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DETERMINATION OF REACTIVE OXYGEN SPECIES IN ISOLATED ERYTHROCYTES

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ABSTRACT

Determination of reactive oxygen species (ROS) in erythrocytes have been extensively studied due to its important pathophysiological role in many diseases. Also, studies on the potential of many antioxidants compounds are important to improve human healthy. This paper describes a simple and fast method to quantify ROS in erythrocytes with induction of oxidation and antioxidant protection. Tert-butyl hydroperoxide (tBOOH)-induced ROS was measured in erythrocytes isolated from 60 healthy adults between 21 and 39 years old, using microplates methods in which 2,7-dichlorofluorescein (DCF) was produced. Fluorescence was monitored at 530 nm after excitation at 495 nm. Data points were taken after 15 and 30 min of incubation at room temperature and at 37°C. Ascorbic acid was used to evaluate the protection of an antioxidant compound. Results were analyzed using the Statistica 10.0 (StatSoft). No outlier was identified and all parameters were normally distributed. Data were expressed as mean \pm standard deviation and one-way analysis of variance (ANOVA) and Tukey test were performed. Results were considered statistically significant when $p < 0.05$. Different concentrations of t-BOOH solutions induced ROS generation with significant differences in all concentrations. Time and temperature interfere in the method that has to be well standardized. Dose-dependent protection of ascorbic acid on ROS production was observed. ROS-induced oxidative stress in erythrocytes and antioxidant protection can be determinate using an easy and fast method, with little blood volume under microplate methods.

Keywords: Reactive oxygen species, Erythrocytes, Tert-butyl hydroperoxide, Dichlorodihydrofluorescein-diacetate.

INTRODUCTION

Oxidative stress is defined as an imbalance between the production of ROS and the ability of the biological system to repair these damage [1]. Numerous exogenous sources, like xenobiotics, radiation, endogenous aerobic respiration and metabolic reactions can induce ROS formation [2-4].

ROS-mediated oxidative stress results in oxidation of membrane lipoproteins, glycooxidation, and oxidation of DNA [5]. The important pathophysiological role of oxidative damage is now well-established in a variety of disease states [6,7] including cardiovascular dysfunction, neurodegenerative diseases, atherosclerosis, metabolic dysfunction, cancer, premature aging, hypertension [8] and hemoglobinopathies [9].

Erythrocytes, which circulate at constant high oxygen and iron concentrations, have been used as a model for the investigation of ROS-induced oxidative stress [10]. Erythrocytes damage occurs due to oxidation of hemoglobin, followed by the conversion of methemoglobin to hemichromes, and free radical attack on membrane components [11-13].

Antioxidants are biochemical compounds that dispose, scavenge, and suppress the formation of free radicals [14] and, although cells are equipped with these defense mechanisms, they undergo a drastic decline in physiological and environmental conditions that cause overproduction of ROS [15]. There are a variety of known antioxidant systems which protect erythrocytes. Some are

non-enzymatics, such as the reduced glutathione (GSH), and enzymes such as superoxide dismutase (SOD) and catalase (CAT) [16,17].

The potential role of ROS and antioxidants in the etiology of chronic diseases has stimulated extensive studies in current years and much interest has been focused mainly on the use of natural antioxidants for the human health improvement [18]. Also, substances involving in the biological oxidative stress process can be used as representative biomarkers in the evaluation of different individuals, with perspectives to apply in clinical anamnesis, diseases diagnoses and accomplish of antioxidants treatments [12, 19].

The aim of the present study was to standardize an easy and fast method of ROS determination in erythrocytes and to evaluate the effects of time and temperature in the method. Effects of an oxidizing agent, tert-butyl hydroperoxide (t-BOOH), and the protection of antioxidant ascorbic acid, were also analyzed.

MATERIAL AND METHODS

Chemicals

Tert-butyl hydroperoxide (t-BOOH); dichlorodihydrofluorescein-diacetate (DCFDA) and ascorbic acid were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Sodium chloride, sodium phosphate dibasic and sodium phosphate monobasic were supplied by Vetec Ltda (Rio de Janeiro, RJ, Brazil). All organic solvents were of high quality and twice-distilled and all the other chemicals were of analytical grade.

Blood samples

Human blood (5 mL), obtained from 60 normal volunteers (24 males and 36 females) with an average age of 24 years old (ranging from 21 to 39 years old), was collected via venopuncture into EDTAK₃. The use of human subjects was approved by the Ethical Committee for Research Involving Humans, Hospital de Clínicas of Universidade Federal do Paraná. Informed consent was obtained from all the volunteers and the exclusion criteria was any hematological alteration in cell blood count or in hemoglobin electrophoresis. Samples were centrifuged at 3000xg for 10min. Plasma and buffy coat were removed by aspiration, and the erythrocytes were washed three times with phosphate buffered saline (PBS) [NaCl (150 mM), NaH₂PO₄ (1.9 mM) and Na₂HPO₄ (8.1 mM), pH 7,4] to obtain a constantly packed cell volume. Red blood cells were diluted to 10% v/v suspension in PBS.

Determination of intracellular ROS with oxidation by t-BOOH

A method described by Lopez-Revuelta *et al.*, (2006) was adapted to small volumes for microplates reading. Erythrocytes (995µL of 10% v/v suspension in PBS) were incubated with 5µL of 10M DCFDA at room temperature and at 37 °C for 30 min, to evaluate the effect

of temperature. After incubation, the suspension was diluted in 9.0ml of PBS and 37.5µL was added to 112.5µL of PBS, in appropriate 96-well plates. Determination of free radicals was performed with the exposure to t-BOOH solutions (25, 75, 125, and 175 µM), in a GloMax®-Multi Microplate Multimode Reader fluorimeter (Promega Corporation, Winconsin, USA). Under these conditions, DCFDA is hydrolyzed to 2',7'-dichlorodihydrofluorescein (DCFH₂), which is available for oxidation by ROS to produce fluorescent 2,7-dichlorofluorescein (DCF). Fluorescence was then monitored at 530 nm after excitation at 495 nm. Data points were taken after 15 and 30 min of incubation, to evaluate the interference of time in the method.

Determination of antioxidant activity

Ascorbic acid, a well known antioxidant compound, was used to evaluate its influence in ROS production. In the same method described above, 20 µL of ascorbic acid solutions (10, 50 and 100 µM) was added to the reaction medium with incubation at 37 °C for 30 min, before the additions of t-BOOH solution (175 µM).

Statistical analyses

Data were analyzed using the Statistica 10.0 (StatSoft). No outlier was identified. All parameters were normally distributed (Kolmogorov-Smirnov test) and data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey test were performed. Results were considered to be statistically significant for p<0.05.

RESULTS

ROS generation induced in oxidized erythrocytes by t-BOOH was monitored by the fluorescence emitted by 2,7-dichlorofluorescein. Figure 1 shows ROS formation in the presence of different concentrations of t-BOOH incubated at room temperature and at 37°C for 15 and 30 min. Both, time and temperature interfere in the method. It was observed significant differences (p<0.0001) in all tested concentrations of t-BOOH at room temperature and at 37°C and for 15 and 30 min.

Dose-dependent effects of ascorbic acid on ROS production in control erythrocytes exposed to 175µM t-BOOH are shown in Figure 2. All differences were significant (p<0.001).

DISCUSSION

Tert-butyl hydroperoxide (t-BOOH) is an organic peroxide widely used in various studies as a model of oxidative stress [20]. It is supposed that t-BOOH cause disruption of intracellular calcium homeostasis, followed by the oxidation of reduced glutathione and protein thiols in the cell, as well as appearance of DNA strand breakage, peroxidation of lipids, and an increase in intra-globular mobility of membrane proteins [10].

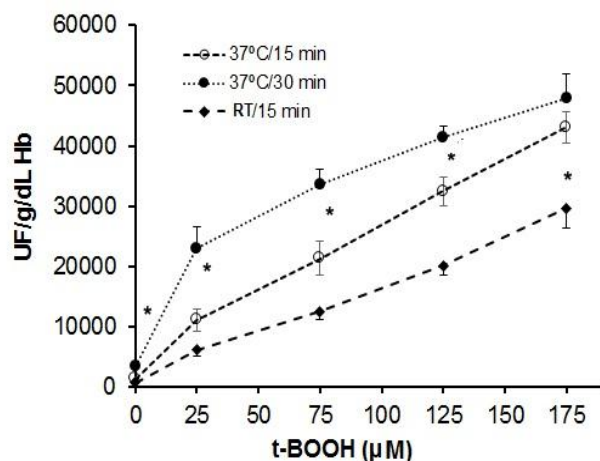
Exposure of erythrocytes to t-BOOH has been shown to cause the oxidation of hemoglobin and profound cellular damage through a heme-catalyzed ROS-mediated process involving tert-butoxyl radicals [16], which induce the toxic effects and morphologic transformations in the cell [21].

Under normal conditions, erythrocytes are protected by the powerful action of its endogenous antioxidants. However, under pathological conditions and in toxic insult occurring during environmental contaminations, the available endogenous antioxidants are depleted, and hence, exogenous antioxidants are required for protection [19].

In this study, normal human erythrocytes were used to investigate the ROS production in oxidative damage induced by t-BOOH *in vitro*, and the capability of ascorbic acid to protect erythrocytes. It was standardized an easy and fast method to assess ROS generation inside cells, with measurement of DCFDA oxidation. This non-fluorescent reagent diffuses passively through the membrane into the cell, and can be oxidized by ROS to form fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence intensity is proportional to ROS concentration, leading to a quantitative measurement of intracellular ROS [22].

Oxidative injury was induced in erythrocytes by a 15 and 30 min exposure to t-BOOH solutions at room temperature and at 37°C. The protective effects of ascorbic acid were assayed at different concentrations.

Figure 1. ROS generation in erythrocytes incubated with t-BOOH solutions



RT – room temperature
*- statistically significant

CONCLUSION

Because ROS have been highly correlated with many diseases in the recent years and with lots of new

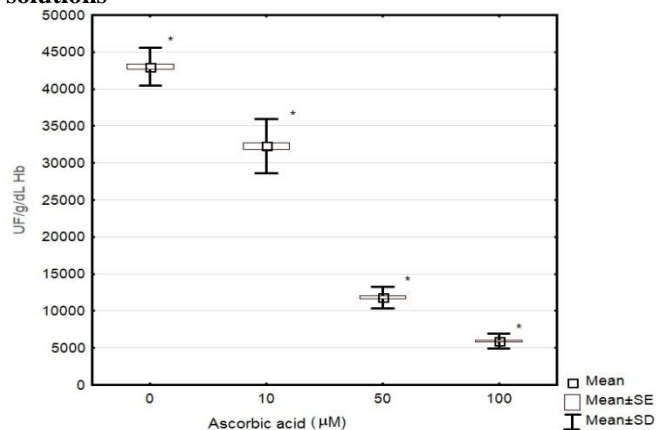
incubation of erythrocytes with increased concentrations of t-BOOH resulted in pronounced toxicity, inducing ROS formation with a marked and statistically significant increase in intracellular DCFDA oxidation. It was observed statistical differences between incubation at room temperature and at 37°C and by 15 and 30 min. These implicates that the methodology must have to be well established to avoid variations.

Co-treatment with ascorbic acid at all concentrations used resulted in a statistical significant reduction in fluorescence Intensity, clearly indicating a protection to erythrocytes against t-BOOH-induced oxidative stress. This compound is extensively used as an antioxidant because it can be oxidized by most of ROS that are formed in human cells, becoming less reactive species [2].

The powerful antioxidant activity of many different possible antioxidant substances have been shown in several studies [8, 10, 16, 18, 23, 24] suggesting that these compounds could play a protective role in oxidative stress-mediated diseases. Recent attention has focused on their potential uses for the prevention and treatment of these pathologies. The success of these investigations is related directly to a better human quality of life.

The results presented show that temperature and time of incubation influence directly in ROS production, and should be considered for better performance in future researches. The proposed method presents a promising diagnostic tool for the assessment of oxidative stress in various research models.

Figure 2. Antioxidant effect of ascorbic acid in ROS generation in erythrocytes incubated with t-BOOH solutions



Error bar represents standard deviation
*- statistically significant (Tukey test)

antioxidant treatments under investigation, we presented here an easy and fast method to direct evaluate ROS formation into the cells.

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