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THE RESPONSE OF HUMAN BODY AT OXIDATIVE STRESS IN SUBJECTS WITH TYPE 2 DIABETES: AMMONIA BREATH ANALYSIS BY LASER PHOTOACOUSTIC SPECTROSCOPY

Mioara PETRUS,[a,*] Ana M. BRATU[a] and Cristina POPA[a,b]

[a]Department of Lasers, National Institute for Laser, Plasma, and Radiation Physics,
409 Atomistilor St., PO Box MG-36, 077125 Bucharest, Roumania

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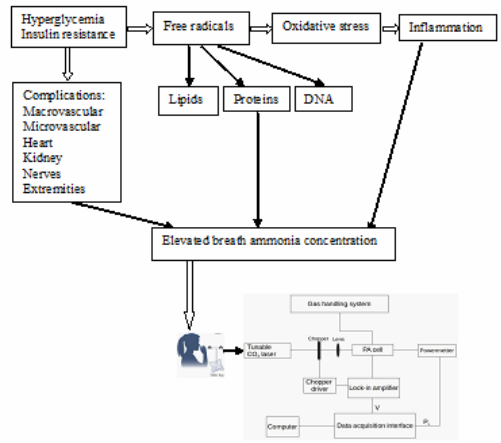
Diabetes is a complex syndrome, heterogeneous disorder that is characterized by hyperglycemia associated with major changes in lipids and proteins. The pathophysiology of the link between diabetes, hypertension, inflammatory syndrome and oxidative stress is complex and multifactorial. Oxidative stress has been implicated in the major complications of diabetes mellitus. There is a need for a marker of oxidative stress that could be associated with complications in diabetics. Various organic compounds (VOCs) from the human breath have been associated with pathological states of the diabetes. Breath ammonia concentrations was measured from subjects with type 2 diabetes and from a healthy control group using laser photoacoustic spectroscopy. Higher ammonia concentration was determined in the diabetic group compared to the healthy subjects, especially at diabetics that present hypertension and/or inflammatory syndrome.



INTRODUCTION

Type 2 diabetes mellitus (T2DM) has reached epidemic proportions with explosive increase in incidence worldwide over the past few decades, particularly in developing countries, in conjunction with increased obesity rates and westernization of lifestyle. The major characteristic of T2DM is hyperglycemia, and it is known as non-insulin dependent or maturity diabetes which generally develops at age after 40 year, but is increasingly at younger ages. The causes if T2DM are inadequate insulin secretion and resistance to insulin action. In most cases, peripheral insulin resistance, defined as the attenuated response to insulin in fat tissue, liver, and skeletal muscle, appears long before the development of hyperglycemia. Systemic insulin resistance has been implicated as one possible factor that links visceral obesity to adverse metabolic consequences; however, the mechanism whereby adipose tissue causes alterations in insulin sensitivity remains unclear. T2DM is associated with long-term metabolic complications, and also with microvascular and macrovascular complications.[1-7] Good control of blood glucose prevents microvascular complications, but macrovascular changes are related more to disturbances in blood pressure, endothelial function and lipids. Diabetes has long been considered a state of chronic, low-level

* Corresponding author: mioara.petrus@inflpr.ro

inflammation,[8] and there is some evidence to suggest that this immune activation may precede insulin resistance in diabetic and pre-diabetic states and ultimately may be the factor that initially increases cardiovascular risk.[9] Infection and inflammation are commonly associated with insulin resistance, and visceral obesity is associated with a chronic, low-grade inflammatory state, suggesting that inflammation may be a potential mechanism whereby obesity leads to insulin resistance.[10]

Numerous studies demonstrated that oxidative stress, mediated by hyperglycemia contributes to the development and progression of diabetes.[11-13] Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) that include free radicals as well as nonradical species. This "oxidative shielding" acts as a defense mechanism for either decreasing cellular uptake of toxic pathogens or chemicals from the environment, or to kill the cell by apoptosis and thus avoid the spreading to neighboring cells. Therefore, ROS formation is a physiological response to stress. Being highly reactive molecules, the pathological consequence of ROS excess is damage to proteins, lipids and DNA. Consistent with the primary role of ROS formation, this oxidative stress damage may lead to physiological dysfunction, cell death, pathologies such as diabetes and cancer, and aging of the organism. Oxidative stress has been proposed as a major participant in the patophysiology of diabetic complications.[11-13] Changes in protein metabolism in diabetes have been less studied. Proteins are the principal target of ROS, whose structure and function can be affected.[14-17] Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, alteration of the electrical charge and increase susceptibility to proteolysis. Thus the structure is destroyed, functions of essential proteins and enzymes and whole cell metabolism are blocked. Ammonia (NH_3) originates in the catabolism of amino acids (that are primarily produced by the degradation of proteins) and can penetrate the blood-lung barrier and appear in exhaled breath.[11] The ammonia is absorbed into the portal circulation, taken up by the liver and converted into urea, via the urea cycle.[18,19] Ammonia is processed in the liver, kidneys and skeletal muscles and plays an significant role in the human body and is considered to be an important biomarker monitored in the blood, or noninvasively in urine, salive or breath. The main sources of ammonia in human body are the deamination of amino acids, transamination of most amino acids with α-ketoglutaric acide to form glutamic acide, and operation of glutaminase enzyme in the kidney. In addition, this compound can be produced during purine and pyrimidine catabolism, the action of intestinal bacteria on the non-absorbed dietary amino acids, and the action of monoamine oxidase enzyme. The ammonia concentration is dependent on a range of factors including the health status of the patient, the route of sampling (nasal or oral), contribution from oral bacteria, as well as diet, pharmaceutical use and levels of metabolic activity.[20] Elevated breath ammonia could be due to liver disease, kidney disease, genetic diseases, periodontal disease and physical exercises. Normal concentration of ammonia in breath is 50-2000 ppb (parts per billion).[21,22] Breath is a complex mixture of gases, vapors and aerosols. The bulk matrix of breath is a mixture of nitrogen, oxygen, carbon dioxide, water vapor and gases: -inorganic gases (*e.g.*, NO, CO_2, and CO); volatile organic compounds- VOCs (*e.g.*, isoprene, ethane, pentane, and acetone); other typically non-volatile substances (isoprostanes, peroxynitrite, cytokines, and nitrogen). More than 3,500 different components have been identified in exhaled breath, from which more than 1,000 VOCs and 35 as breath biomarkers.[23,24] In breath analysis, biomarkers give information very quickly compared to blood or urine tests. Since ammonia passively diffuses from blood to both salivary and sweat glands, it can be detected in oral fluid and sweat.[25,26] In addition to body fluids, NH_3 has been detected in exhaled breath using various methods, including selected ion flow tube mass spectrometry,[27] ion mobility spectrometry,[28] cavity ring-down spectroscopy,[28] and photoacoustic spectroscopy.[30]

MATHERIALS AND METHOD

We have analyzed ammonia concentration from the exhaled breath in the subjects with T2DM and a healthy control group using laser photoacoustic spectroscopy (LPAS).[31-35] Photoacoustic (PA) detection provides also the necessary selectivity for analyzing multicomponent mixtures by the use of line-tunable CO_2 lasers. The kind and number of detectable substances is related to the spectral overlapping of the laser emission with the absorption bands of the trace gas molecules in the wavelength region 9-11 μm. The experimental system of the photoacoustic detection method is presented in Fig. 1. As a radiation source is used a home-built, line-tunable and frequency-stabilized

CO_2 laser, which emits continuous wave radiation with an output power of 2-5 W, tunable between 9.2 and 10.8 μm on 57 different vibrational-rotational lines and its emission spectrum overlaps with the absorption fingerprint of ammonia. The cw, tunable CO_2 laser beam is chopped, focused by a ZnSe lens, and introduced in the PA cell (the external resonator home-build). The PA cell is made of stainless steel and Teflon to reduce the outgassing problems and consists of an acoustic resonator (pipe), windows, gas inlets and outlets, and microphones. Inside the cell, the four microphones (sensitivity of 20 mV/Pa each), connected in series are mounted flush with the internal surface of the resonator tube. The light beam is modulated by a high quality, low vibration noise and variable speed (4–4000 Hz) mechanical chopper model DigiRad C-980 and C-995 (30 aperture blade), operated at the appropriate resonant frequency of the cell (564 Hz). After passage through the PA cell, the power of the laser beam is measured by a laser radiometer Rk-5700 from Laser Probe Inc. with a measuring head RkT-30. Its digital output is introduced in the data acquisition interface module together with the output from the lock-in amplifier (Stanford Research Systems model SR 830). All experimental data are processed in real-time and stored by a computer. Another important element in LPAS system is the vacuum/gas handling system. The Teflon/stainless steel system can perform several functions without requiring any disconnection. It can be used to ensure PA cell and gas purity, to pump out the cell, to introduce the sample gas in the PA cell at a controlled flow rate, and monitor the total and partial pressures of gas mixtures.

The subjects involved in this study are persons diagnosed with T2DM (n = 16), age between 42 and 71 years, that present high body mass index (BMI = 31.4 – 35.3) and, healthy control group (n = 9), age between 29 and 42 years, non-smokers, non-diabetics and normal weight (BMI = 19.8 - 23.4). All samples were given in the laboratory between 09:00 and 11:00 in the morning and analyzed after 2 to 6 hours. Breath samples were collected in special sample bags, aluminized multi-patient collection bags from QuinTron (750 mL aluminum-coated bags), designed to collect multiple samples from patients and hold a sample for maximum 6 hours. After the collection, the sample gas is transferred to the PA cell to be measured. The sample bags is connected to the system and the stored breath samples are transferred from bags into the measuring cell by the gas flow controller #25(MKS 1179A) at a controlled flow rate 600 sccm (standard cubic centimeter per minute). Before entering the PA cell, the gas mixture passes through a potassium hydroxide (KOH) scrubber, which retains most of the interfering carbon dioxide and water vapors (Merck KOH pellets),[35] scrubbers that are replaced after each measurement. To ensure the quality of each measurement, an intensive cycle of N_2 washing was performed between samples, in order to have a maximum increase of 10% for the background PA signal. The ammonia concentration was measured on 9R(30) CO_2 laser line at 9.22 μm, where ammonia absorption coefficient has the maximum value of 57 $cm^{-1}atm^{-1}$.[31]

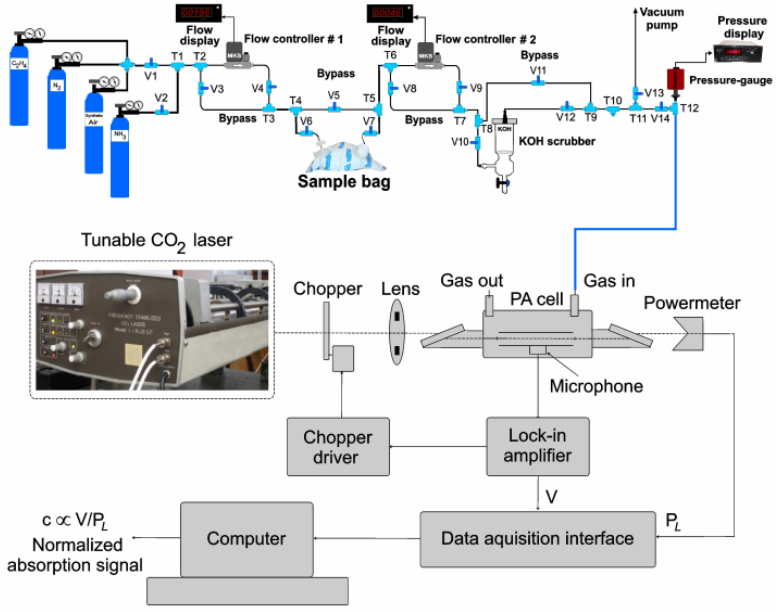


Fig. 1 – CO_2 laser photoacoustic spectroscopy system.

RESULTS AND DISCUSSION

Breath samples were collected from subjects diagnosed with T2DM, and from a healthy control group, without changes in glucose tolerance. People in the control group were carried out the same investigation as those of the diabetic group. All the patient results evaluated in this study were known stable cases of T2DM whose medical therapy had been unaltered over the last 12 months. The participants with hormonal disorders, benign or malignant disorders, renal failure, transplant rejection, central nervous system disorders, and/or other chronic diseases, and also smokers were excluded from the study. No patients were on supplements with antioxidants. Informed consent was obtained from all individuals. Samples were obtained without any adverse effects and were assayed for breath ammonia. The ammonia concentration of healthy subjects was in the normal range and the values obtained in our measurements were between 0.832 ppm (parts per million) and 1.76 ppm, but at the subjects with T2DM the ammonia concentration range was between 2.74 ppm and 10.16 ppm. Our measurements showed a significantly increase of ammonia concentrations in the exhaled breath at diabetic subjects compared to healthy subjects. The differences in exhaled breath ammonia concentration are presented in Fig. 2, where the mean values of breath ammonia concentration in healthy control group and subjects with T2DM are presented. Ammonia is a biomarker of protein metabolism,[11,17,36,37] and at subjects with T2DM, insulin deprivation is associated with an increase in amino acids and by an accelerated protein catabolism.[10,16,17,38,39] Ammonia is a biomarker of protein metabolism and at subjects with T2DM, insulin deprivation is associated with an increase in amino acids and by an accelerated protein catabolism. In diabetic subjects, amino acid catabolism is exaggerated in the fasting state as reflected by increased uptake of alanine by the liver for gluconeogenesis and accelerated branched chain amino acid catabolism in muscle. Protein feeding also exaggerates the hyperglycemia of diabetes by causing an increase in hepatic glucose production.[40] In hyperglycemia there is an overproduction of active oxygen that lead to specific vascular disorders. Under hyperglycemic conditions, intracellular concentration of glucose is elevated and is followed by an accelerated catabolism of proteins and an increased hepatic glucose production.[41] Breath ammonia concentrations in subjects with diabetes can be a consequence of oxidative stress after ROS attack on proteins, accelerated catabolism of proteins due to insulin deprivation and hepatic glucose production, because after Gurzov *et al.*, oxidative stress accompanying obesity inactivates protein-tyrosine phosphatases (PTPs) in the liver to activate select signaling pathways that exacerbate disease progression.[42] Also, there is known that oxidative stress is an important factor that contributing to the development and progression of diabetes. Most of the subjects involved in this study with T2DM present complications such as hypertension and/or inflammatory syndrome. Moreover, we found that breath ammonia concentrations are higher in T2DM subjects that present hypertension and/or inflammatory syndrome than in those without complications, and the mean values are presented in Fig. 3. It is known that T2D lead to complications like kidney failure, heart disease, cerebrovascular disease, but there is a lack of information of ammonia level in subjects with T2DM and the relationship between ammonia level and diabetes complications.

Diabetes mellitus and hypertension are interrelated diseases, and hypertension is about twice as frequent in individuals with diabetes as in those without diabetes. T2DM is an inflammatory disease, and inflammation is caused by insulin resistance correlated with obesity, or by hyperglycemia and hyperlipidemia, also there is a close link between metabolism and immunity Inflammatory signaling pathways can be activated by metabolic stresses and it has been demonstrated that obesity leads to the activation of inflammatory signaling pathways and thus contributes to insulin resistance. Additionally, increased glucose metabolism can lead to a rise in mitochondrial production of ROS. ROS production is elevated in obesity, which causes enhanced activation of inflammatory pathways.[10] Studies show that inflammatory markers are associated with the degree of T2DM.[10,43] Inflammatory markers (eg, C-reactive protein (CRP)) are increased in patients with diabetes, hypertension, and the metabolic syndrome, and also predict the development of these diseases.[44-46] Inflammation is a normal response to tissue injury or pathogen exposure and is a critical factor in the body's ability to heal itself or to fight off infection. The inflammatory response involves the activation of leukocytes (white blood cells) and is mediated, in part, by a family of cytokines and chemokines.

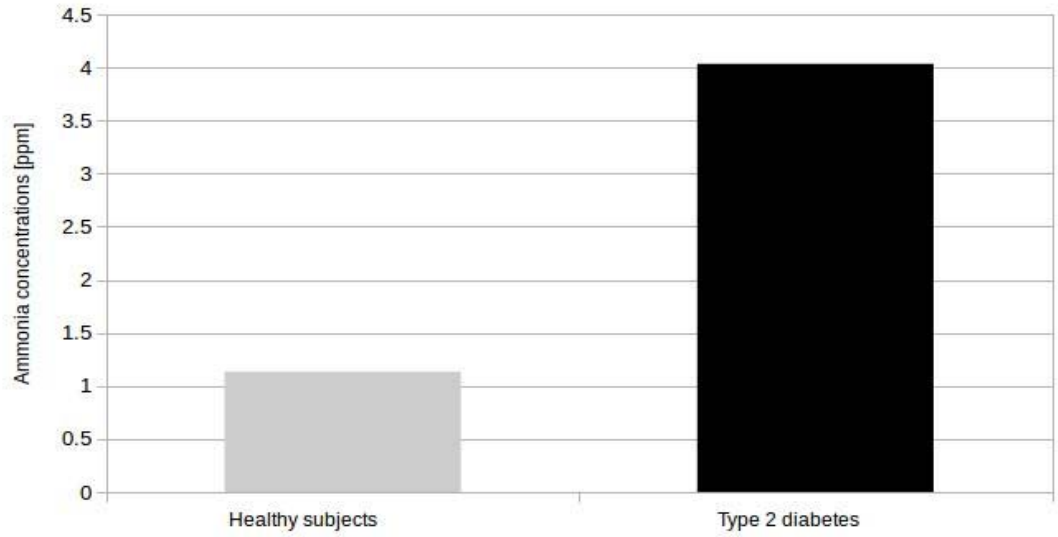


Fig. 2 – Mean breath ammonia concentrations in healthy control group and in subjects with type 2 diabetes.

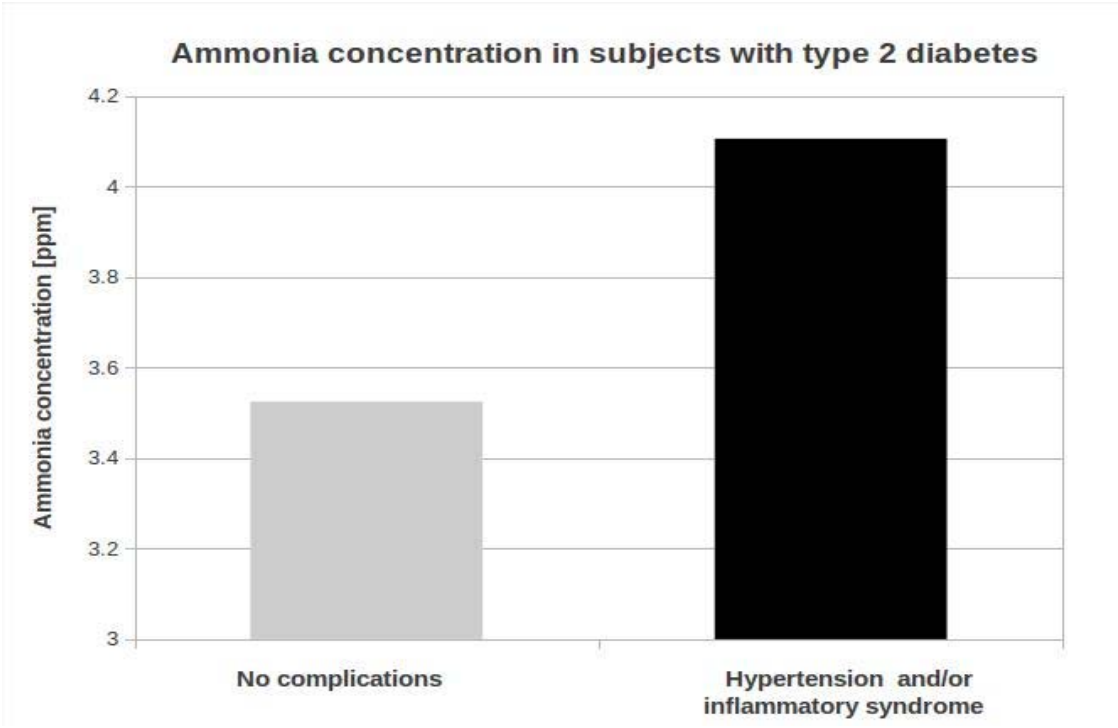


Fig. 3 – Mean values of breath ammonia concentration in subjects with type 2 diabetes without any complication and subjects with type 2 diabetes that preset hypertension and/or inflammatory syndrome.

In this study, the hypertension and inflammatory syndrome represent the progression of the diabetes disease, inflammation is involved in the pathogenesis of T2DM.[47-50] The relation between level of ammonia in the exhaled breath and T2D could be explained by the inadequate insulin control with disease progression by development of complications such as oxidative stress, inflammatory syndrome, and hypertension . Also, hyperglycemia increase ROS production and oxidative stress, leading to the activation of signaling molecules which increases insulin resistance and will lead to diabetic complications during the longer term. The studies show a relation between hyperglycemia, oxidative stress and inflammation coexist in pathological processes,[50] but also that hyperglycemia and free radicals increase the oxidative stress which will then activate the inflammatory processes.[51-53]

Between oxidative stress, inflammation and insulin resistance exists a vicious circle, since they are mutually reinforcing. Future studies are needed

to understand the relationship between them and the importance of breath ammonia biomarker.

CONCLUSIONS

A quantitative research on breath ammonia in the people with T2DM was presented. In the present work the CO_2 laser-photoacoustic spectroscopy technique was applied to measure ammonia from exhaled breath of patients diagnosed with T2DM vs. healthy subjects, due to its high sensitivity and selectivity. The purpose of this study was to determine the breath ammonia concentration in subjects with T2DM and a healthy control group. We observe a higher level of ammonia in the breath of diabetics subjects compared to healthy persons, but also an increase in ammonia concentration in diabetic subjects that present hypertension and/or inflammatory syndrome. Not all factors influencing ammonia are known in T2DM, but progress can be difficult without accounting for these factors. The higher level of ammonia in the exhaled breath of T2DM subjects may indicate that the body is not effectively eliminating ammonia as a byproduct of the metabolism of protein, as a consequence of oxidative stress in body or complications due to insulin resistance and high blood sugar. The molecular basis for the pathway-specific insulin resistance remains unknown. The studies show that there is a relation between hyperglycemia, oxidative stress and inflammation coexist in pathological processes, but also that hyperglycemia and free radicals increase the oxidative stress which will then activate the inflammatory processes.

Exhaled ammonia levels is correlated with severity of T2DM that include hypertension and inflammatory syndrome. Future studies are needed for understanding the role of ammonia breath biomarker in the progression of T2DM.

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