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Measuring the Antioxidant Capacity Induced by Reduced Thiols in Human Erythrocytes Using SW-Voltammetry

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This study examines the use of square wave voltammetry (SWV) to measure the antioxidant capacity induced by reduced thiols in human erythrocytes using a static mercury drop (working), a platinum wire (auxiliary) and the Ag/AgCl saturated electrode. The erythrocytes were isolated from fresh collected blood of 15 subjects aged between 20-30 years old. The applayed voltage ranged from -0.8 to -0.4 V. The current intensity peak (Ip) increases with the hemoglobin concentration (11.3 – 86.0 µmol/L) of the erythrocyte mixture (line equation R=0.9), increasing additions of reduced glutathione (0-90 µmol/L) to 23 µmol/L of hemoglobin. The signal decreases when increasing concentrations of diamide (a specific thiol ligant) (0 – 46 mmol/L) was added to the erythrocyte preparation (70.0 µmol/L hemoglobin). This decreasing was fitted by a quadratic equation (R^2=0.99). The SWV method demonstrate to be more adequate to quantify the oxidative attack promoted by small concentrations of H_2O_2 (0-53 µmol/L) to the erythrocyte preparation (65 µmol/L hemoglobin), when compared to the spectrophotmoetric method that detect the diminishment of oxyhemoglobin and increase of methemoglobin and hemichrome. These results allow the use of the proposed method to quantify alterations in the antioxidant status of the human erythrocytes.

Keywords: SW voltammetry, erythrocyte oxiadtive status, reduced thiols.

1. INTRODUCTION

Thiol reduced groups, present mainly in the cysteine residues of proteins (-SH) and in reduced gluthatione (GSH) are among the most important non-enzymatic antioxidants in cells and biological tissues [1,3]. Alteration on the reduced thiols concentrations are a suitable marker of the cellular redox status status, because GSH is the cofator of the enzymes glutathione peroxidases (GPx) that detoxify

peroxides. These family of enzymes present a low K_M for both H_2O_2 and lipoperoxides acting in the onset of the oxidative stress situation. GSH can also be used to reduce the dissulfides produced during the oxidative attack to the cysteine residues of proteins [1,4,5,6]. The cellular redox status can be defined as the ratio between the oxidized and reduced cellular components altering the activity of enzymes of cellular metabolism and its regulation [7-11]. Furthermore, GSH is also the substract of the enzyme gluthatione-S-transferase that conjugates xenobiotics [12,13].

Even small changes in GSH concentrations are related to oxidative stress status, which depending on its intensity, has been associated to the onset or progression of many diseases such as cancer, atherosclerosis, psoriasis, hypertension, Alzheimer's, heart and liver deseases and with important physiological processes like adaptation, exercise trainning and aging [1,13-19].

Erythrocytes are the blood cells that carry oxygen throughout whole body. The presence of O_2 and transition metal ions, transforms the erythoricites in easy targets for the reactive species and are very sensible to systemic redox system [1,16,20]. Blood and erythrocytes by consequence are easy collection samples and are used to detect and monitor many parameters to control physiologic and phatologic processes [21].

Many methodologies were presented in the literature to acess and quantify GSH and other biological reduced thiols in tissues. Including HPLC with various detecting methods [22,23,24], capilary electrophoresis, mass spectrometry, NMR [25], fluorescence [26], spectrophotometry using the Elman's reagent [27,28] and electrochemical methods [29-31]. Most of these processes require prior preparation of the analytical sample and equipment that complicates its use for quick analysis.

The electrochemical methods have the advantages of not requiring a long process of sample preparation, speed of aquisition and good signal acquisition [32]. Mladenov *et al.* [12] have detected the SW voltammogram and Corrêa-da-Silva *et al.* [33] presented a method to quantify GSH near biological conditions. These authors used a static mercury drop as the working electrode, a platinum wire as the auxiliary and Ag/AgCl (KCl 3 mol/L) as the reference electrode.

The objective of this study was to measure the antioxidant capacity induced by reduced thiols in isolated human erythrocytes submitted to alterations ("in vitro") in it oxidative status using a SW voltammetry method.

2. EXPERIMENTAL

2.1 Voltammetric measurements

All voltammograms were recorded using an Autolab multimode potentiostat/galvanostat (PGSTAT) 101 (Metrohm, Netherlands) connected to a static mercury drop electrode (SMDE) model 663 VA (Metrohm, Netherlands). A platinum wire was used as the auxiliary electrode and the Ag/AgCl (3 mol/L KCl) (MME- Metrohm, Netherlands) was used as the reference electrode. To minimise noise, the cell was kept inside a grounded Faraday cage. Pure nitrogen was used to purge the electrolyte solutions for 5 min prior each measurement.

2.2 Subjects, sampling and assays

The human erythrocytes were prepared from blood obtained from 15 healthy subjects aged between 20 and 30 years. Each experiment was done with the blood of at least 5 individuals. The fraction containing erythrocytes was separated from the blood freshly collected (2 mL each subject) by usual centrifugation method, and washed twice with PBS buffer (phosphate 0.1 mol/L pH 7.4 plus 0.9% NaCl). All erythrocytes were mixed to ensure the homogeneity of the sample. 1.5 mL of the erytrocyte moisture was dissolved in 30 mL PBS buffer, and the obtained solution was used as sample for the experiments. The hemoglobin concentration was quantifyed using the spectrophotometric method proposed by Winterbourn [34].

To increase the –SH groups of the samples the erythrocyte amount were varied from 11.3 to 86.0 μmol/L hemoglobin equivalent. After, were adedd to fixed amount of erythrocyte samples (23,0 mol/L hemoglobin) increasing concentrations of GSH dissolved in PBS buffer (0 – 90 μmol/L).

To decrease the –SH groups concentrations the erythrocyte samples (70,0 μmol/L hemoglobin) were incubated for 30 min, 25^{o}C, with increasing concentrations of diamide (0-0.46 mmol/L) dissolved in phosphate buffer.

To induce "in vitro" oxidative attack, the erythrocyte samples were submitted to two distinct experiments: In the first, a fixed amounth of erythrocytes (26 μmol/L hemoglobin) were incubated for 30 min, 25^{o}C, under stirring to provide enough oxygen, with incresing milimalar concentrations of H_2O_2 (0-350 mmol/L). After, the samples were submitted to spectrophotometry (500-700 nm scan) to quntify oxyhemoglobin, methemoglobin and hemichrome [34] and to the TBARS assay [35,36]. In the second experiment, a fixed amount of erythrocytes were incubated with increasing micromolar concentrations of H_2O_2 (0-53 μmol/L hemoglobin) under the same conditons of the first experiment. The samples were submited to spctrophotometry (scan 500 to 700 nm) [34] and to SW voltammetry. Before the spectrophotometric measuremet the erytrocytes were hemolyzed using water to prevent light scattering induced by the membranes.

2.3 Ethics and data processing.

This experiment was previously submitted and aproved by the Institutional Ethics Commitee on Human Research (CEP/UNIARARAS – Protocol 083/2010). Origin 6.0 was used to make the average SW voltammograms, the average spectrophotometric scans and the correlation plots.

3. RESULTS AND DISCUSSION

3.1 SW voltammetric responses to increasing concentrations of human erythrocites.

Figure 1 shows the SW voltammogram at a static mercury drop electrode, obtained when human erythroscites (20.2 μmol/L hemoglobin), dissolved in PBS buffer (0.1 mol/L phosphate pH 7.4 plus 0.9% NaCl). The net SW component, calculated as the difference between the cathodic and

anodic currents, provides information on both the reduction and oxidation half electrode reactions and can be observed as a single bell-shaped curve with a peak potential of Ep=-0.580. Mladenov *et al.* [12] and Corrêa-da-Silva *et al.* [33] related similar behavior when submitedd reduced gluthatione (GSH) to the same voltammetric system. Small differences in Ep could be related to sample differences, the cited authors had used GSH dissolved in adequate buffer and this work used a more complex system: the human erythrocites.

The human erythrocites are the blood cells specialized to carry oxygen in the bloodstream. Hemoglobin, the protein with the highest concentration within the erythrocytes, have Fe^{2+} in the heme group, the site where binds oxygen. This protein readly undergoes one-electro oxidations, and it can act as a source or sink of free radicals. Hemoglobin also interacts with redox-active xenobiotics and metabolites, generating reactive species than can, depending on its concentraction, attack the hemoglobin itself and other biomolecules [1,34] To face this situation, erithrocites have a suitable antioxidant system like the other cells as described in the Introduction.

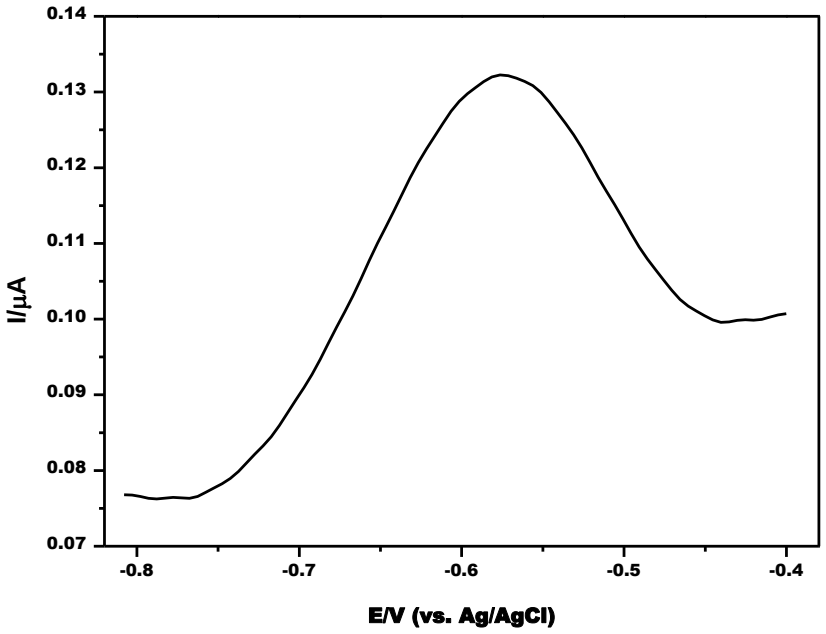

Figure 1. SW voltammogram (δ current) of erythrocytes (20.2 μmol/L hemoglobin) dissolved in PBS buffer (0.1 mol/L phosphate pH 7.4 plus 0.9% NaCl). The experimental conditions were: SW frequency *f*=50 Hz; SW amplitude E_{sw}=0.02 V; step potential dE=0.005 V; deposition potential E_{dep}=1.1 V and deposition time t_{dep}=5 s.

The antioxidant species that have –SH groups, mainly GSH and thiol groups of cysteine protein residues, can donate electrons in negative potentials, to suitable electrodes as for example the static drop mercury electrode using the Ag/AgCl electrode as the reference [33,34,36]. Mladenov et al [12] studied the electrode reaction of GSH at the static mercury drop by means of square-wave voltammetry (SWV). At potentials more positive than -0.350 V, GSH reacts with the mercury electrode producing a sparingly soluble mercury-GSH complex that deposits onto the electrode

surface. Under cathodic potential scan, the deposited complex acts as a reducible reactant generating the SW response.

When increasing amounts of erythrocytes (11.3 to 86 µmol/L hemoglobin equivalent), in the same conditions are submitted to the voltammetric cell, the obtained voltammograms are presented in Figure 2A. Figure 2B presents the correlation (R = 0.99) between hemoglobin concentrations and the peak current intensity (Ip). Ip was the highest current peak in the potential (E) range of -0.8 to -0.4 V.

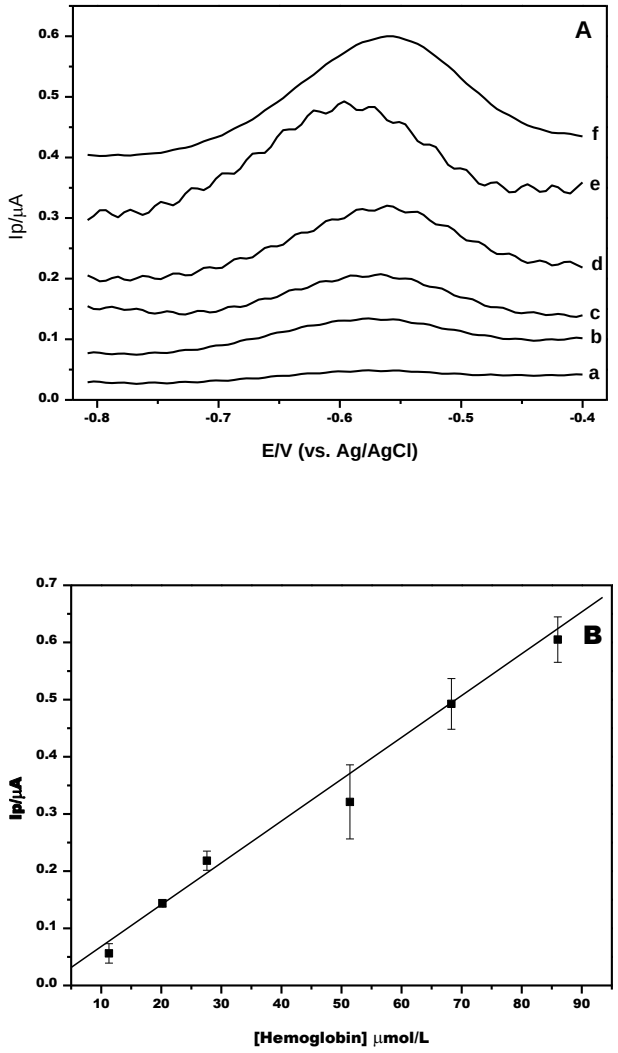

Figure 2. SW voltammograms (A) and plot of the value of Ip (peak current intensity) (B), referent increasing concentrations of erythrocytes dissolved in PBS buffer (0.1 mol/L phosphate pH 7.4 plus 0.9% NaCl). a=11.3, b=20.2, c=27.6, d=51.4, e=68.3, f=86.0 µmol/L hemoglobin. Each voltammogram represents the mean of five independent experiments. Error bars denote the standard deviation. Other experimental conditions were the same as those described in Fig. 1.

These data do not allow us to say that the obtained signal was only from the concentration of GSH in the sample. Biological samples present large diversity of molecules that can interact with the mercury electrode interfering in the SW voltammogram.

3.2 SW voltammogram response of addition of GSH to erythrocytes.

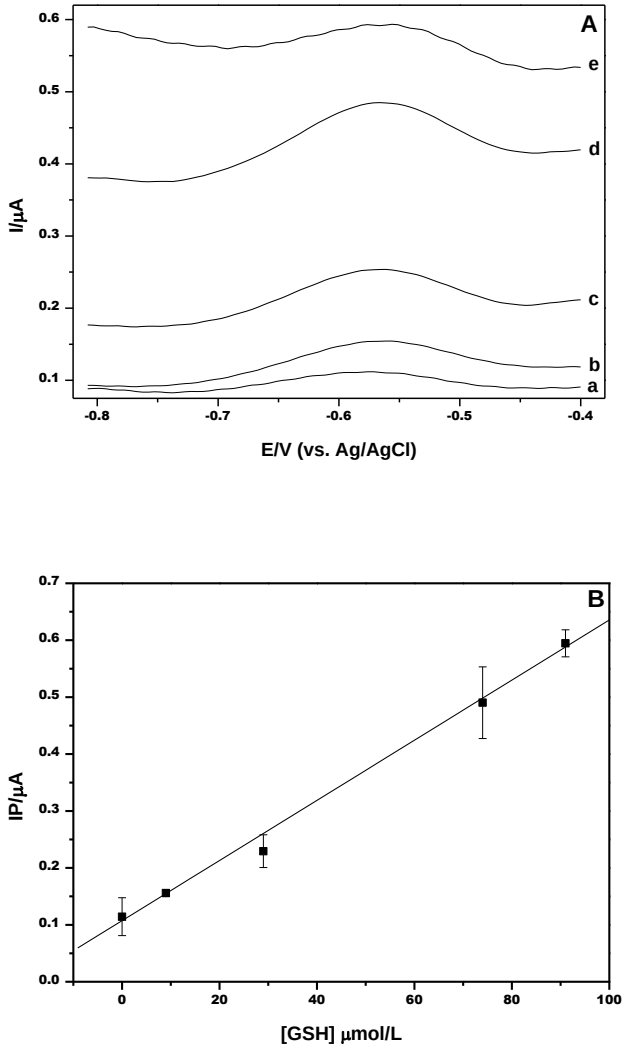

Figure 3. SW voltammograms (A) and plot of the value of Ip (peak current intensity) (B), referent addition of increasing concentrations of reduced glutathione (GSH) to erythrocytes (23.0 µmol/L hemoglobin) dissolved in PBS buffer (0.1 mol/L phosphate pH 7.4 plus 0.9% NaCl). a=0, b=10, c=30 d=70, e=90 µmol/L GSH. Each voltammogram represents the mean of five independent experiments. Error bars denote the standard deviation. Other experimental conditions were the same as those described in Fig. 1.

When was added to the erythrocytes (23 µmol/L hemoglobin), increasing concentrations of GSH (0 – 90 µmol/L) was observed an increasing in Ip. Figure 3 demonstrate the SW voltammograms (A) and the line equation correlating (R=0.99) the GSH concentrations and the Ip (B). Corrêa-da-Silva *et al* [33] described the same behavior when increasing concentrations of GSH were add to phosphate buffer pH 7.5. Thus, these data seem to indicate that the GSH concentration of erythrocytes contribute significantly to the SW voltammograms shown in Figures 1 and 2. Other reduced thiols such as cysteine residues of the proteins should also be contributing to the signal.

3.3 Depletion of erythrocyte –SH groups by diamide.

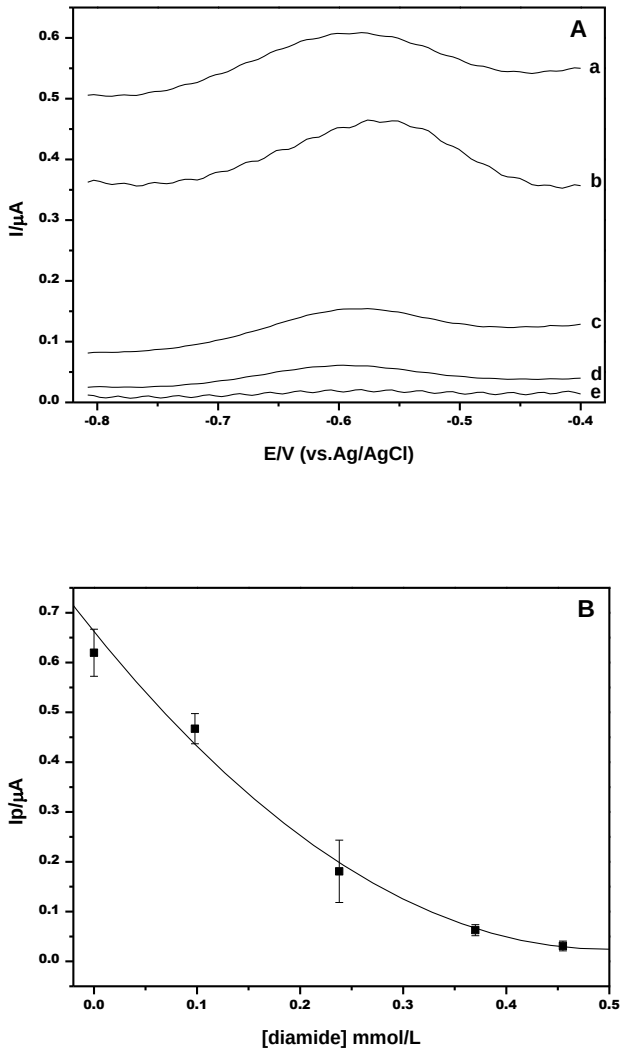

Figure 4. SW voltammograms (A) and plot of the value of Ip (peak current intensity) (B), referent addition of increasing concentrations of diamide to erythrocytes (70.0 μmol/L hemoglobin) dissolved in PBS buffer (0.1 mol/L phosphate pH 7.4 plus 0.9% NaCl). a=0, b=0.10, c=0.24, d=0.37, e=0.46 mmol/L diamide. Each voltammogram represents the mean of five independent experiments. Error bars denote the standard deviation. Other experimental conditions were the same as those described in Figure 1.

Diamide (diazenedicarboxylic acid bis(N,N-dimethylamide) is a specific thiol oxidant [36] and was used to decrease GSH concentration in rat erythrocytes both *in vitro* and *in vivo* [37] and isolated rat liver [38]. yeast mitochondria [39] and GSH dissolved in phosphate buffer [33]. When increasing concentrations of diamide (0 – 46 mmol/L) were added to the human erythrocytes preparation (70.0 μmol/L hemoglobin equivalent), was induced a decrease in Ip of the SW voltammograms, as presented in Figure 4. The decay in Ip can be fitted by a quadratic equation ($R^2 = 0.99$) compatible with a second

order reaction. The reaction between GSH and diamide is a second order reaction [36]. Diamide is an oxidant probe for thiols and is not specific for GSH [36].

It was not possible to remove all of O_2 to obtain the voltammograms shown here. Total hypoxia could induce an oxidative stress that leads to erythrocyte membrane damage [37]. In this condition, all of antioxidant system must have been deplete, including the reduced thiols, making assay impossible. Mladenov et al. [12] recommended the purge with N_2 during 8 min to obtain voltammograms without O_2 interference. Here was used 5 min to preserve the erythrocyte functions.

However, the reason why the voltammograms described here did not starts in zero cannot be attribute only to the presence of O_2. When the erythrocyte amount and probably the concentration of O_2 was maintained (Fig. 3 and 4), the voltammograms started always above zero. The Ip values in -0.8 V increased proportionally to GSH addition (Fig. 3) and decreased proportionally to thiols oxidation (Fig. 4). For these reasons, it should be understood that the all voltammogram points not just Ip were affected by the sample redox status. Then it should be note that the Ip variations correlate directly with the sample redox status, allowing its use instead of the voltammogram area.

3.4 Oxidative stress induced by H_2O_2

Because of the absorption spectrum of hemoglobin be quite complex and have high absorption coefficients, it is difficult to determine the concentration of thiols in erythrocytes, or follow reaction with diamide without the use of complex sample preparation. However, events that decrease the antioxidant capacity can decrease the –SH groups concentration [1]. Thus, if erythrocytes were submitted to an oxidant attack induced by H_2O_2, it is expected diminishment of the -SH groups. H_2O_2 is oxygen-based reactive specie that can cross biological membranes and can oxidize biomolecules [1]. In erythrocytes, hemoglobin is an important target of the H_20_2, mainly by the presence of ferrous ion within its binding site. The oxidation of hemoglobin generates methemoglobin that is not able to bin oxygen [34]. This process can be monitor for changes in the absorbance spectrum between 500 and 700 nm. The typical absorption peaks of hemoglobin (560 and 577 nm) decrease, whereas the 630 nm peak increases [34].

When were used millimolar concentrations of H_2O_2 (0-325 mmol/L) to induce the oxidative stress to a fixed amount of erythrocytes (26.8 μmol/L hemoglobin), were obtained the data described in Fig. 5. As can be seen, the oxidative stress caused disruption in the hemoglobin structure (Fig. 5A), indicated by the decrease in oxyhemoglobin and the increase in methemolglobin concentrations as function of the increasing H_2O_2 additions. The hemichrome changes only slightly at the H_2O_2 highest concentrations.

Moreover, occurred attack to the erythrocyte membranes causing lipid peroxidation, which can be view in Fig. 5B. The oxidative attack to the membrane lipids generates lipoperoxides and aldehydes that are thiobarbituric acid reactive substances and can be detect by the TBARS assay [14,40].

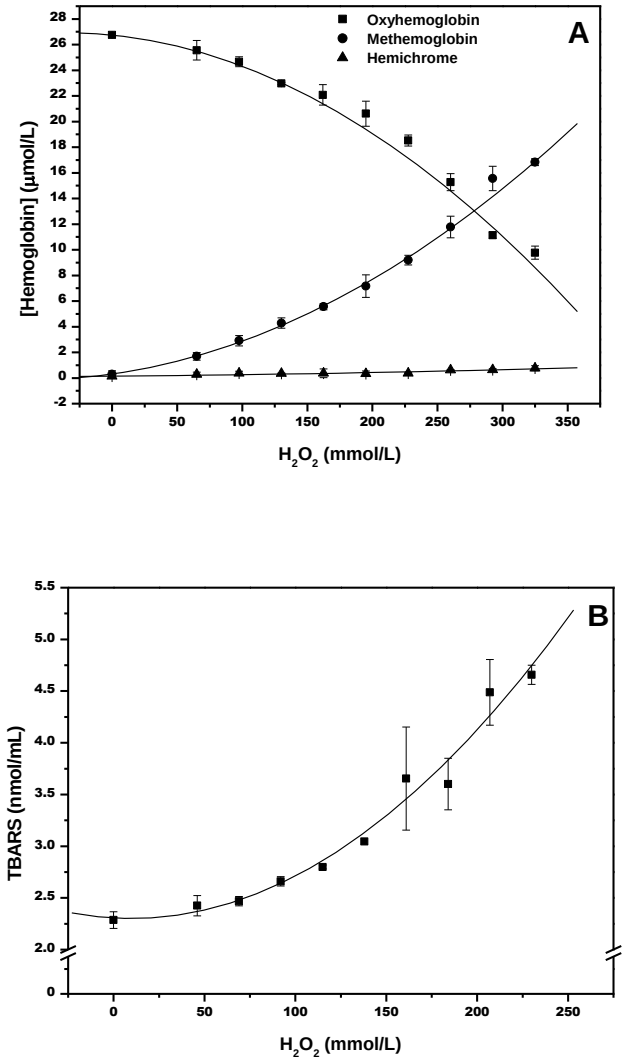

Figure 5. Alterations induced by the incubation (30 min, 25°C) of human erythrocytes (26.8 μmol/L hemoglobin), dissolved in PBS buffer pH 7.4, with increasing concentrations of H_2O_2 (large concentrations). (A) - Oxyhemoglobin (■), methemoglobin (●) and hemichrome (▲) concentrations. (B) – TBARS increasing concentration. The H_2O_2 concentrations ranged from 0 to 325 μmol/L (A) and 0 to 225 mol/L in (B). Error bars denote the standard deviation of five independent experiments.

When has added to the erythrocyte moisture increasing micromolar concentrations of H_2O_2 (0-53 μmol/L), the results in the hemoglobin absorbance spectra are presented in Figure 6A. Can be noted diminishment in 530 and 577 peaks, indicating decreasing in oxyhemoglobin concentrations. It is a sign of an oxidative damage that unsettles the functionality of hemoglobin. No changes are detectable at 630 nm. In the other hand, the same event was viewed by the alterations in the SW-voltammograms (Fig 6B) and the results demonstrate a significant decrease in the reduced thiols-induced antioxidant capacity. Table 1 compare the two methodologies and highlights the greater sensitivity of the SW-voltammetry method (decreasing 88.8% of Ip) when compared to the spectrophotometric (decreasing

7.5% of oxyhemoglobin concentration) to evaluate the damage to the erythrocytes induced by H_2O_2 in the concentrations studied here.

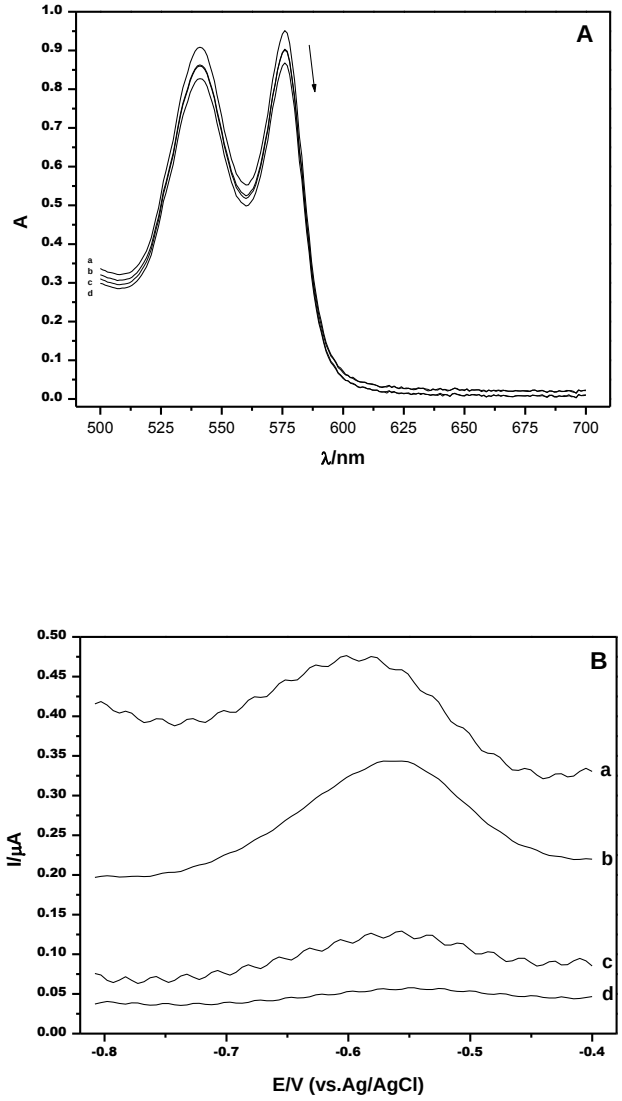

Figure 6. Alterations induced by the incubation (30 min, 25°C) of human erythrocytes (65 μmol/L hemoglobin), dissolved in PBS buffer pH 7.4, with increasing concentrations of H_2O_2 (small concentrations). (A) - In the absorption spectra (500-700 nm) a=0, b=7, c=34, d=53 μmol/L H_2O_2. (B) - In the SW voltammograms (some H2O2 concentrations). Each spectrum or voltammogram represents the mean of five independent experiments. For voltammetry the other experimental conditions were the same as those described in Figure 1.

These results demonstrate the dynamics of the clash between oxidative damage and the antioxidant systems of the erythrocytes. In the cells, H_2O_2 can generate hydroxyl radical ($^{\cdot}OH$) mainly by reactions Fenton-type (interaction with reduced transition metal ions) and Haber Weiss (interaction with $O_2^{\cdot-}$). $^{\cdot}OH$ can attack biomolecules starting the lipoperoxidation process whose byproducts, high reactive carbon based radicals and aldehydes, can attack other cellular structures. The $^{\cdot}OH$ and H_2O_2

themselves, can attack directly proteins and other biomolecules. To face this oxidative attack, in this H_2O_2 concentration, cells mobilize their antioxidant systems, mainly the glutathione peroxidase enzymes. These enzymes reduce H_2O_2 to water by oxidizing GSH. The oxidative species can also attack other –SH groups. Glutathione reductase and other reductases that regenerate other –SH groups use NADPH as electron donor. This coenzyme as also used by the methemoglobin reductase. In our experiment, there was no extra source of glucose, so the NADPH was used preferentially for the activity of methemoglobin reductase, thus causing depletion in SH-groups [1,41].

Table 1. Comparison between the alterations in (*) oxyhemoglobin concentration (measured by spectrophotometry) and in (**) the antioxidant capacity provided by reduced thiols (measured by SW voltammetry) when human erythrocytes (65 μmol/L hemoglobin), dissolved in PBS buffer pH 7.4, were incubated (30 min, 25^{o}C) with increasing concentrations of H_2O_2. The spectra and voltammograms are described in Fig. 6.

[H_2O_2] (μmol/L)	[Oxyhemoglobin] (μmol/L)	%*	Antioxidant Capacity (Ip/μA)	%**
00	62.3 ± 0.27	100	0.507 ± 0.10	100
07	59.8 ± 0.47	96.1	0.342 ± 0.01	67.5
34	58.6 ± 0.27	94.1	0.123 ± 0.09	24.3
53	57.6 ± 0.21	92.5	0.057 ± 0.01	11.2

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4. CONCLUSIONS

The results presented here indicates the use of the Ip of the SW voltammograms obtained in the same conditions described, to quantify the alterations in the concentration of –SH groups within the

human erythrocytes. The method proved be suitable to identifying differences induced by addition or decreasing in –SH concentrations without previous sample preparation. Moreover, when compared with other method that provides the same information about the antioxidant status, the proposed method proved to be much more sensitive to identify and quantify the oxidative stress induced by H_2O_2.

As the blood passes through whole body, carrying nutrients and waste products, is targeted and at the same time contributes to distribute the reactive species and antioxidant products. The erythrocytes are presented as samples easily collected and whose oxidative state may reflect the oxidative status of the organism as a whole. Differences in oxidative status and antioxidant capacity of red blood cells may be an important tool to evaluate the antioxidant capacity of patients to identify, track the progression or regression of physiological and pathological processes in which oxidative stress is an important factor.

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