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Metalloprotein and multielemental content profiling in serum samples from diabetic and hypothyroid persons based on PCA analysis

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ABSTRACT

Diabetes and hypothyroidism are both metabolic diseases with great incidence worldwide. Metalloproteins and metals play key roles in normal glucose metabolism and thyroid hormone synthesis, which are altered in their respective pathologies. The aim of this work was to establish the corresponding multielemental and metalloprotean profiles in a control group (n = 20) compared with a diabetic (n = 20) or hypothyroidism group (n = 20), by exploring a multivariate principal components model. Classification to discriminate these groups was possible based in the quantification of 23 elements (Mg, Al, K, Ca, V, Cr, Zn, Fe, Se, Rb, Pb, Cu, Mn, Co, Ni, U, Sr, Mo, Sb, Ba, Tl, Cd, Ag), and alternatively on the metalloprotein profiles obtained by SEC-ICPMS. Determinations were assessed by means of QQQ-ICP and SEC-ICPMS for total and metalloprotean content, respectively. Samples were classified using Principal Component Analysis chemometric tool. Results showed that there were statistical differences in transitional elements concentrations, such as Zn, Cu, Co, Mn, V, and Cr. For the metal associated protein study, the expression of the fractions of the same transitional elements also were statistically different when compared between control vs diabetic patients, and control vs hypothyroid patients. Se levels showed no differences in both studies among groups. This screening study demonstrates that mass spectrometry methods and data analysis with chemometrics tools may be valuable in order to find possible biomarkers in serum samples of diabetic and hypothyroid patients. Future proteomics analysis are necessary to complete these findings.

1. Introduction

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces [1]. Hyperglycemia, or raised blood glucose, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels [1]. In 2012 diabetes was the direct cause of 1.5 million deaths and high blood glucose was the cause of another 2.2 million deaths, and by 2014, 8.5% of adults aged 18 years and older had diabetes [2]. Hypothyroidism is the result of inadequate production of thyroid hormone or inadequate action of thyroid hormone in target tissues. It develops with a great variety of symptoms, altogether effecting general metabolism and dysfunction in multiple organ systems. Primary hypothyroidism is the main manifestation of hypothyroidism, but other causes include central deficiency of thyrotropin-releasing hormone (TRH) or thyroid-stimulating hormone (TSH). Subclinical hypothyroidism (SCH) manifests when there is evidence of primary hypothyroidism with an elevated TSH but a normal free thyroxine (FT4) level [3].

The increasing evidence indicating the potential for antioxidant metals as adjunct therapy for diabetes supports the concept that most of these metals play an important role in the physiological mechanisms related to glycemic control and redox homeostasis [4]. For example, Zn, Mg and Mn are cofactors of hundreds of enzymes, and Zn is involved in the synthesis and secretion of insulin from the pancreatic beta cells [5–15]. Similarly, Cr enhances the insulin receptor activity on target tissues, especially in muscle cells [16–20]. Understanding of the essential role of Se in the synthesis of thyroid hormones, metabolism and action, as well as for normal thyroid function, increased substantially during the last decades [21–23]. High content of Se found within the thyroid tissue is consequence of the expression of several Se-dependent enzymes [22–25].

Abbreviations: SEC-ICPMS, size exclusion chromatography inductively couple plasma mass spectrometry; QQQ-ICP, triple quadrupole inductively couple plasma mass spectrometry; OS, oxidative stress; ROS, reactive oxygen species; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; SCH, subclinical hypothyroidism; HbA1C, glycosylated hemoglobin; MARS, multi affinity removal system; PCA, principal component analysis.

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The study of the metallome and metal homeostasis in biological environments brings challenges regarding the development of improved detection methods with high sensitivity, and robust enough to be performed in physiological conditions. There is not much research on accurate quantification of metal and metalloprotein species in serum samples of diabetic nor hypothyroid individuals. Nevertheless, most speciation studies were focused on revealing functionality and profiling of metalloprotein content in neurodegenerative processes [26–33], while others paid their attention on other diseases such as acute and chronic arthritis [34], Wilson's disease [35], cutaneous melanoma [36], degenerative joint diseases [37], among others.

A number of studies were carried out in order to classify diabetic, hypothyroid and control serum samples based on their total elemental and metallopreotean content. To this aim, total elemental and metalloprotean profiles were evaluated with QQQ-ICP and high-performance liquid chromatography (HPLC) coupled to QQQ-ICP, respectively. The functional metalloproteins are usually less abundant than the non-active albumin; effective depletion of this abundant fraction allows observing small differences in metalloprotean contents. For this reason, affinity chromatography was used to remove albumin along with gamma immunoglobulin, removing approximate 70% of proteins from the plasma samples.

Quantitative data from total content and integrations of peak areas were processed using chemometrics methods to elaborate the corresponding profiles, which allowed a statistical classification of samples.

2. Experimental

2.1. Instrumentation

The human Multiple Affinity Removal System column 4.6 × 50 mm (MARS) was purchased from Agilent Technologies (Santa Clara, CA, USA) to deplete HSA and IgG. For the size exclusion chromatography separation, a TSK Gel 3000SW 7.5 × 300 mm (Tosoh Bioscience, Germany) was used. The pre-concentration of samples was done by centrifugation (Sorvall instruments RC5C, Buckinghamshire, England). An Agilent 8800 QQQ-ICP equipped with a plasma frequency RF generator and an octopole collision/reaction system (ORS3) with collision/reaction cell (CRC) was coupled with an Agilent 1100 LC (Agilent Technologies, Santa Clara, CA, USA). An Agilent 1100 series HPLC system equipped with a binary pump, vacuum membrane degasser, thermostated auto sampler, column oven, and diode array detector with a semi-micro flow UV–Vis cell was used for all chromatographic analysis (Agilent Technologies, Santa Clara, CA, USA). The QQQ-ICP was controlled by the Mass hunter version 4.1 from Agilent Technologies, while the HPLC system was controlled using Chemstation software (Agilent Technologies, Santa Clara, CA, USA).

2.2. Reagents and solutions

Methanol, ammonium acetate, bovine serum albumin, and formic acid were analytical grade purchased from Sigma (St. Louis, MO, USA). Ammonium bicarbonate and acetic acid were purchased from Fluka (St. Louis, MO, USA). Nitric acid (trace metal grade) was purchased from Fisher scientific (Pittsburgh, PA, USA). Double deionized (DDI) water was prepared by passing distilled water through a NanoPure (18 MΩ) treatment system (Barnstead, Boston, MA, USA) and was used to prepare all solutions used in the experiments. For calibrating the SEC column, the SEC standard (Bio-Rad Laboratories, Hercules, CA) mixture of molecular weight markers ranging from 1300 to 670,000 Da was used. For the sample concentration after the MARS system, spin concentrators for proteins (3 kDa MWCO, Amicon Ultra Centrifugal Filters) from Millipore (Billerica, MA, USA) were used. Buffer A and buffer B mobile phases for MARS column and 5 kDa MWCO were purchased from Agilent (Agilent Technologies, Santa Clara, CA, USA).

2.3. Procedures

2.3.1. Serum samples

Diabetic and hypothyroid patients who volunteered for this project were enrolled under an informed consent approved by an Ethics Committee Board from the Universidad Nacional de Rosario (Argentina). Blood draws were performed in a private Biochemical Clinic from San Luis, Argentina, were every medical history was recorded (data not shown). The samples were collected into metal free collection tubes, and placed in a thermostatic bath (37 °C) for an hour and then centrifuged for 10 min at 2000*g*. in order to obtain the serum. After this, the serum was pipette out into polypropylene cryovials and stored in −80 °C until further analysis.

For the present work, sixty serum samples were collected during a 3-month period: Twenty (20) samples from diabetic patients, twenty (20) from hypothyroid patients, and twenty (20) from individuals with no ostensible symptoms or disease, which were set as controls. In Table 1 are presented some clinical and biochemical characteristics for the selected cases. Limitations of our study lie on the fact that study subjects are ambulatory patients and, for the diabetic ones, glycosylated hemoglobin (HbA1C) determination was not available for monitoring and control. In addition, most of these patients are under treatment (metformin for diabetic and levothyroxine for hypothyroid ones), therefore, it is not expected significant variations on some of the clinical features, specially blood glucose and TSH, in comparison with a non-treated individual. However, there was found subtle differences between groups that are consistent with the diagnosis. For example, average concentration of blood glucose found in diabetic is higher than hypothyroid and control ones, as expected.

2.3.2. Multi affinity removal system

In order to determine the content of functional metalloproteins in serum samples, and subsequently develop the corresponding profiles, the first step was the removal of albumin and immunoglobulin gamma. Albumin has no metals as prosthetic groups or cofactors and, by definition [38], is not considered metalloprotein with specific biological function, yet it will bund a wide range of metals un-specifically. The MARS column is specially designed to remove the unwanted fractions from the serum. First, the HPLC system was flushed with isopropyl alcohol for 10 min, followed by flushing with water for 2 h. After that, 2 blanks with and without column were injected into the system. The mobile phase used in the affinity chromatography to remove the HSA and IgG is a solution of

Table 1
Clinical characteristics from volunteer subjects. Complementary biochemical determinations are presented, such as blood glucose and TSH, which are specific biomarkers of diabetic and hypothyroidism diagnosis, respectively.

	Healthy control (n = 20)	Diabetic (n = 20)	Hypothyroid (n = 20)
Age (years)	46	44	44
Median (range)	(29–57)	(29–57)	(27–70)
Gender (% female)	55	40	60
Body Mass Index (kg m^{-2})	22	26	24
Median (range)	(13–28)	(14–34)	(17–28)
Creatinine (mg dL^{-1})	0.81	0.99	0.78
Median (range)	(0.5–1.1)	(0.45–1.67)	(0.45–1.14)
Cholesterol (mg dL^{-1})	189	197	196
Median (range)	(139–256)	(113–235)	(113–250)
Triglycerides (mg dL^{-1})	118	147	129
Median (range)	(56–280)	(55–289)	(59–280)
TSH (IU L^{-1})	2.14	2.75	3.00
Median (range)	(1.5–2.89)	(1.62–4.6)	(0.7–7.7)
Blood glucose (mg dL^{-1})	80	130	79
Median (range)	(56–136)	(100 − 200)	(56–100)