Video Article

Assessment of Gastric Emptying in Non-obese Diabetic Mice Using a [^{13}C]-octanoic Acid Breath Test

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Abstract

Gastric emptying studies in mice have been limited by the inability to follow gastric emptying changes in the same animal since the most commonly used techniques require killing of the animals and postmortem recovery of the meal^{1,2}. This approach prevents longitudinal studies to determine changes in gastric emptying with age and progression of disease. The commonly used [^{13}C]-octanoic acid breath test for humans³ has been modified for use in mice⁴⁻⁶ and rats⁷ and we previously showed that this test is reliable and responsive to changes in gastric emptying in response to drugs and during diabetic disease progression⁸. In this video presentation the principle and practical implementation of this modified test is explained. As in the previous study, NOD LtJ mice are used, a model of type 1 diabetes⁹. A proportion of these mice develop the symptoms of gastroparesis, a complication of diabetes characterized by delayed gastric emptying without mechanical obstruction of the stomach¹⁰.

This paper demonstrates how to train the mice for testing, how to prepare the test meal and obtain 4 hr gastric emptying data and how to analyze the obtained data. The carbon isotope analyzer used in the present study is suitable for the automatic sampling of the air samples from up to 12 mice at the same time. This technique allows the longitudinal follow-up of gastric emptying from larger groups of mice with diabetes or other long-standing diseases.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50301/>

Introduction

This manuscript describes the technical and methodological considerations involved in non-invasive measurement of gastric emptying in mice. By following the protocol described here, investigators can reliably and reproducibly follow changes in gastric emptying due to development of disease, study the impact of pharmacological agents on gastric emptying and follow the response of gastric emptying to treatment of underlying diseases or defects^{6,8,11,12}. In previous publications, the application of ^{13}C octanoic acid breath tests was shown to be a useful way of measuring gastric emptying in humans and animals^{3,8}. This paper describes in detail, the procedures necessary to obtain reliable data over the 6 to 8 months necessary for a longitudinal study of gastric emptying in mice with diabetes. The advantages of following this protocol when compared to previously published methods are that the investigator can be assured the data obtained will be reliable and reproducible. In addition, the automated system for collecting and analyzing the gas samples described here increases the number of animals that can be followed simultaneously in a study. Overall, the goal of this paper is to identify the key factors that maintain habituation of the mice to the test and that reduce variability in obtained results.

For the *in vivo* measurement of gastric emptying, mice are fasted overnight and put into the transparent plastic testing chambers with constant airflow. After the mice get habituated to the tubes, baseline exhaled $^{13}\text{CO}_2$ levels are determined and airflow adjusted accordingly. Next, we administer a test meal consisting of egg yolk mixed with ^{13}C -labeled octanoic acid. Because the mice are fasted and trained, they generally eat the test meal within 2 min. The administered octanoic acid is not absorbed in the stomach but will be taken up in the duodenum and will get metabolized in the liver into $^{13}\text{CO}_2$, which is released and exhaled, resulting in an enrichment of $^{13}\text{CO}_2$ in the surrounding air. Air samples are collected at determined time intervals and are analyzed by the carbon isotope analyzer. The rate-limiting step in this whole process is gastric emptying and the pulmonary excretion of $^{13}\text{CO}_2$ directly corresponds with gastric emptying of the labeled meal.

Figure 1. Schematic of gastric emptying apparatus. After overnight fasting, mice are placed in transparent chambers allowing them to move and turn freely. An inlet tube allows fresh and constant air influx and an outlet leads to the isotope analyzer to measure the ^{13}C -to- ^{12}C ratio in the exhaled breath. The chamber also has a central port for delivery of food containing ^{13}C -octanoic acid.

Protocol

1. Training and Habituation of the Mice

1. Prior to analysis, put all mice in the testing chambers for 2-4 hr with constant airflow in order to habituate them to the testing conditions. This markedly reduces stress levels that might otherwise cause aberrant detection of delayed gastric emptying. Treat the mice the same way as if the gastric emptying experiment was running. Prepare egg yolk (see further) without adding octanoic acid and feed 0.2 g to each mouse.
2. Repeat this process till the mice are sufficiently trained (typically 2-3 times). The mice typically are easily habituated as long as the environmental conditions are kept the same.

Note: Non-habituated mice continue to move around for about 1 hr after transfer to the chamber, and defecate and urinate frequently, while habituated mice quickly settle in their new environment and rest quietly.

Note: During the experiment: Monitor animals for signs of loss of habituation such as excess urination, defecation, lack of interest in eating the egg. If this is the case consider re habituating in an empty chamber 1-2 times prior to obtaining gastric emptying data. Consistency is extremely important while doing this experiment. Doing things exactly the same way every time is the only way to get reliable and reproducible results. This includes giving treatment (e.g. insulin) every day at the same time, not separating the mice from their cage-mates unless absolutely necessary, fasting the mice and starting the gastric emptying test at the same time, and handling the mice the same way.

2. Preparation of the Isotope Containing Test Meal

1. Start with weighing out 5 g of egg yolk in a 50 ml falcon tube. Repeat these steps each experimental day to prepare a fresh test meal.
2. Add 10 μl of octanoic acid with a concentration of 2 $\mu\text{g/g}$ to the 50 ml falcon tube containing the egg and mix vigorously for 1 min with a spatula in the falcon tube.
3. The egg is then transferred to a glass beaker and heated over a Bunsen burner until it coagulates and its consistency is suitable to make small balls. This typically takes about 30 sec.

Note: The balls of egg yolk should weigh 0.2 g per mouse. This is important to keep the cumulative dose constant in all the mice.

3. Starting the Experiment

1. Once trained and ready for gastric emptying, fast the mice overnight (12 hr) on a metal "mesh-bottom" fasting rack to prevent coprophagia. Make sure they have free access to drinking water. Since diabetic mice are used in the current experiment, they should not be fasted for more than 16 hr.
2. Start by setting up the gastric emptying chambers. Use clean chambers and covers that have been air-dried. Also, any tubes connecting the chambers to the analyzer or the CO_2 air supply should be moisture free; water can interfere with the signal read by the analyzer
3. Connect the chambers to the inlet tubes that provide a constant air flow. Then connect the outlet tubes from the chambers to the machine. Close the tubes and turn on the airflow.

Note: Apply a very small amount of Vaseline at the end of the cover lids so they close easily and are securely sealed. This tight seal is necessary to collect all the carbon dioxide produced by the mice.

4. Experimental Procedures

1. Start out by weighing each mouse. Body weight is a measure of their continuing good health. Then place each mouse in the appropriate chamber. It is of course important to have the air flowing into the chambers at this time.
2. To start the measurement, allow the mice to acclimate to the chambers before adjusting the air levels.
3. Once the mice appear calm, which may take a few minutes, adjust the air flow rate for each mouse chamber. This may be different for each mouse. Typically, the air flow is adjusted at the beginning of the experiment to make sure that exhaled CO₂ reaches levels detectable by whatever equipment is being used, and to make sure that the level stays low enough to ensure healthy air turnover. We use initial CO₂ levels between 1,000 and 1,500 parts per million.
4. If having difficulty with adjustments, check for air leaks. Then repeat the process for each of the chambers and watch for another round of measurements to see if adjustments made to airflow have corrected the CO₂ level. It is important to obtain a steady baseline reading prior to feeding the mice. We use a machine with a self calibration feature. If this is not the case calibration should be checked.
5. When this is achieved, administer the egg meal to the first mouse and record the time each mouse receives the food.
6. We run the procedure for 4 hr to obtain enough values for fitting the ¹³CO₂ enrichment curve for each mouse. Check on the mice every 30-60 min to make sure that the CO₂ levels are still safe for the mice.
7. Prepare new boxes containing food before the end of the test so the mice can start eating immediately after the test is over.

Representative Results

A representative data set from three different mice is shown in **Figure 2**. The black graph represents the data points from a mouse with normal gastric emptying. It shows the fraction of ¹³C that is recovered in the exhaled air expressed as a percentage of the administered dose per hour expressed as a function of time. The blue curve is from a mouse with an accelerated gastric emptying with a T half value of 40 min and the red curve is from a mouse with a delayed gastric emptying with a T half value of 168 min. As established in a previous study in our lab⁷, the normal gastric half emptying time for a non-diabetic NOD mouse age 9-15 weeks ranges from 62 to 131 min as represented by the gray box. The data points are then fitted by a non-linear regression curve with the following equation⁸:

$$y = at^be^{-ct}$$

Where y is the percentage of the ¹³C recovered in the breath per hour (t) and a, b and c are regression constants estimated for each breath vs. time curve. The half emptying time (T_{1/2})

is calculated from a numerical integration procedure using an inverse gamma function. We have obtained results from several thousand studies with a biological intra-mouse variability of about 10%.

Figure 2. Representative gastric emptying data from three different mice with normal (black curve), delayed (red curve) and accelerated (blue curve) gastric emptying. The grey box highlights normal values. The equation of the non-linear regression curves is shown in the box.

Discussion

The described technique herein allows for repeated and non-invasive *in vivo* measurement of solid gastric emptying in mice. This system has the advantage that the animals are not restrained in the measurement chamber, allowing them to move and turn freely. Since this is an unfamiliar environment, the mice still need to be trained and habituated to the testing chambers to prevent effects of stress on gastric emptying. In general, we assume the gastric emptying data are reliable if the intra-mouse variability between consecutive gastric emptying tests is less than 10%.

This report is a more detailed description of our previous reports on gastric emptying measurements in mice^{8,11,12} and includes additional information on trouble-shooting problems with the system. The main modification is the use of the LGR isotope analyzer for measuring gas levels. The analyzer we used in the present study measures ¹²CO₂, ¹³CO₂, and H₂O concentrations every second. The computer controlled valves in the multiple-input unit automatically switch flow to the detector between mouse chambers every 25 sec. Therefore 12 mice can be simultaneously analyzed with a 5 min interval between readings. It has to be noted that the data can also be obtained by manual sampling of the exhaled air and subsequent analysis. If manually sampling or using another device, make sure readings are obtained at about 10 min intervals. The sensitivity of our system and the frequency of sample collection means that the gastric emptying curves are more reliable, easier to fit and so we have found that we get more reproducible data with this improved method.

The major pitfalls to this technique occur when the mice fail to eat the meal within the allotted time, when the mice lose their habituation to the chamber and when technical problems arise with the detection system. Failure to eat the meal and loss of habituation are usually a consequence of not following the critical step of having a regular schedule for testing. After 3 consecutive tests separated by 7 days or less, our mice become habituated to the test and eat the meal. Once habituated, reliable data are obtained if the mice are tested at least once every 2 weeks for the remainder of the experiment. If for some reason the mice have not been tested for 3 or more weeks then two training sessions within a week will usually ensure habituation. If the personnel handling the animals change then re-training of the mice is advised and it is particularly important to discourage use of strongly scented soaps and/or perfumes by the people handling the mice. Difficulties with the detection system are often due to the low levels of ¹³CO₂ generated by a small animal like a mouse. The detector must be calibrated and for some detectors such as infra-red based systems, it is very important to reduce water content of the sample to a minimum prior to measurements.

There are few limitations to this technique unless the investigator wishes to study gastric emptying more frequently than once every 3 days in the same mouse. It is not advised to fast the mouse so often and such dietary restriction on a regular basis can alter gastric function. Modifications to the technique can be made to measure liquid gastric emptying and that is a useful potential application of the method.

Other methods for measuring gastric emptying, including scintigraphy or measuring retained content after a fixed time have significantly more limitations including issues with the stress of the process or due to the need to kill the animal at the end of each test. Stressors include gavage of the test meal or restraint of the animal during measurement. The advantages of the breath test are that the mouse is freely moving and not anesthetized or sedated in any way.

In summary, the ¹³C-octanoic acid breath test allows the study of disease progression with age and treatment and these data can then be correlated with changes in other physiological parameters and histological evaluation in the same animal. Among other applications, the test also allows study of how drugs can directly alter gastric emptying as well as investigating the response to treatment in animals that develop changes in gastric emptying due to disease or other interventions.

Disclosures

The authors declare that they have no competing financial interests.

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