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Non-invasive monitoring of diabetes through analysis of the exhaled breath condensate (aerosol)

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ABSTRACT

We show that exhaled breath condensate (EBC) contains glucose (≈ 0.01 mM for healthy subjects), in contrast to previous works reporting minimal glucose content in EBC. The evaluated breath condensate glucose levels correlate positively with blood glucose levels, thus offering the prospect of a non-invasive approach to the monitoring of diabetes.

1. Introduction

According to the World Health Organization, 347 million people (≈ 5% of the world's population) suffer from diabetes. Predicted to become the seventh leading cause of death, diabetes is dangerous because of its complications: cardiovascular diseases, blindness, risk of amputation, kidney failure etc. Glucose concentration in blood is the key parameter for diabetic patients: maintaining it at an appropriate level allows these complications to be postponed.

Non-invasive methods, which exclude not only injury to blood vessels, but also damage to the skin surface, are preferred for diagnostics: such methods are painless and avoid potential infection and trauma to patients. However, despite continuing efforts, the problem of non-invasive evaluation of blood glucose concentration has not yet been solved. The most promising approach appeared to be detection of blood glucose through the skin using near-IR spectroscopy. However, this method did not achieve the required sensitivity [1–4]. Transcutaneous glucose delivery, for example by 'iontophoresis' [5,6] has not been successful in commercialized devices.

Except for detection of glucose, it is possible to monitor diabetes on the basis of secondary metabolites. In particular, the detection of exhaled acetone has been considered for this purpose. However, despite a number of existing analyzers, the question of how the breath acetone measurement is related to the blood glucose level remains to be answered [7].

Exhaled breath condensate (EBC) is already known as an excreted liquid with high diagnostic potential [8–11]. It is sampled by condensation of breath aerosol, which is formed through respiratory fluid film or bubbles bursting during opening of the bronchioles [10]. The airway lining fluid metabolite concentrations are dependent on their content in the blood [8,10].

Unfortunately, breath condensate has not been considered an appropriate candidate for monitoring diabetes due to the reported small levels of glucose content in EBC (0.1–0.2 μM) [12,13]. By comparison, despite the lactate concentration in blood (0.5–2 mM) being lower than that of blood glucose (> 4 mM), the lactate content in EBC has been found to be two orders of magnitude higher (≈ 0.02 mM [14,15]).

Another contradiction has been found when considering the dilution rate (ratio of substance content in blood to its concentration in EBC) of inorganic ions. The latter (particularly sodium and chloride ions) can be employed as an 'internal standard' for excreted liquids due to their concentration in blood remaining nearly constant over a short time scale. The concentrations of Na^+, K^+, and Cl^- in breath condensate have been reported to be a hundred [16] to a thousand [17,18] times lower than in blood. We note the correspondence of breath condensate dilution rates for inorganic ions and lactate (100–400, above). On the basis of the reported EBC dilution rates for both lactate and inorganic ions, one would expect glucose concentration in exhaled breath condensate to be above 4–5 μM.

We report here that breath condensate actually contains glucose at the level of 0.01 mM for healthy human subjects. We have found the reason for the previously reported low glucose content: it is glucose assimilation (metabolizing) in the conventionally collected EBC. Most importantly, we demonstrate that the breath condensate glucose levels

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determined by our method correlate positively with blood glucose levels, thus offering the prospect of a non-invasive approach to the monitoring of diabetes.

2. Experimental

Informed consent was obtained from all subjects (healthy human volunteers from 20 to 25 years old). EBC samples were collected using a commercially available condenser ECoScreen® (Erich Jaeger GmbH, Germany) during the morning, as recommended by the ATS/ERS Task Force [19]. All work was carried out in accordance with GCP regulations. All experimental protocols were approved by the Ethical Committee of Pulmonology Research Institute (Moscow).

Experiments were carried out with Millipore Milli-Q water. All of the inorganic salts were obtained at the highest purity from Reachim (Moscow, Russia) and used as received. D-Glucose was purchased from ICN Biomedicals, USA. Sodium lactate, 40% solution, was purchased from ICN. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (lyophilized powder, activity 270 IU) was purchased from Sigma, Germany. Lactate oxidase (EC 1.1.3.2) from *Pediococcus* sp. (lyophilized powder, activity 72 IU) was supplied by Sorachim, Switzerland.

Glucose was detected in a stirred 0.25 ml cell equipped with a hydrogen peroxide sensor (Rusens Ltd., Russia) [20–22]. The sensitivity of the sensors in batch mode was $1 \pm 0.1\ A{\cdot}M^{-1}{\cdot}cm^{-2}$ (0.00 V, Ag|AgCl).

Capillary blood glucose was measured using a home-made analyzer equipped with an advanced glucose biosensor [23]. The system was validated for blood analysis versus both Biosen C Line (EKF, Germany) and Humastar 300 (Human Diagnostics, Germany).

3. Results and discussion

Glucose concentration in EBC was detected using the enzyme glucose oxidase. Hydrogen peroxide (H_2O_2), the product of the enzyme reaction, was monitored using a sensor based on nano-scaled films of Prussian Blue, the best electrocatalyst for H_2O_2 reduction. As we have already reported, Prussian Blue is three orders of magnitude more active and selective than the conventionally used platinum [20–22]. In order to subtract the background caused by presence of H_2O_2 in EBC [24–26], the glucose-oxidase-catalyzed reaction was initiated by injection of the enzyme into the cell.

Investigation of breath condensate collected conventionally from five healthy subjects showed no presence of glucose (resolution 0.1 μM). This confirms the generally accepted view that there is minimal glucose content in EBC. However, as mentioned above, based on the dilution rates of both lactate and inorganic ions, glucose concentration in breath condensate would be expected to be > 5 μM. This contradiction can only be explained by glucose assimilation in EBC.

The stability of glucose concentration in breath condensate was investigated by spiking 12 EBC samples collected from 8 healthy subjects with 50 μM–100 μM of glucose. The typical kinetics of glucose assimilation in breath condensate at room temperature is shown in Fig. 1. Nearly half of the glucose was consumed during the first 5–6 h of incubation. After 24 h, glucose was present only at a level of 10–15%. Hence, the reported minimal glucose content [12,13] is due to its assimilation in conventionally collected breath condensate.

The pseudo first-order kinetic constants of glucose assimilation in EBC were evaluated from the slopes of kinetic curves in semi-logarithmic plots. The kinetic constants for the same EBC samples were found to be rather similar. A slight decrease in the assimilation constants with increased spiking of glucose concentration is observed: $k = 2.2 \pm 0.3{\cdot}10^{-5}\ s^{-1}$, $1.9 \pm 0.6{\cdot}10^{-5}\ s^{-1}$, and $1.4 \pm 0.8{\cdot}10^{-5}\ s^{-1}$ for 50 μM, 75 μM, and 100 μM glucose additions, respectively.

Glucose consumption in breath condensate has been confirmed independently by observing the accumulation of lactate, the known product of glucose metabolism in living cells. As seen in Fig. 1, after the

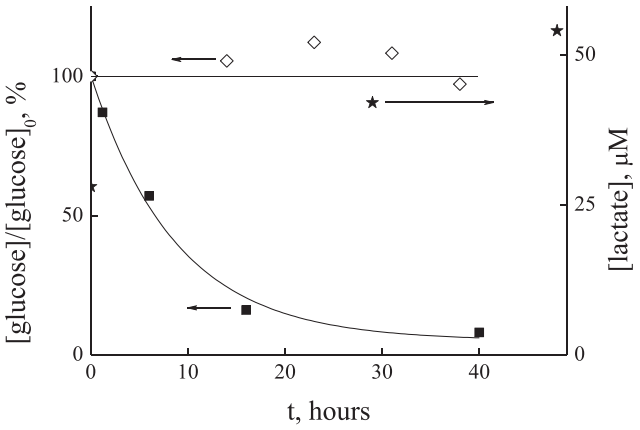

Fig. 1. Kinetics of glucose consumption (■) and lactate accumulation (★) in exhaled breath condensate spiked with 70 μM glucose; EBC spiked with 35 μM glucose in the presence of 1 mM of chloramphenicol (◇).

EBS samples had been spiked with glucose, the lactate concentration increased significantly.

Hence, for accurate glucose detection, the exhaled breath condensate must be collected in the presence of a compound that inhibits glucose metabolism. Chloramphenicol was selected from the range of potential inhibitors, because in concentrations of up to 1–2 mM it does not affect the glucose oxidase activity used in our assay. Fig. 1 shows that in the presence of 1 mM chloramphenicol no decrease in glucose concentration is observed in spiked EBC samples for almost 40 h of incubation.

Accordingly, prior to collection, a dry chloramphenicol sample was placed into the EBC receiver to achieve a final inhibitor concentration of 1–2 mM after dilution directly with breath condensate. Analysis of the breath condensate samples collected in this way from 30 human volunteers has shown an average glucose concentration of 11.5 μM. This is approximately 500 times lower than the glucose concentration in blood samples collected from the same subjects. The EBC dilution rate obtained for glucose now corresponds to the dilution rates for inorganic ions [16–18] and lactate [14,15] (above). Moreover, mass-spectrophotometric investigation of EBC probes frozen immediately after collection showed that two out of three samples had a glucose content in the range of micro moles per liter [27]. Literature data thus confirm that the true EBC glucose concentration in healthy subjects (normal) is at the level of 0.01 mM.

This clearly shows that glucose is present in breath condensate at a concentration detectable by biosensors (≈ 0.01 mM for healthy human subjects). The reason for the minimal glucose content reported previously is due to glucose assimilation in EBC collected by conventional means.

Non-invasive monitoring by means of chemical analysis is often considered unreliable since none of the excreted liquids directly replicates the metabolite content of blood. However, we note that existing long-term glucose monitors referred to as “low-invasive” require calibration after every two or three days of operation. Such ‘calibration’ involves a conventional blood probe with subsequent analysis of the sample for glucose content. Hence occasional blood probing for calibration does not appear to devalue a diagnostic tool referred to as ‘non-invasive’. Accordingly, a sufficient requirement for non-invasive diagnostics would be a correlation in variation rates between metabolite concentrations in the excreted liquid and the corresponding values in blood. We determine the variation rate as the ratio of glucose concentration after an excitation to its value before the excitation.

A blood glucose increase was produced using a “glucose tolerance” test [28]. After an overnight fast, a healthy subject breathed through an ECoScreen® condenser for condensate collection (10 min). Simultaneously a blood sample was taken from his finger. These EBC and blood

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