

Urinary Estrone Sulfate for Monitoring Pregnancy of Dairy Cows

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ABSTRACT : The purpose of this study firstly was conducted to establish a radioimmunoassay (RIA) of estrone sulfate (E₁S), secondly to monitor the reproductive status of dairy cows using their urine samples. Urine and blood samples were collected in series within a day from four pregnant Holstein-friesian cows to evaluate the relationship between E₁S levels in blood and urine with or without urinary creatinine basis. The urine was then collected biweekly from three cows in estrous and those artificially inseminated; collection from pregnant cows was made on a monthly basis. Results indicated that sensitivity for the E₁S RIA was 5 pg/tube and the recovery rate was 100%. The daily urinary creatinine concentrations fluctuated within a day, but changes were slighter in midday, whereas the changes of concentrations of E₁S in urine were relatively smaller. The concentrations of serum E₁S during the estrous cycle were undetectable due to the limitation of assay, but the urinary E₁S level could be measured with no obvious changes during the cycle. The urinary E₁S levels increased remarkably around 7.7 to 8.3 ng/ml, 80 to 100 days after pregnancy but the serum E₁S levels did not elevate until 120 to 150 days. The level of E₁S increased gradually during pregnancy and eventually reached its peak before parturition at around 40 ng/ml and finally decreased to its basal level 2 days postparturition. During pregnancy, E₁S concentrations of urine increased earlier than those in blood. The correlation coefficients between urinary and serum E₁S concentration during pregnancy and postpartum were higher than those adjusted with creatinine (creatinine ratio). The concentrations of E₁S in urine could be maintained unchanged for 8 days storing the samples in room temperature, which was extended to 8 days when the samples were pretreated by boiling for 30 minutes or treated with autoclave. In conclusion urinary E₁S concentrations can be used directly for monitoring the pregnant status and fetal viability of dairy cows and can assist accurate confirmation of pregnancy in cows at least 80 to 100 days after insemination much earlier than by serum E₁S. (*Asian-Aust. J. Anim. Sci.* 2003, Vol 16, No. 9 : 1254-1260)

Key Words : Dairy Cow, Estrone Sulfate, Progesterone, Urine

INTRODUCTION

Reproductive physiology of the mammals is regulated by neuroendocrine system. It plays a vital role in the development of sex glands, maturation of germ cells, stimulation of estrous and mating behavior, and the growth of conceptus and parturition. Thus an assessment of sex hormone concentrations in blood is the most informative and widely used methods for understanding reproductive status of the animals. However, the secretory hormonal dynamics (pulse, amplitude and frequency) can be disrupted temporarily by specific anaesthetic drugs (Johnson and Gay, 1981; Clarke and Doughton, 1983) or interference with or block ovulation in the domestic cat (Howard et al., 1982), nor can it be practical for samplings in long-term basis.

The primary advantage of non-invasive urinary hormone metabolite monitoring technique is that a sample can be collected without the animal being sedated or restrained. This approach also provides a representative "pooled" value of hormonal activity. The hormone secretion naturally is dynamic and circulating levels can fluctuate markedly, sometimes even within minutes, while a single blood sample may produce a value that represents the nadir, peak or mid-point of a pulsed secretion. In contrast, a

urinary sample represents excreted hormonal metabolites over a period of hours (Schwarzenberger et al., 1996). Thus, the ability to distinguish between normal secretory dynamics and a genuine physiological response is improved by measuring the minor oscillation of the urine (Kasman et al., 1986). The benefit of urine monitoring is also that metabolite concentrations frequently are two to four times higher in magnitude than that of the parent steroid in blood; those higher concentration allows a wide range of assays to be employed (Lasley and Kirkpatrick, 1991). Another advantage of this approach is that samples can be collected on a long term basis permitting longitudinal assessment of reproductive activity so that gonadal status for any given individual can be clearly characterized.

Several studies have showed that estrone sulfate (E₁S) is the major estrogen in the maternal circulation, which is produced by the fetal placental unit as a form of estrone (Gaiani et al., 1982; Jainudeen and Hafez, 1993) and measurement of E₁S can therefore be used for evaluation of the viability of fetus. Urinary estriol profiles in women have been used to evaluate the endocrine function of the fetoplacental unit (Klopper, 1969). Thus measurement of E₁S concentration in urine samples taken from those cows offers a potential, non-invasive, complementary approach to determine their pregnancy status. The purpose of this study firstly was to establish radioimmunoassays (RIA) for both serum and urinary E₁S and then to characterize long-term urinary E₁S excretion during estrous cycle and pregnancy in

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the dairy cows by using this technique and also attempt to find a practical way for urine preservation.

MATERIALS AND METHODS

RIA for urinary estrone sulfate (E₁S)

Reagents : Estrone sulfate used as assay standard was purchased from Sigma. (E9145 Germany). The antiserum obtained from ICN Biomedicals USA, was raised in a rabbit to estrone sulfate for E₁S assay and [2, 4, 6, 7-³H] estrone (sp. act. 110 ci/nmol). The assay buffer was phosphate buffer (10.86 g NaHPO₄ and 5.38 g NaH₂PO₄ · 2H₂O per 2 liter) containing 17.5 g NaCl, 2 g gelatin and 0.2 g thimerosal and made up in deionized water to pH 7.0 charcoal/dextran suspension, consisting of 625 mg norit A charcoal and 62.5 mg dextran in 100 ml assay buffer; the suspension was continuously stirred on ice water during dispensing. The scintillation fluid was purchased from BDH, England (Scinutra, 14509).

Procedure : The assay for urinary E₁S was modified from the RIA system of progesterone developed in our lab (Lin et al., 1988). Urine samples were diluted properly with 0.1 M assay buffer (usually 1:10). The diluted samples were mixed with a vortex, and then aliquots of 500 µl in a tube of 12×75 mm. The urinary sample, labeled steroid and antiserum each with 100 µl, were incubated together at 4°C overnight, the free steroid was removed by the addition of 200 µl charcoal/dextran for 15 minutes standing. After 1000×g centrifugation for 10 minutes, the supernatants were poured into the counting vial with 6ml of scintillation fluid, then the samples were counted using a Beckman LS 6,000 K. A standard curve of counts bound was plotted against the logarithm of the concentration of E₁S in the standard. The E₁S concentration in urinary samples was 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 E₁S were measured through RIA, the binding percentages were expressed as logit value, and calculated as the mean±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 E₁S/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 E₁S samples. The inter-assay variation was obtained following the determination of the added E₁S 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 E₁S (10, 25 and 50 pg/tube) added to native sample of urine with various dilution (1:1, 1:5, 1:10)

Recovery : Five different amounts of E₁S 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 E₁S was determined by the RIA of all five samples in five replicates, and then compared with the added amount for the recovery.

Creatinine assay of the urine

The creatinine content of each urinary sample was measured using a modification of the method described by Yeh (1973). The procedure for the assay was as follows: creatinine standards (range 5 to 300 µg/ml) and samples were used in 0.1 ml for the test, then added 0.1ml saturated picric acid, 0.075 ml 10% sodium hydroxide, and deionized distilled water for total volume of 5 ml. The sample in test tube was left in the dark for 10 minutes, after which optical density (absorbance) was read at 530 nm spectrophotometry.

The enzyme immunoassay (EIA) for progesterone

A valid competitive enzyme immunoassay for progesterone was established in our laboratory using monoclonal antibody G7 (Wu et al., 1997). In brief, a 50 µl of diluted tested medium together with 150 µl of progesterone-horseradish peroxidase were added to 96 wells of microtiter plate (Costar 3590) coated with G7 monoclonal antibody. After incubation at room temperature for 15 min and washing with PBS twice, the color was developed in 200 µl of 2.2 mM o-phenylene-diamine in 0.003% H₂O₂ at room temperature for 30 min. The reaction was stopped by adding 50 µl of 8 N sulphuric acid to the wells. Absorbance rate at 490 nm was compared with a progesterone standard curve. The variation coefficients within and between assays were approx. 7 and 10% respectively. The sensitivity was 0.3 pg/well.

Sampling

Samples collection for 24 h : The urine and blood samples were collected in series within a day from four pregnant Holstein cows (#7911 at 9 years old, #8523, #8532 at 3 and #8626 at 2 yrs old) housed in the dairy cattle farm of National Taiwan University for evaluating the relationship between E₁S in blood and urine with or without the base of urine creatinine. The cows were kept in a paddock during the time waiting for urination, with fresh water provided *ad libitum*. Blood samples (2 ml) were obtained from the tail vein using no.21 gauge needles immediately after urine collection.

Sample collection during the estrous cycle and the pregnant period : The urine and blood samples were collected twice a week and monthly for estrous cows on cycle (#8402 at 4 yrs old, #8524 and #8528 at 3 yrs old) and pregnant cows after artificial insemination (#8329 and #8303 at 5 yrs, #8015 at 8 yrs old) respectively; the sampling procedure was the same as those mentioned in

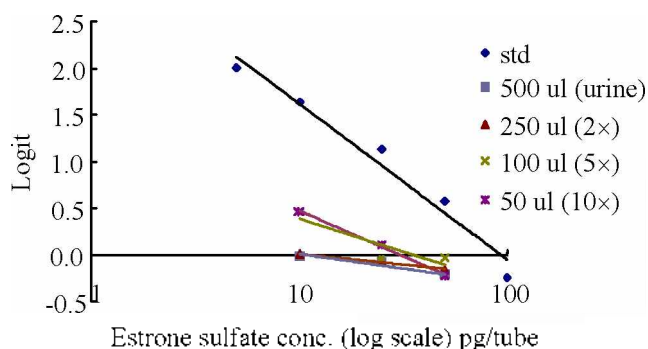


Figure 1. The estrone sulfate parallelism test for various volumes of urine.

samples collection above.

Sample treatment : The urinary samples were obtained after centrifugation (4°C, 800×g for 15 minutes), and the clotted blood samples were centrifuged (4°C, 1000×g for 10 minutes); both the supernatants were stored at -20°C until conducted EIA and RIA for measuring the levels of P₄ and E₁S of the samples, respectively.

Urine preservation study

To determine the ability of preservatives to maintain urinary E₁S 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). They were categorized into groups containing no-treatment, boiling in water bath for 30 minutes and autoclave (121°C, 20 min, 1.2 kg/cm²) treatments respectively, then analyzed for the change of E₁S after 0, 1, 2, 4 and 8 days of storing in dark.

Statistical analysis

The E₁S concentrations of urine samples were adjusted with creatinine concentration, and were expressed as ng/mgCr. Data obtain from the experiment with creatinine basis or without creatinined basis were tested with serum E₁S for statistical significance by using Spearman's correlation coefficient and Z* test (Steel and Torrie, 1981). A value of p<0.05 was considered statistically significant different.

RESULTS

A system established for E₁S RIA

Sensitivity : The limit of urinary E₁S detection significantly different from zero concentration was 5 pg/tube corresponding to 10 pg/ml, and is sensitive enough for the monitoring of urinary E₁S RIA.

Precision : The precision of the E₁S RIA was analyzed

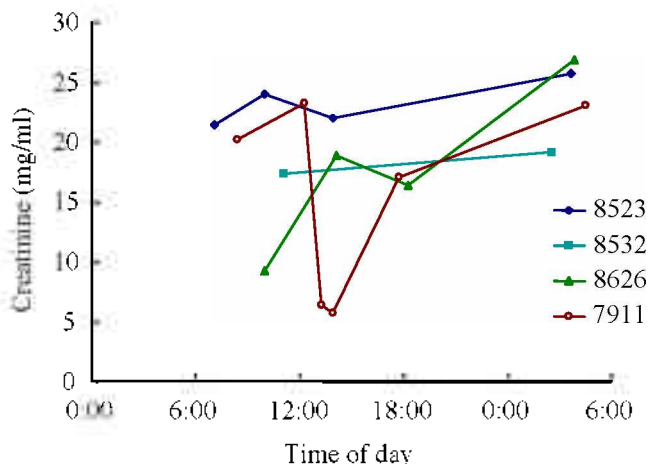


Figure 2. The diurnal shift of urinary creatinine concentration in pregnant Holstein dairy cows.

Table 1. The diurnal shift of serum estrone sulfate and urinary estrone sulfate concentrations in pregnant Holstein cows

Steroids (ng/ml)	Cow*			
	No.8523 (n=4)	No.8532 (n=2)	No.8626 (n=4)	No.7911 (n=6)
Serum estrone sulfate	17.44±0.98	21.55±1.43	21.53±1.98	14.46±2.42
Urinary estrone sulfate	12.98±1.14	13.91±0.06	11.74±3.62	18.45±7.23

* Mean±SD.

by using the method of Wilson and Miles (1978). For the intra- and the inter-assay precisions were 8.49 to 12.96% and 12.04 to 13.43%, 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 estimating E₁S with 10, 25, and 50 pg/tube. Each dilution was not significantly different from the standard (p>0.05) (Figure 1.) and showed parallelism with the standard linear. It appears that there is no interference in the urine dilution, no matrix effect was found (Shah et al., 1988). The recovery of various concentrations of E₁S ranging from 87 to 113%, and the average at 100%, is within the acceptable range 80 to 120% (Abraham, 1975).

The number of urinations, daily change of the urinary creatinine, steroid in the blood and urine

The daily urinary creatinine concentrations of cows are show in Figure 2. The number of urinations of the four cows within 24 h varied from two to six times. Two urine samples of Cow #7911 collected between 1 to 2 PM, showed the lowest level of creatinine at 5 ng/ml. If these were to be excluded, the variation changed slightly during the nudday Cow #8626 showed great variation in creatinine concentrations in four samples of urine, and it appeared that urinary creatinine concentrations varied individually. The diurnal shift of serum E₁S and urinary E₁S concentrations in

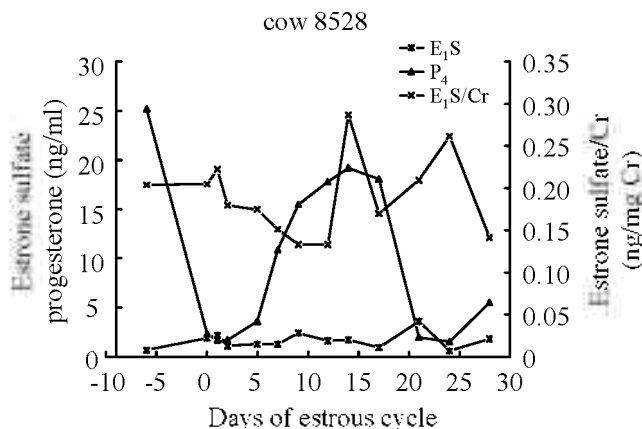


Figure 3. Changes of serum progesterone (P₄), urinary estrone sulfate (E₁S) concentrations and the ratio of estrone sulfate/creatinine (E₁S/Cr) in a cyclic Holstein cow (No. 8528).

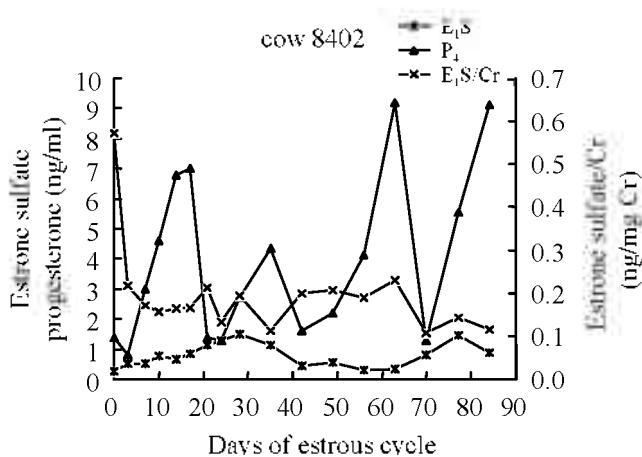


Figure 4. Changes in serum progesterone (P₄), urinary estrone sulfate (E₁S) concentrations and the ratio of estrone sulfate/creatinine (E₁S/Cr) in a cyclic Holstein dairy cow (No. 8402).

pregnant Holstein cows are shown in Table 1; both did not vary with the time of urination.

The changes of urinary E₁S and serum E₁S concentrations during the estrus of cows

The serum E₁S concentration during the estrous cycle could not be detected, however the concentrations of urinary E₁S were under 4 ng/ml without any obvious change (Figure 3, 4).

The concentrations of E₁S in urine and serum during the period of mating and parturition

The changes of E₁S concentrations in serum and urine with and without creatinine basis in the Holstein cows during the period of mating and parturition are shown in Figures 5, 6, 7. Cow #8329 showed 90 pg/ml in serum E₁S

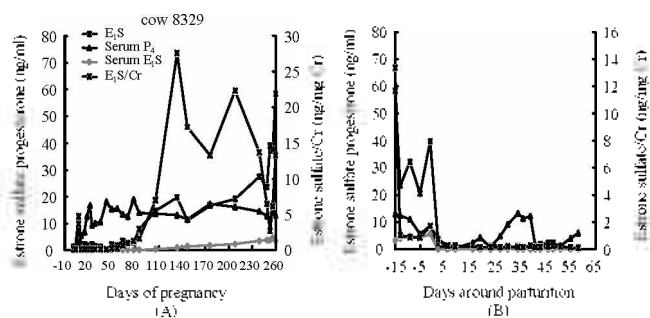


Figure 5. Changes in serum progesterone (P₄), serum and urinary estrone sulfate (E₁S) concentrations and the ratio of estrone sulfate/creatinine (E₁S) during the period of pregnancy (A) and days around parturition (B) of the Holstein dairy cow (No. 8329).

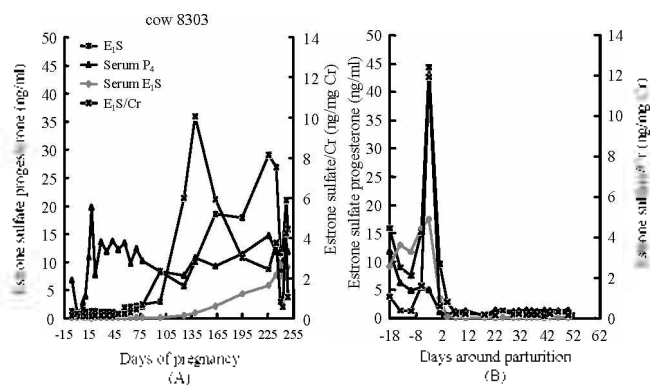


Figure 6. Changes in serum progesterone (P₄), serum and urinary estrone sulfate (E₁S) concentrations and the ratio of estrone sulfate/creatinine (E₁S) during the period of pregnancy (A) and days around parturition (B) of the Holstein dairy cow (No. 8303).

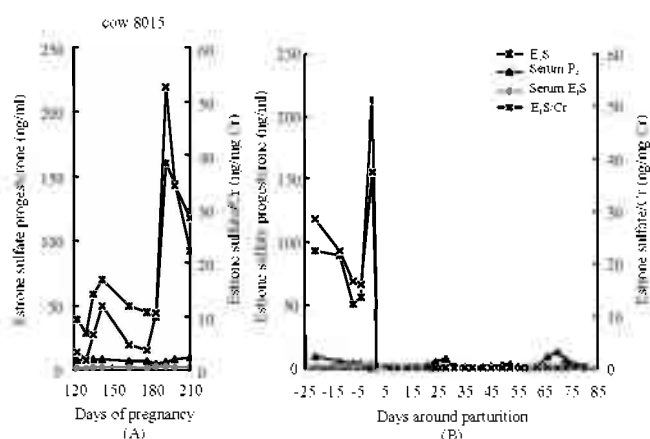


Figure 7. Changes in serum progesterone (P₄), serum and urinary estrone sulfate (E₁S) concentrations and the ratio of estrone sulfate/creatinine (E₁S) during the period of pregnancy (A) and days around parturition (B) of the Holstein dairy cow (No. 8015).

Table 2. The correlation between urinary and serum estrone sulfate during the period of pregnancy and postpartum of Holstein cow

Cow	Sample size	Spearman correlation coefficient		Z
		(r)	(r')	
8329	49	0.306	0.206	0.511
8303	47	0.708	0.669	0.346
8015	36	0.453	0.336	0.560

r, not indexed by creatinine; r', indexed by creatinine.

at the 78th day of pregnancy, but reached 0.86 ng/ml at the 147th day of pregnancy, its concentrations increased gradually and reached the peak one day prior to the parturition at the level of 5.36 ng/ml, then dropped to nadir two days after parturition. However the concentration of urinary E_1S increased apparently as early as 78th day of pregnancy with concentration of 7.7 ng/ml and then raised gradually, reaching concentration of 40 ng/ml periparturition, about eight-fold higher than those of the serum concentration. The concentration of urinary E_1S changed variously with creatinine base; two cows revealed the same pattern of E_1S , but urinary concentrations of E_1S in cow #8015 were much higher than the other two.

The correlation between urinary E_1S and serum E_1S from pregnancy to post parturition of Holstein dairy cows is shown in Table 2. The correlation coefficient was higher if without creatinine basis tested, no significant difference between them with Z* test, suggesting that creatinine adjustment was not needed.

The changes of E_1S concentration in urine preservation study

After storing for 8 days at room temperature, urinary E_1S changed insignificantly ($p>0.05$), and after being boiled for 30 minutes or treated with autoclave, the urine samples could be preserved for 8 days without affecting change of E_1S concentrations (Figure 8).

DISCUSSION

The daily urinary creatinine concentrations fluctuated within a day, but slightly changed in midday. These findings were similar to the beef cattle (Albin and Clanton, 1966) and sheep (Hogen et al., 1967), though two urine samples of cow # 7911 collected between 1-2 PM, revealed the lowest level of creatinine at 5 ng/ml, but were not found in the three cows. If the data is to be excluded, the variation changes slightly during the midday, thus the sample collected from throughout would be acceptable.

Concentrations of serum E_1S during the estrous cycle were undetectable due to the limitation of the assay, and cyclic variation can be revealed from urinary E_1S . It appeared that E_1S is not the major estrogen in cycling cows (Dobson and Dean, 1974); the Eld's deer (*cervus eldi*

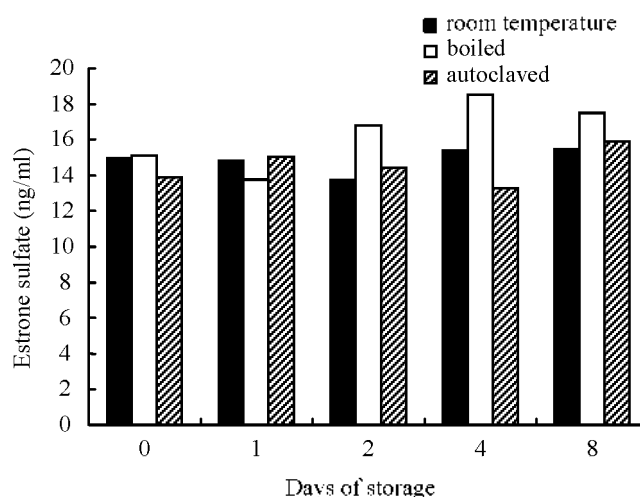


Figure 8. Changes in urinary estrone sulfate concentration under different treatments and storage time. The main effects and joint effect of treatment and day are nonsignificant ($p>0.05$).

thamin) in estrus also showed the similar situation (Monfort et al., 1990).

E_1S is the major estrogen by conceptus, diffusing from the chorionic vesicle at a time where there is a tight apposition to the uterine caruncles. As the embryo and its membranes expand and further elongate, new attachment sites continue to be formed between day 30-70, during which time high concentrations of E_1S are to be found in the fetal fluids of the pregnant ewe (Carnegie and Robertson, 1978), and E_1S is also detectable in the allantoic fluid of the cow between days 35 and 67 of pregnancy (Robertson et al., 1979). However, E_1S can also be measured in maternal plasma, milk or urine in all farm species; the concentrations of E_1S increased with fetus growth, and would provide conformation of pregnancy in animals (Heap and Hamon, 1979; Holdsworth et al., 1982; Jainudeen and Hafez, 1993). The date of serum E_1S detectable after breeding showed species variation, the cow serum able to detect at day 80 (Gaiani et al., 1982), day 70 in ewe (Tsang, 1975), day 35 to 40 in mare (Kindah et al., 1978) and day 22 to 24th in gilt (Tzeng and Lin, 2000). There is also breed difference because of the difference in fetal size (Mohamed et al., 1987; Abdo et al., 1991). The concentration of serum E_1S of cow #8329 and cow #8303 showed 90 pg/ml at 78th day and 165 pg/ml at 77th day after breeding respectively, showed similarity with the finding of Robertson and King (1979), but the levels of serum E_1S didn't change until 120 to 150 days afterward and reached the plateau at day 240 to 260 after breeding. That variation also showed similarity with that of Heap and Hamon (1979), Hatzidakis et al. (1993), Zhang et al. (1999). The pattern of serum E_1S between the period of mid-gestation and postparturition showed similarly as the

research of Hoffman et al. (1997). The pattern of E₁S concentrations in urinary is similar to that reported in plasma except that large amounts (7.7 to 8.3 ng/ml) were detectable in urine samples as early as days 80 to 100 after pregnancy. It revealed that the concentrations of urinary E₁S could afford the result much better than that of the serum E₁S when making the pregnancy confirmation. The levels of urinary E₁S increased gradually as pregnancy progress and eventually reach its peak right before parturition at 40 ng/ml and finally decreased to its nadir 2 days after parturition.

During the end of gestation, the concentration of E₁S in serum raised dramatically which can be linked to the initiation of parturition as a result of estrogens synthesis by fetus placenta (Edqvist et al., 1973; Fairclough et al., 1981). E₁S is linked to enhance the contraction of myoendometrium for the initiating labor (Hoffman et al., 1997). The concentration of urinary E₁S of Pere David's deer (*Elaphurus davidians*) increased dramatically to the level of 5.5 to 7.5 ug/mg Cr 1 to 2 weeks before parturition (Monfort et al., 1991), much higher than our study in Holstein cow with the level between 14 to 40 ng/mg Cr. The E₁S concentrations in urine of Dall sheep (*Ovis dalli dalli*) increased apparently to the level of 705 to 4,431 ng/mg Cr 1 to 3 days before parturition and dropped rapidly to <2.5 ng/mg Cr after parturition (Goodrowe et al., 1996). In sheep there is a sharp increase in free circulating estrogens 2 days before parturition (Challis, 1971), but no peak estrogen concentration was revealed in the urine of the Suffolk sheep at the same time. These variation indicate species difference in the metabolism of urine estrogen (Goodrowe et al., 1996). However the level of urinary E₁S changes much earlier and clearer than those of the serum, thus the concentration of urinary E₁S preparturition might be beneficial for the evaluation of fetal status.

The preservation of E₁S in urine samples stored at room temperature after boiling in water is due primarily to their antibacterial properties. The ability to maintain samples at room temperature for 8 days offers a practical advantage for field study, especially for long distance shipment.

In conclusion, the result confirmed that urinary E₁S excretion accurately reflects the parent serum steroid level and hence the status of viable conceptus. In these respects, E₁S concentrations in urine of pregnant cows can provide a useful laboratory diagnostic tool for confirmation of pregnancy and fetal viability.

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