^{13}C isotopic abundances in natural nutrients: a newly formulated test meal for non-invasive diagnosis of type 2 diabetes

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Abstract

A new method to replace commercially prepared ^{13}C-labelled glucose with naturally available ^{13}C-enriched substrates could result in promotion of the clinical applicability of the isotopic breath test for detection of type 2 diabetes (T2D). Variation of the carbon-13 isotope in human breath depends on the ^{13}C enrichment in the diet taken by subjects. Here, we formulated a new test meal comprising naturally available ^{13}C-enriched foods and subsequently administered it to non-diabetic control (NDC) subjects and those with T2D. We found that the new test meal-derived ^{13}C enrichment of breath CO_2 was significantly lower in T2D compared with NDC. Furthermore, from our observations T2D exhibited higher isotopic enrichment of oxygen-18 (^{18}O) in breath CO_2 compared with NDC following ingestion of the new meal. We determined the optimal diagnostic cut-off values of ^{13}C (i.e. $\delta^{13}C‰ = 7.5‰$) and ^{18}O (i.e. $\delta^{18}O‰ = 3.5‰$) isotopes in breath CO_2 for precise classification of T2D and NDC. Our new method involving the administration of naturally ^{13}C-abundant nutrients showed a typical diagnostic sensitivity and specificity of about 95%, suggesting a valid and potentially robust global method devoid of any synthetically manufactured commercial ^{13}C-enriched glucose which thus may serve as an alternative diagnostic tool for routine clinical applications.

1. Introduction

Type 2 diabetes (T2D) is one of the major global health concerns of the 21st century. T2D involves a series of complications including cardiovascular disease, kidney damage (nephropathy), blurred vision (retinopathy) and nerve damage (neuropathy) during the course of the disease [1, 2]. Epidemiological studies have shown that about 415 million people worldwide are currently afflicted with T2D and the risk factors associated with the disease could affect several millions of people in the near future. Unfortunately, half of the world's population is totally unaware of the onset of the disease due to the asymptomatic nature of T2D in the early stages, suggesting the need to implement a preventive health care strategy. Traditionally, T2D is diagnosed by the oral glucose tolerance test (OGTT), which requires repeated sampling of blood following oral administration of a certain amount of glucose. Glycosylated haemoglobin (HbA1c) has been proposed to be a superior method to blood glucose estimation [3], but the results of HbA1c (%) may vary from patient to patient because of different haemoglobin levels present in the subjects. Sometimes, insulin resistance (IR) is targeted to identify the diabetic condition of patients, and consequently a hyperinsulinemic-euglycemic clamp study is performed to evaluate the IR of T2D patients. But the clamp study is difficult for routine clinical applications because it not only requires several blood samples but is also time-consuming and expensive [4].

Recently, a ^{13}C-glucose breath test has been proposed as an alternative non-invasive method to monitor insulin resistance in T2D [4–10]. The subject under

Table 1. Clinical parameters of the subjects (data are expressed as mean ± SD).

Parameters	Non-diabetic controls (NDC) ($n = 28$)	Type 2 diabetes (T2D) ($n = 23$)	p-value
Sex (male/female)	17/11	14/9	
Age (years)	33.2 ± 10.1	36.1 ± 8.5	>0.01
Weight (kg)	67.3 ± 9.5	63.74 ± 8.3	>0.01
Body mass index (kg m^{-2})	23.7 ± 1.9	24.8 ± 1.7	>0.01
Fasting plasma glucose (mg dl^{-1})	93.2 ± 10.3	138.6 ± 12.8	<0.001[a]
2-hr post-dose plasma glucose (mg dl^{-1})	119.6 ± 13.4	237.2 ± 49.2	<0.001[a]
HbA1c (%)	5.3 ± 0.3	8.6 ± 1.1	<0.001[a]

[a] Statistically significant difference ($p < 0.05$) between NDC and T2D.

investigation is allowed to take an oral dose of carbon-13 (^{13}C)-labelled glucose, which is metabolized in the body to produce isotope-enriched carbon dioxide ($^{13}CO_2$) which is subsequently detected in exhaled breath. Insulin assists in the promotion of glucose uptake into cells [11, 12]. In the post-dose state, the utilization of exogenous glucose for cellular energy production is decreased due to IR and the decrease in take up of exogenous glucose in T2D is monitored non-invasively from the recovery rates of ^{13}C-enriched CO_2 levels in exhaled breath. However, the poor availability and high cost of the synthetically labelled ^{13}C-enriched glucose have limited the widespread clinical applicability of the ^{13}C-GBT for non-invasive diagnosis of T2D. Therefore, there is a need to develop an alternative and effective test meal comprising easily available naturally ^{13}C-enriched substrates for early detection of T2D by means of ^{13}C breath tests. So far, to our knowledge, no study has reported any particular test meal derived from ^{13}C-enriched natural nutrients in the diagnosis of T2D.

However, as reported by Duchesne and co-workers [13, 14], the $^{13}C/^{12}C$ isotopic ratio in exhaled breath is largely dependent on the composition of the diet administered. In general, photosynthesis proceeds in such a way that the ^{12}C isotope in plants is slightly more enriched compared with the ^{13}C isotope. In nature, plants that have four carbon cycles (C4 plants) fix more ^{13}C atoms than C3 plants. Therefore, C4 plants such as maize and sugarcane are more highly enriched in ^{13}C than other vegetables and foods. Consequently, subjects will exhibit quite different $^{13}C/^{12}C$ isotope ratios in their exhaled breath CO_2 depending upon the consumption of ^{13}C-enriched foods [15]. The main aim of this study was therefore to explore a newly formulated test meal comprising naturally available ^{13}C-enriched foods which can be used in the ^{13}C glucose breath test for precise classification of non-diabetic control (NDC) and T2D subjects.

Moreover, some early studies showed that the oxygen-18 (^{18}O) isotope in breath CO_2 is associated with altered metabolism in T2D, regulated by the enzymatic activity of carbonic anhydrase (CA), a ubiquitous metalloenzyme present in the human body [16–18]. Therefore, another aim of the present study was to investigate the clinical feasibility of using analysis of ^{18}O isotopes in breath CO_2 for non-invasive diagnosis of T2D after ingestion of a naturally available ^{13}C-enriched test meal.

2. Materials and methods

2.1. Subjects

Fifty-one subjects comprising NDC ($n = 28$) and T2D ($n = 23$) were recruited in the current study. Fasting and post-prandial blood glucose as well as HbA1c (%) were recorded for the study. Individuals suffering from hypertension, interstitial lung diseases or chronic respiratory disorders were excluded from the study. Subjects with a previous history of diabetes or who were taking any medication that could alter carbohydrate metabolism were also excluded from the study. The study subjects were classified into two groups: NDC (HbA1c < 5.7% and 2-h OGTT < 140 mg dl^{-1}) and T2D (HbA1c > 6.5% and 2-h OGTT > 200 mg dl^{-1}), according to the standard protocol of the American Diabetes Association [19, 20]. Table 1 demonstrates the clinical parameters of the study subjects. The study protocol was approved by the Institutional Ethics Committee of Vivekananda Institute of Medical Sciences (registration no. ECR/62/Inst/WB/2013). Written informed consent was obtained from each subject prior to participation in the study.

2.2. Study protocol

We first selected a few control subjects ($n = 10$) for standardization of the test meal in our study. After overnight fasting (~10–12 h), we administered 150 ml of sugarcane juice (test meal 1) and collected post-dose breath samples every 30 min. The breath samples were analysed by a laser-based spectroscopic technique to measure the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotopic ratios of breath CO_2. We enrolled the initial 10 control subjects to ingest the following three test meals: test meal 2 (150 ml sugarcane juice and 50 g normal glucose), test meal 3 (150 ml sugarcane juice, 50 g normal glucose and 40 g cornflakes) and test meal 4 (150 ml sugarcane juice, 50 g normal glucose, 40 g cornflakes, 100 g yogurt and 20 g baby corn). Test meal 4 was considered as the final composite meal for our study. This final composite meal was administered to a few more NDC ($n = 28$) and T2D

($n = 23$) subjects. Post-dose breath samples were collected 30 min up to 2.5 h, whereas blood samples were collected 2 h after meal ingestion. We also performed the same breath test in the presence of 75 mg U-$^{13}C_6$-labelled D-glucose (CLM- 1396-CTM, Cambridge Isotope Laboratories, Inc., USA) along with 75 g of normal glucose dissolved in 150 ml of water. During the duration of the administration of the breath test subjects were not allowed to consume any kind of food or drink. Blood samples were used for the measurements of different clinical parameters such as plasma glucose concentration and HbA1c (%).

2.3. Breath sample analysis by an integrated cavity output spectrometer

Breath samples were analysed by a laser-based high-resolution integrated cavity output spectrometer (ICOS) (CCIA 36-EP, Los Gatos Research, USA) exploiting cavity-enhanced absorption spectroscopy for the simultaneous measurement of $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$ isotopes in breath CO_2 [21, 22]. The principle of cavity-enhanced absorption spectroscopy has been described elsewhere [23]. The ICOS system comprised a continuous wave diode laser (distributed feedback), operating at ~2.05 μm and a high-finesse optical cavity of length ~59 cm with two high-reflectivity ($R \sim 99.98\%$) mirrors attached at its two ends. Such an arrangement allows the laser light to reach an effective path length of ~3 km. The absorption spectra of $^{12}C^{18}O^{16}O$, $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$ were recoded at the wavenumbers 4874.178 cm^{-1}, 4874.448 cm^{-1} and 4874.086 cm^{-1}, respectively by probing the P(36), R(28) and P(16) ro-vibrational lines in the (2, 0^0, 1) ← (0, 0^0, 0) vibrational combination band of the CO_2 molecule [24].

2.4. Statistical method

For the statistical calculations, Origin Pro 8.0 (Origin Lab Corporation, USA) and Analyse-it Method Evaluation software (Analyse-it Software Ltd, UK, version 2.30) were utilized. A normality test was performed to check whether the data were normally distributed or not. To compare the dataset, one-way analysis of variance (ANOVA) (for normally distributed data), the Kruskal–Wallis test and the Mann–Whitney test (for non-normally distributed data) were performed. Receiver operating characteristic (ROC) curve analysis was performed to estimate the optimal diagnostic cut-off values for the tests. A p-value of <0.05 was assumed to be statistically significant.

3. Results

No significant difference was found in weight, height or body mass index (BMI) between the NDC and T2D. The plasma glucose (fasting and post-prandial) and HbA1c (%) were significantly higher in T2D than NDC.

To standardize the composition of the test meal, initially a test dose containing sugarcane juice (1.0971% excess of ^{13}C isotope) [25] was administered to a few control subjects ($n = 10$) in a fasting state. We found very little increment of mean $^{13}CO_2/^{12}CO_2$ isotope ratio [mean delta-over baseline (DOB) i.e. $\delta_{DOB}{}^{13}C = 4.2 \pm 1.1‰$ at 2 h of study] from the basal value in exhaled breath of the subjects (figure 1(a)). Moreover, natural glucose has been reported to be enriched with 1.0958% excess of ^{13}C [25]. Therefore, we next administered a test meal containing 150 ml sugarcane juice and 50 g normal glucose to the same control subjects in a fasting state. This time we observed a slight increment of mean $^{13}CO_2/^{12}CO_2$ isotopic ratio (mean $\delta_{DOB}{}^{13}C = 6.6 \pm 0.7‰$ at 1.5 h of study) in the breath CO_2 of these subjects (figure 1(a)). Furthermore, maize glucose has been reported to be associated with more ^{13}C isotopic enrichment (1.0995% excess of ^{13}C isotope) than normal C3 plant foods [25]. To check whether the incorporation of maize in our test meal would also increase $^{13}CO_2$ excretion in exhaled breath, we further added cornflakes (maize) to the composite test meal. Here, we found that after addition of 40 g cornflakes to the test meal, the $\delta_{DOB}{}^{13}C‰$ value was further enhanced to reach a mean value of $9.8 \pm 0.9‰$ at 1.5 h after administration of the test meal (figure 1(a)). Moreover, a few studies [25, 26] have reported that yogurt and baby corn are highly enriched with ^{13}C isotopes compared with normal foods. Therefore, we added these two foods to the test meal and made a composite meal consisting of 150 ml sugarcane juice, 50 g normal glucose, 40 g cornflakes, 20 g baby corn and 100 ml yogurt. After administration of the modified meal, we found a marked enhancement in the $^{13}CO_2/^{12}CO_2$ isotopic ratio (mean $\delta_{DOB}{}^{13}C = 14.7 \pm 1.3‰$) after 2 h of the study (figure 1(a)). We considered this as the final naturally ^{13}C-enriched composite test meal for our study on the diagnosis of T2D.

To address the feasibility of our new standardized test meal for detecting diabetes mellitus, we administered the final composite meal to several NDC ($n = 28$) and T2D ($n = 23$) patients after overnight fasting. We observed that the enrichment of $\delta_{DOB}{}^{13}C$ (‰) from the basal value was significantly higher in NDC than T2D 1 h after meal ingestion. To check the efficiency of our proposed method, we performed the whole study separately in the presence of the artificially prepared ^{13}C-enriched glucose meal and the naturally produced ^{13}C-enriched composite meal (figure 1(b)). Our study reveals that although the separation of $\delta_{DOB}{}^{13}C$ (‰) values between the two groups (NDC and T2D) for the artificial ^{13}C-glucose load was larger than the utilization of naturally ^{13}C-enriched foods, we can still selectively distinguish the T2D from NDC after 1 h of the excretion kinetics when the naturally available ^{13}C-enriched composite meal is administered.

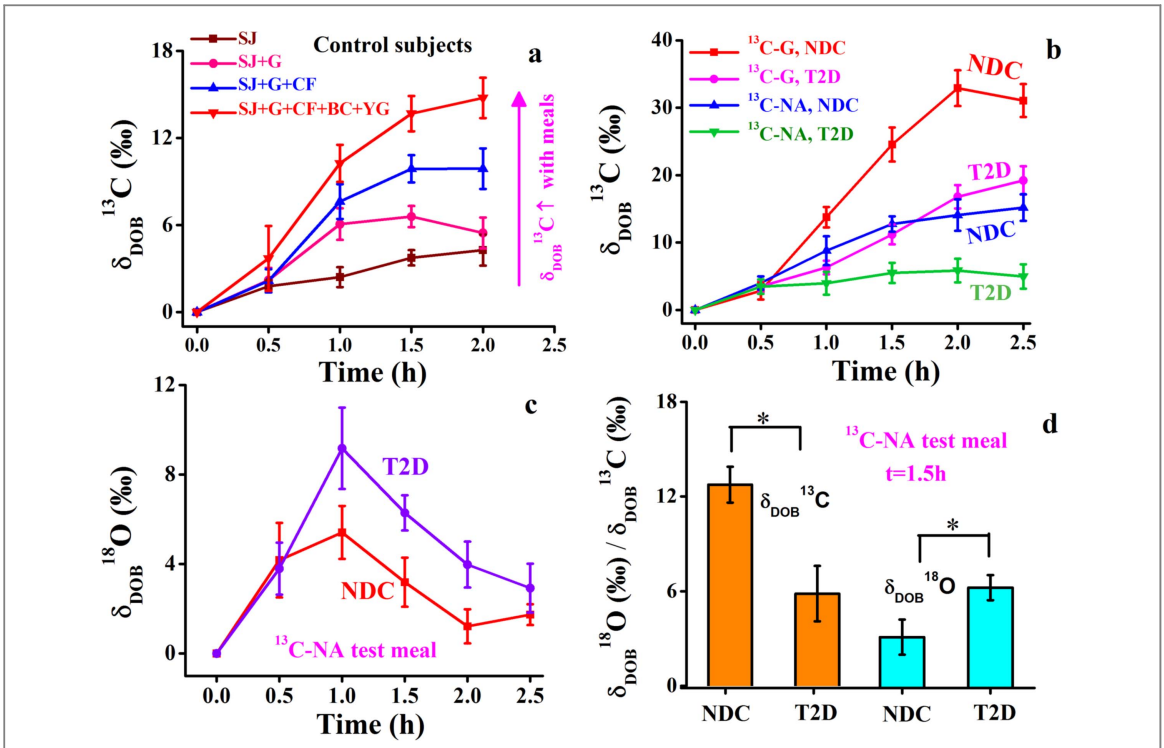

Figure 1. (a), (b) The excretion kinetic pattern of $\delta_{DOB}{}^{13}C$ (‰). (c) The $\delta_{DOB}{}^{18}O$ (‰) in NDC and T2D after administration of the natural abundance (NA) test meal . (d) A comparative representation of $\delta_{DOB}{}^{13}C$ (‰) and $\delta_{DOB}{}^{18}O$ (‰) at 1.5 h after the test meal load. SJ, sugarcane juice; G, glucose, CF, cornflakes; BC, baby corn; YG, yogurt. $^{*}p < 0.05$.

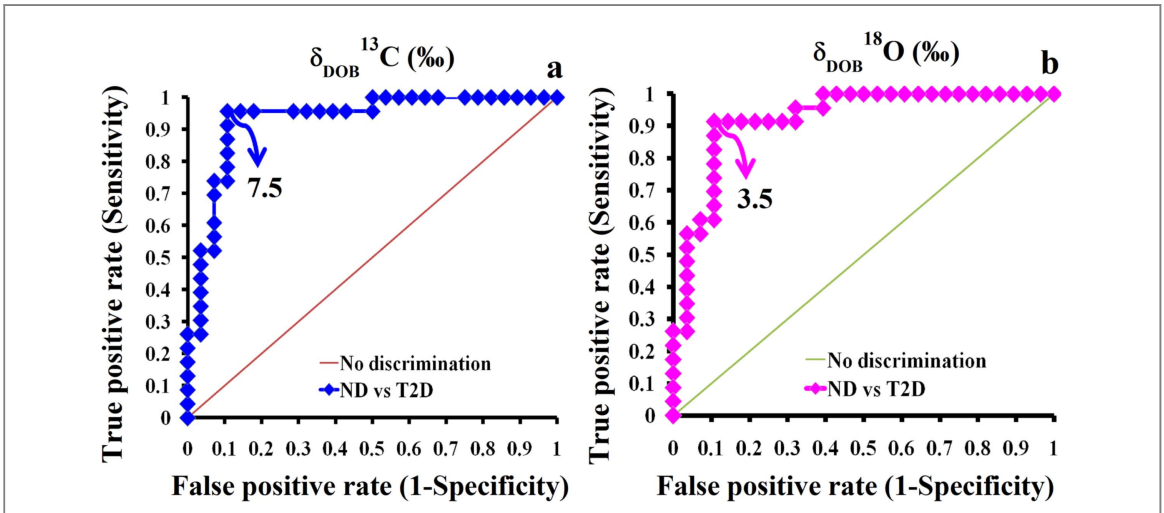

Figure 2. The optimal diagnostic cut-off values of $\delta_{DOB}{}^{13}C‰$ (a) and $\delta_{DOB}{}^{18}O‰$ (b) were estimated by utilizing ROC analysis for screening NDC and T2D subjects after the natural abundance test meal load.

We next investigated the isotopic fractionations of ^{18}O isotopes in breath CO_2 in response to the ingestion of our newly formulated naturally ^{13}C-enriched test meal. In the human body, the ^{16}O isotopes of $^{12}C^{16}O_2$ and ^{18}O isotopes of body water ($H_2^{18}O$) are rapidly exchanged during metabolism to produce $^{12}C^{18}O^{16}O$ in exhaled breath. We therefore measured the $^{18}O/^{16}O$ isotope ratios of breath CO_2 (i.e. $\delta_{DOB}{}^{18}O$ ‰) in T2D and NDC (figure 1(c)). Our results showed that the enrichment of ^{18}O isotopes in breath CO_2 was significantly higher in T2D compared with NDC, and this difference can clearly distinguish the two types of subjects 1 h after meal administration.

We finally determined the optimal diagnostic cut-off values of both ^{13}C and ^{18}O isotopes of breath CO_2 for precise classification of T2D and NDC. We utilized the ROC curves to determine the cut-off levels. We found that individuals with $\delta_{DOB}{}^{13}C‰ < 7.5$ and $\delta_{DOB}{}^{18}O‰ > 3.5$ were considered as T2D (figure 2), with a diagnostic sensitivity and specificity of about 91% and 89%, respectively (table 2), suggesting the broad clinical efficacy of our method exploiting a

Table 2. Optimal diagnostic parameters corresponding to cut-off values of δ_{DOB} ^{13}C (‰) and δ_{DOB} ^{18}O (‰) by the ICOS method for screening NDC and T2D individuals after administration of the naturally available ^{13}C-enriched test meal (AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value).

Group	Cut-off point	Sensitivity	Specificity	PPV	NPV	AUC	Accuracy
NDC versus T2D	$\delta^{13}_{DOB}C = 7.5‰$	95.7%	95.8%	88.0%	96.0%	0.93	92.1%
NDC versus T2D	$\delta^{18}_{DOB}O = 3.5‰$	91.3%	89.3%	88.0%	93.0%	0.92	90.1%

newly formulated test meal for non-invasive diagnosis of T2D.

4. Discussion

During the last few years the ^{13}C breath test has been considered for non-invasive assessment of IR. To enable the isotopic breath test to be used for routine clinical applications, considerable efforts have been devoted to formulate an alternative test meal rather than synthetically manufactured ^{13}C-labelled glucose. Here, our study demonstrates that a newly formulated test meal containing naturally available ^{13}C-enriched foods can detect differences in $^{13}CO_2$ abundances in exhaled breath of NDC and T2D subjects which has never been explored before. When an oral dose of naturally ^{13}C-enriched food is administered, entry of the isotope-enriched glucose into the cells is restricted due to IR in T2D, and consequently the production of $^{13}CO_2$ is impaired in exhaled breath. In our study, the data show a slower rate of excretion of $^{13}CO_2$ in individuals with T2D than in NDC subjects, supporting our assumption that the insulin-resistant T2D patients utilize the isotopic substituent at a slower rate than NDC individuals after the administration of a composite meal enriched in naturally available ^{13}C.

However, the study needs to be carried out in a large cohort of diabetic patients worldwide to confirm the accuracy of the ^{13}C-enriched composite meal as a cost-effective substitute for ^{13}C-labelled glucose to identify patients with IR (T2D). In our study, we prepared the test meal from the foodstuffs obtained from south-east Asia (India). Therefore, it would be interesting to perform further study with variations of foodstuffs produced from different parts of the world. The meal will also need to be standardized and packaged to make it commercially available in all parts of the world. The incorporation of ^{13}C-enriched C4 crops like corn/maize (*Zea mays*), sugarcane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*) and millet in the meal would enhance the accuracy of the breath test to identify patients with IR. However, the need for an expensive mass or laser spectrometer to analyse breath samples limits the clinical applicability of the breath test and negates the benefits of substituting expensive $^{13}C_6$-glucose with the naturally ^{13}C-enriched composite meal. Therefore further research is necessary to develop a simple cost-effective system for breath analysis to validate this method as a potential diagnostic tool for practical clinical application.

5. Conclusion

In conclusion, we have formulated a new test meal comprising naturally available ^{13}C-enriched foods which can be used for accurate evaluation of T2D without ingestion of commercially available ^{13}C-labelled glucose. By utilizing the newly formulated test meal, we were able to clearly distinguish T2D patients from NDC through the excretion kinetics of breath $^{13}CO_2$, thus opening a new pathway for the non-invasive diagnosis of T2D. Moreover, the determination of new cut-off values of ^{13}C and ^{18}O isotopes of breath CO_2 may assist in tracking NDC and T2D for practical clinical utilization. Finally, our new protocol for the ^{13}C breath test exploiting naturally ^{13}C-enriched foods is simple, thus suggesting the widespread clinical applicability of non-invasive diagnosis of T2D in a more robust way.

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