Radioimmunoassay of Saliva Estrone Sulfate in Pregnant Sows

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ABSTRACT. The aim of this study was to establish radioimmunoassay (RIA) for saliva estrone sulfate (E1S), and to elucidate changes in saliva E1S during pregnancy in the sow. Saliva E1S was extracted using a commercially available solid phase column, and the E1S fraction obtained was subjected to RIA. The sensitivity of the RIA was 29.7 pg/tube. The intra- and inter-assay coefficients of variation were 5.5–8.4% and 13.1–19.5%, respectively. Mean recovery for E1S added to saliva samples was as high as 99.9%. A significant positive correlation (r=0.54, n=69, p<0.01) existed between saliva and plasma E1S concentrations. During gestation, the changing patterns of saliva and plasma E1S concentrations were essentially the same, and two peaks of E1S concentrations were observed, one around day 30 and another just before parturition, although E1S concentrations in saliva remained at only 2.4–38.1% (mean 11.4%) of those in plasma E1S. Thus, the present study has made it possible to measure saliva concentrations of E1S and demonstrated a high degree of positive correlation between saliva and plasma E1S concentrations. These results suggest that diagnosis of early pregnancy and of normal or abnormal fetal development could be made by measurements of E1S in saliva. — KEY WORDS: estrone sulfate, pregnancy, radioimmunoassay, saliva, sow.

In pregnant sows, estrone sulfate (E1S) in peripheral blood is first detectable around day 16 of pregnancy and fluctuates showing 2 peaks, one on days 23–30 and another just before parturition [14]. Since Robertson and King [14] suggested that E1S concentration in the maternal blood reflects the amount of estrogens synthesized and secreted by the blastocyst or fetus [14], early pregnancy diagnosis [2, 19, 20] and the diagnosis of normal or abnormal fetal development [3] could be made in terms of maternal blood E1S concentrations.

Because saliva samples can be more easily and safely collected compared to blood samples, various steroid hormones in saliva have been measured in humans, cows, horses and pigs [4, 5, 8, 9, 11–13, 15]. In pigs, saliva progesterone and cortisol have been determined for early pregnancy diagnosis [9] and for an adrenocortical function test [11], respectively. An attempt has been made to determine saliva E1S in horse [15] but not in the pig.

Therefore, the present study was undertaken to measure saliva concentrations of E1S by radioimmunoassay (RIA) and to correlate the changing pattern of saliva E1S with that of plasma E1S in pregnant sows.

MATERIALS AND METHODS

Saliva and blood collections: Five hybrid parous sows raised in this laboratory were used for the present study. Saliva samples were collected from each sow at intervals of 2–10 days during pregnancy, as described previously [9]. Briefly, a wooden chopstick (approximately 20 cm long) tipped with 2 g of absorbent cotton was inserted into the mouth. Subsequently, the cotton soaked with sufficient amounts of saliva was removed from the stick and compressed in a 10–20 ml disposable syringe to squeeze the saliva from the cotton. Saliva samples obtained were mixed with sodium azide (5 mg/ml) and kept frozen at -20°C until assayed for E1S. Immediately after saliva collection, blood samples were also collected from the median tail vein into heparinized test tubes. Plasma was separated by immediate centrifugation (1,700 × g, 15 min) and kept frozen at -20°C until assayed for E1S.

Extraction of E1S: Saliva and plasma E1S was extracted by a slight modification of the method described by Nakamura [10]. Briefly, saliva and plasma samples (1–4 ml) were diluted with 2 ml borate buffer (0.2 M, pH 8.0) containing 0.05% bovine serum albumin and 0.06% bovine y-globulin (BSA buffer), and then poured into a 5 ml disposable syringe attached to solid phase columns (Sep-Pak® PLUS C18 Cartridge, Millpore Co., Ltd., U.S.A.). The sample solutions were allowed to flow into the columns at a rate of 0.2–1 ml per min. The columns were then washed with 4 ml distilled water and with 3 ml diethylthlether, successively and the effluents were discarded. Finally, five ml aceton was poured into the columns, and the resultant effluent (E1S fraction) was recovered into test tubes. The effluents were evaporated to dryness at 50°C under nitrogen gas, and the residues were dissolved in 200 µl of BSA buffer.

RIA for E1S: Saliva and plasma E1S were measured by a modification of RIA described by Nakamura [10], as shown in Fig. 1, using 1, 3, 5 [10]- Estratrien-3-ol-17-one sulfate (E1S) as a standard, estrone sulfate ammonium salt [6.7–3H(N)] (6.7–H-E1S; specific activity, 1.48–2.22 TBq/mM) as a tracer and anti-estrone-3-sulfate rabbit serum (anti-E1S serum). The standard E1S preparation was provided by Dr. A. Kanbegawa (Teikoku Hormone Manufacturing Co., Ltd., Japan) and 3H-E1S was prepared by Daiichi Pure Chemicals Co., Ltd., Japan. The anti-E1S serum was generated by
Cosmo Bio Co., Ltd. Japan using anti 6-oxo-estrone-3-sulfate-6-CMO-BSA as the antigen. According to the information from the manufacturer, the cross-reactivities of the anti-E1S serum with E1S, estrone glucuronide, estrone, estradiol-3-sulfate, estradiol-3-glucuronide and estriol sulfate were 100, 5.4, 4.0, 1.5, 0.8 and 0.8%, respectively. Prior to use, standard E1S and 3H-E1S were diluted with BSA buffer at concentration of 3.9–1,000 pg/200 µl and 250,000 cpm/ml, respectively. Anti-E1S serum was diluted 1:36,000 with BSA buffer for use.

Standard E1S (3.9–1,000 pg) and saliva or plasma extracts dissolved in 200 µl BSA buffer were each mixed with 100 µl anti-E1S serum solution and allowed to react at 4°C for 12–24 hr. The mixtures were then incubated with 100 µl 3H-E1S solution at 4°C for 12–24 hr. The incubated solutions were mixed 250 µl of dextran-coated charcoal solution [0.2 M borate buffer solution containing 0.5% charcoal (Norit A; Kishida Chemical Industries, Ltd., Japan) and 0.05% dextran (DextranT70; Pharmacia Fine Chemicals AB, Uppsala, Sweden), pH 8.0], left at 4°C for 10 min and subsequently centrifuged at 4°C for 15 min (1,700 × g). Two-hundred µl of the supernatant was emulsified with 3 ml scintillator (Scintisol® EX-H, Wako Pure Chemical Industries, Ltd., Japan), and radioactivity was assayed by a scintillation counter (Liquid scintillation system LSC-703, Aloka Co., Ltd., Japan). The quantities of E1S in saliva and plasma samples were estimated by interpolation from a standard curve.

RESULTS

Parameter for reliability of E1S RIA: The standard curve obtained by the assay of standard E1S in quantities ranging from 3.9 to 1,000 pg is shown in Fig. 2. The sensitivity was estimated as 29.7 pg/tube. Recovery of E1S in swine saliva samples was determined by addition of 500, 1,000 or 3,000 pg E1S to 2 ml saliva samples, as shown in Table 1. Mean recovery ranged from 92.3 to 108.4%, and for all the 9 measurements the value was 99.9%.

The intra- and inter-assay coefficients of variation were each calculated from quintuplicate results on 2 pooled saliva

Table 1. Recovery of varying amounts of estrone sulfate (E1S) added to swine saliva samples

<table>
<thead>
<tr>
<th>Added E1S (pg/ml)</th>
<th>No. of assays</th>
<th>Assay value (pg/ml)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>11 ± 0.6*</td>
<td>–</td>
</tr>
<tr>
<td>250</td>
<td>3</td>
<td>241 ± 21.6</td>
<td>92.3</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>506 ± 57.4</td>
<td>99.0</td>
</tr>
<tr>
<td>1,500</td>
<td>3</td>
<td>1,638 ± 162.4</td>
<td>108.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total mean</td>
<td>99.9</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
samples with different mean E1S concentrations. The intra-assay coefficient of variation was 5.5% for the sample with the lower E1S concentrations (302 \( \text{pg/ml} \)) and for the other sample with the higher E1S concentrations (2,233 \( \text{pg/ml} \)) it was 8.4%, while the inter-assay coefficient of variation for the sample with the lower E1S concentrations (324 \( \text{pg/ml} \)) was 19.5% and 13.1% for the sample with the higher E1S concentrations (2,598 \( \text{pg/ml} \)).

**Correlation between saliva and plasma E1S:** Correlation between saliva and plasma E1S concentrations were examined in 69 pregnant sows. The regression line obtained from a plot of the two parameters is given in Fig. 3. A significant positive correlation existed between saliva and plasma E1S concentrations. The correlation coefficient was 0.547 (p<0.01).

**Changes in saliva and plasma E1S concentrations during pregnancy:** As shown in Fig. 4, mean E1S concentrations in saliva and plasma fluctuated exhibiting two peaks. The first peak of E1S concentrations was evident on days 26–32 in saliva and on days 24–30 in plasma. The steroid in both saliva and plasma remained at baseline levels during mid-pregnancy. Saliva and plasma E1S, however, began to rise gradually around days 70–80 and reached a peak concentration (the second peak) on days 110–114 (just before parturition). Thus, the changing patterns of saliva E1S concentrations resembled closely the plasma pattern, although E1S concentrations in saliva remained at only 2.4–38.1% (mean 11.4%) of those in plasma.

**DISCUSSION**

In the present study, first the reliability of the RIA method as a procedure for measuring saliva E1S in sows has been demonstrated. Variations in individual recovery rates for E1S added to saliva samples are in small range, and mean recovery for steroid is as high as 99.9%. The intra- and inter-assay coefficients of variation obtained for saliva E1S RIA in the present study are rather small and closely comparable with the values reported for plasma E1S assay in cows [10] and humans [1]. The sensitivity of the assay is 29.7 \( \text{pg/tube} \) and high enough to measure E1S concentrations not only in the plasma but also in the saliva.

Two peaks of plasma E1S concentrations are evident in

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Fig. 3. Correlation between saliva and plasma estrone sulfate (E1S) concentrations in sows.

Fig. 4. Changes in saliva and plasma estrone sulfate (E1S) concentrations during pregnancy in sows.
the present study, the first peak around day 30 and the second peak just before parturition. This agrees quite well with the finding of Robertson and King [14], although they carried out serial estimations of plasma E\textsubscript{1S} in only a single gilt at implantation, throughout pregnancy, and at parturition. The present study has first described changes in saliva E\textsubscript{1S} concentrations during pregnancy in the sow. Moriyoshi et al. [9] have reported that saliva and plasma progesterone concentrations are nearly comparable in pregnant sows. However, E\textsubscript{1S} concentrations in saliva remain at only 2.4–38.1% of those in plasma in present study. This is consistent with the previous finding that only a small fraction of blood E\textsubscript{1S} can pass into saliva in pregnant horses [15]. Thus, in contrast to the striking similarity in progesterone concentrations between swine saliva and plasma, a great difference in E\textsubscript{1S} concentrations between saliva and plasma are apparent in pregnant sows and horses. In general, steroid hormones are transported in the blood through association with various types of proteins, and the free form usually less than 10% of the total amount of hormone in the plasma [16]. The steroid hormones must be in the free or unbound form before it can pass into saliva [21]. This is accomplished by the establishment of an equilibrium between bound and free form in plasma. The lower concentrations of saliva E\textsubscript{1S} measured in the present study might be related to fairly tight association of the greater amount of the steroid with plasma proteins in the blood [18] which prevents the plasma E\textsubscript{1S} molecules from passing into saliva.

It has been suggested that E\textsubscript{1S} concentrations in maternal blood reflect not only the amount of estrogens synthesized and secreted by the blastocyst or fetus [14] but also the litter size [17]. On the other hand, Kasman et al. [7] suggested that the amount of E\textsubscript{1S} in the urine may be of best use as an indicator of fetal demise in nontractable, feral, or exotic equine species. As described above, E\textsubscript{1S} concentrations remain much lower in saliva than in the blood, and concentrations of the steroid in individual saliva samples tend to represent slightly wide variations. However, E\textsubscript{1S} concentrations in the saliva are positively correlated with those in the plasma, and the changing patterns of saliva and plasma E\textsubscript{1S} concentrations are essentially the same. Since, compared to the blood or urine collection, the saliva collection can be performed more readily and safely, with little or no stress, diagnosis of early pregnancy and of normal or abnormal fetal development could be made in terms of measurements of E\textsubscript{1S} in saliva but not in blood. For this purpose, the more sensitive and simple method for measurement of saliva E\textsubscript{1S} such as Hatzidakis et al. [6] has reported should be preferably developed in the near future.

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REFERENCES


