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 applied 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 (R²=0.99). The SWV method demonstrate to be more adequate to quantify the oxidative attack promoted by small concentrations of H₂O₂ (0-53 µmol/L) to the erythrocyte preparation (65 µmol/L hemoglobin), when compared to the spectrophotometric 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 oxidative status, reduced thiols.

1. INTRODUCTION

Thiol reduced groups, present mainly in the cysteine residues of proteins (-SH) and in reduced glutathione (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$_2$O$_2$ 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 substrate of the enzyme glutathione-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 desease and with important physiological processes like adaptation, exercise training 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 acces and quantify GSH and other biological reduced thiols in tissues. Including HPLC with various detecting methods [22,23,24], capillary 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 erythrocyte moisture was dissolved in 30 mL PBS buffer, and the obtained solution was used as sample for the experiments. The hemoglobin concentration was quantified 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 added 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°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 amount of erythrocytes (26 µmol/L hemoglobin) were incubated for 30 min, 25°C, under stirring to provide enough oxygen, with increasing milimolar concentrations of H₂O₂ (0-350 mmol/L). After, the samples were submitted to spectrophotometry (500-700 nm scan) to quantify 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 H₂O₂ (0-53 µmol/L hemoglobin) under the same conditions of the first experiment. The samples were submitted to spectrophotometry (scan 500 to 700 nm) [34] and to SW voltammetry. Before the spectrophotometric measurement the erythrocytes were hemolyzed using water to prevent light scattering induced by the membranes.

3. RESULTS AND DISCUSSION

3.1 SW voltammetric responses to increasing concentrations of human erythrocytes.

Figure 1 shows the SW voltammogram at a static mercury drop electrode, obtained when human erythrocytes (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 \( E_p = -0.580 \). Mladenov et al. [12] and Corrêa-da-Silva et al. [33] related similar behavior when submitted reduced glutathione (GSH) to the same voltammetric system. Small differences in \( E_p \) 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 erythrocytes.

The human erythrocytes are the blood cells specialized to carry oxygen in the bloodstream. Hemoglobin, the protein with the highest concentration within the erythrocytes, have \( \text{Fe}^{2+} \) in the heme group, the site where binds oxygen. This protein readily 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 concentration, attack the hemoglobin itself and other biomolecules [1,34] To face this situation, erythrocytes have a suitable antioxidant system like the other cells as described in the Introduction.

![SW voltammogram](image)

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

The antioxidant species that have \( \text{–SH} \) 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 \(-0.350 \text{ V}\), 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.

![SW voltammograms](image)

**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.

![SW voltammograms (A) and plot of the value of Ip (peak current intensity) (B)](image)

**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$_2$O$_2$

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$_2$O$_2$, it is expected diminishment of the -SH groups. H$_2$O$_2$ 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$_2$O$_2$, 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$_2$O$_2$ (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 methemoglobin concentrations as function of the increasing H$_2$O$_2$ additions. The hemichrome changes only slightly at the H$_2$O$_2$ 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 H2O2 (large concentrations). (A) - Oxyhemoglobin (■), methemoglobin (●) and hemichrome (▲) concentrations. (B) – TBARS increasing concentration. The H2O2 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 H2O2 (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$_2$O$_2$ 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$_2$O$_2$ (small concentrations). (A) - In the absorption spectra (500-700 nm) a=0, b=7, c=34, d=53 µmol/L H$_2$O$_2$. (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$_2$O$_2$ can generate hydroxyl radical (OH) mainly by reactions Fenton-type (interaction with reduced transition metal ions) and Haber Weiss (interaction with O$_2^-$). OH can attack biomolecules starting the lipoperoxidation process whose byproducts, high reactive carbon based radicals and aldehydes, can attack other cellular structures. The OH and H$_2$O$_2$
themselves, can attack directly proteins and other biomolecules. To face this oxidative attack, in this 
H$_2$O$_2$ concentration, cells mobilize their antioxidant systems, mainly the glutathione peroxidase 
enzymes. These enzymes reduce H$_2$O$_2$ 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°C) with increasing concentrations of H$_2$O$_2$. The 
spectra and voltammograms are described in Fig. 6.

<table>
<thead>
<tr>
<th>[H$_2$O$_2$] (μmol/L)</th>
<th>[Oxyhemoglobin] (μmol/L)</th>
<th>%*</th>
<th>Antioxidant Capacity (Ip/μA)</th>
<th>%**</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>62.3 ± 0.27</td>
<td>100</td>
<td>0.507 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td>07</td>
<td>59.8 ± 0.47</td>
<td>96.1</td>
<td>0.342 ± 0.01</td>
<td>67.5</td>
</tr>
<tr>
<td>34</td>
<td>58.6 ± 0.27</td>
<td>94.1</td>
<td>0.123 ± 0.09</td>
<td>24.3</td>
</tr>
<tr>
<td>53</td>
<td>57.6 ± 0.21</td>
<td>92.5</td>
<td>0.057 ± 0.01</td>
<td>11.2</td>
</tr>
</tbody>
</table>

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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 [41].

### 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 H₂O₂.

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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References