[Frontiers in Bioscience, Scholar, 5, 638-649, January 1, 2013]

Urban $PM_{2.5}$ activates GAPDH and induces RBC damage in COPD patients

Araceli Montoya-Estrada[1,2], Yessica D Torres-Ramos[1], Arturo Flores-Pliego[1], Alejandra Ramirez-Venegas[3], Guillermo M Ceballos-Reyes[2], Alberto M Guzman-Grenfell[1], Juan J Hicks[1]

[1]Departamento de Bioquimica y Biologia Molecular, Instituto Nacional de Perinatologia, Isidro Espinosa de los Reyes (INPerIER), [2]Laboratorio multidisciplinario Escuela Superior de Medicina, IPN, Mexico, [3]Clínica de EPOC, Instituto Nacional de Enfermedades Respiratorias (INER). Ismael Cosio Villegas Mexico

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
3.1. Collection of particulate matter
3.2. Patients
3.3. Treatment strategy
3.4. Hydroxyl (HO) radical generation
3.5. Biochemical analysis
3.6. Enzymatic assays
3.6.1. Glutathione peroxidase activity
3.6.2. Glutathione reductase activity
3.6.3. Glucose 6 phosphate dehydrogenase activity
3.6.4. Glyceraldehyde 3 phosphate dehydrogenase activity
3.6.5. Western blot analysis
3.7 Statistics
4. Results
4.1. Respiratory probes
4.2. Oxidation and enzymatic activities
5. Discussion
6. Conclusion
7. Acknowledgement
8. References

1. ABSTRACT

During Chronic Obstructive Pulmonary Disease (COPD) progression, the intracellular antioxidant defence in RBCs must preserve the integrity of the plasmalemma through $NADPH^+$ generation to obtain a sufficient number of reduced non-protein SH-groups. Here, we studied the activities of enzymes in RBCs that are related to glutathione metabolism under conditions of increasing oxidative stress, which are associated with COPD progression, by increasing cellular damage *in vitro* with $PM_{2.5}$, a ROS generator. The study included 43 patients, who were separated according to their GOLD classification into moderate and severe groups, along with 11 healthy volunteers (HV). Blood samples were analysed for G6PD, GAPDH, GPx, and GR. The results showed significant decreases in the oxidation of the G6PD, GR and GPx proteins, resulting in decreased enzymatic activity. By contrast, an increase ($p<0.05$) in GAPDH was observed, suggesting a pool of ATP on the membrane. However, it is evident that RBCs are damaged during the progression of COPD, although their integrity is preserved, and they retain limited function, thus allowing patient survival without haemolysis.

2. INTRODUCTION

Among all lung diseases, Chronic Obstructive Pulmonary Disease (COPD) is the most strongly correlated with exposure to air pollution and has been linked to urbanisation and population density. The Mexico City Metropolitan Area (MCMA) has 18 million inhabitants according to the 2000 census (1). The MCMA is an elevated basin approximately 2240 metres above sea level that is surrounded by mountains to the south, west, and east. At this altitude, 23% less oxygen is available than at sea level, which makes combustion less efficient.

The Global Initiative for Chronic Obstructive Lung Disease (1) outlines a simple classification system that divides disease severity into four stages: GOLD I-Mild, GOLD II-Moderate, GOLD III-Severe, and GOLD IV-Very Severe. Oxidative stress (OS) has been implicated in the pathogenesis and progression of COPD (2). OS causes structural changes to essential components of the lung, leading to irreversible damage to both the parenchyma and the airway wall. Moreover, a serious imbalance between reactive oxygen species (ROS) production and antioxidant defence leads to the activation

of transcription factors (such as nuclear factor NF-KB), the inactivation of anti-proteases, an increased sequestration of neutrophils in the pulmonary microvasculature, and oxidation-induced cellular injury (3). ROS are generated principally from leukocytes in the blood and air spaces or are inhaled in the form of environmental oxidant pollutants, including cigarette smoke, particulate matter (3), and wood smoke.

An increase in RBC oxidative stress has been reported in many pathological conditions associated with various diseases (4). This increase has been proposed as a biosensor of COPD progression because it is possible to detect oxidative modifications in cells during the GOLD II stage and after experimental exposure to ROS and reactive nitrogen species (RNS). Typically, these RBC modifications are studied by scanning electron microscopy and flow-cytometry analysis. RBCs exhibit a moderate OE response that is characterised by a decrease in the ion exchange functional capacity of the cells. Some affected exchanges include the O_2 and CO_2 uptake from the lungs or peripheral tissues, respectively, and CO_2 extrusion as HCO_3^- through the band 3 anion exchanger (5). The band 3 anion exchanger forms a scaffold for the assembly of a protein complex that can transmit extracellular signals and modulate the transport and mechanical properties of the erythrocyte. These altered RBCs have reduced transport capacities and reduced peripheral release of O_2 (4).

The mechanisms by which particulate matter (PM) produces adverse effects, such as COPD on the respiratory system are related to chemical compounds in the ambient PM (transition metals and aromatic organic compounds) that can contribute to the intrinsic generation of ROS. The increase in oxidative biomarkers due to COPD progression in RBC ghosts exhibited significant levels of $PM_{2.5}$, which is a ROS generator, while total SH groups in RBCs and the activities of band 3 phospho-tyrosine phosphatase (PTPase) and glucose-6-phosphate-dehydrogenase (G6PD, E.C 1.1.1.49) were diminished.

In this study, RBCs from COPD patients were incubated with $PM_{2.5}$ or a ROS generator system, and membrane biomarkers of oxidative injury were measured by assessing the activities of the following enzymes: glyceraldehyde 3 phosphate dehydrogenase (GAPDH, E.C 1.2.11.2), glucose 6 phosphate dehydrogenase (G6PD, E.C 1.1.1.49), glutathione reductase (GR, E.C 1.6.4.2) and glutathione peroxidase (GPx, E.C 1.11.1.9). These enzymatic activities are related to band 3 phospho-tyrosine phosphatase (PTPase) activity (5), which decreased under the experimental conditions in this study, thus affecting intracellular signalling and increasing activity in the glycolytic pathway (6).

The results presented here provide strong evidence that there is increased vulnerability to oxidative damage in the RBCs of COPD patients because chemical changes oxidise G6PD, GR and GPx, reducing their activities and rendering the antioxidant systems of the cell less effective. By contrast, an increase in GAPDH activity was observed, suggesting a pool of ATP on the membrane that can be replenished by glycolysis (7). However, it is evident that the membranes of RBCs are damaged during COPD progression, although their integrity is preserved and they retain limited function, thus allowing patient survival without haemolysis.

3. MATERIALS AND METHODS

Unless otherwise specified, all reagents used in this study were obtained from Sigma Chemical Co. (St. Louis, MO).

3.1 Collection of particulate matter

Respirable particles [aerodynamic diameter < 10 μm (PM_{10})] and fine particles [< 2.5 μm ($PM_{2.5}$)] were collected at the Centro Nacional de Investigación y Capacitación Ambiental (National Centre for Environmental Research and Training, CENICA). The samples were collected using Andersen-Graseby high volume samplers onto quartz fibre filters (Whatman). Before and after sample collection, the filters were conditioned at 22 ± 3°C and 40 ± 5% RH during a 24-hour period and weighed with an analytical balance (Sartorious, sensitivity 10^{-4} g). The filters were weighed, characterised and analysed following previously reported procedures. The composition of the particulate matter used includes a high concentration of metals; iron was the most abundant particle. (5).

3.2. Patients

A total of 43 patients with a COPD diagnosis and 11 healthy volunteers (controls) were enrolled in this study. COPD patients were ex-smokers and belonged to a cohort of subjects who were evaluated during a clinically stable period that was exacerbation free in the preceding six weeks. These subjects had a history of tobacco use, smoking at least 10 packs/year. A COPD diagnosis was established according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (1) with the patients divided into moderate and severe groups (Table 1). Subjects who met the criteria for COPD but had an alternative respiratory disorder (i.e., bronchiectasis or asthma) were excluded. The spouses of COPD patients that had no history of chronic illness, including diabetes, rheumatoid arthritis, or lung disease, were invited to participate as controls. Controls with a history of smoking tobacco were excluded. The protocol for this study (B10-08) was approved by the Ethics Committee at the National Institute of Respiratory Diseases. All subjects were informed, and their written consent was obtained.

3.3. Treatment strategy

Subjects underwent treatment for post-bronchodilator spirometry following the procedures recommended by the ATS and ERS (8). We used Mexican standard reference equations for predicted values (8). For spirometry, we used a dry rolling-seal volume spirometer (Sensormedics, Yorbalinda, CA). Subjects also underwent testing to assess their blood count and lipid profile.

Blood samples (5 mL) from both controls and COPD patients were obtained by venipuncture and centrifuged. The plasma and erythrocyte ghosts were obtained and used for stimulation with particles and in the Fenton reaction.

Table 1. Demographics and lung function characteristics

	Age (years)	Sex (F-M)	TS (Pack/yr)	BMI	FEV1 p (%)	FVC p (%)	FEV1/FVC
HV n=11	61.00±8.8	10-1	-------	27.6± 3	106±21.88	104.7±21	79.0±4
Moderate n=26	69.95±10	9-17	40.5±18	26.7± 4	78.55± 12	103.86± 8	57.99± 8
Severe n=17	68.02± 7.5	4-13	46± 15.5	26.18± 2.5	58.15± 6*	61.33± 17*	36.27± 9.5*

The data are the mean ± SD; TS= Tobacco smoking; BMI=body mass index; FEV =first forced expiratory volume; FVC=forced vital capacity. *Chi square test. Statistical significance $p<0.0001$

3.4. Hydroxyl (HO) radical generation

Hydroxyl radicals were generated by the Fenton reaction (9) using 1 mM H_2O_2 and 5 mM $CuSO_4$. After 5 min. of incubation at 37°C, the reaction was stopped by the addition of 5 mM citrate.

Following stimulation, open erythrocyte membranes were prepared by the hypotonic lysis of RBCs that were obtained from whole blood, as originally described by Steck (10), and these membranes were subsequently used for biomarker analyses.

3.5. Biochemical analysis

To evaluate lipoperoxidation, 1-methyl-2-phenylindole (Sigma-Aldrich, MO) was used as a standard. Aliquots of plasma were used to measure malondialdehyde (MDA) as per Gerard *et al.* (11). To evaluate lipid peroxides, we used the assay conditions described by El-Saadani *et al.* (12). Protein damage was evaluated by the carbonyl group content of the erythrocyte membranes, which was determined by treatment with 2,4-dinitrophenylhydrazine (DNPH) and measured according to Amici *et al.* (13). PTPase activity was determined using p-nitrophenyl phosphate (p-NPP) as a substrate, according to previously published procedures (14). Glucose 6 phosphate dehydrogenase (G6PD, E.C 1.1.1.49) activity was measured at an absorbance of 340 nm, as previously described (15).

3.6. Enzymatic assays

3.6.1. Glutathione peroxidase activity

Glutathione peroxidase (GPx, E.C 1.11.1.9) catalyses the reduction of hydroperoxides, including hydrogen peroxide, by using reduced glutathione, and it functions to protect the cell from oxidative damage. With the exception of phospholipid-hydroperoxide GPx, which is a monomer, all of the GPx enzymes are tetramers with four identical subunits. Each subunit contains a selenocysteine in the active site that participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine (16). The activity was measured by the Cayman Glutathione Peroxidase Assay Kit (No. 703102).

3.6.2. Glutathione reductase activity

Glutathione (GSH) is a tripeptide that is widely found in both plants and animals. GSH serves as a nucleophilic co-substrate to GSH transferases in the detoxification of xenobiotics and is an essential electron donor to GSH peroxidases in the reduction of hydroperoxides. GSH is also involved in the transport of amino acids across membranes.

Glutathione reductase (GR, E.C 1.6.4.2) is a homodimeric flavoprotein disulphide oxidoreductase that catalyses the NADPH-dependent reduction of oxidised glutathione (GSSG) to GSH. This enzyme is essential to the GSH redox cycle, which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress.

Glutathione reductase activity was evaluated by measuring the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm, thus providing a spectrophotometric means of detection that is directly proportional to the GR, E.C 1.6.4.2 activity in the sample. The reaction is thus measured by a decrease in the absorbance at 340 nm using the extinction coefficient 6220 M^{-1} cm^{-1} for NADPH (17).

3.6.3 Glucose 6 phosphate dehydrogenase activity

Glucose 6 phosphate dehydrogenase (G6PD, E.C 1.1.1.49) activity was measured as the absorbance at a wavelength of 340 nm, as previously described (15).

3.6.4. Glyceraldehyde 3 phosphate dehydrogenase activity

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH, E.C. 1.2.1.1.2) is a highly conserved protein with a key role in the glycolytic pathway. GAPDH catalyses the oxidative phosphorylation of glyceraldehyde 3 phosphate in the presence of NAD+ and inorganic phosphate. GAPDH, E.C. 1.2.1.1.2 activity was evaluated by the rate formation of NADH, which was measured at an absorbance of 340 nm at 37°C in a Beckman spectrophotometer (18).

A typical reaction mix consisted of 30 mM sodium pyrophosphate at pH 8.4, 0.04 mM cysteine hydrochloride, 30 mM sodium arsenate and 2.5 mM of β-NAD, for a final volume of 0.5 millilitres.

All enzymes are expressed in microkatals (µKat): µKat = 1 µmol substratum per minute with a reference value of mg protein (1 µmol/min/mg protein).

3.6.5. Western blot analysis

In all western blot analyses, 1 mg of protein was separated by SDS-polyacrylamide gel electrophoresis under reduced conditions and electroblotted to Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Medford, MA, USA) in a semidry blotting apparatus, according to the method described by Hirano and Watanabe (19). Electroblotting was performed for 90 min. at a constant current of 0.8 mA/cm^2. After electroblotting, the

membrane was blocked with 3% non-fat milk in TBS. The membrane was incubated with mouse anti-human G6PD antibody (Abcam cat no. ab91034) in 3% non-fat milk in TBS-T. For the detection of the mouse antibody, the membrane was incubated with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Abcam cat no. ab6728). The peroxidase activity was visualised by chemiluminescence using the ECL western blotting detection system (ECL ® Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions. Lysate from MCF7 cells (a human breast adenocarcinoma cell line) was used as a positive control.

3.7. Statistics

Data are expressed as the mean ± standard deviation. A one-way ANOVA and Bonferroni's Multiple Comparison Test, linear regression, and Pearson's correlation were used for the statistical analyses. Differences were considered to be significant for p-values < 0.05. Data analyses were performed using the Statistical Package for Social Sciences (version 10.0 for Windows; SPSS Inc., Chicago, Ill).

4. RESULTS

4.1. Respiratory probes

There were significant differences between the control and patient groups with respect to the post-bronchodilator FEV_1 per cent predicted, the forced vital capacity (FVC) per cent predicted, and the FEV_1/FVC ratio (Table 1).

There were no significant differences between the two groups with respect to age and body mass index (BMI). The mean cumulative tobacco consumption for COPD patients was 46 ± 26 pack-years. COPD patients had statistically higher hematocrit values in their blood counts.

This study included a total of 26 patients in the "moderate" group (7 patients (16.2%) in GOLD stage I and 19 (44.1%) in GOLD stage II) and 17 patients in the "severe" group (10 patients (23.2%) in GOLD stage III and 7 (6.2%) in GOLD stage IV). The only difference between the GOLD stage groups was in the spirometry parameters, as calculated using ANOVA (Table 1).

4.2. Oxidation and enzymatic activities.

Figure 1-A shows that the enzymatic activity of glucose 6 phosphate dehydrogenase decreased in all patient groups when PM was added, including the healthy volunteer (HV) group; however, only the moderate group showed a significant difference ($p < 0.05$) in enzymatic activity levels before and after the addition of PM. Figure 1-B shows a high correlation ($r = -0.6966$) that is statistically significant ($p < 0.0001$) between enzymatic activity and the degree of protein carbonylation. The western blot analysis carried out for G6PD is shown in figure 1-C. Western blots showed that there was no change in protein concentration, suggesting that the results obtained are due only to changes in enzymatic activity. Figure 2-A shows that the enzymatic activity of GR was significantly different between the studied groups when the PM was added. These decreases might be related to the level of oxidation ($p < 0.01$) (Figure 2-B) of the enzyme, which is evident as protein carbonylation ($r = -0.2798$). A similar oxidative effect was observed when GPx activities were compared between the B (basal) and PM groups, where there was a decrease in enzymatic activity ($p < 0.05$) (Figure 3). The oxidative correlation when enzymatic activity was compared to the amount of protein oxidation ($r = -0.2662$) had a p value < 0.01. The enzymatic activities of GAPDH are shown in Figure 4-A, in which there are significant increases in all of the studied groups when comparing healthy volunteers with COPD patients; the increase was greater when PM was added: 60 % for the HV group, 33 % for the moderate group, and 50 % for the severe group ($p < 0.05$). Figure 4-B shows the protein carbonylation compared with enzymatic activity, showing a significant increase ($p < 0.05$) in the activity and oxidation ($r = 0.2340$). Figure 4-C shows the inverse correlation between phospho-tyrosine phosphatase (PTP) and GAPDH activities ($p < 0.01$, $r = -0.2897$). Figure 5 shows the ratio of G6PD/GAPDH activities that were compared within the studied groups, showing a significant difference between the obtained values for the HV group before and after the PM addition ($p < 0.001$), decreasing by 56.9 %. The obtained values, when compared to the COPD values, were lower in the patient groups than in the HV group. The relationship between lipoperoxidation (malondialdehyde) versus protein carbonylation is shown in Figure 6. There were differences in the ratio when comparing the HV and patient groups ($p < 0.001$), as well as in the moderate group, when B was compared with PM values ($p < 0.05$). Figure 6-B shows the regression values when comparing MDA to carbonylation ($p < 0.01$, $r = -0.3143$).

5. DISCUSSION

COPD is a progressive inflammatory process in the lung that is caused predominantly by the inhalation of noxious gases and particulate material (PM). (2). Cigarette smoking (active and passive) is thought to be responsible for the vast majority of COPD cases; however, other sources of exposure, such as burning of biomass fuels for heating and cooking, working in polluted indoor and outdoor environments, and exposure to ambient PM from internal combustion engine exhaust, industrial emissions, and forest fires, are increasingly recognised as significant contributors to the development, progression, and exacerbation of COPD (3, 5, 20). Among all lung diseases, COPD is the most strongly correlated with exposure to air pollution and has been linked to rapid urbanisation in cities such as the Metropolitan Area of Mexico City (5). Oxidative stress has been implicated in the pathogenesis and progression of COPD (2). Oxidative stress causes structural changes to essential components of the lung, leading to irreversible damage to the parenchyma and the airway wall and affecting the transfer of oxygen to blood. Erythrocytes from COPD patients also exhibited severe structural damage, particularly to the membrane, that resulted in a decreased uptake of oxygen.

In RBCs, membrane and cytoskeletal proteins are particularly susceptible to oxidative damage, which is

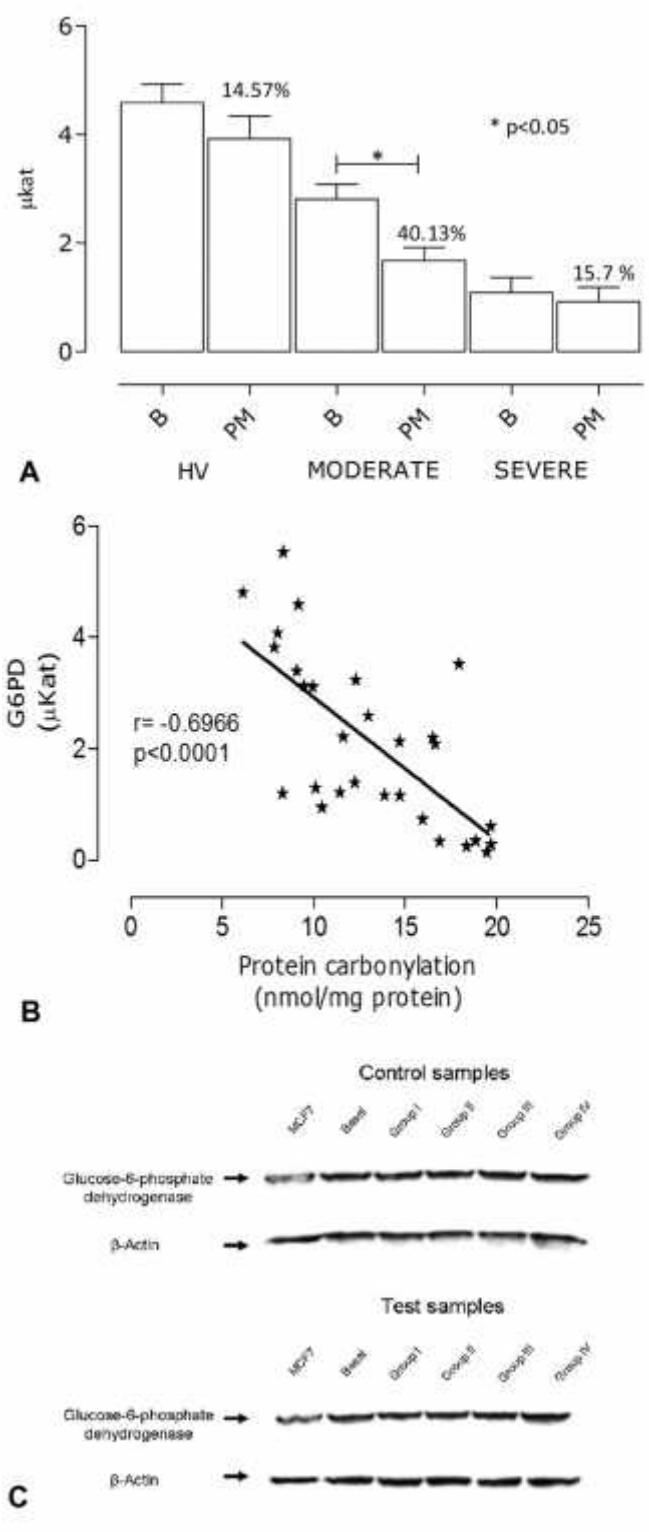


Figure 1. (1-A) The glucose 6 phosphate dehydrogenase (G6PD) activity expressed in µKat (1 µmol/min/mg protein) in RBCs measured in healthy volunteers (HV) or moderate and severe COPD patients. The values shown are before (B) and after (PM) incubation with $PM_{2.5}$. Percentages represent the decrease in enzymatic activity with respect to B for each group. (1-B) Protein carbonylation in RBCs and its correlation with negative G6PD activity. (1-C) Western blot analysis carried out for G6PD. β-actin was used as a control.

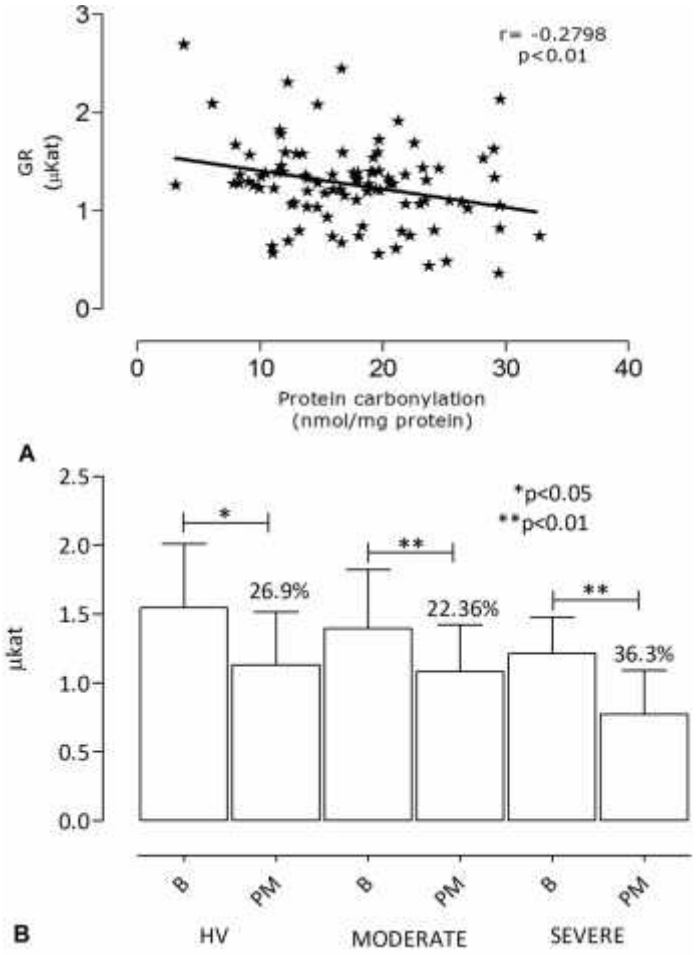


Figure 2. (2-A) The glutathione reductase (GR) activity expressed in µKat (1 µmol/min/mg protein) in RBCs measured in healthy volunteers (HV) compared with that of moderate and severe COPD patients. The values shown are before (B) and after (PM) incubation with $PM_{2.5}$. Percentages represent the decrease in enzymatic activity with respect to B for each group. (2-B) Protein carbonylation in RBCs and its correlation with GR activity in two COPD stages: before and after $PM_{2.5}$ addition.

thought to reduce whole-cell deformability and cause microrheology abnormalities that are similar to those observed in sickle RBCs. An increase in oxidative stress in RBCs leads to their clearance by naturally occurring antibodies that are directed to the band 3 protein; RBCs are also cleared in different hereditary haemolytic malaria disorders and in other pathological conditions and diseases, including COPD. In all of the aforementioned situations, RBCs undergo oxidative stress, and hemichromes (haemoglobin degradation products) are formed. Hemichromes possess a strong affinity for band 3 cytoplasmic domains and, following their binding, lead to band 3 oxidation and affect enzymatic activities, including that of the band 3-associated phospho-tyrosine phosphatase (PTPase) (21). Therefore, the decrease in PTPase activity is considered to be an excellent indicator of oxidative injury (2, 5, 21).

The phosphorylation of tyrosine residues in human RBC band 3 proteins is regulated *in vivo* by constitutively active tyrosine-kinases (PTKs), including p72(syk) and p56/53(lyn), and phosphor-tyrosine-phosphatases (PTPs), including PTP1B and SHPTP-2. (22) Tyr-phosphorylation of band 3 increases upon a reduction of cell volume, as occurs in hypertonic media (NaCl) (14) or by the inhibition of PTPs (vanadate, thiol oxidation, and peroxynitrite (23)). Quercetin and its analogue, (+)-catechin, inhibit the peroxynitrite-dependent upregulation of band 3 tyrosine phosphorylation. (23). Our results demonstrate that ROS generated by $PM_{2.5}$ *in vitro* significantly reduced (55 %) the activity of PTPs in RBCs from the group of healthy volunteers (0.72 to 0.33 nmoles/min) and COPD patients (Figure 4-C), inducing a release of glycolytic enzymes from band 3.

Figure 4-A shows an increase of GAPDH activity (60 %) when comparing the control treatment (B) with the PM treatment in the HV group. A similar effect was obtained using RBCs from moderate or severe COPD patients. It is noteworthy that the oxidation of the RBC proteins, including GAPDH, exhibited (Figure 4-B) a significant correlation with an increase in enzymatic

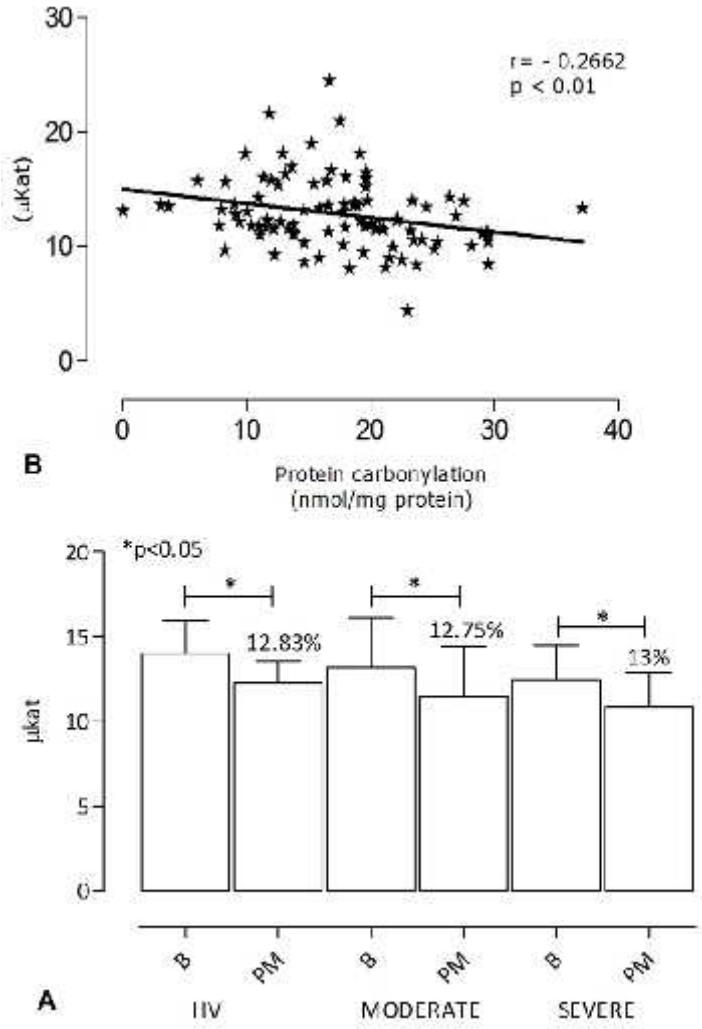
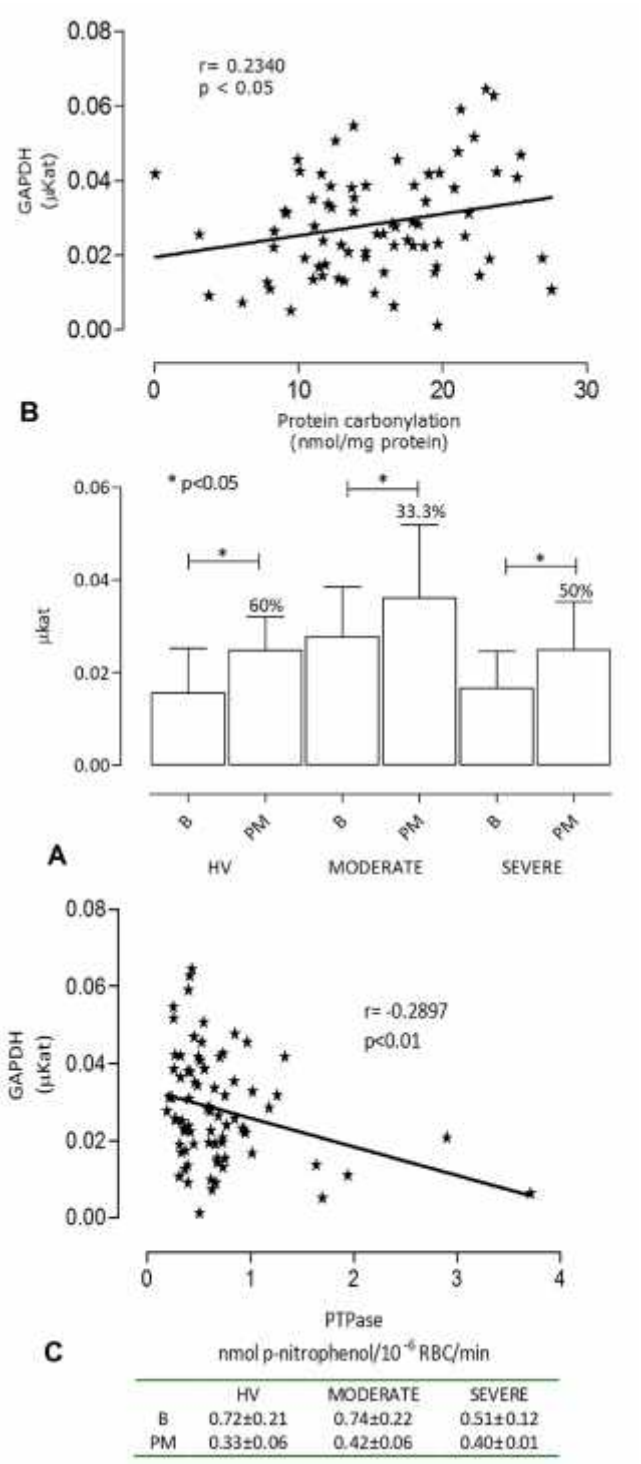


Figure 3. (3-A) The glutathione peroxidase (GPx) activity expressed in µKat (1 µmol/min/mg protein) in RBCs measured in healthy volunteers (HV) compared with that of moderate and severe COPD patients. The values shown are before (B) and after (PM) incubation with $PM_{2.5}$. Percentages represent the decrease in enzymatic activity with respect to B for each group. (3-B) Protein carbonylation in RBCs and its correlation with GPx activity in two COPD stages: before and after $PM_{2.5}$ addition.

activity. RBC oxidative stress was evident during COPD progression, and the increased damage to the red blood cells *in vitro* might cause the oxidation of PTPase SH groups, reducing their ability to facilitate GAPDH release and activation. The oxidation (carbonylation) of the enzyme was insufficient for oxidising the four catalytic SH groups, however, resulting in the activation of the enzyme (Figure 4-A) and leading to increased substrate accessibility to the catalytic site and/or relieving the allosteric inhibition of one or two subunits. (24). Alternatively, GAPDH might contribute to polarity modifications as a result of decreased G6PD activity under low RBC oxygenation (25); these modifications could facilitate the release of the enzyme from the membrane, increasing its activity and consequently increasing the activity of the glycolytic pathway over a less-favourable pentose shunt (Figure 5).

The oxygenation state of the erythrocyte regulates multiple RBC properties, including changes in the activities of many membrane solute transporters in response to the oxygen content of the cell. Some examples include volume regulatory transporters (e.g., Na^+/H^+ exchanger), cation-coupled Cl^- co-transporters, amino acid transporters and ion channels (26). The K/Cl co-transporter, for example, is reported to be 20-fold more active in oxygenated RBCs than in deoxygenated RBCs (26). Erythrocytes are modulated by the O_2 tension of the medium. Thus, glucose flux through the pentose phosphate pathway proceeds twice as rapidly in oxygenated cells as in deoxygenated cells, and glycolysis is inhibited by oxygenation (27). The deoxygenation of RBCs dislodges all glycolytic enzymes from the membrane (28), which is consistent with the established ability of deoxyHB but not oxyHb to bind the N-terminus of band-3. Tyrosine



	HV	MODERATE	SEVERE
B	0.72±0.21	0.74±0.22	0.51±0.12
PM	0.33±0.06	0.42±0.06	0.40±0.01

Figure 4. (4-A) The glyceraldehyde 3 phosphate dehydrogenase (GAPDH) activity expressed in µKat (1 µmol/min/mg protein) in RBCs measured in healthy volunteers (HV) compared with that of moderate and severe COPD patients. The values shown are before (B) and after (PM) incubation with $PM_{2.5}$. Percentages represent the increase in enzymatic activity with respect to B for each group. (4-B) The protein carbonylation in RBCs and its correlation with GAPDH activity in two COPD stages: before and after $PM_{2.5}$ addition, presented as a positive correlation. (4-C) The phospho-tyrosine phosphatase (PTPase) activity in RBCs and its correlation with GAPDH activity, showing a negative correlation. The data shown in the table are PTPase activity expressed as nmol p-nitrophenol/10^6 RBC/min (mean ± SD).

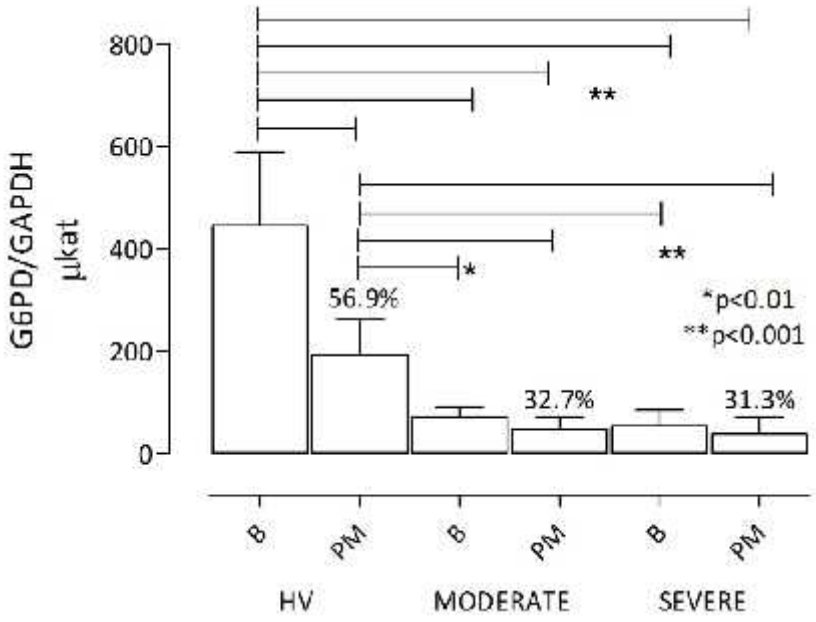


Figure 5. Ratio obtained from G6PD (representative of the pentose shunt) compared with the GAPDH enzyme (glycolysis); the activity is expressed as µKat/mg protein in RBCs measured in healthy volunteers (HV) versus the activity in moderate and severe COPD patients. The values shown are before (B) and after (PM) incubation with $PM_{2.5}$. Percentages represent the decrease in enzymatic activity with respect to B for each group.

phosphorylation (4, 14) of the N-terminus of band-3 also reversibly releases all of the glycolytic enzymes, including the key enzyme GAPDH, which interacts specifically with an acidic sequence at the N-terminus of band 3.

The results shown here suggest that the metabolic response in COPD patients is characterised by a progressive decrease in G6PD activity, which was diminished in both the moderate and severe COPD groups (38.7 and 76.21 %, respectively, Figure 1-A) when compared with the HV group, as well as in all of the studied groups compared to their controls before the PM was added *in vitro* (14.5, 40.13 and 157 % in the HV, moderate and severe groups, Figure 1-A). This inhibition might be related to catalytic-SH oxidation and not to changes in the amount of enzyme (shown by western blot), which remained constant (Figures 1-A and 1-C). The enzymatic activity was affected both by GOLD status and by the additional incubation with a ROS generator (PM) (5). The inverse correlation of enzymatic activity and carbonylation ($p<0.0001$, $r = -0.6966$, Figure 1-B) addressed the decrease in activity with an increase in protein oxidation, which resulted in their molecular modification (carbonylation), leading to functional alterations, as demonstrated with free-circulating hormones and particulate or soluble enzymes (2, 5, 29).

As COPD progresses in patients and damage to their RBCs increases due to the addition of ROS generators, the consequences of oxidative stress are often reflected in protein modifications, including the production or addition of carbonyl groups (CO), aldehydes and ketones on oxidised protein side chains (especially of Pro, Arg, Lys, and Thr). These moieties are chemically stable, which allow their detection. In addition, CO groups may be introduced into proteins by secondary reactions of the nucleophilic side chains of Cys, His, and Lys residues with aldehydes (4-hydroxy-2-nonenal, malondialdehyde (MDA), 2-propenal (acrolein)) produced during lipid peroxidation. The ratio of lipid to protein oxidation was markedly different when comparing the HV group to the moderate and severe COPD groups, exhibiting a decrease in the ratio of MDA to carbonylation (67 % and 46 %, respectively, Figure 6-A); the HV group showed twice as much oxidation of lipids (MDA) compared to that of proteins. The predominant oxidation of lipids in the HV group might be considered a pattern for moderate oxidative injury, in contrast to the pattern of major protein oxidation, which is related to both newly formed CO and additional previously formed groups that are products of an active oxidation of lipids. The increase in protein carbonyls showed an inverse correlation with MDA at the measured concentration ($r = -0.3143$, $p < 0.01$) due to the formation of MDA-protein adducts with the exposure of new carbonyl groups on the protein (Figure 6-B). The major molecular alterations were observed in RBCs from severe COPD patients after the rigorous addition of PM (Figure 6-A).

In human RBCs, there are several catalytic differences between GAPDH and G6PD, including the Michaelis constant (Km) values and the number of catalytic sites, depending on their quaternary structure; GAPDH is a tetramer and has one catalytic SH group for each monomer, and G6PD is a dimer with two active SH-dependent sites. These differences affect the regulation of the metabolic function of the respective enzymes. The membrane

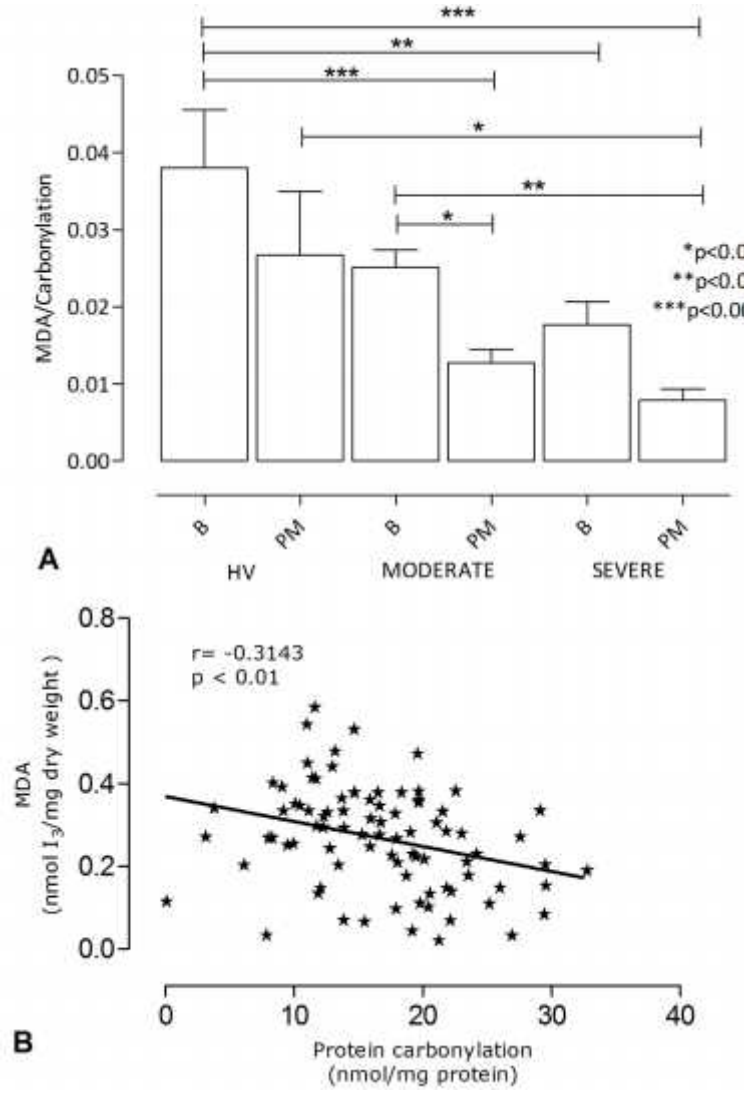


Figure 6. (6-A) Ratio of lipoperoxidation (MDA) to protein oxidation (carbonylation) measured in RBCs from healthy volunteers (HV) versus that of moderate and severe COPD patients; the values shown are before (B) and after (PM) incubation with $PM_{2.5}$. (6-B) shows the protein carbonylation in RBCs and its negative correlation with MDA.

integrity of the cell is in important factor to consider because the higher basal activity of G6PD produces sufficient NADPH for antioxidant and repair mechanisms. ATP generation and oxygen uptake/release are related to GAPDH efficiency. Under basal conditions, G6PD is 100 times more active than GAPDH. In Figure 5, the comparative ratio of the enzymatic activities of G6PD/GAPDH is shown with the specific activity of both enzymes expressed in µKat (1 µmol/min/mg protein), demonstrating comparative preferences for the metabolic use of glucose. The HV group showed a metabolic shift (30) in glucose metabolism from a pentose shunt (G6PD) to glycolysis (GAPDH) (Figure 5) after the PM addition, decreasing G6PD activity (14.6 %) (Figure 1-A) and increasing GAPDH activity (60 %) (Figure 4-A). The major decrease in G6PD activity occurred when HV controls were compared with severe patients (76.21 %); however, the increase in GAPDH activity was only 6.6 %. Lower ratios were obtained from COPD patients (Figure 5).

Glycolytic enzymes reversibly associate with the human erythrocyte's plasmalemma as part of their regulatory mechanism. The site for this association has been described as the amino terminus of band 3, a transmembrane anion transporter. Band 3 protein (anion exchanger 1, or AE1) is made of two distinct domains: (i) a membrane-spanning segment that catalyses the exchange of anions across the membrane and (ii) a cytoplasmic segment that serves as the locus of multiple interactions between the integral domain of the protein, the membrane skeleton and various cytosolic proteins, including haemoglobin and glycolytic enzymes. Because anion transport across the red blood cell membrane is modulated by the ATP concentration, we postulate that ATP may act primarily through the phosphorylation of a specific membrane

tyrosine kinase, leading to the majority of its phosphorylation.

The deficient antioxidant capacity of RBCs that results from the reduced availability of non-protein sulfhydryl group (SH) during COPD progression was previously reported (5) and has been confirmed by three of the enzymes related to metabolism. G6PD generates the necessary NADPH to serve as a substrate for glutathione reductase (GR), which has reduced activity (due to its oxidation) when comparing the RBC activity from COPD patients with that of the HV group; significant differences were obtained when comparing PM groups (Figure 2-A) because the correlation between the enzymatic activity in µKat versus carbonylation was significant (Figure 2-B). The number of non-protein SH groups that are available for glutathione peroxidase activity (Figure 3-A), which reduces the peroxidation of the membrane, is limited because both the glutathione-SH that is available as a substrate and its oxidation reduce the enzymatic activity by at least 12.8 % compared with the induced injury (PM).

6. CONCLUSION

In summary, we have demonstrated that COPD progression and ROS generation during oxidative stress together cause continuous damage to RBCs, diminishing their intracellular oxygenation. This damage results from both low oxygen availability and the alteration of transporters, such as the PTPase-mediated decrease in AE1 function, which caused two events: i) glycolytic enzymes were released from the membrane and consequently activated, inducing a pentose shunt (NADP generation) to glycolysis and ii) oxidation and decrease of glutathione metabolism-related enzymatic activities. However, the erythrocyte membranes were damaged as a result of the oxidation; consequently, the metabolic function of these RBCs adapted to the new substrate, cofactor and low oxygen conditions. Therefore, these adapted cells are able to maintain the survival of the COPD patient by preventing RBC lysis and increasing ATP generation through glycolysis instead of through anti-oxidant enzymatic mechanisms that are dependent on NADPH generation from an active pentose shunt.

7. ACKNOWLEDGEMENT

This work was part of a PhD by Araceli Montoya-Estrada, Escuela Superior de Medicina. IPN-Mexico. This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT) Register: 200221.

8. REFERENCES

1. KF Rabe, S Hurd, A Anzueto, PJ Barnes, SA Buist, P Calvery, Y Fukuchi, C Jenkins, R Rodriguez-Roisin, C Van Weel & J Zielinski: Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 176(6), 532-555 (2007)

2. YD Torres-Ramos, ML Garcia-Guillen, IM Olivares-Corichi & JJ Hicks: Correlation of Plasma Protein Carbonyls and C-Reactive protein with GOLD Stage Progression in COPD Patients. *Open Respir Med J* 3, 61-66 (2009)

3. MP Sierra-Vargas, AM Guzman-Grenfell, S Blanco-Jimenez, JD Sepulveda-Sanchez, RM Bernabe-Cabanillas, B Cardenas-Gonzalez, G Ceballos & JJ Hicks: Airborne particulate matter PM2.5 from Mexico City affects the generation of reactive oxygen species by blood neutrophils from asthmatics: an in vitro approach. *J Occup Med Toxicol* 29, 4-17 (2009)

4. G Lucantoni, D Pietraforte, P Matarrese, L Gambardella, A Metere, G Paone, E Li Bianchi & E Straface: The red Blood Cell as a Biosensor for Monitoring Oxidative Imbalance in Chronic Obstructive Pulmonary Disease: An Ex vivo and In vitro Study. *Antioxid Redox Signal* 8(7-8), 1171-1182 (2006)

5. YD Torres-Ramos, A Montoya-Estrada, AM Guzman-Grenfell, J Mancilla-Ramírez, B Cardenas-Gonzalez, S Blanco-Jimenez, JD Sepulveda Sanchez, A Ramirez-Venegas & JJ Hicks: Urban PM2.5 induces ROS generation and RBC damage in COPD patients. *Front Biosci* E3, 808-817 (2011)

6. SB Walsh & GW Stewart: Anion exchanger 1: Protean function and associations. *Int J Biochem Cell Biol* 42(12), 1919-1922 (2010)

7. JF Hoffman: ATP compartmentation in human erythrocytes. *Curr. Opin. Hematol* 4, 112–115 (1997)

8. R Perez-Padilla, G Valdivia, A Muino, MV Lopez, MN Marquez, M Montes de Oca, C Talamo, C Lisboa, J Pertuze, B Jardim JR & B Menezes AM: Spirometric reference values in 5 large Latin American cities for subjects aged 40 years or over. *Arch Bronconeumol* 42, 317-325 (2006)

9. C Ortega, AM Guzmán-Grenfell & JJ Hicks: Oxidation of PMSG by oxygen-free radicals, change its structure, affecting the hormonal activity. *Mol Reprod Dev* 52, 264-268 (1999)

10. TL Steck & JA Kant: Preparation of imperineable ghost and inside-out vesicles from human erythrocyte membranes. *Methods Enzymol* 231, 172-180 (1974)

11. D Gerard-Monnier, I Erdelmeier, K Reganrd, N Moze-Henry, JC Yadan & J Chaudiere: Reactions of 1-Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Analytical Applications to a Colorimetric Assay of Lipid Peroxidation. *Chem Res Toxicol* 11(10), 1176-1183 (1998)

12. M El-Saadani, H Esterbauer, M El-Sayed, M Goher, AY Nassar & G Jürgens: A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J Lipid Res* 30, 627-630 (1989)

13. A Amici, RL Levine, L Tsai & ER Stadtman: Conversion of amino acids residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed reactions. *J Biol Chem* 264(6), 3341-3346 (1989)

14. Y Zipser, A Piade, A Barbul, R Korenstein & NS Kosower: Ca2+ promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. *Biochem J* 15(368), 137-144 (2002)

15. NH Kirkman: Glucose 6-phosphate dehydrogenase from human erythrocytes. *J Biol Chem* 23, 2364-2370 (1962)

16. DE Paglia & WN Valentine: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 70, 158-169 (1967)

17. I Calberg & B Mannervick: Glutathione reductase. *Methods Enzymol* 113, 484-490 (1985)

18. R Medina-Navarro, G Duran-Reyes & JJ Hicks: Pro-oxidating properties of melatonin in the in vitro interaction with the singlet oxygen. *Endocr Res* 25, 263-280 (1999)

19. H Hirano & T Watanabe: Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis: application to an insoluble protein. *Electrophoresis* 11, 573–580 (1990)

20. A Ramírez-Venegas, RH Sansores, R Pérez-Padilla, J Regalado, A Velázquez, C Sánchez & ME Mayar: Survival of patients with chronic obstructive pulmonary disease due to biomass smoke and tobacco. *Am J Respir Crit Care Med* 173(4), 393-397 (2006)

21. L Bordin, C Fiore, G Doná, A Andrisani, G Ambrosini, D Faggian, M Plebani, G Clari & D Armani: Evaluation of erythrocyte band 3 phosphotyrosine level, glutathione content, CA-125, and human epididymal secretory protein E4 as combined parameters in endometriosis. *Fertil Steril* 94(5), 1616-1625 (2010)

22. A Ciana & G Minetti, C Balduini: Phosphotyrosyne phosphatases acting on band 3 in human erythrocytes of different age: PTP1B processing during cell ageing. *Bioelectrochemystry* 62(2), 169-173 (2004)

23. A Maccaglia, C Mallozi & M Minetti: Differential effects of quercetin and resveratrol on band 3 tyrosine phosphorylation signalling of red blood cells. *Biochem Biophys Res Commun* 305(3), 541-547 (2003)

24. FO Sale, E Vanzulli, S Caielli & D Taramelli: Regulation of human erythrocyte glyceraldehyde-3-phosphate dehydrogenase by ferriprotoporphyrin IX. *FEBS Lett* 579, 5095-5099 (2005)

25. DJ Jollow & DC McMillan: Oxidative stress, glucose-6-phosphate dehydrogenase and the red cell. *Adv Exp Med Biol* 500, 595-605 (2001)

26. C Drew, V Ball, H Robinson, J & Clive Ellory, JS Gibson: Oxygen sensitive of red cell membrane transporters revisited. *Bioelectrochem* 62, 153–158 (2004)

27. I Messana, M Orlando & L Cassiano: Human erythrocyte metabolism is modulated by the O2- linked transition of hemoglobin. *FEBS Lett* 390, 25-28 (1996)

28. ME Campanella, H Chu & PS Low: Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. *PNAS* 102, 2402-2407 (2005)

29. R Medina-Navarro, AM Guzmán-Grenfell, I Olivares-Corichi & JJ Hicks: Structural and functional changes in the insulin molecule produced by oxidative stress. *Methods Mol Biol* 594, 141-53 (2010)

30. R Dybkaer: The tortuous road to the adoption of katal for the expression of catalytic activity by the General Conference on Weights and Measures. *Clin Chem* 48(3), 586-590 (2002)

Key Words: Antioxidant enzyme, Glycolysis, red blood cell, Oxidative Stress, COPD, Particulate matter, Glutathione.

Send correspondence to: Juan J. Hicks, Instituto Nacional De Perinatologia, Isidro Espinosa de los Reyes, Montes Urales, 800. Colonia Lomas Virreyes C.P 11000, Del, Miguel Hidalgo, Tel: 01152 55209900, ext 438 Fax: 01152 55200034 E-mail: jjhicks2002@yahoo.com.mx