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A Preliminary Study of ^{13}C-Phenylalanine and ^{13}C-Dipeptide Breath Tests in Horses

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This study aimed to establish a standard dose and sample collection time for ^{13}C phenylalanine and ^{13}C-Dipeptide breath test in horses. To evaluate dose-dependent effects, healthy horses received 2.5 mg/kg, 5 mg/kg, and 10 mg/kg ^{13}C phenylalanine dissolved in 1 ml/kg distilled water and 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg ^{13}C dipeptide dissolved in 2 ml/kg distilled water. Tmax was observed during the sample collection time. For ^{13}C phenylalanine, the standard deviation of Cmax at 5 mg/kg was lower than that of 10 mg/kg. For ^{13}C dipeptide, the standard deviation of Tmax was the lowest at 5 mg/kg. This study revealed that an optimal dose for breath tests with ^{13}C phenylalanine and ^{13}C dipeptide may be 5 mg/kg in horses.
Key words: *^{13}C- breath test, dipeptide, horses, phenylalanine*

Equine hepatitis and gastrointestinal (GI) diseases such as pancreatitis are associated with anorexia and innutrition, and the resulting unfavorable performance can lead to economic loss. They may also progress to serious conditions [1, 10], and establishment of a more accurate method of diagnosis is important. In recent years, ^{13}C breath test was established as a diagnosis method for delayed equine gastric emptying [15] and is expected to be applied to diagnose other GI diseases. In humans, ^{13}C phenylalanine and ^{13}C dipeptide are clinically applied for liver and pancreas function tests, respectively [3–5, 7] and they appear promising for equine liver and pancreas function tests. However, since basic study on equine ^{13}C breath test has not been adequately conducted, dose finding and sample collection time finding experiment as well as establishment of evaluation index are necessary to use these tests in horses. For these reasons, preliminary study on ^{13}C breath tests using ^{13}C phenylalanine and ^{13}C dipeptide were conducted.

Six healthy adult thoroughbred horses (1 stallion, 3 mares and 2 geldings), aged 5.7 ± 2.1 years and 485.0 ± 40.9 kg body weight were used (mean ± SD). The horses had no history of GI diseases. They were kept inside of a horse barn and fed with 1.8 kg oats, 0.6 kg bran, and 7 kg hay per day, twice a day at 8am and 6pm. Water was given *ad libitum*. Reagents were administered using a retaining stall and breath samples were collected in the barn. This study was approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine.

Reagents used in this study were ^{13}C phenylalanine (C6H5CH2CHNH213COOH, molecular weight 166, Tokyo Gas Chemicals, Japan) and ^{13}C dipeptide (Bz-Tyr-13C-Ala, molecular weight 379, Tokyo Gas Chemicals, Japan). For ^{13}C phenylalanine, 3 doses of 2.5 mg/kg, 5 mg/kg, and 10 mg/kg were tested by dissolving each dose with 1 m*l*/kg distilled water and delivering it with a nasogastric catheter. ^{13}C dipeptide was tested for 3 doses of 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg by dissolving each dose with 2 m*l*/kg distilled water and delivering it with a nasogastric catheter.

Breath samples were collected via a silicon tube, which was attached to a plastic plate, inserted into the horses' nasal cavity into a breath sampling bag at expiration (200 m*l* and 1,300 m*l* UBit®-specialized breath sampling bags, Otsuka Pharmaceutical, Japan).

Horses were muzzled for fasting from 12 hr before reagent administration. Breaths collected into five

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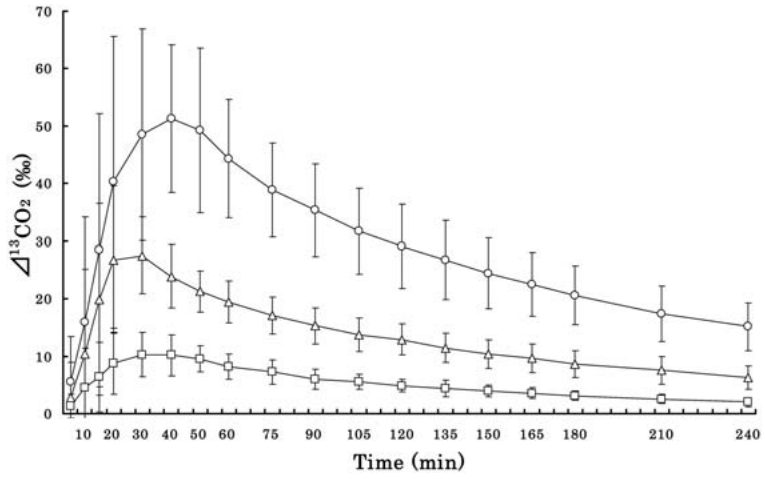

Fig. 1. Changes in $\Delta^{13}CO_2$ following 13C phenylalanine administration. □: 2.5 mg/kg group, △: 5 mg/kg group, ○: 10 mg/kg group. Data are shown as mean ± SD. Rapid $\Delta^{13}CO_2$ increases were observed from 0–20 min in the 10 mg/kg and 5 mg/kg groups. Tmax was observed at 40 min in the 10 mg/kg group.

1,300 m*l* bags before reagent administration was used as control. Breath samples were collected in duplicate into 200 m*l* bags at 5 min intervals from 0–20 min, at 10 min intervals from 20–60 min, at 15 min intervals from 60–180 min, and at 30 min intervals from 180–240 min after reagent administration. A report by Sasaki *et al.* was used as a reference for the timing of breath sampling [14].

The breaths collected were analyzed using a $^{13}CO_2$-infrared spectrophotometry analyzer (POC One®, Otsuka Pharmaceutical, Japan). $\Delta^{13}CO_2$, the difference of breath $^{13}CO_2$ measured before and after administration, was calculated [14]. $\Delta^{13}CO_2$ of a particular timing was defined as the mean value of the $\Delta^{13}CO_2$ collected from the 2 samples at that timing. Tmax (min) was the time the peak $\Delta^{13}CO_2$ was obtained and Cmax (‰) was the $\Delta^{13}CO_2$ at that peak.

Figure 1 shows the results of the ^{13}C breath test using ^{13}C phenylalanine. $\Delta^{13}CO_2$ increased dose dependently following ^{13}C phenylalanine administration. Peak $\Delta^{13}CO_2$ levels were clearly observed soon after 5 mg/kg and 10 mg/kg administration, but no clear peak level was observed for 2.5 mg/kg. Cmax for 10 mg/kg and 5 mg/kg were 57.8 ± 16.3‰ and 32.1 ± 11.0‰, respectively, and that of 5 mg/kg was lower than that of 10 mg/kg.

Figure 2 shows the results of the ^{13}C breath test using ^{13}C dipeptide. $\Delta^{13}CO_2$ increased soon after 5 mg/kg administration and it clearly reached the peak level. However, no clear peaks were observed when 1.25 mg/kg and 2.5 mg/kg ^{13}C dipeptide were administered. Cmax of 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg were 5.6 ± 1.1‰, 11.5 ± 1.1‰, and 23.5 ± 2.4‰, respectively, indicating dose-dependent increase in $\Delta^{13}CO_2$. Tmax of 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg were 71.7 ± 35.9 min, 71.7 ± 23.9 min, and 58.3 ± 13.1 min, respectively, and that of 5 mg/kg was the lowest.

^{13}C phenylalanine breath test is used to evaluate liver diseases such as chronic hepatitis and liver functions of patients with liver cirrhosis in humans. [5, 13]. Orally administered ^{13}C phenylalanine is absorbed in the intestine and then delivered to the liver. Phenylalanine hydroxylase, enzyme that is associated with phenylalanine metabolism, exists only in the liver. ^{13}C phenylalanine absorbed in the intestine is metabolized specifically in the liver and changes from phenylalanine to tyrosine [6]. When ^{13}C phenylalanine is metabolized in the liver, the end product $^{13}CO_2$ is produced, which is excreted into expired air via the lung. Thus, the difference in breath $^{13}CO_2$ levels measured before and after ^{13}C phenylalanine administration ($\Delta^{13}CO_2$) can be used to evaluate liver functions indirectly. Clinical use of this method has started in human medicine.

Sample collection time used for gastric emptying evaluation was referred to for the breath sample collection of the present study. Since peak

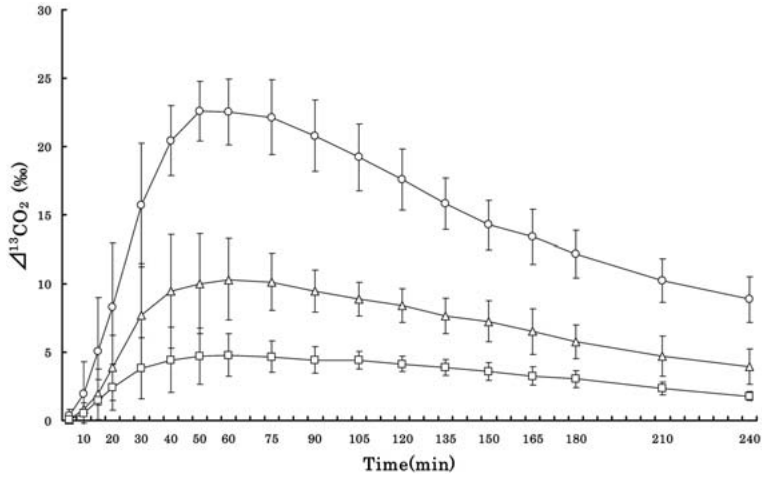

Fig. 2. Changes in $\Delta^{13}CO_2$ following 13C dipeptide administration. □: 1.25 mg/kg group, △: 2.5 mg/kg group, ○: 5 mg/kg group. Data are shown as mean ± SD. $\Delta^{13}CO^2$ markedly increased and Tmax was observed at 50 min in the 5 mg/kg group.

concentrations and subsequently, a course of $\Delta^{13}CO_2$ decrease were observed within this time frame, the sample collection time used in this study was considered appropriate.

Healthy adult thoroughbred horses received ^{13}C phenylalanine at different doses and the effects were studied. Tmax was observed clearly when 5 mg/kg and 10 mg/kg were administered, but not for 2.5 mg/kg. In studies in humans and non-equine animals, unclear Tmax and decreased Cmax were observed in patients with liver diseases [2, 8, 11]. Dose 2.5 mg/kg was thus considered inappropriate for evaluation of liver diseases. Tmax was observed for 5 mg/kg and 10 mg/kg, and the standard deviation of Cmax at 5 mg/kg (11.0‰) was lower than that of 10 mg/kg (16.3‰). Therefore, 5 mg/kg was considered an optimal dose for equine ^{13}C phenylalanine breath test, since the standard deviation of Cmax and the dose itself were lower.

^{13}C dipeptide breath test has been studied as a pancreatic exocrine secretion test for humans, and a correlation with diseases such as chronic pancreatitis, which accompany exocrine pancreatic insufficiency, was found [7, 9, 12]. N-benzoyl-L-tyrosyl-P-aminobenzoic acid (bentiromide) is used as a substrate in the BT-PABA method that tests pancreatic exocrine secretion in humans. ^{13}C dipeptide is a substance that substitutes the PABA of bentiromide with the ^{13}C-labelled amino acid alanine. In breath tests that use ^{13}C dipeptide as a reagent, orally administered ^{13}C dipeptide is degraded by carboxypeptidase A, a digestive enzyme in pancreatic juice, and ^{13}C alanine is released [20]. Free ^{13}C alanine is absorbed in the intestine, metabolized mainly in the liver, and $^{13}CO_2$ is produced. In the breath tests that use ^{13}C alanine as a reagent, no difference was found between patients with chronic pancreatitis and healthy subjects. The difference observed between patients with chronic pancreatitis and healthy subjects in ^{13}C dipeptide breath test is thought to be associated with exocrine pancreatic insufficiency [12]. Present study used the sample collection time for gastric emptying evaluation as a reference for breath collection. Since peak levels and subsequently, a course of $\Delta^{13}CO_2$ decrease were observed, the sample collection time was considered adequate.

Healthy adult thoroughbred horses received ^{13}C dipeptide at doses 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg and the effects were studied. Clear Tmax was observed when 5 mg/kg was administered, but no clear peaks were seen for 1.25 mg/kg and 2.5 mg/kg. In studies in humans and non-equine animals, unclear Tmax and decreased Cmax are observed in patients with pancreatic diseases [7]. Therefore, the dose of 5 mg/kg that produced clear peak levels was considered an optimal dose for equine ^{13}C dipeptide breath test. Further, since the standard deviations of Tmax for doses of 1.25 mg/kg, 2.5 mg/kg, and 5 mg/kg were

35.9 min, 23.9 min, and 13.1 min, respectively, this result also supported that dose 5 mg/kg, which had the lowest standard deviation, may be optimal.

This study demonstrated that the optimal dose for equine ^{13}C phenylalanine and ^{13}C dipeptide breath test may be 5 mg/kg.

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