Differentiation of Pregnant Shiba Goats Using Plasma Amino Acid Concentrations and Mathematical Analysis

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Abstract. Development of convenient techniques to diagnose pregnancy status is important for efficient animal production. Considering that pregnancy is an exciting biological event in the maternal body, the metabolism of amino acids during this period might be different from other periods in life. Pregnant animals would then be distinguished from non-pregnant ones, if relative differences in plasma amino acid concentrations could be identified. In the present investigation, a linear discriminant analysis was performed to characterize specific and possibly unique aspects of plasma amino acid concentrations in pregnant animals. Pregnant group (PG, n=5) and non-pregnant group (NPG, n=5) of Shiba goats were subjected to this study at the Experimental Station for Bio-Animal Science, The University of Tokyo. On approximately 50th day after mating (day 0 = day of estrus), the animals’ pregnancy status was diagnosed by using an ultrasonography. In addition to body weight (BW) measurements, blood samples, from which plasma amino acid concentrations were determined, were obtained every week via the jugular vein from two groups of animals. Because variations of plasma amino acid concentrations (Ile, Leu, Glu and Tau) and BW in PG among pregnant days after mating were high (P<0.01), they were adjusted to the average days passed after mating (adjusted to 70th, 77th, 84th, 91st, 98th, 105th and 112th days). An effect of pregnant status was significant only for Glu (P<0.01). Correlations between BW and plasma amino acid concentrations of PG and NPG were low, suggesting that concentrations of these plasma amino acids were not affected directly by BW gain. Groups of PG and NPG were discriminated significantly using a linear discriminant model with plasma amino acid concentrations in the middle period of pregnancy (P<0.05). These results suggest that the pregnancy status can be evaluated easily and at low cost by using mathematical models and suitable weighted variables of biological factors such as plasma amino acid concentrations.

Key words: Amino acids, Discriminant analysis, Pregnancy, Shiba goat

In addition to food resources for human beings, domestic animals such as cattle, sheep and goats have supported human life in various ways, and one of recent contributions is to serve as biological factories for producing useful medical materials. In the last two centuries, ruminants have been studied extensively for the promotion of their economic ability maximally. To produce such useful ruminants, it is important to develop techniques to diagnose whether or not the animals are pregnant as early as possible.

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Green et al. reported that a family of specific glycoproteins is secreted by the trophoblast after 25th day of ovine and bovine pregnancy. Because this protein family is continuously detected until 250th day, this could be used to diagnose pregnancy status in the early stage [11]. In fact, many biological factors have been characterized for the purpose of diagnosing pregnancy status in the early stage of mammalian pregnancy, however, with the exception of human chorionic gonadotropin (hCG), most materials are biosynthesized long after the pregnancy is recognized. Thus, the detection of the pregnancy status in early stages is still difficult in most mammals. In the human, Deutinger et al. tried to predict the pregnancy status using a combination of statistical methods with multiple factors such as the beta subunit of hCG, pregnancy-specific glycoprotein and progesterone concentrations [9].

In our preliminary experiment with Shiba goats, an imbalance of specific free amino acid concentrations in the maternal plasma was identified during the gestational period. Amino acids are metabolized drastically in the liver and consumed as energy source without accumulating in the body. Free amino acids occupy only 2–3.0% in the plasma and this volume is very low as compared to those that have been consumed from food, suggesting that plasma concentrations of free amino acids are tightly controlled. Essential amino acids in the maternal plasma are transported across the placenta and umbilical cord to the fetus [4, 6, 18, 21], and placental trophoblast at the maternal-facing surface, which includes the microvillous brush-border, is the site of amino acid absorption and transport. In addition, the permeability of trophoblast at maternal and fetal surfaces is likely to be determined by the concentration of specific amino acid carrier proteins on the cell surface and the affinity characteristics of these proteins [4]. Due to the active utilization of amino acids by placental and fetal compartments, plasma amino acid concentrations in the maternal system during the gestational period might have unique characters [1–3, 7, 15]. It would, therefore, be possible to diagnose the pregnancy status through the efficient evaluation of such characters if suitable and biologically meaningful amino acids could be selected.

As a mathematical method to evaluate plural variables such as amino acids, multivariate analysis as explanatory variables of the specific amino acid concentrations in the plasma would be useful [13, 14, 16, 17, 20, 22]. Especially, a linear discriminant analysis could be used to classify such variables into valuable factors and to identify whether or not the animal is pregnant by weighting those factors [5, 8, 10]. The aims of our study were to characterize plasma amino acid concentrations, isoleucine (Ile), leucine (Leu), glutamic acid (Glu) and taurine (Tau) that had been selected based on statistical analysis and biological significance, and to use such information to diagnose the pregnancy status of Shiba goats.

Materials and Methods

Animals

Pregnant group (PG, n=5) and non-pregnant group (NPG, n=5) of Shiba goats were subjected to this study at the Experimental Station for Bio-Animal Science, The University of Tokyo. Animals were raised together in a free stall with sufficient room during the experimental period and fed ad libitum haylages composed of Orchard grass and Italian ryegrass. Universal concentrates consisting of corn, soybean and alfalfa pellets were fed to each animal (60 g/head/day). On approximately 50th day after mating, the pregnancy status was determined by using an ultrasonography (ECHO VISION, ALOKA Co., Ltd., Tokyo, Japan). Average body weights (BW) at the initiation of the study were 22.3 and 20.2 kg for PG and NPG animals, respectively. When the study began, these animals were 25.8 month old and had an average of 1.2 pregnancies. All animals were treated with 3 mg of prostaglandin F2α (Panacelan-Hi, Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan) to synchronize their estrous cycles.

Blood sampling and body weight measurements

From the beginning of this experiment, weekly blood samples (10 ml each) from each goat were collected from the jugular vein into a tube containing 90 units of heparin sodium. Weekly blood samples at the same time points were also collected from NPG animals after estrous synchronization. Plasma was separated by centrifugation and kept at −20 C until the measurement of free amino acid concentrations. At the time blood samples were collected, body weight
of the animals was also measured.

**Free amino acid concentration (µg/ml) measurements**

Plasma was deproteinized by centrifugation following the addition of an equal volume of 5% trichloroacetic acid loading buffer, from which the supernatant was separated. The supernatant was analyzed for free amino acids by using a High Speed Amino Acid Auto-analyzer (L-8500A, HITACHI Co. Ltd., Tokyo, Japan) according to the manufacturer’s instructions. All measurements were made with duplicated samples.

**Statistical analyses**

Analysis of variance (ANOVA): All data were analyzed using the least squares analysis with the General Linear Model (GLM) procedure of SAS [19]. In addition to BWs, plasma concentrations of four amino acids, isoleucine (Ile), leucine (Leu), glutamic acid (Glu) and taurine (Tau) in PG animals were adjusted to averages of days passed after mating; adjusted to 70th, 77th, 84th, 91st, 98th, 105th and 112th days. The same parameters were adjusted to the initial day of estrus after estrous synchronization in NPG animals. Thus, cluster pooled data formed from 70th to 112th days were used to evaluate the pregnancy status in the middle of the gestational period. The mathematical model for analysis of variance that included an interaction between pregnant status and adjusted days after mating was:

$$ Y_{hijkl} = \mu + \alpha_h + \beta_i + \gamma_j + a_j(\delta_{ijkl} - \overline{\delta}) + \varepsilon_k + \alpha(\gamma_{hij} + c_{ijkl}) $$

Where,

- $\overline{\delta}$ = Means of days after mating.
- $\varepsilon_k$ = Effect of the $k$th AM as deviation from the overall mean.
- $a_j$ = The $j$th first-degree regression coefficient of each amino acid to days after mating in each PS.
- $\alpha(\gamma_h)$ = Interaction between effects of PS and AD.
- $c_{ijkl}$ = Random error.

On the result of analysis of variance for plasma amino acid concentrations, mean differences for each dependent variable were tested for significance by Student’s $t$-test. The degrees of variance in amino acid concentrations of PG and NPG were examined by the $\chi^2$-test.

Linear discriminant analysis with pregnant status as an objective variable and plasma amino acid concentration as an explanatory variable: For cluster pooled data formed from 70th to 112th days, two-group discriminant analysis was performed. The first group included the data from pregnancies resulting in delivery, and the second group included the data from non-pregnancies. The discriminant model ($Z$) was,

$$ Z = a_0 + \sum_{k=1}^{n} a_k \chi_k (n = 4) $$

where $a_k = (a_1, a_2, a_3, a_4)$: weighted vectors of Ile, Leu, Glu and Tau in order,

$$ \chi_1: \text{vectors of observation of Ile, Leu,} $$

$$ \chi_2: \text{Glu and Tau in order,} $$

$$ a_0: \text{intercept.} $$

Variance-covariance matrices of PG ($\Sigma_{PG}$) and NPG ($\Sigma_{NPG}$) used to calculate parameters for the discriminant model were given by

$$ \Sigma_{PG} = \begin{bmatrix} \sigma_{11PG} & \sigma_{12PG} & \sigma_{13PG} & \sigma_{14PG} \\ \sigma_{21PG} & \sigma_{22PG} & \sigma_{23PG} & \sigma_{24PG} \\ \sigma_{31PG} & \sigma_{32PG} & \sigma_{33PG} & \sigma_{34PG} \\ \sigma_{41PG} & \sigma_{42PG} & \sigma_{43PG} & \sigma_{44PG} \end{bmatrix} $$

and

$$ \Sigma_{NPG} = \begin{bmatrix} \sigma_{11NPG} & \sigma_{12NPG} & \sigma_{13NPG} & \sigma_{14NPG} \\ \sigma_{21NPG} & \sigma_{22NPG} & \sigma_{23NPG} & \sigma_{24NPG} \\ \sigma_{31NPG} & \sigma_{32NPG} & \sigma_{33NPG} & \sigma_{34NPG} \\ \sigma_{41NPG} & \sigma_{42NPG} & \sigma_{43NPG} & \sigma_{44NPG} \end{bmatrix} $$

respectively. In this analysis, the STEPWISE and REG procedure of SAS [22] were used.
Results

Analysis of variance for BW and plasma amino acid concentrations

To analyze variables that would have influenced BW and plasma amino acid concentrations, analysis of variance was conducted (Table 1). For BW, all effects were highly significant (P<0.01). An interaction between PS and AD (PS × AD) was significant (P<0.01). Effect of PS on Glu was highly significant (P<0.01). Effects of AD and PS × AD were significant at P<0.01 and P<0.05, respectively. Since the effects of days passed after mating for Ile and Leu were highly significant (P<0.01), all variables within the treatment group were adjusted to the averages of each blood samples collected on days (70th, 77th, 84th, 91st, 98th, 105th and 112th days).

Relationship between BW and plasma amino acid concentrations in PG and NPG

Least squares means of BW and plasma amino acid concentrations in PG and NPG are shown in Table 2, and gradual changes in BW and Glu are shown in Figs. 1 and 2, respectively. BW of PG increased (P<0.01) whereas BW remained constant in NPG. Differences between mean BWs of PG and NPG were significant (P<0.05). Glu in PG animals changed with increasing days significantly. Though the results of Ile and Leu are not shown here, concentrations of these amino acids in PG changed slightly, which were not statistically significant. In contrast to Glu, Ile and Leu, changes in Tau were not detected. Simple correlations between BW and plasma amino acid concentrations during pregnancy are shown in Table 3. Correlations in changes between Glu and BW in PG and NPG were negative, but they were very low.

Table 1. Analysis of variance for BW, Ile, Leu, Glu and Tau

<table>
<thead>
<tr>
<th></th>
<th>BW</th>
<th>Ile</th>
<th>Leu</th>
<th>Glu</th>
<th>Tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN</td>
<td>P&lt;0.01</td>
<td>N.S</td>
<td>N.S</td>
<td>N.S</td>
<td>N.S</td>
</tr>
<tr>
<td>PS</td>
<td>P&lt;0.01</td>
<td>N.S</td>
<td>N.S</td>
<td>P&lt;0.01</td>
<td>N.S</td>
</tr>
<tr>
<td>AM</td>
<td>P&lt;0.01</td>
<td>N.S</td>
<td>N.S</td>
<td>N.S</td>
<td>N.S</td>
</tr>
<tr>
<td>AD</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
<td>N.S</td>
</tr>
<tr>
<td>PS × AD</td>
<td>P&lt;0.01</td>
<td>N.S</td>
<td>N.S</td>
<td>P&lt;0.05</td>
<td>N.S</td>
</tr>
</tbody>
</table>

PN: number of pregnancies, PS: pregnant status, AM: month of age, AD: adjusted days after mating in each PS by a linear covariance analysis, PS × AD: interaction between PS and AD, N.S: not significant.

Table 2. Least squares mean (± standard error) of BW (kg), and Ile, Leu, Glu and Tau (µg/ml) in the middle period of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>PG</th>
<th>NPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>25.5 ± 0.25a</td>
<td>20.5 ± 0.55b</td>
</tr>
<tr>
<td>Ile</td>
<td>15.4 ± 0.96</td>
<td>13.3 ± 2.03</td>
</tr>
<tr>
<td>Leu</td>
<td>23.3 ± 1.62</td>
<td>16.8 ± 3.42</td>
</tr>
<tr>
<td>Glu</td>
<td>24.0 ± 2.18a</td>
<td>17.1 ± 4.61b</td>
</tr>
<tr>
<td>Tau</td>
<td>9.5 ± 1.40</td>
<td>13.9 ± 2.97</td>
</tr>
</tbody>
</table>

Different superscripts in the same line show significant difference (P<0.05).

Correlations between BW and Ile were 0.21 in PG and 0.14 in NPG whereas those of BW and Leu were 0.36 in PG and 0.15 in NPG. During this experimental period, apparent relationships between BW and plasma amino acid concentrations were not identified.

Discriminant analysis for pregnant status with plasma amino acid concentrations

Distribution of functional values (Z) predicted by a linear discriminant model with plasma amino acid concentrations in the middle of the gestational period is shown in Fig. 3. The linear mathematical model to discriminate pregnant status using plasma amino acid concentrations was estimated as:

\[ Z = -3.80 - 0.036 \times \text{Ile} + 0.07 \times \text{Leu} + 0.12 \times \text{Glu} + 0.01 \times \text{Tau} \] (P<0.05).

For error probability of discrimination in these groups, the probability to diagnose NPG animals as pregnant ones was estimated at 34.0%. The \( \chi^2 \)-test was performed to examine the degree of variance in amino acid concentrations in PG and NPG animals, which resulted in 8.88 and was not significant.
AMINO ACID CONCENTRATIONS IN PREGNANT SHIBA GOATS

Discussion

Identification of pregnant animals from non-pregnant ones and subsequent management of these animals often determines the productivity of domestic animals. However, it is still difficult in the ruminant ungulates to diagnose the pregnancy status easily, accurately and early in a cost effective manner. During the pregnant period, essential amino acids in the maternal plasma are continuously transported across the placenta and umbilical cord to the fetus, resulting in amino acid balances slightly different from other periods [4, 6, 18, 21]. If such differences in plasma amino acid concentrations could be identified using a multivariate analysis of a linear discriminant analysis, pregnant animals would be distinguished from non-pregnant ones.

In this study, since the differences in pregnant days after mating were highly significant for plasma amino acid concentrations (P<0.01), they were adjusted to the averages of increasing days after mating; adjusted to 70th, 77th, 84th, 91st, 98th, 105th and 112th days. The effect of pregnant status was significant only for Glu (P<0.01). An interaction between pregnant status and adjusted day of pregnancy was significant (P<0.05). Since Glu levels in PG were higher than those in NPG (P<0.05), it was possible that those changes were unique in this period. During the experimental

Table 3. Simple correlations between BW and amino acid concentrations in the middle period of pregnancy

<table>
<thead>
<tr>
<th></th>
<th>PG</th>
<th>NPG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile</td>
<td>0.21</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>Leu</td>
<td>0.36</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Glu</td>
<td>-0.35</td>
<td>-0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>Tau</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Total: Total data from PG and NPG were used to calculate correlation coefficients.
period, BW of PG (25.5 kg) increased significantly (P<0.01) and that of NPG (20.5 kg) remained unchanged. Correlations between BW and Glu of PG and NPG were low and exhibited negative values of –0.35 and –0.15, respectively. Correlations of BW gain and other amino acid concentrations were also low. These results suggest that concentrations of the plasma amino acids examined during this period of pregnancy may not be affected directly by BW gain and feed intake, which might be one of potential indicators that the pregnancy presents.

The variance of plasma amino acid concentrations in PG and NPG animals was determined to be similar because the $\chi^2$-value was calculated at 8.88 and was not significant [12]. More importantly, changes in plasma amino acid concentrations were not directly associated with BW gain or feed intake. For these reasons, a linear multivariate analysis of discriminant analysis applied to amino acid concentrations would be possible to distinguish PG from NPG animals. In fact, we were able to distinguish PG from NPG using the amino acids and the linear mathematical model in the middle of the gestational period (P<0.05). It should be noted that the probability to include an animal of NPG as a pregnant one was estimated at 34.0%, although the error probability should decline as the number of animals analyzed increases.

The aims of this study were to characterize biological factors specific to the gestational period and to diagnose pregnancy status with the use of these information efficiently, accurately and economically. The results from this investigation suggest that pregnancy status could be evaluated using weighted variables of plasma amino acids and suitable mathematical models. This was performed without searching for only one remarkable factor such as a pregnancy-associated glycoprotein that needs to be detected using complicated techniques with a high cost [11]. The technique established in the present investigation could be applied to diagnose pregnancy status in earlier stages of gestation. However, because this method might be influenced by unknown environmental factors, suitable adjustments to field data might be required. In order to increase the accuracy of pregnancy diagnosis using this method, it would be necessary to collect additional data, to select some best explanatory variables, and to adjust data collected suitably.

Acknowledgements

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References


