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Study of breath acetone and its correlations with blood glucose and blood beta-hydroxybutyrate using an animal model with lab-developed type 1 diabetic rats

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Breath acetone has long been known a biomarker for diabetes. However whether breath acetone analysis can be used for clinical applications ultimately depends on how breath acetone concentration is quantitatively and accurately related to an established clinical diagnostic parameter(s). Numerous studies of breath acetone using human subjects have been done, yet this fundamental question remains unaddressed because complex physiological processes and various conditions of humans are relevant to change of breath acetone. We report for the first time on the study of breath acetone and its correlations with blood glucose (BG) and blood β-hydroxybutyrate (BHB) using an animal model of rats. 18 non-diabetic healthy rats and 20 lab-developed Type 1 diabetic (T1D) rats were used as two subject groups. Breath gas samples from the rats were collected using a noninvasive oral intubation method that was compared with the intrusive and time-consuming method of tracheostomy. Breath acetone concentrations were measured using a cavity ringdown spectroscopy based breath analyzer without using additional sample preparation, sample pre-concentration, or calibration. The measured breath acetone concentrations were in the ranges of 1.9 – 4.3 ppm (part per million by volume) for the 20 T1D rats and 1.4 – 2.8 ppm for the 18 non-diabetic healthy rats. Simultaneous BG and blood BHB levels were also obtained. Results show that breath acetone, BG, and blood BHB in the T1D rat group all have significant difference from the one in the healthy rat group (P<0.05). A significant positive relationship (Pearson's r=0.644, P<0.05) between breath acetone and blood BHB was found to exist in both subject groups. A significant negative relationship ((Pearson's r=–0.678, P<0.05) between breath acetone and BG was found in the T1D rats only. However, the relationship between breath acetone and BG shifts from negative to weakly positive when T1D rats were treated by insulin. Furthermore, results from a multiple linear regression model analysis reveal that breath acetone has the predictive nature for blood BHB in T1D rats, which confirms the sole report in T1D human subjects. This result suggests that the animal model can be used for large scales of clinical study to help address fundamentals in human breath analysis in some cases.

1 Introduction

Breath analysis offers a stress-free method for potential disease diagnosis and therapeutic status monitoring through testing abnormal concentration of a biomarker or a specific pattern of multiple exhaled breath components. Breath acetone has long been known as a biomarker for Type 1 diabetes (T1D).[1–4] Quantitative measurements of breath acetone in well-defined T1D and Type 2 diabetes (T2D) were reported more than 60 years ago.[4] The published data to date using clearly-defined T1D patients concur that the mean elevated breath acetone concentration exists in T1D patients.[4] However the question of a quantitative correlation of breath acetone concentration with blood glucose (BG) level in T1D or T2D patients remains to be unaddressed. Of more than 40 independent studies that have involved more than 3,000 human subjects (T1D, T2D, and healthy), results fall mainly into one of the three categories[4]: some degree of positive correlation,[5–7] some degree of negative correlation,[8] and no correlation.[9,10] It is now clear

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that the relationship between a breath acetone level and a BG level and other bioinformatic parameters needs to be further investigated.

In addition to hyperglycemia, diabetic (T1D and some of T2D) patients frequently experience ketosis (hyperketonemia), in which serum concentration of the ketone bodies (acetoacetate (AA), β-hydroxybutyrate (BHB), and acetone) can exceed 25 mmol/L in severe cases.[11,12] Measurement of capillary levels of BHB has become routine in the management of young T1D patients.[9,13] In some cases, e.g. ketogenic diet treatments for intractable seizures,[14–16] blood BHB can be predicted by breath acetone; however relationship between breath acetone and blood BHB in T1D patients has not been explored, except a recent study that has illustrated the predictive nature of the breath acetone–blood ketone (BHB and AA) relationship in the cohort of 113 young T1D patients.[9]

Natural intra-individual biological and diurnal variability including age, gender, and diet was found to be correlated to the breath acetone concentration in previous publications.[17-19] Therefore, the objective of the present study was to measure breath acetone and explore its correlations with BG level and blood BHB level under the condition that has minimal to no baseline effect resulting from individual physiological heterogeneity. Breath analysis carried out using an animal model increases reliability of the information obtained from a particular experiment significantly because experimental parameters for animals can be more readily controlled than humans.[20–22] Some animals such as rats that are frequently used in research share more than 98% DNA with humans; thus an animal model of T1D rats that are developed artificially can be used to help tackle the fundamental question of correlations of breath acetone with BG and blood BHB. Studies of exhaled breath in rats with asthma,[22,23] ketogenic diet,[16] sepsis,[24,25] parkinsonism disease,[20] neurofibromatosis,[26] allergic airway inflammation,[27] lung disease,[28,29] and steatohepatitis[30] have been reported. However, to the best of our knowledge, no study has reported on exhaled breath acetone analysis in diabetic rats.

In this work, breath acetone from 20 T1D and 18 non-diabetic healthy rats were measured using a cavity ringdown spectroscopy (CRDS)-based breath acetone analyzer. The advantage of cavity ringdown breath analyzer used for breath analysis compared with gas chromatography–mass spectrometry (GC–MS) that is a widely accepted analytical method for trace-gas analysis was discussed in our previous works.[31] Basically, current GC-MS is not suitable for developing a point-of-care (POC) clinical instrument for real- time on-line breath analysis, partially due to its complicated sample preparation, time-consuming test procedure, and large instrument geometry. Concurrent BG levels and blood BHB in T1D and non-diabetic healthy rats were also measured. Here we report our findings in breath acetone in rats and relationships of breath acetone with BG and blood BHB.

2 Materials and Methods

2.1 Development of an animal model of rats

38 Sprague-Dawley male rats (200 – 300 g, 10 weeks old) were obtained from the Beijing HFK Bioscience. An experimental protocol was approved by the Ethical Committee of Animal Research at the Institute of Biomedical Engineering, Chinese Academy of Medical Sciences (IBME). All rats were housed in a temperature and humidity controlled room (18–25 ºC, relative humidity of 40%–70%) in the Laboratory of Animal Center at IBME. No more than five rats were housed in each polycarbonate cage containing wood shavings. The rats were fed by a commercially available rat pellet diet and tap water freely throughout the experimental period.

A rat model of T1D was developed via a single intravenous injection of streptozotocin (STZ, Sigma-Aldrich, USA) in a rat, which caused a severe damage of islet β cells and resulted in less quantity of insulin secretion. In this study, 20 rats received the single intravenous injection of STZ with a dose of 45 mg/kg by weight. The STZ were dissolved in a citric acid-sodium citrate buffer (pH 4.4) at the concentration of 20 mg/mL and used within 10 minutes. One week after the STZ injection, the BG level in the blood collected from the tail vein was measured with a miniature glucose meter (Roche, Switzerland). Rats with a non-fasting BG level >16.7 mmol/L were considered as diabetic. Also, pancreas slices from one non-diabetic healthy rat and 4 T1D rats developed by the injection of STZ were analyzed individually using the hematoxylin-eosin staining method (Tianjin Yishengyuan Biotech Co. Ltd, China).

Figs. 1 (a), (b), and (c) show images of pathophysiological analysis of the pancreas biopsies from the non-diabetic healthy rat. The images in (a), (b), and (c) have magnifications of 40, 100, and 400, respectively. Figs. 1 (d), (e), and (f) show images of pathophysiological analysis of the pancreas biopsies from the T1D rat with the magnification of 40, 100 and 400 respectively. The

pathophysiological analyses of the pancreas biopsies from another 3 T1D rats with a magnification of 400 were shown in Figs. 1 (g), (h), and (i). As can be seen in Fig. 1, the normal pancreas islet from the non-diabetic healthy rat has the clear islet boundary and a relatively large amount of healthy β cells; but the pancreas islets from the 4 T1D rats have collapsed, which showed atrophied tissue structures, reduced amount of normal shape β cells, and local inflammation. This pathophysiological difference indicates that the T1D rat model was developed successfully.

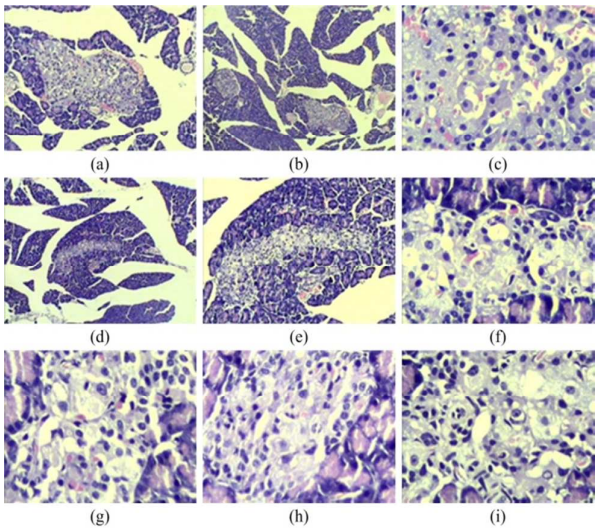
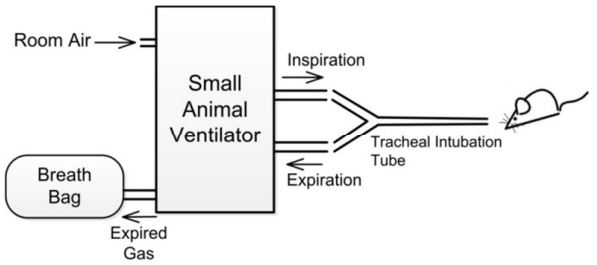


Fig. 1 Pathoghysiology of pancreas slices from one non-diabetic healthy rat (a – c) and four T1D rats (d – i).

2.2 Methods of breath gas collection in rats

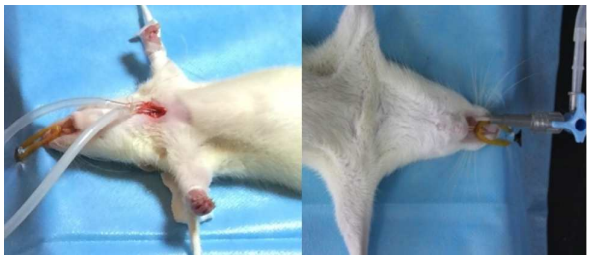
Unlike breath gas sampling in human subjects, breath gas collection in rats requires additional effort with well-designed procedures. The schematic diagram and the experimental setup for breath gas sampling were shown in Figs. 2 (a) and (b), respectively. In this work, both methods of intrusive tracheostomy and nonintrusive oral intubation were used to collect breath gas samples, as shown in Fig.3. In each method, rats were anesthetized via an intraperitoneal injection of chloral hydrate aqueous solution (10%) with a dose of 0.03 mL/kg by weight.



(a)

(b)

Fig. 2 The schematic diagram (a) and experimental setup (b) for breath gas sampling from a rat.



(a) (b)

Fig. 3 Breath gas sampling from a rat by (a) tracheotomy and (b) oral intubation.

There are three steps to be carried out for the tracheostomy of the rat, as illustrated in Fig.4. First, a slit incision (5 – 8 mm) was made in the neck of an anesthetized rat (see Fig.4 (a)). Second, a small incision (~ 1 mm) was picked open on the trachea with circular cartilage tissue (see Fig.4 (b)). Third, one port of a customized glass stopcock (a 3-way tubing from Tianjin Yishengyuan Biotech Co. Ltd, China) of 1 mm in diameter was inserted into the trachea of the rat through the small incision (see Figs.4 (c) and (d)). The whole procedure took about 5 minutes.

In the non-invasive oral intubation method, a flexible tube connected to one port of a customized glass stopcock was intubated into the trachea of the anesthetized rat through the oral. The other two ports of the stopcock were connected to the inspiration and expiration (see Figs. 2 (a) and (b)) of the small animal ventilator (ALC-V8S， Shanghai Alcott Biotech Co. Ltd， China) by the

section of the tubing with an inner diameter of 3 mm. In the experiment, breath frequency, breath ratio, and tidal volume of the small animal ventilator were adjusted for each individual rat to make the rat breath spontaneously. There are two ports on the back panel of the small animal ventilator, as shown in Fig. 2, one was used as the gas inlet that was connected to room air directly, the other was the gas outlet that was connected to a fluorinated ethylene propylene (FEP) breath gas bag (1 L) for breath gas collection from the rat. In this work, all breath samples were collected from the rats with non-fasting. The FEP sampling bag can keep breath gas fresh for up to 6 hours (h). The breath samples were stored in a portable thermal isolated box (17 L), and all samples were tested using the breath acetone analyzer within 6 h after a breath gas collection. BG and blood BHB were measured using a standard diabetic management BG meter (Roche, Switzerland) and a blood BHB meter (Optium Xceed, Abbott, USA), respectively. All rats were euthanized (via with 1–2 mL of air injected into the tail vein) after breath gas collection.

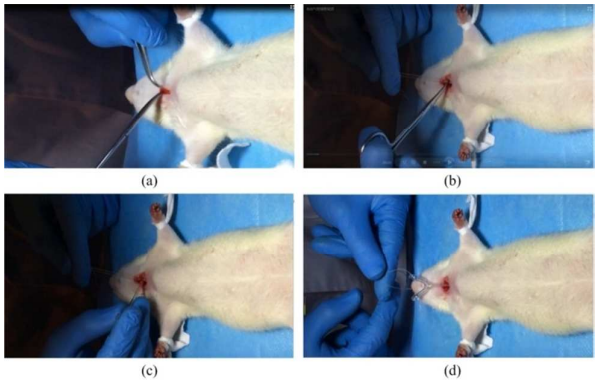


Fig. 4 The main three steps of the tracheostomy for the rat.

2.3 Ringdown breath acetone analyzer

Fig. 5 shows a schematic diagram of the near-real time CRDS-based breath acetone analyzer. Details of the breath analyzer can be seen in elsewhere [e.g. Ref. 32]. A Q-switch Nd:YAG laser (Changchun New Industries Optoelectronics, MPL-F-266nm, China) was used as the light source. The laser operated at 266 nm with a repetition rate of 1 kHz and single-pulse energy of 4.5 μJ, with a compact laser head (238.5 mm × 88 mm × 74 mm). Except for an iris and two reflective mirrors, which were used to direct the laser beam, no mode-matching optical components were used to couple the laser beam into the ringdown cavity. The gas cell consisted of a 50-cm long stainless steel pipe with an inner diameter of 3.81 cm (CRD Optics Inc., U.S.), one pair of mirror mounts (CRD Optics Inc., U.S.), and one pair of high-reflectivity UV mirrors (Los Gatos Research, U.S., R = 99.9956%, radius of curvature = 1 m). Each mirror mount held a mirror whose position could be adjusted in multiple dimensions via three alignment screws. The gas cell was equipped with three ports, as shown in Fig. 5, one was used as a gas inlet, and another two were gas outlets that were connected to a vacuum pump (Oerlikon, SC5D, Germany) and a micro pressure manometer (MKS870B, U.S.). In order to make sure the gas cell was not contaminated, high-purity nitrogen (>99.99%, DaFang Special Gas, China) was used to flush the gas cell each time after one breath sample was tested. The ringdown decay waveform captured by a photo multiplier tube was input to an oscilloscope for display. A ringdown waveform was digitalized to 10,000 data points and transferred to a laptop computer via a GPIB-USB cable (National Instrument GPIB-USB-HS). The in-house developed ringdown software was installed in the computer to obtain a ringdown time by fitting the ringdown data points to a single exponential decay.

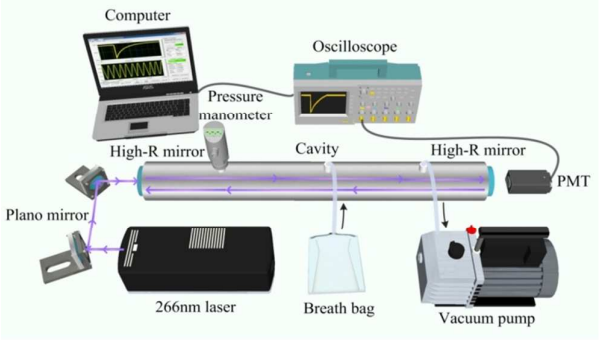


Fig. 5 Schematic diagram of the ringdown breath analyzer.

2.4 Determination of acetone concentration

Absolute concentration of acetone in a breath sample was determined by the background subtraction method which was described in detail in our previous work.[7, 33, 34] The absorbances of atmosphere (A_{atm}) and breath gas (A_{breath}) were expressed by equations (1) and (2),

$$A_{atm} = \sigma(\nu)nd = d/[c(1/\tau_{atm} - 1/\tau_0)] \quad (1)$$

$$A_{breath} = \sigma(\nu)nd = d/[c(1/\tau_{breath} - 1/\tau_0)] \quad (2)$$

where $\sigma(\nu)$ is the absorption cross-section of the transition line at frequency ν, n is the sample concentration, d is the distance between the two mirrors, c is the speed of light, τ_{atm} and τ_0 are the ringdown times when the gas cell is filled with laboratory air to 1 atm and

under vacuum, respectively. τ_{breath} is the ringdown time detected when the gas cell is filled with breath gas.

In this study, absorbance of the atmosphere is used as the background baseline. Then the absolute concentration of breath acetone (the upper limit[7, 33]) in a breath gas is obtained by

$$\Delta A = A_{breath} - A_{atm} = \sigma(\nu)nd \quad (3)$$

where ΔA is the absorbance difference. The absorption cross-section of acetone at 266 nm is 4.5×10^{-20} cm^2/molecule at atmospheric pressure and room temperature.[35]

The rationale of the background subtraction method includes an assumption that the absorbance difference is attributed to the absorption of acetone alone. The reliability of this assumption has been evaluated in the previous study by investigating the possible absorbance from other atmospheric molecules and high abundance breath volatile organic compounds (VOCs).[33] The gas exchange based on the inhalation of air in rats is assumed to be the same as in human, because the rats that are frequently used in research share more than 98% DNA with humans. Breath gas components in normal human beings have been well known, e.g. including about 300 high abundance chemicals or up to more than 3000 species in total; however, no similar database is available for rats (the rats often used for research). Since more studies of breath in rats have been done, quantification and identification of breath gas components in rats may be an interesting subject of future study. In the current work, we cautiously assumed that the method can be used for rats sampled.

Prior to the full scale of measurements of breath acetone in the 38 rats, the CRDS-based breath analyzer was validated using a certified GC-MS facility through comparing breath acetone concentrations from human breath samples measured using both methods, which was conducted in our previous works.[32, 36] A linear fitting of the results from both methods (CRDS and GC-MS) indicated that the obtained acetone concentrations using both methods were consistent, which confirmed that this fast (~1 s), sample pre-concentration-free ringdown breath acetone analyzer was a reliable instrument for measurement of breath acetone.

3 Results and Discussion

3.1 Comparison of two different methods for breath gas collection

The method of tracheotomy that requires incision of rats is intrusive and more time consuming. The nonintrusive oral intubation method has no physical damage to rats.[22, 23] Comparatively, the intubation method is preferred if it is as reliable as the intrusive method. In order to investigate reliability of the oral intubation method, 15 breath samples collected from T1D rats using both methods were tested. Fig. 6 shows the result of the comparison of the measured acetone concentrations using the different sampling methods. The slope (k = 1.04) in the fitting equation suggests that the obtained acetone concentrations using both sampling methods are consistent. This test confirmed that breath gas collection using the non-invasive oral intubation method was reliable; subsequently, the oral intubation method was used for the rest of measurements of breath acetone.

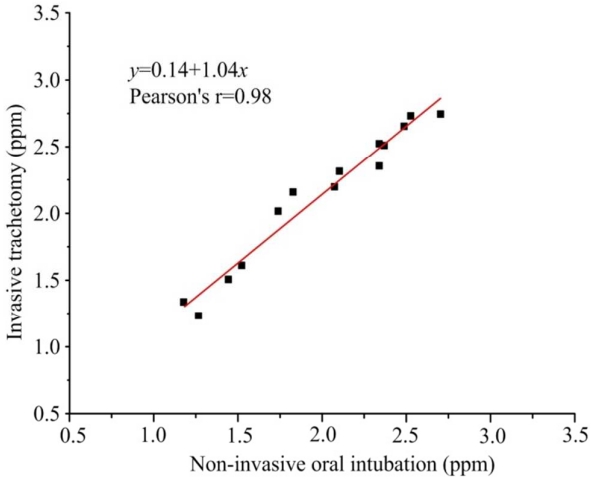


Fig. 6 Comparison of breath acetone measurements using the invasive tracheotomy sampling method with that from the non-invasive oral intubation sampling method.

3.2 Relationship between breath acetone and blood glucose

The data analysis was carried out using a statistical software program (IBM SPSS Statistics19.0). Significance was set at P<0.05. Breath acetone, BG, and blood BHB from the 20 T1D rats (group n1) and the 18 non-diabetic healthy rats (group n2) were measured. The statistical information and test results were listed in Table 1.

Table 1. Statistical information and test results of the healthy and T1D rats.

Factor	Numbers	T1D (n1)	Healthy (n2)	P value
Acetone (ppm)	n1=20, n2=18	3.0±0.6 (1.9–4.3)[a]	2.3±0.4 (1.4~2.8)	<0.001

BLG (mmol/L)	n1=20, n2=18	30.1±3.7 (21.6–33.3[b])	12.6±4.0 (5.6–18.4)	<0.001
BHB (mmol/L)	n1=12, n2=11	1.2±0.3 (0.8–1.8)	0.9±0.2 (0.5–1.1)	<0.05
Weight (g)	n1=20, n2=18	248±31 (196–313)	261±20 (240–308)	0.137

[a] Values presented as mean±SD (min–max). Acetone and weight were normally distributed and were evaluated using Independent Samples Test, In contrast, BGL and BHB were not normally distributed and were analyzed by Kruskal-Wallis test.
[b] The number of 33.3 is the upper limit of the BG meter used.

As can be seen in Table 1, breath acetone in the T1D rat group has a significant difference from the one in the healthy rat group (P<0.001). Ranges of the breath acetone concentration were 1.9 – 4.3 ppm (part per million by volume) for the 20 T1D rats and 1.4 – 2.8 ppm for the 18 non-diabetic healthy rats. The determined mean value of 3.0 ± 0.6 ppm in the 20 T1D rats is higher than that (2.3 ± 0.4 ppm) in the 18 non-diabetic healthy rats.

Determination of breath acetone in T1D human subjects was conducted in numerous previous studies, in some of which parallel BG levels were also determined. The reported relationships between breath acetone and BG level are arguably inconsistent. There have been a few datasets showing some degree of positive correlation of breath acetone concentration with BG level under particular data process algorisms[7] and or using a particular sampling control,[5,6] Whereas some studies reported no correlation of breath acetone concentration with BG level.[9,10] Nevertheless, in this animal-model study we found a negative relationship between breath acetone concentration and BG level in the 20 T1D rats (Pearson's r=−0.678 , P<0.05), as shown in Fig.7 (a). It should be noted that the BG level in the T1D subjects, as shown in Fig. 7 (a), was extremely high, which is far higher than the BG level that most diabetic patients would be able to tolerate. However, no correlation was found between breath acetone concentration and BG level in the 18 non-diabetic healthy rats, as shown in Fig.7 (b).

It should be stressed that this negative relationship between breath acetone concentration and BG level found in the T1D rats cannot be used to mislead a conclusion that this trend is agreeable or disagreeable with the trends reported for human subjects. To date, numerous studies and publications about breath acetone in human subjects (healthy, T1D, or T2D subjects) have been reported, however, whether breath acetone concentration and BG level have a positive or negative correlation still remains to be a unanswered question; and no single study has been considered overwhelmingly a taxogram in terms of sufficiency in the scale of sampling, longitudinal experimental time, cross sectional comparison, interference control, sampling precision, and calibration of a measuring method, classification and clarification of different types of subjects, control in physiological heterogeneity, etc. From this point of view, a rat model, as a standalone sampling target, may provide an invaluable venue to tackle the fundamental, e.g. whether or not there exists a relationship between the two quantities, not necessarily be compared with those in human beings.

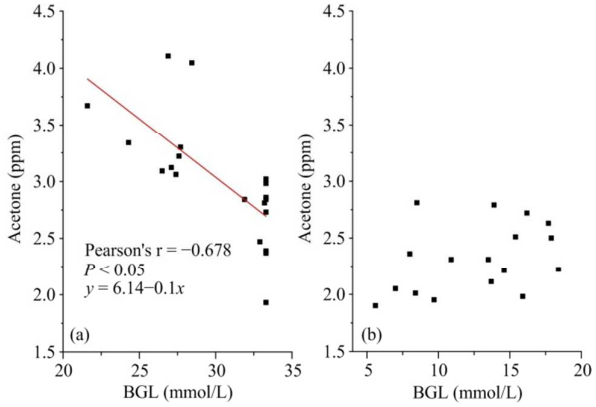


Fig. 7 The measured breath acetone concentration versus BG level (a) in T1D rats and (b) in healthy rats.

Variations of breath acetone concentration versus BG level at different times during a day were also investigated in the current study. A healthy rat, a rat on the 3rd day after STZ injection (3DSI), and two STZ-induced T1D rats were randomly selected for continuous measurements of breath acetone for 6 h.

Observed variations in breath acetone and the simultaneous BG levels in the healthy rat, the 3DSI rat, and the STZ-induced T1D rats during the 6 h monitoring are shown in Fig. 8. The ranges of breath acetone concentration were 1.9 – 2.5, 1.9 – 6.1, 2.1 – 7.5, and 1.9 – 4.3 ppm, for the healthy, 3DSI, and two T1D rats, respectively. The highest BG levels of 20.1, 26.5, 25.4, and 29.6 mmol/L occurred at 1:43 pm, 11:33 am, 10:15 am, and 9:41 am for the healthy, 3DSI, and two T1D rats respectively. Negative correlation patterns were found in the two T1D rats (Figs. 8 (c) and (d)) and the 3DSI rat (Fig. 8 (b)), which are in general agreeable with the finding of negative

relationship between breath acetone concentration and BG level in the 20 T1D rats.

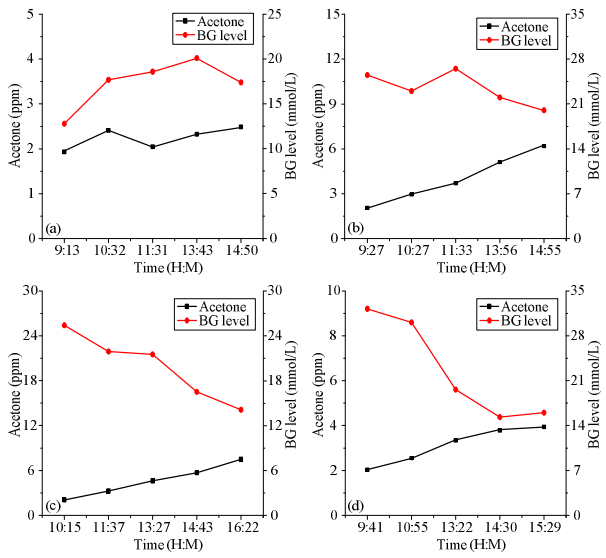


Fig. 8 The variations in breath acetone and the simultaneous BG level in (a) the healthy, (b) the 3DSI, and (c and d) two T1D rats during 6 h monitoring.

Further, five T1D rats were randomly selected for the insulin therapy to explore how breath acetone changes along with varying BG levels. Insulin was injected into each of the five rats with a dose of 8 u/kg for five days. Breath gas samples from the T1D rats were collected and measured on the third day and the fifth day repeatedly. As can be seen from Fig. 9, it is very interesting that the relationship between breath acetone and BG shifts from negative to positive when BG from exceedingly high concentrations (Fig.7 (a), to the concentrations that are more close to those for normal subjects (Fig.7 (b)). And even a very weak positive relationship between breath acetone and BG can be found. This result suggests that the relationship between breath acetone and BG changes with the BG range. To the best of our knowledge, this finding was the first time to be reported.

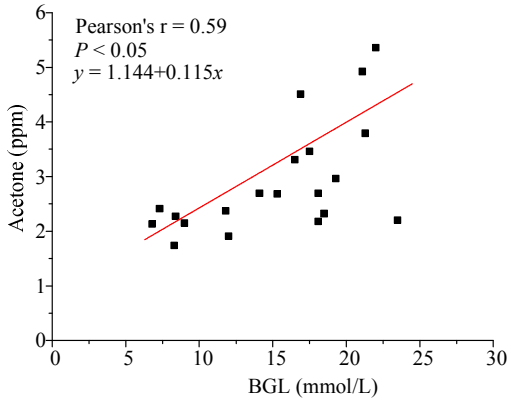


Fig. 9 The measured breath acetone concentration versus BG in the rats under an insulin therapy.

3.3 Relationship between breath acetone and blood BHB

Blood BHB in the T1D rat group has a significant difference from the one in the non-diabetic healthy rat group (P<0.05), as can be seen in Table 1. The determined mean value of blood BHB in T1D was 1.2 ± 0.3 mmol/L, which is higher than that in the non-diabetic healthy rats, 0.9 ± 0.2 mmol/L. Although this result was obtained from the rats, the trend is consistent with the one from human subjects. For instance, Type 1 diabetic patients have frequent episodes of ketosis and have elevated levels of ketone bodies in their circulations. Even at the time of a routine check-up visit in a clinic, ketonemia levels at 1 – 2 mmol/L are frequently seen in Type 1 diabetic patients while the concentration in healthy individuals is lower than 0.5 mmol/L.[11]

Fig. 10 (a) shows a significant positive relationship between breath acetone concentration and blood BHB level in the 20 T1D rats (Pearson's r=0.644, P<0.05). This result, although it is obtained from the rats, further confirms the very recent report on a significant positive relationship between the two parameters in young T1D patients.[9] In that study breath gases from 113 T1D human subjects on a single visit in a routine hospital clinic were analyzed using a soft-ionization mass spectrometer. The results were compared with capillary blood ketone with relatively low levels of 0 – 0.4 mmol/L of BHB taken at the same time. A significant positive relationship (P<10^{-4}) was found between breath acetone and blood BHB in that study. Except for the positive relationship between breath acetone and blood BHB found in T1D human subjects, similar positive relationships between the two parameters were also

found in humans with fasting ketosis[37, 38] and nutritional ketosis[14,15] where ketone is produced in healthy individuals by fasting or nutritional interventions.

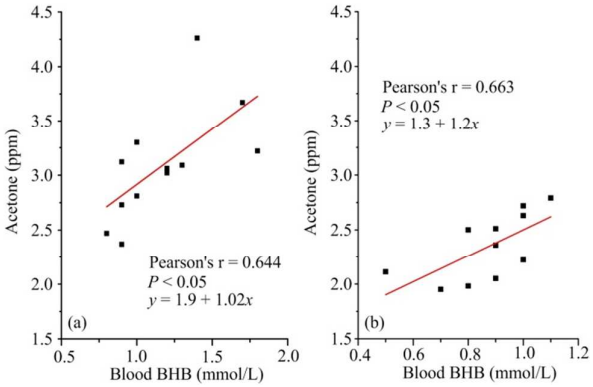


Fig. 10 The measured breath acetone concentration versus blood BHB (a) in T1D rats and (b) in healthy rats.

In order to further confirm the predictive nature of the breath acetone for BHB, one healthy rat, one 3DSI rat, and two STZ-induced T1D rats were randomly selected for continuous measurements of breath acetone for 6 h. The observed variations in breath acetone and the simultaneous blood BHB in the healthy, the 3DSI, and the STZ-induced T1D rats during the 6 h monitoring are shown in Fig. 11. The ranges of breath acetone concentrations were 1.5 – 2.2, 1.9 – 6.1, 2.5 – 8.0 and 2.0 – 3.6 ppm for the healthy, the 3DSI, and two T1D rats, respectively. As can be seen in Fig. 11, BHB increased with time, and positive correlated patterns were found in the four rats, which further confirmed that blood BHB is predictable using breath acetone.

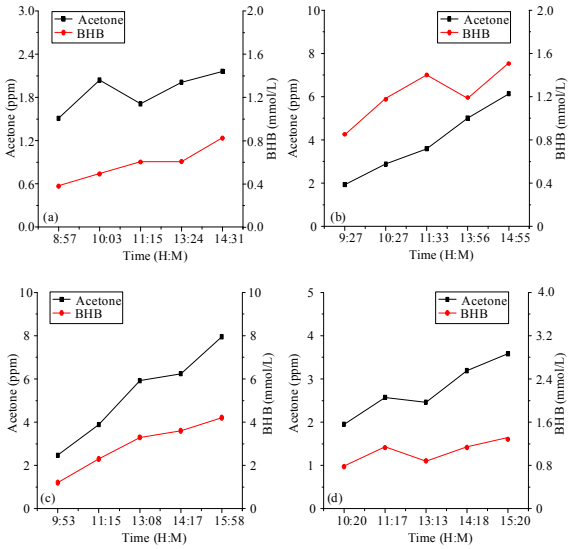


Fig. 11 The variations of breath acetone and the simultaneous blood BHB level in (a) the healthy, (b) the 3DSI, and (c and d) two STZ-induced T1D rats during the 6 h continuous monitoring.

3.4 Multiple linear regression analysis

Multiple linear regression analysis[39] was used to develop a model for sensitivity analysis. The purpose was to examine how breath acetone concentration is sensitive to a measured variable, e.g. BG, blood BHB, Type (Assignment: 0 for healthy, 1 for diabetes), or body weight. . The model consisting of blood BHB and Type had a significantly predictive value for breath acetone (Decision coefficient R^2= 0.684, ANOVA comparison of models produced P<0.0001); but when including BG and body weight the model showed no significant improvement. As we can see in Table 2, blood BHB was by far the most significant variable (Regression coefficient: blood BHB 1.05, Type 0.43). This multi-variation analysis showed that blood BHB and Type served as the independent factors associated with breath acetone concentration. Therefore, the result suggests the predictive nature of breath acetone concentration for blood BHB level in the T1D rats.

Table 2. Multiple linear regression analysis of the measured variables.

	Regression coefficient	Standard error	T-test value	*P* value
Intercept	1.43	0.26	5.48	<0.001

BHB	1.05	0.28	3.80	<0.05
Type	0.43	0.16	2.65	0.137

From Table 3, the regression equation for the predictive model was expressed by $y = 0.43x_1 + 1.05x_2 + 1.43$, where y is breath acetone concentration, x_1 is Type, x_2 is blood BHB. In order to verify the predictable ability of the model, breath gas from additional 10 healthy rats and 10 T1D rats were collected and tested using the ringdown breath acetone analyzer. Simultaneous blood BHB for the health and T1D rats was also measured. The correlation between measured breath acetone concentration and predictive breath acetone concentration was shown in Fig. 12. The coefficient of correlation R, 0.95 indicates that the model have a good prediction ability for breath acetone using BHB and Type.

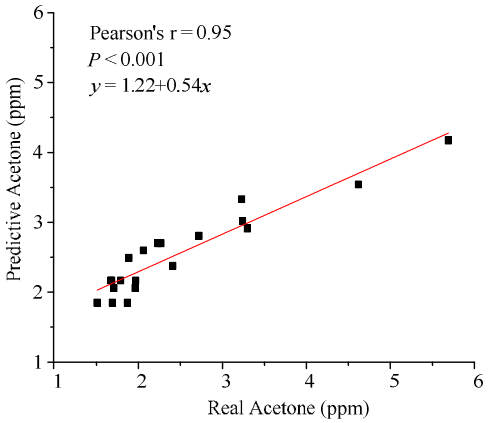


Fig. 12 The measured breath acetone concentration versus predictive breath acetone concentration.

4 Conclusions

In this work, determination of breath acetone in T1D and healthy rats using the GC-MS validated ringdown breath acetone analyzer was conducted. The method of the breath gas collection in rats using the non-invasive oral intubation method was compared to the invasive tracheotomy method. For the first time, breath samples from the 20 T1D and 18 non-diabetic healthy rats were collected and breath acetone was determined. Simultaneous BG and blood BHB levels were also measured.

It was found that breath acetone, BG, and blood BHB in the T1D rat group all have significant difference from the one in the healthy rat group (P<0.05). A significant positive relationship between breath acetone and blood BHB was found to exist in both the 20 T1D and the 18 healthy rats. Furthermore, a positive correlation of breath acetone with BHB was also found during the 6 h continuous monitoring. A significant negative relationship between breath acetone and BG was found in the 20 T1D rats. Meanwhile, negative correlation patterns of breath acetone versus BG were found in all T1D rats used for the 6 h continuous monitoring. However, the relationship between breath acetone and BG shifts from negative to weakly positive when T1D rats were treated by insulin. No correlation between breath acetone and BG was found in the 18 non-diabetic healthy rats. The results from a multiple linear regression model analysis reveal that breath acetone has the predictive nature for blood BHB in T1D rats, and the predictable ability of the model was verified by testing the breath acetone and simultaneous blood BHB from additional 10 healthy rats and 10 T1D rats. The new findings suggest the predictive nature of breath acetone for blood BHB in T1D rats though further study with a larger number of subjects needs to be carried out. This result further confirms the very recent report on the similar finding in T1D human subjects.[9]

This study supports that conducting breath analysis using an animal model of rats is a useful protocol for breath research. Additionally, study of breath acetone using the combination of the T1D rat model and the near-real time on-line ringdown breath acetone analyzer, as described in this work, may help address the question of quantitative correlations in breath analysis, because (1) the T1D animal model has a higher rate of standardization than T1D human patients due to reduction in variations of breath acetone concentration caused by different physiological conditions and (2) the ringdown breath analyzer, with sample pre-concentration-free and high data throughput, allows breath analysis to be conducted with a large number of samples in a short period of experimental time.

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