progesterone (P₄), urinary pregnanediol glucuronide (PdG) concentrations and the ratio of pregnanediol glucuronide/creatinine (PdG/Cr) in the cyclic Holstein dairy cows (No. 8524, 8402).

The change of urinary PdG and serum P₄ concentrations during the estrus of cows

The urinary PdG concentration showed periodic variations like the serum P₄ levels in cows on estrus (Figure 2). Samples from cow #8524 40 days post parturition showed serum P₄ level rising from the fourth day of the cycle, with the plateau phase maintained at 11th to 18th day and rapid decrease to the baseline at 22nd day for the estrus cycle of 24 days. The urinary PdG and the urinary PdG with creatinine basis (PdG/Cr) of the estrus cycle also showed the same pattern as those of the serum P₄. Samples from cow #8528 were collected daily from the day of heat sign and it showed a cycle of 22 days. Samples from cow #8402 showed three estrus cycles within a span of 70 days; the average estrus cycle duration being 23 days.

The correlation between urinary PdG and serum P₄ in cyclic Holstein dairy cows was slightly higher in creatinine basis, however no significant difference was seen between them with Z* test (Table 2).

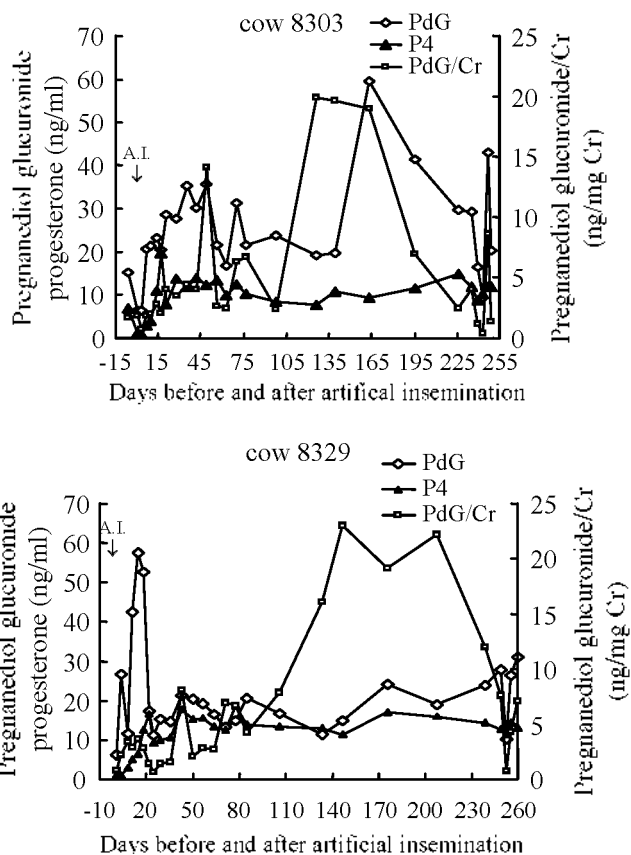


Figure 3. Changes in serum progesterone (P₄), urinary pregnanediol glucuronide (PdG) concentrations and the ratio of pregnanediol glucuronide/creatinine (PdG/Cr) during the pregnancy of the Holstein cow (No. 8303, 8329).

Table 2. The correlation between urinary pregnanediol glucuronide and serum progesterone in Holstein cyclic dairy cows and during the period of pregnancy and postpartum of Holstein cows (#)

Cow	Sample size	Spearman correlation coefficient		Z*
		(r)	(r')	
8528	14	0.70	0.84	0.86
8402	17	0.69	0.68	0.03
8524	31	0.45	0.65	1.07
8329 [#]	49	0.32	0.03	1.45
8303 [#]	47	0.79	0.66	1.29
8015 [#]	36	0.79	0.50	2.11

Note: r (not indexed by creatinine), r' (indexed by creatinine).

The concentrations of urinary PdG and serum P₄ during the period of mating and pregnancy

The changes of serum P₄, urinary PdG concentrations and urinary PdG concentrations with creatinine basis of the Holstein cows during the period of mating and pregnancy are shown in Figure 3. Cow #8303 showed the lowest concentration of serum P₄, urinary PdG and PdG/Cr during the day of AI, they were 0.86 ng/ml, 6.7 ng/ml and 0.7 ng/mg Cr respectively. The levels increased after AI, serum P₄ reached the peak at 12th to 16th day, however urinary

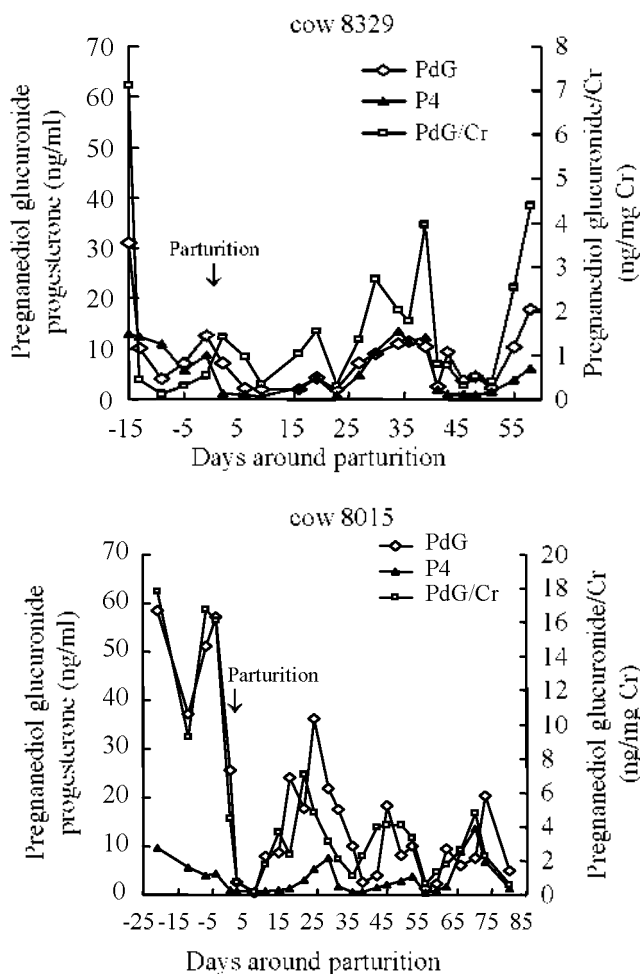


Figure 4. Changes of serum progesterone (P_4), urinary pregnanediol glucuronide (PdG) concentrations and the ratio of pregnanediol glucuronide/creatinine (PdG/Cr) around parturition of the Holstein cow (No. 8329 No. 8015).

PdG reached the peak at 16th day and their concentrations were 19.8 ng/ml, 28.4 ng/ml and 4.05 ng/mg Cr respectively. The average concentration of serum P_4 of #8303 and #8329 cows between 21 days post mating and 30 days pre-parturition was 14.03 ± 2.7 ng/ml and 11.23 ± 2.13 ng/ml respectively. In cow #8015 the average concentrations of serum P_4 with 7.46 ± 1.18 ng/ml between 120 day of gestation and 30 days pre-parturition was much higher than that of the luteal phase and the concentrations of PdG and PdG/Cr were 28.93 ± 5.97 ng/ml and 4.94 ± 3.45 ng/mg Cr respectively. Both of which were also much higher than those of the luteal phase and clearly reflected the changes in serum P_4 level. The gestation period of cows #8329, #8303 and #8015 were 275, 266 and 231 days respectively.

The concentrations change of urinary PdG and serum P_4 in pre-parturient cows

The concentrations of serum P_4 of cows #8303, #8329 and #8015 began to decrease from the 18th, 9th, and 12th

day before parturition respectively. The levels of serum P_4 for the former two cows were around 10ng/ml, while that of the latter one was around 6 ng/ml. However, serum P_4 dropped dramatically 1 to 3 days before parturition, finally falling to its basal value after parturition. The concentration of urinary PdG also showed similar patterns with those of the serum P_4 (Figure 4).

The correlation between urinary PdG and serum P_4 from pregnancy to post parturition of Holstein dairy cows was higher if tested without creatinine basis, the difference being significant between them when tested with Z^* test (Table 2).

Changes of PdG concentration in urine preservation study

After storing for 4 days at room temperature, urinary PdG changed significantly ($p < 0.05$), while boiling for 30 minutes at 100°C extended the preservation duration of urine for 8 days without affecting change of PdG concentrations.

DISCUSSION

This study provided fundamental information on the utility of non-invasive monitoring of reproductive status in dairy cow. Daily urinary creatinine concentrations fluctuation within a day was also seen here as in our earlier work (Yang et al., 2003), but the changes were slight in samples from midday. This findings correlates with other reported findings from beef cattle (Albin and Clanton, 1966), sheep (Hogen et al., 1967). The change of serum P_4 during the estrus cycle of the test cows showed similarity with the finding of Stabenfeldt et al. (1969), but the concentrations of urinary PdG and the ratio of PdG/Cr showed much wide variation than those of serum P_4 . However, the concentrations of PdG during the luteal phase varied from 8.2 to 17.4 ng/ml ($n=3$), 3 to 5 times higher more than that during the follicle phase. Other herbivorous such as bison (*Bison bison*) showed much higher concentration of PdG, with 42.5 ± 7.5 ng/mg Cr at the follicular phase, and 587 ± 108.8 ng/mg Cr at the peak of luteal phase (Kincy et al., 1990); Dall's sheep (*Ovis dallidalli*) is also reported with 80 to 200 ng/mg Cr in luteal phase (Goodrowe et al., 1996); Eld's deer showed 40 to 180 ng/mg Cr in luteal phase and < 10 ng/mg Cr in follicle phase (Monfort et al., 1990). However in white-tailed deer (*Odocoileus virginianus*) it was reported 0.15 to 1.57 ng/mg Cr at estrus, 0.92 to 5.27 ng/mg Cr at luteal phase (Knox et al., 1992). Equids showed 3 ng/mg Cr at the time of ovulation to nearly 400 ng/mg Cr at the mid-luteal phase peak (Arthur et al., 1990) indicating that there exists a wide variation among different animal species.

The estrus cycle of the Holstein cows was determined at

23±1 (n=3) days in this experiment based on urinary PdG. It is identical to the assessment through from serum P₄, though there exists an excretion lag time of <12 h between serum and urine (Schwarzenberger et al., 1996). The findings are within the normal ranges of dairy cow (Cupps, 1991), and support our finding that urinary PdG could reflect its parent blood P₄ correctly. The correlation between urinary PdG and serum P₄ in cyclic Holstein dairy cows was slightly higher in creatinine basis, however no significant difference was seen between them with Z* test, suggesting that the adjustment was not needed. The determination of woman ovarian function using morning urine steroid assays also revealed that the relation coefficient between urine and serum steroid is better without creatinine basis (Denari et al., 1981).

The comparison of serum P₄ and urinary PdG between Holstein cows in different phase of estrous cycles and gestations also revealed interesting findings. It was found that the concentrations of urinary PdG reached 14.2 ng/ml level 21 days post AI and they were 3 to 4 times higher than those of non-pregnant cows. This finding clearly suggests that the determination of urinary PdG could be used for early pregnancy detection. The urinary PdG could aid early pregnancy detection by at least three days compared to the milk P₄ test which could only be reliably employed by 24th day after mating at the earliest (Pennington et al., 1985).

Parturition again is a complex phenomenon, much of which yet remains to be understood. The concentration of serum P₄ decreases in pre-parturition due to the rise of fetal cortisol, which stimulates the conversion of placental derived P₄ to estrogen. Estrogen then act upon the cotyledon-carcuncle complex to stimulate the production and the release of prostaglandin F₂α (PGF₂α) which play a key role in initiating parturition and luteolysis that cause serum P₄ to drop dramatically (Arthur et al., 1990; Thatcher et al., 1992). The urinary PdG showed similar pattern as those of serum P₄, the latter is in agreement with these reporting by Donaldson et al. (1970) and Stabenfeldt et al. (1970). However, the urinary PdG of cow #8303 and #8015 increased greatly one week before parturition, not correlating the slight rise of the serum P₄, this might be the accumulated effect of the urine metabolite which showed that possibility of deviation from serum P₄ correlation exists. Nevertheless, this should not affect the parturition prediction from the urinary PdG following long-term sampling procedure. The post-partum period of cow #8329 and #8015 exhibited both an increase of serum P₄ and urinary PdG of short duration before full cycle and this incidence is similar to that observed by Schams et al. (1978) and Lamming et al. (1981), suggesting the ovarian activity recovered soon after parturition. However, cow #8303 was assisted with the delivery as it failed with normal parturition and that might be responsible for the uncyclic nature of the

cow after parturition.

The correlation between urinary PdG and serum P₄ from pregnancy to post parturition of Holstein dairy cows was significantly higher (Z* test) when tested without creatinine basis over the creatinine basis adjustment. This suggests that creatinine adjustment was not needed as reported by Denari et al. (1981). Beyond the potential logical application of this assay to domestic and zoo animals, the ability to extract urine from the soil and to measure steroid metabolites without interference permits a more precise evaluation of ovarian dynamic in free-ranging wild life as well (Kirkpatrick et al., 1990). The concentration of PdG in urine samples was maintained with no significant difference for two days at room temperature. When the urine was simply boiled in water bath for half an hour the storage duration extended to 8 days without any significant change in concentration of PdG. This provide another advantage as the urine sample can be handled, transported and stored even in situations with marginally available facilities common in field conditions.

In conclusion, the results confirmed that urinary PdG excretion accurately reflected serum P₄ concentrations and the presence or absence of a functional CL. This enables the monitoring of the estrous cycle and pregnancy in dairy cows by using urinary PdG analysis directly and does not require urinary creatinine adjustment. This method combined with the urine E₁S for evaluation the viability of fetus (Yang et al., 2003) may be successfully applied for monitoring reproductive status of farm, zoo and wild animals.

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