ALTERATION OF SOME BIOPHYSICAL PROPERTIES OF ERYTHROCYTES OF SILICOTIC PATIENTS

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Abstract. Long-term exposure to silica has been reported to be the principal cause of silicosis which is perhaps the best-known occupational disease. In the present study, we investigated plasma malondialdehyde (MDA) level as an index of lipid peroxidation and erythrocyte reduced glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities levels as an index of antioxidant status in silicotic patients. The exposed workers were divided into three categories according to the intensity of their exposure, which had been weak, moderate and strong. Results showed that there is a significant decrease of erythrocyte GSH, GPx and SOD as well as a significant increase in MDA level in comparison with the controls. Moreover, a functional disturbance in RBC membrane occurred as revealed by alteration in hemorheological and dielectric properties. Thus, the mentioned techniques seem to be reliable, rapid and sensitive for detecting erythrocytes damage cells and fulfil the requirements of biological markers for silica exposure.

Key words: Silicosis, biophysical properties, antioxidants, dielectric spectroscopy.

INTRODUCTION

Silica is one of the most documented workplace contaminants. Long-term exposure to silica has been reported to be the principal cause of silicosis. A recent review [11] estimates 8,800 silicosis deaths a year worldwide. Occupational exposure to dust containing crystalline silica occurs in mining, ceramics production, etc. [3].

Because the body cannot clear or metabolize a respirable portion of inhaled mineral dust particles, fibrosis develops in the upper regions of the lungs, which interferes with their normal expansion. Alveolar macrophages are destroyed, with fibrotic nodules forming around them. Alveolar and interstitial macrophages are activated after particle uptake and produce reactive oxygen species (ROS).

The high polyunsaturated fatty acid content of the erythrocyte membrane and the continuous exposure to high concentrations of oxygen and iron in hemoglobin are factors that make erythrocytes very sensitive to oxidative injury, making them an appropriate model to study oxidative stress [24]. Fortunately, the cells are able

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to protect themselves against oxidative stress, as they developed numerous defensive mechanisms based on the antioxidative enzymes activity and action of low molecular antioxidants such as glutathione [26]. ROS attack on cell membranes results in formation of lipid peroxidation products such as malondialdehyde (MDA). In the present study, we investigated erythrocyte reduced glutathione (GSH), erythrocyte glutathione peroxidase (GPx) and erythrocyte superoxide dismutase (SOD) activities levels as an index of antioxidant status in silicotic patients. Orman *et al.* [30] showed that direct measurement of MDA and GSH could be accepted as an indicator of oxidative injury in workers exposed to cement dust.

With more stringent regulations of environmental exposure, a variety of modifications have been integrated into the traditional approach to increase the sensitivity and specificity of epidemiological studies. Perera and Weinstein [31] suggested the incorporation of laboratory analytical techniques with traditional epidemiological surveys to elucidate the biochemical or molecular basis of disease etiology. Other studies have been conducted to provide useful analytical data, such as internal exposure doses and biological effects [6]. Clearly there is a need for methods to monitor early adverse effects, exposure and/or susceptibility of individual subjects due to occupational causes.

Dielectric spectroscopy is a promising technique for investigating cell structure in a non-invasive manner. The aim of the present work is to apply dielectric spectroscopy, biochemical analysis and hemorheological properties as biophysical-biochemical probes to detect perturbations and early pathological manifestations associated with silica occupational hazards.

MATERIALS AND METHODS

STUDY SUBJECTS

This study was conducted at Kasr El-Eini Hospital after approval of Occupational Medicine Department (Faculty of Medicine – Cairo University) and the Local Ethics and Research Committee. Subjects were consecutively enrolled if informed consent had been given by them.

Silicosis presents radiographically with small opacities, more commonly rounded but occasionally irregular, in the upper or mid zones of the lung. As disease advances, opacities may become visible throughout all lung zones and may become confluent.

In the present study a person is considered to have silicosis if there is: 1) a history of exposure to silica for at least 3 years, and 2) a chest X-ray interpretation showing rounded opacities of 1/0 or greater profusion per the International Labour Office's (ILO) classification system for pneumoconiosis.

For each subject, the earliest chest radiograph at the time of initial diagnosis or thereafter and the most recent chest radiograph, both of acceptable quality, were selected. The X-ray films were read independently by three experienced readers according to the international standard classification of radiographs of pneumoconiosis 1980. Miniati *et al.* [29] suggested that the information derived from standardized reading of chest radiographs is comparable to that obtained by quantitative analysis of high-resolution computed tomography scans.

Selected silicotic patients were without clinical cardiovascular disorders or malignant respiratory disease at the time of survey. The exposed workers (with age range of 40–60 years) were divided into three categories according to the intensity of their exposure, which had been weak (mild, GpI) for a first group of 52, moderate (GpII) for a second group of 47 and strong (GpIII) for a third group of 33. GpII have the longest durations of exposure, i.e., generally more than 20 years. A standard questionnaire [5] was used as part of the process to confirm the diagnosis of silicosis. The control group (C) consisted of 30 healthy male persons matched with the silicotic for age.

BIOCHEMICAL MEASUREMENTS

For biochemical measurements, whole blood was collected into EDTA-coated tubes and centrifuged at 3500×*g* at 4°C for 10 min. The erythrocyte pellet was separated and stored at –70°C until analyzed (≤ 24 hours). All calorimetric measurements were carried out, using UV-1601 spectrophotometer with CPS-Controller 240A temperature control attachment (Shimadzu, Japan). Hemoglobin concentration of the samples was measured by Drabkin's method [20] to express erythrocyte GSH, GPx and SOD activity values in units per gram hemoglobin.

Glutathione assay

RBCs were thawed and resuspended in 4 volumes 5% ice cold metaphosphoric acid. After the sample was mixed by vortex and centrifuged at 3000×*g* for 10 min at 4°C, the upper aqueous layer was collected and assayed with the use of a GSH-400 spectrophotometric assay kit (Bioxytech; OXIS International, Portland, OR).

Glutathione peroxidase assay

Cells were washed with 10 volumes cold buffer, which consisted of 50 mmol tris-HCl/L [Tris (hydroxymethyl) aminomethane hydrochloride; pH = 7.5] containing 5 mmol EDTA/L and 1 mmol dithiothreitol/L. Samples were centrifuged again at 8500×*g* for 10 min at 4°C and the supernatant fluid was discarded. Cells

were then lysed by adding exactly 4 volumes of ice-cold deionized water. After centrifuging again at 8500×*g* for 10 min at 4°C, the supernatant fluid was collected and stored at –70°C for analysis. GPx activities were measured by using a GPx-340 spectrophotometric assay kit (Bioxytech; OXIS International, Portland, OR).

Superoxide dismutase assay

RBCs were thawed, resuspended in 4 volume ice-cold water, and mixed thoroughly by vortex. An ice-cold extraction reagent of ethanol: chloroform (62.5:37.5, by volume) was added to the erythrocyte suspension and the suspension was mixed by vortex for 30 sec. After centrifugation again at 3500×*g* for 10 min at 4°C, the upper phase was collected and stored at –70°C until analyzed. Cu/Zn superoxide dismutase activities were measured by using a superoxide dismutase-525 spectrophotometric assay kit (Bioxytech; OXIS International).

Serum malondialdehyde

For measurement of MDA, 0.25 ml of serum was treated to 2.5 ml of 20% trichloroacetic acid, and then 1ml of 0.67% thiobarbituric acid. The mixture was incubated at 100°C for 30 min. After cooling, the sample was extracted with 4ml *n*-butanol and centrifuged at 3000×*g* for 20 min. The absorbances of extract were measured at 535 nm, and the results were expressed as nmol/ml. MDA standards were prepared from 1, 1, 3, 3-tetraethoxypropane [21].

ERYTHROCYTE MEMBRANE PROPERTIES

RBC membrane properties were estimated using the osmotic fragility test [9]. In the differential osmotic fragility curves, *C*(%) is the NaCl concentration at which hemolysis starts to occur. This value indicates the relative permeability of RBC membrane. Also, the width at half maximum (W_{hmax}) of the differential curves is observed to estimate the relative elastic limit of the RBC membrane. Erythrocyte sedimentation rate (*ESR*) was measured using Westergreen's method [9].

DIELECTRIC MEASUREMENTS

Measurements of dielectric properties of erythrocytes were carried out by means of a resonance technique. This method made use of a commercially available LCR bridge model HIOKI "3532 LCR HiTESTER HIOKI E.E. Corporation manufactured in Japan; together with a sample cell type PW 9510/60

manufactured by Philips. The sample cell has two squared platinum black electrodes, each having an area of one cm^2 with an inter-electrode distance of one cm. The sample cell was kept at 37±0.1°C in a temperature controlled incubator; then the dispersion characteristic in the frequency range 20 Hz to 10 MHz was investigated. Correction of residual electrode polarization and determining dielectric parameters were performed in accordance with Ali [2].

The conductance of the sample G_s is given by

$$G_s = 1/R_s = \sigma A/d = 2\pi f \varepsilon_\circ \varepsilon'' A/d \tag{1}$$

where d is the distance between the two electrodes, A is the area of each electrode, ε_o is the permittivity constant of free space and ε'' is the dielectric loss.

The capacitance of the sample is given by

$$C_s = \varepsilon' \varepsilon_o \frac{A}{d} \tag{2}$$

where ε' is the relative permittivity of the sample.

The Cole-Cole equation [8] is given by

$$\varepsilon^* - \varepsilon_\infty = (\varepsilon_o - \varepsilon_\infty)/[1 + (i\omega\tau_o)^{1-\alpha}] \tag{3}$$

where α ($0 < \alpha < 1$) is the Cole-Cole spread parameter, τ is the relaxation time which is related to the critical frequency (f_c) by $\tau = 1/(2\pi f_c)$.

Knowing that $i^{(1-\alpha)} = e^{i(1-\alpha)\pi/2}$, $e^{i\theta} = \cos(\theta) + i \cdot \sin(\theta)$, and using the trigonometric relations for the subtraction of two angles, it could be shown that the real and imaginary parts of the Cole-Cole equation can take the forms

$$\varepsilon'(f) = \varepsilon_\infty + \sum_n \frac{\Delta\varepsilon_n \left[1 + \left(f/f_{c_n}\right)^{1-\alpha_n} \sin\left(\alpha_n \pi/2\right)\right]}{1 + \left(f/f_{c_n}\right)^{2(1-\alpha_n)} + 2\left(f/f_{c_n}\right)^{1-\alpha_n} \sin\left(\alpha_n \pi/2\right)} \tag{4}$$

$$\varepsilon''(f) = \sum_n \frac{\Delta\varepsilon_n \left[\left(f/f_{c_n}\right)^{1-\alpha_n} \cos\left(\alpha_n \pi/2\right)\right]}{1 + \left(f/f_{c_n}\right)^{2(1-\alpha_n)} + 2\left(f/f_{c_n}\right)^{1-\alpha_n} \sin\left(\alpha_n \pi/2\right)} + \frac{\sigma_s}{2\pi f \varepsilon_o} \tag{5}$$

The dielectric data were fitted to the summation of multiple Cole-Cole dispersions in addition to a conductivity term [25] in which σ_s is the static ionic conductivity. Origin 6.1 software was used to handle the fitting process.

RESULTS AND DISCUSSION

Fig. 1 shows a comparison of erythrocyte GSH, GPx and SOD and serum level of MDA in silicotic patients with controls.

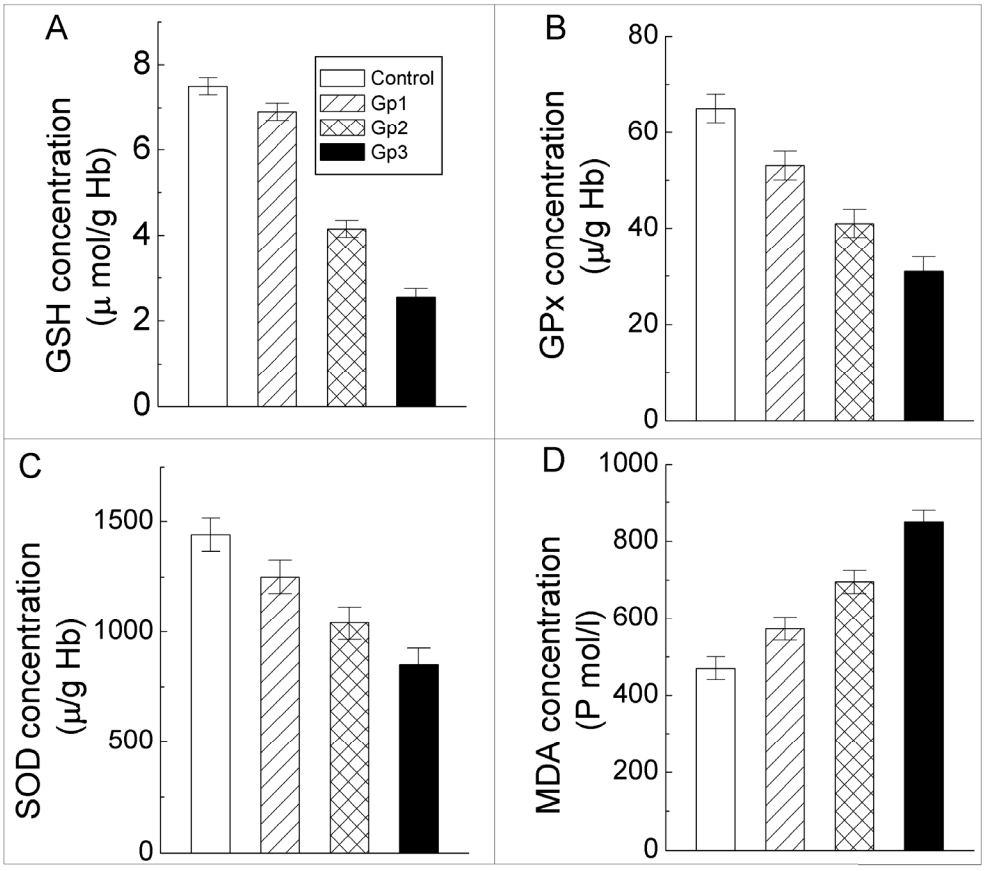

Fig. 1. Comparison of erythrocyte GSH (A), GPx (B) and SOD (C) and serum level of MDA (D) (respectively) in silicotic patients with controls. Data presented as mean and standard error.

The cytotoxic properties of freshly fractured crystalline silica are related to the presence of highly reactive radicals on their surface which exert their action at the level of the cytoplasmic membrane, inducing lipid peroxidation and protein denaturation. They may destabilize the membrane skeleton, thereby compromising cell survival [12]. Lipid peroxidation causes membrane depolarization, disturbs asymmetry of membrane's lipids, induces inhibition of membrane enzymes, modulates transport of proteins and finally causes loss of plasmatic membrane integrity. This may give reasons for the significant decrease ($p < 0.001$) of erythrocyte GSH, GPx and SOD (Fig. 1 (A, B, and C)) in comparison with the controls.

SOD and GPx, both of which sequentially convert superoxide into hydrogen peroxide and molecular oxygen with water [13]. Hydrogen peroxide behaves as a strong inhibitor of SOD activity [33]. Moreover, this reduction may be due to an increased endogenous production of ROS as evidenced by the significant increase in MDA (Fig. 1D).

Mengesha and Bekele [28] showed that inflammatory reactions are sustained in quartz exposed animals resulting in continuum secretions of cellular products, repeated phagocytosis of quartz and enhanced generation of ROS which increase MDA levels. Kamal and El-Khafif [22] and Orman *et al.* [30] showed that MDA levels in asbestos- and silica exposed workers (respectively) were significantly higher than those of controls.

Increased lipid peroxidation causes structural and functional abnormalities in erythrocytes of silicotic patients. The effect of silicosis on RBC's is very complex and involves several stress sources. Besides the biochemical response, a functional disturbance in the red cell membrane occurred as revealed by alteration in hemorheological and dielectric properties.

The rheological properties of RBC play a significant role in determining the fluidity of blood [4]. The two special features of RBC that underlies the non-Newtonian rheological behavior are cellular deformability and aggregation. Deformability is the ability of the entire cell to adopt a new reversible shape in response to a deforming force [18].

Assessments of the osmotic fragility, defined as the sensitivity of RBC to the osmotic shock, are widely used to elucidate mechanisms of ionic and molecular transport across the plasma membrane and for diagnosis of certain hematological diseases [9]. As shown in Table 1 there is a significant decrease ($p < 0.05$) in RBC membrane permeability (C(%) values) and cellular membrane elasticity (W_{hmax} value) for silicotic patients.

Table 1

Parameters of the differential osmotic fragility curves and *ESR* results for RBC's of the studied groups

Variables	C	GpI	GpII	GpIII
C(%)	77.5 ± 5.05	70 ± 4.5	59.1± 3.35	48.7± 6.63
$W_{hmax.}$	19.03± 1.55	15.5± 2.2	10 ± 1.33	5.5 ± 2.65
ESR(mm)				
First hour	20 ± 0.4	17 ± 0.31	12 ± 0.45	8 ± 1.29
Second hour	27 ± 0.7	21 ± 0.55	17 ±1.25	11 ± 2.15

Tvedten and Weiss [36] showed that anything that disrupts the interaction of the proteins within the lipid bilayer of the membrane will lead to decreased deformability and increased osmotic fragility of the cell. Thus lipid peroxidation leads to membrane damage, hemolysis and finally to the death of erythrocyte.

The significant decrease in erythrocyte sedimentation rate, *ESR* (Table 1), is due to RBC aggregation, which is preferentially among more deformable cells, then the formed aggregates settle faster [35].

The high reactivity of crystalline silica to biologic membranes is due to the unique properties of surface –SiOH groups which are hydrogen donors, whereas most biological macromolecules contain lone-pair electrons on oxygen or nitrogen that serve as hydrogen acceptors. The formation of hydrogen bonds would result in a strong interaction between silica and biologic membranes, resulting in possible damage. The surface of SiO is negatively charged which would react strongly with scavenger receptors on alveolar macrophages and activate the generation of ROS and inflammatory cytokines [7]. Oxygen-free radicals attack cellular structures, resulting in metabolic and structural changes, and leading ultimately to damaging major constituents of biological systems and cause diseases [23]. The pathogenicity of silica is linked with its capacity to damage biological membranes. Silica hydrogen bonds to protein components of the membrane and subsequently abstracts these proteins from the membrane [34].

So, silicosis induces oxidative stress in human erythrocytes (RBC's of silicotic patients were more fragile than those from normal subjects). The present results are in agreement with others [15], confirming that cell membrane damage occurred.

Harley and Margolis [17] proposed a two-step interaction model: first, ionic attraction between silica (–SiO) groups and amino groups at the cell surface; second, protein denaturation the extent of which may depend purely on the geometric relationship between colloidal particles and protein molecules. Zhou *et al.* [40] showed that hemorheological indices in silicotic patients increased with the severity of the disease and were all significantly higher than those in the control group.

RBC membrane skeleton mostly determines the shape, deformability (rheological properties) and durability (half-life and resistance to shear stress) of erythrocytes [1]. Modification of cytoskeletal composition and/or organization can alter erythrocytic properties and shapes. By-products of lipid peroxidation cause profound alterations in structural organization and functions of cell membrane including decreased membrane fluidity, increased membrane permeability and inactivation of membrane-bound enzymes [38].

To clarify better the effect of silicosis on RBCs and to characterize the structural changes occurring at the membrane level, their electrical properties were measured by means of frequency domain dielectric spectroscopy. The dielectric data for erythrocytes were fitted to several commonly used functions.

A superposition of two Cole–Cole relaxation processes yielded an excellent fit (data not shown).

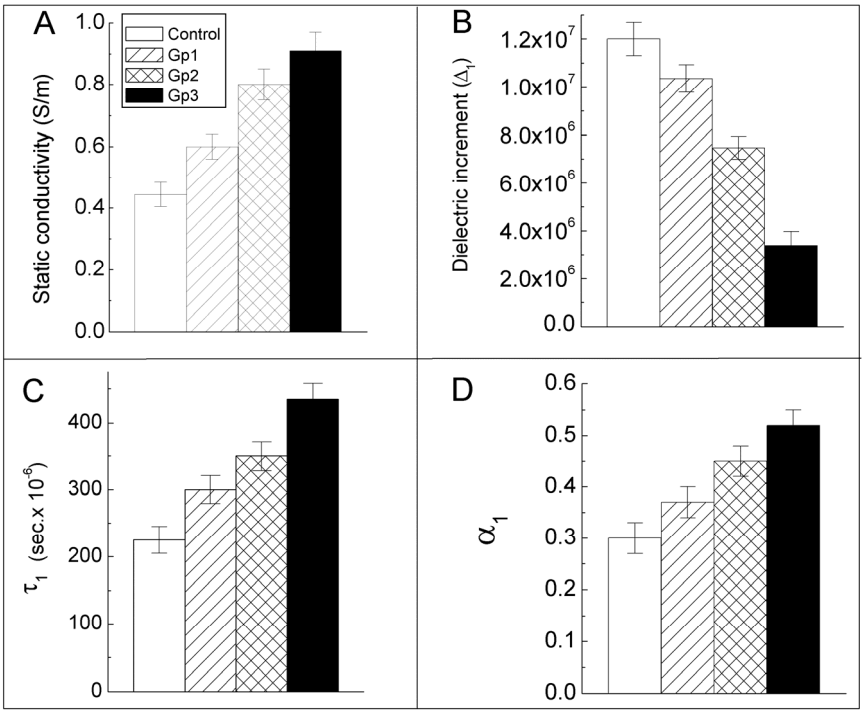

Fig. 2. Changes in the Cole–Cole equation parameters: static conductivity (σ), dielectric increment (Δ_1), relaxation time (τ_1) and spread of relaxation time (α_1) for erythrocyte samples of the studied groups.

Changes in membrane conductivity (a measure of overall ionic transport across cell membrane through ion channels and/or pores) provided support for the hypothesis that silica is incorporated in the lipid bilayer of the erythrocyte membrane, thus modifying the transport properties of the overall membrane. The electrical membrane parameter changes are due to the membrane-silica interactions, which can modify the organization and composition of the lipid bilayer of erythrocyte membrane, resulting in an increase of its electrical conductivity (Fig. 2A) and permittivity.

Ionic transport in the plasma membrane is accomplished by carriers embedded in the phospholipids bilayer. Extraction and/or denaturation of such proteins would lead to the reduction of the ionic transport through the membrane. Harley and Margolis [17] proposed that this interaction is responsible for hemolysis. The extent of this damage is dependent on silica concentration.

Cellular dielectric parameters respond sensitively to changes in cell morphology and physiology [39]. It may therefore be expected that exposure of erythrocytes to silica that lead to modifications in these cellular characteristics will

result in dielectric parameters changes. Hughes *et al.* [19] showed that changes in membrane capacitance by up to 40% can be made by chemical agents. When cells died, eventual rupture of cell membrane occurred. Therefore, impedance between intra- and extracellular space for these cells decreased, since the membrane lost its insulating property [16]. This and the possible collapse of the β-dispersion [27] may give reasons for the significant reduction of Δ_1 (Fig. 2B).

The significant increase in τ with increasing the severity of the disease (Fig. 2C) may be interpreted as follows: As silica incorporates into cell membrane and diffuses into the cell, clusters formation occurred. In such clusters, morphology of cell membranes is changed and the membrane needs to rearrange its structure with the consequent formation of channels and increasing its permeability. This increases cell volume which in turn increased τ.

Pethig and Kell [32] showed that τ depends on effective mobility of ions along macromolecules surface. i.e., permeability change causes a dual elevation in τ and σ values (Figs. 2C and 2A).

The increase in α_1 (Fig. 2D) indicates a broadening of the dispersion and suggests that as different cells respond at a different rate to changes in silica concentration the distribution in cell sizes broadens. The smallness of the change in α and its sensitivity as a fitting parameter make the change the most speculative, although similar changes are seen in other datasets.

Variation of the other Cole–Cole parameters (Δ_2, τ_2 and α_2) (data not presented) for the studied groups is compatible with that of Δ_1, τ_1 and α_1, and may be explained upon the same reasons discussed previously.

Diociaiuti *et al.* [10] showed that freshly fractured silica particles are able to damage cellular membranes and modify their dielectric properties. The present data revealed that the degree of cell disruption as determined by dielectric parameters well corresponded to those obtained by biochemical measurements and hemorheological properties. So, cell's passive electrical properties can be used as a useful sensitive probe for studying the alteration of membrane structure induced by silicosis in a non-invasive manner. This is in agreement with Ulgen and Sezdi [37] who showed that Cole-Cole parameters can be a potential index for evaluating blood in clinical application. Fujii *et al.* [14] showed that electrical properties of blood change with deformation of RBCs.

The present results showed that subjects with severe degree of silicosis (more than 20 years of exposure to silica dust) have the most highly damaged erythrocytes. The electrical membrane parameter changes are due to the membrane-silica interactions, which modify the organization and composition of the lipid bilayer of erythrocyte membrane. The experimental approach used might have relevant clinical implications and offer possibilities to predict silicosis in early stages. Our results also show that dielectric spectroscopy, biochemical analysis and hemorheological properties seem to be reliable, rapid and sensitive methods for detecting erythrocytes damage cells and fulfil the requirements of a biological

marker for silica exposure. Also, these techniques may be considered as promising tools in biomonitoring studies on occupationally exposed human populations.

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