Contribution to Characterization of Oxidative Stress in Diabetic Patients with Macroangiopatic Complications

Gregorio MARTÍNEZ-SÁNCHEZ *,1, Igor POPOV 2, Gema PÉREZ-DAVISON 1, Saied-Mohamed AL-DALAEN 1, Rosemeres HORWAT-DELAPORTE 3, Attilia GIULIANI 4 & Olga Sonia LEÓN-FERNÁNDEZ 1

1 Centre for Research and Biological Evaluations, Institute of Pharmacy and Food Sciences, Havana University, Cuba.
2 Research Institute for Antioxidant Therapy (FAT GmbH), Vivantes Klinikum im Friedricscain, Berlin, Germany.
3 Instituto Superior de Ciências Farmacêuticas e Bioquímica, Universidade Paranaense, Umuarama, PR, Brazil
4 Department of Chemistry and Medical Biochemistry, University of Milan, Via Saldini, 50-20133 Milan, Italy

SUMMARY . The aim of this study was to investigate the status of an extensive array of redox indices in diabetic patients with macroangiopatic complications compared to healthy subjects. Blood samples from 101 diabetic patients with macroangiopatic complications and 60 healthy subjects were tested by spectrophotometric techniques in order to measure oxidative stress indices. All measured biomarkers of antioxidant-proxidant balance and nitric oxide (NO) were significantly (p<0.05) modified in diabetic patients compared to normal subjects. An increase of biomolecule damage markers was noted (e.g. malondialdehyde 8.91 ± 0.64 compared to 1.80 ± 0.07 µM in normal subjects). A significantly (p<0.05) reduction of the soluble antioxidant glutathion (2.83 ± 0.30 vs. 3.32 ± 0.62 mM in control group) and NO levels (51.28 ± 8.58 vs. 67.82 ± 22.44 µM in control group) were also observed in diabetic patients. Antioxidant enzymes were activated in these patients with a disruption of the catalase/superoxide dismutase balance (2.16 ± 0.10) compared to normal subjects (0.11 ± 0.20). In addition, the global indicator susceptibility to lipid peroxidation was increased by 82% in diabetics compared to the control group. The evaluated indicators could be extrapolated to routine clinical analysis and contribute to an integral overview of the oxidant / antioxidant balance in diabetic patients and could also be used as indices of treatment efficacy.

RESUMEN. "Contribución a la caracterización del estrés oxidativo en pacientes diabéticos con complicaciones macroangiopáticas". El propósito del presente trabajo fue el estudio del comportamiento de un grupo de indicadores redox en pacientes diabéticos con complicaciones macroangiopáticas y su comparación con los valores de sujetos sanos. Se tomaron muestras de sangre de 101 pacientes y 60 voluntarios sanos y los indicadores del balance redox fueron analizados mediante técnicas espectrofotométricas. Todos los biomarcadores del estado antioxidantes/proxidentes y las concentraciones de óxido nítrico (NO) fueron significativamente diferentes (p < 0.05) en los pacientes diabéticos cuando se compararon con los sujetos sanos. Se produjo un incremento en los indicadores de daño a biomoléculas (p.ej. el valor del malondialdehído fue de (8,91 ± 0,64 comparado con 1,80 ± 0,07 µM en los controles), una reducción significativa (p < 0,05) de los valores de antioxidantes solubles (glutatión 2,83 ± 0,30 vs. 3,32 ± 0,62 µM en los controles) y una reducción de las concentraciones de NO en los pacientes diabéticos (51,28 ± 8,58 vs. 67,82 ± 22,44 µM en los controles). Las enzimas antioxidantes se activaron en estos pacientes y se observó una disrupción en el equilibrio entre el balance catalasa/superoxido dismutasa (2.16 ± 0.10) con relación al valor alcanzado por los controles (0.11 ± 0.20). Adicionalmente, el indicador global de susceptibilidad a la peroxidación lipídica se incrementó en un 82% en los pacientes diabéticos con respecto al grupo de referencia. Los indicadores evaluados pudieran extrapolarse a los ensayos clínicos de rutina indicados a este tipo de pacientes para contribuir a una visión integral del balance oxidantes / antioxidantes y a la evaluación de la eficacia de diferentes terapias.

INTRODUCTION

Diabetes Mellitus (DM) is characterized by metabolic abnormalities, including a disorder of carbohydrate metabolism, with the presence of hyperglycemia and glycosuria. Changes in human behavior and live style over the last century have resulted in a dramatic increase in the incidence of DM worldwide (over 124 million individuals) 1. These metabolic abnormalities result from inadequate production or utilization of

KEYWORDS: Diabetes mellitus, Lipid peroxidation, Oxidative stress, Peroxidation potential

PALABRAS CLAVE: Diabetes mellitus, Estrés oxidativo, Peroxidación lipídica, Potencial de peroxidación

* Autor a quien dirigir la correspondencia. E-mail: gregorio@cieb.sld.cu

ISSN 0326-2383 197
insulin. Long-term complications, causing morbidity and premature mortality, are characterized by microvascular disease with capillary basement membrane thickening, macrovascular disease with accelerated atherosclerosis, neuropathy involving both the somatic and autonomic nervous systems, neuromuscular dysfunction with muscle weakening, and decreased resistance to infections. Such chronic complications involve the eyes, kidneys, heart, nerves and blood vessels. Accelerated atherosclerosis produces 80% of all diabetic mortality, three fourths of this being caused by coronary disease 2,3.

The role of the reactive oxygen species (ROS) in inducing oxidative damage at the level of lipid peroxidation, DNA injury and protein damage, has been demonstrated in diabetic patients 4-8. Four main molecular mechanisms have been implicated in glucose-mediated vascular damage: 1) increased polyol pathway flux; 2) increased advanced glycation end-product (AGE) formation; 3) activation of protein kinase C isoforms and 4) increased hexosamine pathway flux 9.

An hyperglycemia-induced process of radical anion superoxide (O$_2^-$) overproduction by the mitochondrial electron-transport chain seems to be present. Furthermore, a decrease in the antioxidant defense system, involving the erythrocyte superoxide dismutase and catalase 10,11, has been mentioned, with a simultaneous decrease in leukocytes’ vitamin C concentration, and the scavenger capacity of radicals in plasma also decreases 12.

An important role for O$_2^-$, which is generated during the mitochondrial electron-transport chain, has recently been proposed for these ROS. It is considered to be the unifying factor that links the four pathological events 13. In diabetic patients there seems to be a threshold above which O$_2^-$ production is markedly increased. It has been found that hyperglycemia increases the proton gradient above this threshold value as a result of overproduction of electron donors by the tricarboxylic acid cycle 14. Superoxide anions can be dismutated to hydrogen peroxide which generates very reactive hydroxyl radicals by Fenton chemistry. ROS is able to oxidize lipids, proteins and DNA, with concomitant changes in their structures and function.

The methodology for detecting oxidative stress status at clinical level is hardly to be found in the literature. There are some useful methods for investigating the oxidative profile but they are not applicable to the clinical diagnostic of this study. Before the present work was carried out, this research group had applied a set of methods in order to establish reference values in normal populations 15 or evaluate the effect of antioxidant therapy 16. The aim of the present work was to study the status of an extensive array of oxidative stress indices that would permit examination of the role of oxidative stress in diabetic patients with macroangiopatic complications. Therefore, the blood levels of these markers in DM patients with macroangiopatic complications relative to those in healthy age-matched volunteers group were compared.

MATERIALS AND METHODS

Subjects and blood collection

Blood and serum samples taken from 101 adult patients with a diagnosis of type 2 diabetes and previous history of diabetic foot, aged 20 to 67 years, of both sexes and of varying ethnicity, were used in the present study. It was a requirement of the study that these patients did not meet any of the following criteria: severe septic conditions, hepatic dysfunction, pregnancy, cancer or other serious disease, inability to co-operate with the requirements of the study, recent history of alcohol or drug abuse, current therapy with any immunosuppressive agent or anticonvulsant, participation in another clinical study or current treatment with an investigational drug. In patients between 20-40 years the presence of diabetes type 2 was confirmed by the determination of the HLA-D (by using the allele-specific polymerase chain reaction method) 17, and by the determination of serum cell cytoplasmic antibodies and islet cell surface antibodies by immunoprecipitation and Western blotting 18. Furthermore, BMI (body mass index) was also analyzed.

The control subjects were 60 sex- and age-matched healthy individuals. After verbal and written explanation of the methods and risks involved, subjects gave their informed consent to take part in the study. Study procedures were reviewed and approved by both the Institute “Salvador Allende” (Hospital) and the Pharmacy and Food Science College (Havana University) Committees for Research on Human Subjects, protocol No.02/IPAL808. So first of all, blood samples were obtained after an overnight fast of at least 12 hours, after centrifugation (3,000 g, 15 min, 4 °C) serum was obtained and stored in separate fractions (headspace was flushed with
nitrogen) at -70 °C until analysis. This protocol then measured the following: glucose, fructosamine, advanced oxidation protein products (AOPP), nitric oxide (NO), reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), total oxygenperoxides (TO), peroxidation potential (PP) and malondialdehyde (MDA).

Biochemical measurements

Firstly, Serum GSH was analyzed by using the method described by Sedlak and Lindsay (1968). Then GSH (Sigma, St. Louis, MO., USA) was used to generate standard curves. Following this, MDA concentrations were analyzed with the LPO-586 kit obtained from Calbiochem (La Jolla, CA., USA). In this assay, a stable chromophore is produced after 40 min of incubation at 45 °C and measured at a wavelength of 586 nm using a Pharmacia Spectrophotometer. Therefore, as reference standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, MO., USA) were used and assayed under identical conditions. After this, MDA concentrations in serum samples were calculated using the corresponding standard curve, and values were expressed in µM.

In order to determine susceptibility to lipid peroxidation (PP), serum samples were incubated with a solution of cupric sulfate (final concentration 2 mM) at 37 °C for 24 h. The PP was calculated by subtracting the MDA concentration at time 0 from that obtained at 24 h.

Furthermore, TO was measured by using Bioxytech H2O2-560 kit Cat. 21024 (Oxis International Inc. Portland, USA). The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. Ferric ions bind with the indicator xylenol orange (3,3'-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresolsulfone-phthalein, sodium salt) to form a stable colored complex which can be measured at 560 nm.

Evaluation of GPx activity was determined using Randox Ltd. (Diamond Road, Crumlin, U.K.) Kit Cat. No.SD125. In brief, the method employs xanthine and xanthine oxidase to generate O_{2}^{-}, which reacts with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the inhibition degree of this reaction.

In addition, nitrite/nitrate (NO\textsubscript{2} /NO\textsubscript{3}) levels were determined by the Griess reaction by first converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy) after which the Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added. Samples were incubated at room temperature for 10 min and the absorbance was measured at 540 nm using a microplate reader. Following this, the AOPP were measured spectrophotometrically (calibrated with chloramines-T). Briefly, serum (100 µL) in PBS (1 mL) was treated with 50 µL of potassium iodide 1,16 M followed by 100 µL of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm against reactive blank. The chloramines absorbance at 340 nm being lineal within the range of 0 to 100 µM. AOPP concentration were expressed as µM of chloramines-T.

Relative fructosylsine content (Amadori's product of glycated serum proteins) was measured by reduction of the redox indicator NBT at 530 nm. Glucose concentration was determined by a colorimetric method (the absorbance was measured at 560 nm), according to the procedure described by Schmidt et al. 27.

Finally, Hba1c (glycosylated hemoglobin) and TC (total cholesterol) were determined using standard available diagnostic kits (Sigma, St. Louis, MO., USA). Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO., USA).

Statistics

Initially the OUTLIERS preliminary test for detection of error values was applied as a first step in the statistical analysis. After this, the homogeneity of variance test (Bartlett-Box) was used followed by ANOVA method (single way). In addition, a multiple comparison test was used (Duncan test). Data are expressed as means ± S.D. or median (maximum-minimum). For all experiments the level of statistical significance employed was at least p < 0.05. The SPSS software package was used for all statistical analyses.
RESULTS

In relation to the baseline characteristics, both groups (normal subjects and diabetic patients) were similar in age, ethnicity and sex at randomization (p > 0.05) as shown in Table 1. There was a prevalence (45-51 %) of subjects older than 60 years in both groups, with the race of the great majority being white Caucasian. The diabetic history was mainly characterized by hypertension. Concomitant treatments were those used for the hypertension control (captopril in 31%, nifedipine in 10% and nitropental in 2% of patients respectively), glycemic control (glybenclamide 62% of patients respectively), and cardiovascular disease treatments (aspirin in 17% of patients). For glycemic control, 38% of the patients who did not receive glybenclamide were kept under diet control regimen.

The disease developed in patients between 1 and 42 years of age, and the main blood glucose concentration for all the patients enrolled in the study was 10.11 ± 4.20 mM (min. 2.70; max. 20.60). In normal subject the glucose concentration were within the glucose reference values (3.33 - 7.00 mM); the mean of this value was significantly different (p<0.001) compared to diabetic patients. As expected the BMI of diabetic type II subjects was in the obese status, whereas control subjects were classified as normal. The values of Hb A1c in diabetic indicated poor glucose control during the preceding 1 to 3 months. In addition, Table 1 shows that hypercholesterolemia was detected in these patients (high levels of TC).

Furthermore, it was found that all measured biomarkers of antioxidant-prooxidant balance and NO$\cdot$/NO$^-$ were significantly (p<0.05) modified in diabetic patients compared to normal subjects (Table 2). An increase in markers of biomolecular damage was noted: firstly, increase of protein oxidation product (relative fructolisine content or Amadori’s products precursor of AGE and the AOPP); secondly, increase in lipid peroxidation (MDA); and thirdly, increase in TO. A significant (p<0.05) reduction of the soluble antioxidant GSH, and also in NO$\cdot$/NO$^-$, was observed in diabetic patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control (n=60)</th>
<th>Diabetic Patients$^c$ (n=101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 - 40</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>40 - 60</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>≥ 60</td>
<td>27</td>
<td>52</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>41</td>
<td>69</td>
</tr>
<tr>
<td>Black</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Mixed race</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>56</td>
</tr>
<tr>
<td>Previous History</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Renal dysfunction</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Complementary Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>3±1</td>
<td>12 ±2 *</td>
</tr>
<tr>
<td>BMI $^d$</td>
<td>22.1 ±2.3</td>
<td>29.3 ±3.0 *</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>3.18 ±0.5</td>
<td>7.01 ±1.2 *</td>
</tr>
<tr>
<td>ETD (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X ± S.D.</td>
<td></td>
<td>17 ± 9</td>
</tr>
<tr>
<td>Min.</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Max</td>
<td>-</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 1. Baseline characteristics of control subjects and diabetic patients. ETD, Evolution Time of the Disease; X, mean value; S.D., standard deviation; HbA1c, glycosylated hemoglobin; TC, total cholesterol; BMI, body mass index. $^a$ Hypertension was defined as elevation of systolic (>140 mm Hg) and/or diastolic (>90 mm Hg) blood pressure. $^b$ Cardiovascular disease (CVD) was diagnose by thorough history and physical examination. $^c$ Renal dysfunction: increase in serum creatinine > 1.5 mg/dL or BMI weight (kg)/height (m2). $^d$ All diabetic patients included were previously history of diabetic foot $^* p< 0.05$, when compared to the control group.

Note: No significant statistical differences between both groups (p > 0.05) for age, ethnicity and sex were found.
The enzymes GPx, SOD and CAT were activated in these patients with disruption of the CAT/SOD balance compared with normal subjects. In addition, the global indicator susceptibility to lipid peroxidation PP increased by 82% in diabetic patients relatively to the control group.

On the other hand, in stratification analysis of the diabetic group attending to different criterion (age, with or without hypertension or cardiovascular diseases, under or not hypoglycemic medication) all parameters were found statistically not significant. In normal subjects a positive and significant (p<0.05) correlation was observed between fructolysine - PP, GSH (+0.72) - GPx and CAT (+0.72) - CAT/SOD (+0.85). In addition, a negative and significantly (p<0.05) correlation was detected between GPx - CAT (-0.73) and CPx - CAT/SOD (-0.97). Furthermore, a significant (p<0.05) lineal correlation (r=0.67) was found between glucose level and CAT/SOD index in normal subjects. However, in diabetic patients the correlation between all the biomarkers was lost with the only exception being the CAT - CAT/SOD correlation (0.98 p<0.05). In addition correlation analysis in DM patients revealed a significant (p<0.05) positive correlation between HbA1c and MDA, and between blood glucose and MDA (r=0.63 and r=0.71, respectively).

**DISCUSSION**

Oxidative stress is one of the metabolic events associated with diabetes and its complications. In fact, there is both experimental and clinical evidence proving that the production of ROS increases in both types of diabetes. However, the precise mechanisms by which oxidative stress may accelerate the development of complications in diabetes are only partially known. An efficient clinical diagnostic of the redox balance in diabetic patients is very important in order to control the degenerative damage associated with oxidative stress, or to follow the effect of a nutritional or therapeutic regimen.

Oxidative damage to proteins is of particular importance and is tested both for its main function (affecting the function of receptors, enzymes, transport proteins, etc. and perhaps generating new antigens that provoke immune response), and also for the contribution to secondary damage possible on other biomolecules. Fructolysine content caused by Maillard reactions is associated with oxidative processes. Fructolysine is a precursor of the AOPP, which are both induced by oxidative stress and also induce oxidative stress. Glycosilated proteins inactivate enzymes (for example, the antioxidant enzymes) also affect the functions of binding, transport and protein structure. As expected, the level of AOPP was higher in DM patients than that in normal subjects and the level of fructolisine was twenty-eight fold higher (Table 2). In addition, the protein oxidative damage is connected to the increment in HbA1c (Table 1). Testing of a new parameter for characterization of the degree of oxidative stress by a novel chemiluminescence methods, in diabetes patients, has shown that raised HbA1c is associated with lowered antioxidative protection (Total Antiradical Capacity of Water Soluble Substance, ACW) and raised Antiradical Ability of Plasma Protein (ARAP).

Endogenous defense mechanisms have been identified which use antioxidants or free radical scavengers to neutralize ROS-generated lipid peroxidation. However, in diabetic patients the extensive generation of free radicals appears to overwhelm the natural defense mechanisms, dramatically reducing the levels of endogenous antioxidants, thus leading to the uncontrolled...

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Control subjects (n=60)</th>
<th>Diabetic patients (n=101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructolysine (relative fructolysine content)</td>
<td>50 ±17</td>
<td>1373 ± 117</td>
</tr>
<tr>
<td>AOPP (µM of chloramine)</td>
<td>12.13 ± 0.93</td>
<td>20.49 ± 0.84</td>
</tr>
<tr>
<td>GSH (mM)</td>
<td>3.32 ± 0.62</td>
<td>2.83 ± 0.30</td>
</tr>
<tr>
<td>MDA (µM)</td>
<td>1.80 ± 0.07</td>
<td>8.91 ± 0.64</td>
</tr>
<tr>
<td>PP (µM)</td>
<td>7.63 ± 1.29</td>
<td>13.85 ± 1.34</td>
</tr>
<tr>
<td>GPx (U/mL/min)</td>
<td>30.28 ± 4.12</td>
<td>65.00 ± 8.08</td>
</tr>
<tr>
<td>SOD (U/mL/min)</td>
<td>1.46 ± 0.14</td>
<td>4.10 ± 0.14</td>
</tr>
<tr>
<td>CAT (U/L/min)</td>
<td>161.5 ± 23.11</td>
<td>2496 ± 230</td>
</tr>
<tr>
<td>CAT/SOD</td>
<td>0.11 ± 0.20</td>
<td>2.16 ± 0.10</td>
</tr>
<tr>
<td>TO (µM)</td>
<td>103.7 ± 17.7</td>
<td>142.3 ± 30.6</td>
</tr>
<tr>
<td>NO⁻³/NO⁻² (µM)</td>
<td>67.82 ± 22.44</td>
<td>51.28 ± 8.58</td>
</tr>
</tbody>
</table>

Table 2. Biomarkers of oxidative damage to biomolecules, antioxidant-prooxidant balance and nitric oxide. AOPP, advanced oxidation protein products; GSH, reduced glutathione; MDA, Malondialdehyde; PP, peroxidation potential; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; TO, total organoperoxide; NO⁻³/NO⁻², nitrites/nitrates.

Note: Data are mean ± SD. All means were significantly different (p < 0.05) between groups.
progression of peroxidative damage to cellular membranes. In this line, a lower content of GSH and a higher level of MDA were detected in the investigated patients (Table 2). This is consistent with the finding of Murakami et al., and Memisogullari et al., suggesting that GSH metabolism is altered in type 2 diabetic. Several studies support the hypothesis that in DM, chronic hyperglycaemia increase the polyl pathway as well as AGE formation and free radical generation rate, leading to increase GSH oxidation. A relative depletion of NADPH due to aldose reductase activation and secondary production thought the pentose cycle impairs GSH regeneration and lead to depletion of this free radical scavenger.

High levels of TO were found in diabetic patients compared with normal subjects. The glucose autoxidation is an additional source of H_{2}O_{2} in diabetic patients. In addition, it has been demonstrated that H_{2}O_{2} plays a role in the cross-linking of proteins in diabetes. The H_{2}O_{2}, derived from hyperglycemia has been demonstrated to promote cell death by necrosis in human aortic smooth muscle cells and this effect was reversed only when CAT was added to the culture medium. H_{2}O_{2} is able to activate the transcription factor NF-{\kappa}B, which promotes the generation of cell adhesion molecules, cytokines, and pro-coagulant tissue factor mediators of the vascular complications present in diabetic patients. The oxidative stress involvement in the activation of NF-{\kappa}B was well demonstrated when this factor was inhibited by antioxidants such as vitamin E and \alpha-lipoic acid.

Antioxidant enzymes levels are sensitive to oxidative stress. Both increased and decreased levels have been reported in different disease states in which an enhancement of oxygen species is a cause or a consequence of the disease. Table 2 shows a significant increase in GPx, CAT and SOD activity in the serum of DM patients. The increase in GPx and CAT activities may respond to an induction of the enzyme caused by the high level of its substrate (organoperoxides and H_{2}O_{2}). Our results indicate and the literature proves that there must be a relationship between SOD and CAT levels that may guarantee effective control of the oxidative stress, without the variation of one or the other being sufficient independently. The ratio CAT/SOD is considered to be a biomarker of the glycemic control and to be a risk factor in the development of diabetes complications.

The levels of NO \textsuperscript{-2}/NO \textsuperscript{-3} concentration, as a measure of NO, were depleted in diabetic patients. This depletion might be related to the vascular dysfunction that takes place in these patients. On the other hand, in the face of the impossibility of measuring all the components of the antioxidant / pro-oxidant system, any diagnosis set should count measure by using a global indicator of the redox balance. Serum PP is an expression of the balance between the generation and inactivation of those oxidized metabolites, and increment of PP in diabetic patients has been demonstrated. In the present study, the PP of diabetic patients was approximately twice the value of the non-diabetic subjects.

The physiologic antioxidant system involved in detoxification of organoperoxides and H_{2}O_{2} is basically formed by GPx and its substrate GSH and CAT. As a consequence, the correlation coefficient was higher between these variables in normal subjects. In these subjects, the general equilibrium of the redox system (measured by PP) was also correlated with the SOD/CAT balance and the levels of fructolisine. In addition, the serum glucose levels, as an index of metabolic control, were correlated with CAT/SOD, as reported by Sozmen et al. In diabetic patients these correlations were lost, thus reflecting the disruption of the physiological and metabolic control.

This study provides evidence of oxidative damage to different molecules and its impact on antioxidant / pro-oxidant system in type 2 diabetic patients with macroangiopatic complications. The clinical diagnosis used covers different components making an integrative analysis of the redox balance possible. The methods used are relatively cheap, easily performed, non time-consuming procedures and possess sufficient precision to be extrapolated for routine clinical analysis. The indicators evaluated could contribute to an integral overview in diabetic patients and could be used as indices of treatment efficacy.

Acknowledgements. Authors gratefully acknowledge the support from the Department of Chemistry and Medical Biochemistry, University of Milan, Italy and the technical assistance of Maité Casanova and Yamile Orta. This work was funded partially by Project Alma Mater 2002, Havana University, Havana, Cuba; and Alexander von Humboldt Foundation, Germany. Clive Prestt kindly checked the English of the manuscript.
REFERENCES