Determination of gastric emptying in nonobese diabetic mice

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Choi KM, Zhu J, Stoltz GJ, Vernino S, Camilleri M, Szurszewski JH, Gibbons SJ, Farrugia G. Determination of gastric emptying in non-obese diabetic mice. Am J Physiol Gastrointest Liver Physiol 293: G1039–G1045, 2007. First published September 20, 2007; doi:10.1152/ajpgi.00317.2007.—Animal studies on diabetic gastroparesis are limited by inability to follow gastric emptying changes in the same mouse. The study aim was to validate a nonlethal gastric emptying method in nonobese diabetic (NOD) LtJ mice, a model of type 1 diabetes, and study sequential changes with age and early diabetic status. The reliability and responsiveness of a [13C]octanoic acid breath test in NOD LtJ mice was tested, and the test was used to measure solid gastric emptying in NOD LtJ mice and nonobese diabetes resistant (NOR) LtJ mice. The [13C] breath test produced results similar to postmortem recovery of a meal. Bethanechol accelerated gastric emptying [control: 92 ± 9 min; bethanechol: 53 ± 3 min, mean half emptying time (T1/2) ± SE], and atropine slowed gastric emptying [control: 92 ± 9 min; atropine: 184 ± 31 min, mean T1/2 ± SE]. Normal gastric emptying (T1/2) in nondiabetic NOD LtJ mice (8–12 wk) was 91 ± 2 min. Aging had differing effects on gastric emptying in NOD LtJ and NOR LtJ mice. Onset of diabetes was accompanied by accelerated gastric emptying during weeks 1–2 of diabetes. Gastric emptying returned to normal by weeks 3–5 with no delay. The [13C]octanoic acid breath test accurately measures gastric emptying in NOD LtJ mice, is useful to study the time course of changes in gastric emptying in diabetic NOD LtJ mice, and is able to detect acceleration in gastric emptying early in diabetes. Opposing changes in gastric emptying between NOD LtJ and NOR LtJ mice suggest that NORLtJ mice are not good controls for the study of gastric emptying in NOD LtJ mice.

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relative levels of the specific isotope of carbon reflect changes in gastric emptying (1, 17, 37, 42). However, the \([^{13}C]\)octanoic acid breath test has not been previously in NOD LtJ mice.

The studies described in this paper aimed to establish that the \([^{13}C]\)octanoic acid breath test is a reliable and responsive method for following changes in gastric emptying in NOD LtJ mice. The studies also aimed to determine control values for gastric emptying in diabetic and nondiabetic NOD LtJ mice as well as nonobese diabetes resistant (NOR) LtJ mice. NOR LtJ mice do not develop diabetes (28, 33, 40) and are often used as controls for NOD LtJ mice and to measure sequential changes in gastric emptying with age and early diabetic status in NOD LtJ mice.

METHODS

Animals. Female NOD/LtJ mice (Jackson Laboratories, Bar Harbor, ME) and age-matched NOR/LtJ mice were used in this study. Mice were obtained at 8 wk of age and housed in a room maintained at 20–23°C with 10- to 14-h light-dark cycle. Once mice became diabetic, they were placed in individual cages, and the cages were cleaned daily. Gastric emptying was also determined in nondiabetic Balb/c mice, since this strain is one of the most commonly used mice strains. Protocols were approved by the Mayo Foundation Institutional Animal Care and Use Committee.

Glucose testing. Blood glucose was measured on a weekly basis. NOD LtJ mice were considered prediabetic when the blood glucose exceeded 155 mg/dl and diabetic when the glucose level exceeded 250 mg/dl. A single drop of blood from the tail vein was collected, and the blood glucose was measured by using an ACCU-CHEK device (Roche, Indianapolis, IN).

Measurement of gastric emptying using \([^{13}C]\)octanoic acid breath test. Mice were fasted overnight with free access to water in a metabolic cage. Mice were placed in a chamber (130 ml) flushed with CO₂-free air (flow speed of ~0.5 l/min; Fig. 1). The flow rate was chosen to keep CO₂ levels in the chambers below 3%. The chamber was sufficiently wide to allow the mice the freedom to turn around inside it. After a baseline reading, mice were fed 200 mg scrambled cooked egg yolk containing 2.5 μmol \([^{13}C]\)octanoic acid. This amount of egg was ingested by practically all mice in <5 min. Any remaining food was removed after 5 min and weighed. Air containing the exhaled breath was collected and analyzed to determine the \([^{13}C]/^{12}C\) ratio using an Infra Red Isotope Analyzer (IRIS; Wagner Analysen Technik, Bremen, Germany). Samples were collected every 5 min for 60 min and then every 15 min up to 240 min.

Data analysis. The \([^{13}C]\) enrichment by IRIS was expressed as the change in \([^{13}C]\) (in %) calculated from the sample and standard \([^{13}C]CO_2/[^{12}C]CO_2\) ratios using the isotopic abundance of a limestone standard (Pee Dee Belemnite). The measured \([^{13}C]CO_2\) recovery in the breath was expressed as a percentage excretion per hour of the given \([^{13}C]\) dose. Programs and equations in the IRIS machine were originally designed for use in humans. To correct for the different rate of basal CO₂ generation in mice and body surface area (BSA) of a mouse, the IRIS machine was reprogrammed with a CO₂ production of 40 ml·kg⁻¹·min⁻¹ (12), and the BSA for a mouse was calculated from \(A = kW^{2/3}\) where \(A\) is surface area in square centimeters, \(k\) is 10.5, and \(W\) is body weight in grams (18). The data for \([^{13}CO_2]\) enrichment in the breath vs. time were fitted by nonlinear regression model expressed as:

\[
y = at^b e^{-ct}
\]

where \(y\) is the percentage of \([^{13}C]\) recovered in breath per hour (t), and \(a, b,\) and \(c\) are regression parameters estimated for each breath vs. time curve. The half emptying time (\(T_{1/2}\)) was calculated from a numerical integration procedure using an inverse-gamma function.

Before gastric emptying studies, all mice were trained on arrival to the facility to habituate the animals to the environment of the testing equipment and obtain accurate baseline values for gastric emptying. The mice were individually placed in a chamber used for the \([^{13}C]\) breath test for 1 h. This was repeated six times with at least 1 day between training sessions. After this habituation, a full gastric emptying test was carried out, but the results were not documented. Subsequent gastric emptying tests showed a <10% intramouse variability.

Statistical analysis. Data are presented as means ± SE. Changes in gastric emptying with age were summarized as a (linear) slope for each individual mouse. These slopes were compared against zero (i.e., no change with age) using a two-tailed one-sample t-test at a significance level of 0.05. Comparisons of gastric emptying between pairs of diabetic status categories (e.g., prediabetic vs. 1 wk postdiabetic) were made using paired sample t-tests with Bonferroni adjustment (with 30 potential comparisons that could be made, a P value of <0.0017 was considered significant). This conservative approach was used rather than a repeated-measures ANOVA since specific pairwise comparisons were of interest (gastric emptying was measured at several time points in the same mice). The P values in the text and legends for Figs. 1–6 for the comparisons of gastric emptying are the unadjusted values.

Validation. To determine accuracy of the test, a \([^{13}C]\) breath test was obtained in 6- to 10-wk-old NOD LtJ mice. Later (1 wk), the same mice were given egg yolk (200 mg). After feeding (60 min), the animals were killed. This time point was chosen to allow gastric emptying but also to allow enough egg yolk still present in the stomach to be identifiable and able to be collected. The stomach was removed and opened. All visible yolk was recovered and dried using a centrifugal lyophilizer (Thermo Savant, Holbrook, NY) at 45°C for 15 h, and the weight of the contents was determined and expressed as a percentage of the total dried egg yolk given. To determine sensitivity, the muscarinic agonist bethanechol (20 mg/kg ip) or the muscarinic antagonist atropine (7.5 mg/kg ip) was used to accelerate or delay gastric emptying, respectively. Control values for gastric emptying in these mice were determined following injection of the saline vehicle. NOD LtJ mice were used (n = 6), and the order in which bethanechol, atropine, and saline were given was randomly determined. The drugs were administered 30 min before the start of the breath test for gastric emptying.

RESULTS

Accuracy. To determine the accuracy of the \([^{13}C]\) octanoic breath test, gastric emptying was measured at 60 min in six
NOD LtJ mice and was 28±1.9% (mean ± SE). This was similar to the gastric emptying determined by directly measuring stomach contents at the same time point after ingestion of the same amount of egg yolk (31.5±2.1%, mean ± SE, n = 6 mice, P > 0.05, paired t-test; Fig. 2).

The sensitivity of the [13C]octanoic breath test was determined in six nondiabetic NOD LtJ mice treated with muscarinic receptor agonist bethanechol (20 mg/kg ip), the muscarinic receptor antagonist atropine (7.5 mg/kg ip), and saline as a control. The order of the treatments varied between animals. Bethanechol accelerated gastric emptying in all animals, resulting in a significant decrease in the T1/2 values compared with saline treatment (control: 92±9.1 min; bethanechol: 53±3.1 min, mean T1/2 ± SE, P < 0.05, paired t-test; Fig. 3A). Atropine slowed gastric emptying in all mice, resulting in a significant increase in the T1/2 values compared with saline treatment (control: 92±9.1 min; atropine: 184±30.5 min, mean T1/2 ± SE, P < 0.05, paired t-test; Fig. 3A). The full gastric emptying curves for all three interventions plotted as a percentage of excretion per hour of the given 13C dose are shown in Fig. 3B.

**Normal values.** To determine normal values for gastric emptying, the [13C]octanoic breath test was used to assess gastric emptying in 152 healthy nondiabetic NOD LtJ mice, 38 healthy NOR LtJ mice, and 6 Balb/c mice. The overall T1/2 for NOD LtJ mice 9–15 wk old was 95±2 min, for NOR LtJ mice 83±3 min, and for Balb/c mice 93±4 min. The 5th to 95th percentiles for gastric emptying for nondiabetic NOD LtJ mice ages 9–15 wk were 62 and 131 min, respectively, and for NOR LtJ mice 59 and 102 min. Therefore, for NOD LtJ mice, a T1/2 of <62 min was considered to be accelerated, and a T1/2 of >131 min was considered to be delayed for this age group.

**Effect of age.** To determine the effect of age on gastric emptying, gastric emptying was measured at three time points between 12 and 45 wk in nondiabetic NOD LtJ mice (n = 29) and NOR LtJ mice (n = 10). When plotted against age, the individual T1/2 values for the NOR LtJ mice and the nondiabetic NOD LtJ mice appeared to move in opposing directions (Fig. 4A). Therefore, the data were fit using a linear regression model, and the slopes were estimated for each mouse. The slope for both NOD LtJ and NOR LtJ mice was significantly different from zero (mean slope ± SE; -0.77±0.14 min/wk, P < 0.0001 for NOD LtJ and 0.53±0.23 min/wk, P < 0.05 for NOR LtJ, two-tailed, one-sample t-test; Fig. 4B). Because of this difference, we did not use NOR LtJ mice in the rest of our studies but instead used nondiabetic NOD LtJ mice as age-matched controls for comparison with diabetic NOD LtJ mice. With the use of gastric emptying data from all mice used in this study, the mean T1/2 (5th and 95th percentiles) for NOD LtJ mice ages 9–15 wk was (n = 152) 95 min (62, 131), for ages 16–30 wk (n = 108) 92 min (67, 118), and for 31–45 wk (n = 29) 88 min (62, 107). The mean T1/2 (5th and 95th percentiles) for NOR mice ages 9–15 wk (n = 38) was 83 min (59, 192), for ages 16–30 wk (n = 21) 91 min (66, 112), and for ages 31–45 wk (n = 10) 100 min (64, 139).

**Hyperglycemia and effect of diabetes on gastric emptying.** NOD LtJ mice started to develop diabetes at 11 wk of age [22±0.8 (SE) wk, n = 93 mice]. By 26 wk, 60% of mice were diabetic and by 40 wk 76% of mice were diabetic. Mice that developed diabetes after 26 wk did not always sustain high blood glucose levels (Fig. 5). Therefore, we only studied gastric emptying in mice that developed diabetes at <26 wk old.
To test the effect of development of diabetes on gastric emptying, 34 mice that became diabetic were assigned to study the effect of early diabetes on gastric emptying. The nondiabetic median blood glucose was 105 mg/dl (95–119 mg/dl interquartile (IQR)), and $T_{1/2}$ gastric emptying was 91.3 ± 1.8 (SE) min (Fig. 6A). Blood glucose levels in the mice rose above normal levels (>155 mg/dl, <250 mg/dl) at 19 ± 1.0 wk old (mean ± SE), but gastric emptying was not affected [$T_{1/2} = 92.3 ± 1.7$ (SE) min, $P = 0.6054$ vs. non diabetic, $t$-test with Bonferroni adjustment; Fig. 6A]. Blood glucose levels continued to rise and remained high in all mice for the remainder of the study.

In the 1st wk after development of diabetes [blood glucose levels = 405 (median) and 328–436 (IQR)], gastric emptying, although not accelerated, was significantly faster compared with the control nondiabetic and prediabetic measurements [$T_{1/2} = 80.7 ± 2.2$ (SE) min, $P = 0.0004$ vs. nondiabetic, $P = 0.000009$ vs. prediabetic; Fig. 6A].

At 2 wk of diabetes, gastric emptying was accelerated [$T_{1/2} = 52.5 ± 2.7$ (SE) min, $P < 0.0000001$ vs. nondiabetic, $P < 0.0000001$ vs. prediabetic, $P < 0.0000001$ vs. 1 wk diabetic; Fig. 6A].

At 3–5 wk of diabetes, $T_{1/2}$ was not different from the values obtained before the increase in blood glucose [$T_{1/2} = 85.2 ± 3.2$ min, $P = 0.09$ vs. nondiabetic, $P < 0.0000001$ vs. 2 wk diabetic; Fig. 6A]. The full gastric emptying curves for all five time points plotted as percentage excretion per hour of the given $^{13}$C dose are shown in Fig. 6B. Mice could not be followed for longer in this study, since the severity of their diabetes in the absence of treatment with insulin resulted in a high mortality at later time points, with no mice surviving >10 wk of untreated diabetes.

**DISCUSSION**

In this study, we validate a $^{13}$C-octanoic acid breath test in NOD LtJ mice and show that it is a reliable and responsive method for following changes in gastric emptying of solids in mice. The studies described establish normal values for gastric emptying in NOD LtJ mice and also determine changes in gastric emptying with age and with onset of diabetes. This study strongly suggests that the validated $^{13}$C-octanoic acid breath test can be used to sequentially measure gastric emptying to solids in the same mice and directly correlate gastric emptying changes to changes at the tissue level as modifications in the methodology and equations used in humans (8, 17, 45) allowed us to accurately and serially measure gastric emptying in nondiabetic and diabetic mice. In studies on the effect of development of diabetes on gastric emptying in the diabetic NOD LtJ mice, we observed that gastric emptying to solids was consistently accelerated, not delayed, in the first 1–3 wk after development of hyperglycemia. Mice in this study were not followed for longer than 5 wk because of the high mortality observed at later time points in the absence of insulin treatment. The return to normal emptying levels at week 5 may reflect the early part of a trend toward delayed emptying. Longer studies will require use of insulin to keep the mice from dying. Furthermore, we determined that older NOR LtJ mice were not good controls for comparison with age-matched nondiabetic NOD LtJ mice.

The reliability and responsiveness of the $^{13}$C-octanoic acid breath test as a method for measuring gastric emptying in a noninvasive way has been demonstrated previously (1, 41, 42). However, it was necessary to confirm that this method is accurate and responsive in NOD LtJ mice, since the values obtained for gastric emptying have been shown to vary con-
GASTRIC EMPTYING IN NONOBESE DIABETIC MICE

Fig. 6. Mean $T_{1/2}$ and onset of diabetes. A: mean $T_{1/2}$ are shown for the same cohort of mice ($n = 34$) followed through development of diabetes and for 5 wk after the development of diabetes. The dotted lines show the 5th and 95th percentiles for normal gastric emptying. Gastric emptying was faster 1 wk after the development of diabetes. The dotted lines show the 5th and 95th percentiles for normal gastric emptying. Gastric emptying was faster 1 wk after the development of diabetes. The dotted lines show the 5th and 95th percentiles for normal gastric emptying.

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siderably in different laboratories (1). To do this, we confirmed the accuracy of the breath test by comparing the breath test with a long-established method based on recovering residual gastric contents post mortem (32, 52). The amount of gastric emptying after 60 min determined by the breath test in vivo was not different from the amount of emptying determined by measuring the residual gastric contents. We used this comparator to enable us to use an identical test meal, since the meal content has significant effects on gastric emptying (48, 49).

Compared with other studies that measured emptying of solid, high-fat meals using both isotope-labeled octanoic acid breath tests (42) and scintigraphy (51), the $T_{1/2}$ values we obtained in NOR LtJ and NOD LtJ healthy control mice were similar. Our system has the potential advantage over scintigraphy in that the animals are not restrained in the measurement chamber, allowing the mice to move about and turn around. Despite this advantage, the chamber was still an unfamiliar environment, and it was necessary to habituate the mice to the procedure to avoid effects of this stress on gastric emptying. We routinely habituated the mice before the first full gastric emptying test and did not include the first gastric emptying test in our data. After training, the variability for multiple measurements in individual animals was $<10%$. Furthermore, the ability of the breath test to detect changes in gastric emptying following pharmacological treatment was confirmed by the demonstration that bethanechol accelerated and atropine slowed gastric emptying at doses that have been demonstrated to have these effects in previous studies on mice (24, 32, 52). The ability to make repeated measurements in the same animal further reinforces the potential of the breath test for doing complex pharmacological studies using multiple doses of different compounds.

We identified one major technical problem in calibrating the IRIS machine to reflect the metabolic rate of mice in these studies. The IRIS machine is calibrated according to the CO$_2$ production relative to BSA of humans. To use the IRIS machine for mice, we took the published values for CO$_2$ production by weight (12) and converted them to CO$_2$ production by BSA using a well-validated formula (18).

The ability to follow changes in gastric emptying with progression of age and disease symptoms is a powerful aspect of a noninvasive technique like the breath test. We exploited this feature of the technique to follow the effects of development of diabetes on gastric emptying in NOD LtJ mice. However, it was necessary to identify the appropriate controls for our study and to follow changes in gastric emptying with age in those animals. NOR LtJ mice are commonly used as controls for NOD LtJ mice and are considered better controls than either Balb/c or C57Bl mice because the NOR LtJ strain has a $>80%$ genetic identity to the NOD LtJ mice and has many of the same immunological deficits, albeit without the tendency to develop diabetes (28, 33, 40). Therefore, it was unexpected to observe differences in gastric emptying with age between nondiabetic NOD LtJ mice and NOR LtJ mice. These data suggest that care needs to be taken when comparing gastric emptying times for a particular strain or transgenic animal with a different strain as a control, since false results may be obtained. The incidence of diabetes and levels of hyperglycemia in the NOD LtJ mice at our facility were similar to those reported previously (33, 46). We studied only female mice and observed that about one-half of the mice became diabetic between 8 and 26 wk old. Most of the remaining mice became diabetic between 26 and 40 wk old, but these mice were not reliably diabetic and often did not have sustained hyperglycemia, suggesting that they should not be used in studies on diabetes that require a uniform population.

A consistent finding of the present study was that gastric emptying of solids was reproducibly accelerated in the first 2 wk after the development of diabetes in NOD LtJ mice compared with both the normal range for all mice and with the control gastric emptying values for individual animals. This observation has not been reported previously in animal models of type 1 diabetes. However, faster gastric emptying in patients...
with type 1 and type 2 diabetes has been reported (23, 39), particularly in patients who do not report symptoms of gastrointestinal motility disorders (30). There is also one report of accelerated gastric emptying of solids in diabetic BB/Wor rats and in rats that developed diabetes after treatment with streptozotocin (30). However, these animals had accelerated emptying 36–67 days following onset of diabetes, which is quite different from the time course that we report here for NOD LtJ mice. The mechanism for faster emptying of solids from the stomachs of mice shortly after development of diabetes was not investigated in this study. It is unlikely that changes in blood glucose levels contribute to the observed differences in gastric emptying, since it is established that hyperglycemia slows and hypoglycemia accelerates gastric emptying (15, 38). Also, the average blood glucose levels in the mice was elevated at both 1 and 2 wk as well as at 3–5 wk and did not differ significantly between those time points. Intrinsic changes in the muscle, nerves, and/or interstitial cells of Cajal that regulate motility may account for faster emptying. Loss of nitricergic nerves and interstitial cells of Cajal has been demonstrated in animals with both longer-standing diabetes and delayed gastric emptying (31, 44, 50) and in patients with diabetic gastroparesis (19, 22). Treatment of diabetic mice with insulin resulted in normal gastric emptying and neuronal nitric oxide synthase (nNOS) levels (50), indicating that loss of nNOS leads to slowed gastric emptying. It is possible that a slight reduction in nNOS levels might lead to reduced inhibition of intrinsic excitatory pathways during early diabetes and an initial increase in gastric motility because of a relative increase in excitatory input. This increase in motility could be reversed when depletion of nNOS continues, resulting in loss of coordinated input to interstitial cells of Cajal and smooth muscle and therefore loss of coordinated contractions and pyloric relaxation. In contrast to smaller animals, the effects of inhibition of nitric oxide synthase activity in humans is not as well delineated, with either no effect on gastric emptying or an acceleration of liquid gastric emptying, perhaps reflecting a stiffer fundus, as reported previously (16, 26, 27). Alternatively, changes in extrinsic innervation and loss of either nNOS neurotransmission or interstitial cells of Cajal may occur early during diabetes, leading to accelerated gastric emptying, but later loss of both regulators of motility leads to delayed gastric emptying. Interstitial cells of Cajal in vitro are protected from depletion by insulin treatment, and loss of insulin appears to be the principal cause of interstitial cells of Cajal depletion in diabetic conditions (20). We have established that NO released from nitricergic nerves is necessary for complete development of intact interstitial cells of Cajal networks in the gastric body (7). Therefore, the hypothesis that currently has the most support is that loss of nNOS precedes the loss of interstitial cells of Cajal and that the loss of cytoprotective NO makes the interstitial cells of Cajal vulnerable to injury under diabetic conditions. Detailed studies on expression of neuronal and interstitial cells of Cajal markers will be necessary to test this hypothesis. Another possibility is that development of diabetes is associated with early changes in humoral factors that can change gastric emptying. Several humoral factors such as cholecystokinin, ghrelin, and neurotensin Y and incretins such as glucagon-like protein-1 and others (2, 6, 9, 11, 13, 14, 21, 36, 47) are known to alter gastric emptying, but it is not known whether their release changes in early diabetes.

In conclusion, we show that the [13C]octanoic acid breath test for gastric emptying of solids in NOD LtJ mice is reliable and responsive and have established normal values for this and other mouse strains. Using this method, we found a previously unrecognized reproducible acceleration in gastric emptying during the early stages of diabetes in NOD LtJ mice. This finding is of interest, since it will allow the investigation of cellular and molecular changes that accompany the event and may shed light on the human correlate of similar acceleration in gastric emptying sometimes seen in diabetic patients.

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GASTRIC EMPTYING IN NONOBESE DIABETIC MICE


