European Review for Medical and Pharmacological Sciences 2012; 16: 646-652

A novel approach to study oxidative stress in thyroid diseases: a preliminary study

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Abstract. – *Background and Objectives:* **Recently, several Authors have emphasized the relationship between oxidative stress and thyroid tumors. Several methods have been proposed in the literature for the measurement of oxidative stress in human tissues, although the high reactivity and short half life of Reactive Oxygen and Nitrogen Species make difficult their direct determination. Here we propose a novel approach for the determination of oxidative stress in human tissues, taking into account the relationship between free radicals and thyroid diseases.**

Materials and Methods: **Our goal in this preliminary study, was to demonstrate the opportunity to use the coupling of the EPR-spin trapping technique with the hydroxylamine 1-hydroxy-3-carboxy-pyrrolidine, to detect oxidative stress in the human blood of patients with thyroid disease.**

Results: **Our preliminary findings confirm that this is a sensible, precise and valid method to study the oxidative stress and encourage us to continue the project.**

Conclusions: **Our next goal will be to enroll patients affected by different thyroid diseases and to study the effect of some antioxidants in the management of the disease. This will allow to better understand the pathological path that binds the formation of reactive oxidizing species to the thyroid cancer and eventually to take into account the antioxidant therapy, as a possible additional "therapeutic weapon".**

Key Words:

Oxidative stress, Thyroid cancer, Electron paramagnetic resonance, Thyroid diseases.

Introduction

The overproduction of reactive oxygen and nitrogen species (thereafter collectively called Reactive Oxidizing Species, ROS), and the consequent increase in oxidative stress leading to an irreversible cellular damage, is frequently associated with some diseases like atherosclerosis, hypertension, diabetic nephropathy, pulmonary fibrosis, Alzheimer's disease and in particular with cancer[1-9]. Recently, several Authors have emphasized the relationship between oxidative stress and thyroid tumors, studying macromolecular alterations, typically caused by oxidative stress in thyroid tissue[10] and the activity of some antioxidant enzymes in the blood of patients with thyroid cancer[11]. In the first case, microarray and immunohistochemistry analyses revealed changes in the DNA structure (as the presence of 8-Oxo-2'-deoxyguanosine), typically induced by oxidative stress. In particular, Young et al[10] found significantly higher concentration of 8-Oxo-2'-deoxyguanosine in the neoplastic tissues of patients affected by thyroid neoplasia (follicular and papillary carcinoma), compared to the matched normal tissue. In the second study, the Authors[11] measured the blood concentrations of glutathione peroxidase (an antioxidant enzyme) and malondialdehyde (a product of lipid peroxidation), before and after thyroidectomy. The plasma levels of both markers of oxidative stress were found to be significantly higher in patients with thyroid cancer with respect to the control population. Moreover, it is important to underline that 20 days after thyroidectomy the blood concentrations of glutathione peroxidase and malondialdehyde were similar to those detected before thyroidectomy. We have already shown[12] the importance of the identification of markers to use in the postsurgical follow up of patients with thyroid disease, but for the oxidative stress markers there are some limitations, due to the absence of

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reference standard values universally accepted and to the unreliability of the obtained results with some of them[13]. The aim of this work was to use a highly refined technique, the Electron Paramagnetic Resonance (EPR), to study qualitatively and quantitatively the oxidizing species in the blood of patients with thyroid cancer. This innovative approach is particularly interesting for the high sensitivity in the ROS detection in the human blood. Oxidative stress in whole blood can be studied with the spin trapping and spin probing techniques, using molecules (spin traps and spin probes) able to react with the free radicals to form adducts detectable by EPR analysis. The spectra obtained from EPR analysis are able to provide information on the kinetics, the quantity and the type of reactive species released by neoplastic tissue. The purpose of this preliminary study is to assess the role of oxidative stress in the blood of patients with thyroid cancer or multinodular goiter and eligible for total thyroidectomy with respect the control population (age and sex matched). The same indicators of oxidative damage will be detected also 20 days after the thyroidectomy. Hence, in this complex scenario, the search of serum biomarkers of oxidative stress, which could related to the thyroid cancer is underway in several laboratories. The aim of this preliminary study was to verify the opportunity to study the relationship between thyroid disease and oxidative stress using EPR technique, with the aim to apply this methods subsequently in a perspective study on a larger population with different thyroid diseases. Our preliminary data show that the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpirrolydine (CPH) is a good indicator of oxidative stress in whole blood of healthy donors "activated" to produce ROS (to mime inflammatory conditions). The CPH in fact, is oxidized to the corresponding radical nitroxide (CP•), whose blood levels are measurable by EPR analysis and may be considered to direct expression of ROS production and then the state pro-oxidant of the blood.

Materials and Methods

Patients

All subjects enrolled for the study were recruited in the Department of Surgical Sciences of the Policlinico Umberto I, Sapienza University, Rome, Italy. The control population (healthy donors, HD) was represented by 20 subjects (8 male and 12 female), aged between 40 and 75 years, without any thyroid disease (previous or current) and drugs assumption. The study population (16 patients, sex and age matched) was recruited among those, with multinodular goiter or cytological diagnosis of Thy 4 or Thy 5, (according to the classification of the Italian Society of Pathology and Diagnostic Cytopathology, SIAPEC), eligible for total thyroidectomy. The population enrolled for the study was constituted by 6 patients with multinodular goiter (MNG) and 10 with thyroid neoplasia (8 with papillary adenocarcinoma and 2 with follicular adenocarcinoma). The criteria of exclusion were the presence of debilitating diseases (diabetes advanced stage, immunological diseases and hematological disorders), lack of informed consent and/or authorization form to the processing of personal data.

Analyses and Samples Collection

All subjects recruited for the study underwent: electrocardiogram, chest x-rays and indirect laryngoscopy. Urinary samples were taken to detected urinary steroid profile by gas chromatography-mass spectrometry (Agilent Technologies model 5890/5973, Varian, Palo Alto, CA, USA)[14], while blood samples were taken for the study of oxidative stress and for routine analyses. In fact, each patient underwent blood sampling for the assessment of the following parameters: hemocromocytometric analysis, routine chemistry tests, TSH, FT3, FT4, calcitonin, thyroglobulin, antibodies anti-thyroperoxidase (TPOAb) and anti-thyroglobulin (TgAb). The blood samples to perform the measurement of biomarkers of oxidative stress were taken before surgical intervention (T0) and 20 days after thyroidectomy (T20), using heparinized tubes, to prevent any changes in the redox state of the blood. All analyses for the evaluation and characterization of oxidative stress, have been completed within a 2 hours from collection.

Chemicals

Spin probe 1-hydroxy-3-carboxy-pyrrolidine (CPH), 3-carboxy-proxyl radical (CP•) and Phorbol 12-Myristate 13-Acetate (PMA), were purchased from ENZO Biochem (Laufelfingen, Switzerland). Super Oxide Dismutase (SOD), diethylenetriaminepentaacetic acid (DTPA), H_2O_2, and catalase (Cat), were purchased from Sigma (St. Louis, MO, USA).

Preparation and Processing of Whole Blood and Plasma

Whole blood, collected in heparinized tubes, was transferred to Falcon 15 ml and balanced with air on a shaker for 10 min at room temperature. The plasma was obtained after centrifugation of whole blood, for 5 min at 1000 g/min. The whole blood, Red Blood Cells and the plasma (100 µl), were pretreated with 1 µM PMA or 10 µg/ml SOD or 0.1 mM DTPA or 10 µg/ml Cat, followed by the administration of 1 mM CPH/PBS-Chelex (PBS: phosphate buffered saline).

Analysis of Oxidative Stress Biomarkers by EPR Technique

The EPR spectra were obtained using the instrument Bruker ECS 106 (Bruker, Rheinstetten, Germany) equipped with an external thermostat (ER4111VT) set at 37°C. In brief, 100 µl of sample was placed in a Teflon tube gas-permeable, with an internal diameter of 0.81 mm and a thickness of 0.05 mm (Zeuss Industrial Products, Raritan, NJ, USA). The Teflon tube has been wrapped on itself four times, inserted in a tube of quartz and introduced in the ESR cavity (4108 TMH). Air or N_2 was used as gas flow. The intensity of the signal produced by the formation of CP• radical was calculated 10 minutes after the addition of CPH. This time was chosen to take as reference the intensity of the first peak of the typical spectrum of the CP• radical, because of the possible interference of other radicals, physiologically present in the biological systems. The spectrometer conditions common to all the spectra were: modulation frequency, 100 kHz, microwave frequency, 9.4 GHz, microwave power, 20 mW; 1×10^4 gain, modulation amplitude, 0.1 mT, conversion time, 20.5 ms time constant, 82 ms, sweep time, 21 s, number of scans, 1.

Statistical Analysis

All data were expressed as the mean (± SD) of at least 3 measurements and analyzed using the Student *t* test, with the statistical software Graph Pad Prism 4.0. Values of $p < 0.05$ were considered statistically significant.

Results

Spin-Probing

The radicals are highly reactive species with a half-life usually less than 1 msec and hardly detectable by direct EPR technique alone. This limitation is overcome by combining the EPR technique to spin probing[15-19]. In brief, it consists of adding in the system to study, an organic compound (in our case the CPH), capable of reacting with the radical species present. The reaction between free radicals and CPH causes the oxidation of CPH and the consequent formation of the nitroxide radical, (also called CP• radical), much more stable, with a life time longer than the first radical[20-22] and, therefore, easier to be detected by EPR tecnique. The Figure 1 summarizes the reaction described previously and the changes obtained in the EPR spectra due to the formation of CP• radical.

Oxidation of CPH in Human Blood Activated with PMA

PMA is widely used to activate the respiratory burst in neutrophils and platelets, leading to the formation of ROS through the NADPH oxidase and to cell degranulation through the activation of the PKC-pathway[23-25]. We tested the concentration of CP•, before (Figure 2A) and after (Figure 2B) the addition PMA on whole blood, plasma and Red Blood Cells (RBCs), using CPH as spin probe. The addition of PMA to the plasma and RBCs did not induce any increase of the CPH oxidation, because of the absence of neutrophils and platelets. The results obtained in whole blood, were completely different, in fact PMA was able to induce a significative increase of CPH oxidation with respect to the value detected in whole blood alone (10.1±0.05 µM vs 5.41±0.01 µM).

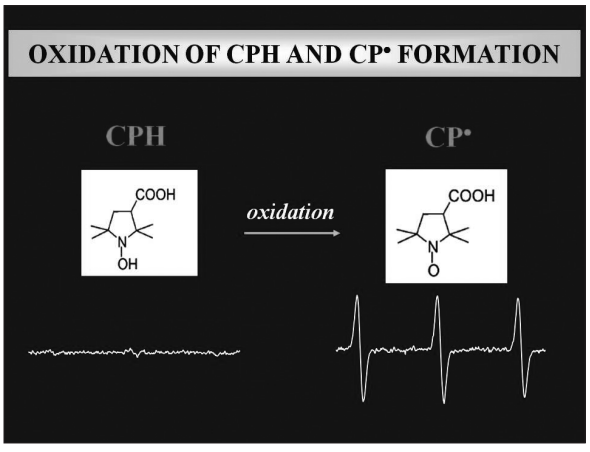

Figure 1. Changing in spectra obtained by EPR analyses after CPH oxidation.

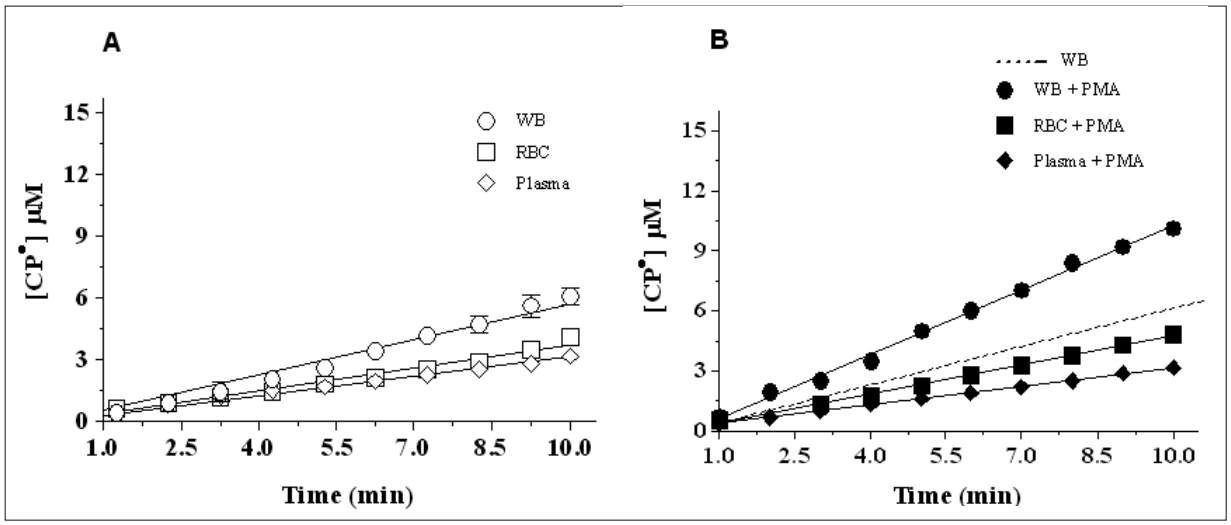

Figure 2. Time course of CP• concentration detected in whole blood, RBCs and plasma before (*A*) and after (*B*) the addition of PMA.

Effect of SOD, Cat, DTPA and NMA on CPH Oxidation

When CPH was added to oxygenated whole blood, the concentration of the CP• detected after 10 min was 5.41±0.01 µM. This basal value was not significantly modified by the addition of SOD, Cat, DTPA, and H_2O_2, used to exclude a direct involvement of oxygen-derived oxidizing species and free metals in the probe oxidation (Figure 3). These results, however, were to be expected, considering that $O_2^{\bullet-}$ and H_2O_2, are rapidly metabolized by endogenous SOD and catalase, present in plasma and in the erythrocytes. On the contrary, the addition of PMA to whole blood, led to a rapid increase in CPH oxidation inhibited by SOD, suggesting that $O_2^{\bullet-}$ was the main oxidant species responsible for PMA-dependent oxidation of CPH in whole blood. Cat, DTPA and NMA were not able to change the oxidation of CPH (Figure 3).

Application of the Spin Probing for the Monitoring of the Oxidative Stress in the Blood of Patients with Thyroid Diseases

Considering the involvement of oxidative stress in thyroid diseases, we decided to measure the rate of CP• formation in whole blood of healthy donors (HD) and to compare these values with those detected in patients with thyroid disease (multinodular goiter MNG and thyroid cancer TC). Figure 4 shows this comparison expressed as CP• concentration after 10 minutes of incubation. In HD (n = 20), the amount of CP• was between

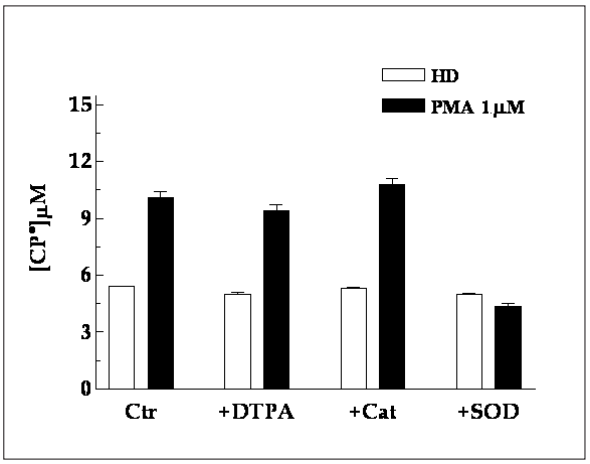

Figure 3. Effects of DTPA, Cat and SOD on PMA-dependent CP• formation, in whole blood of healthy donors.

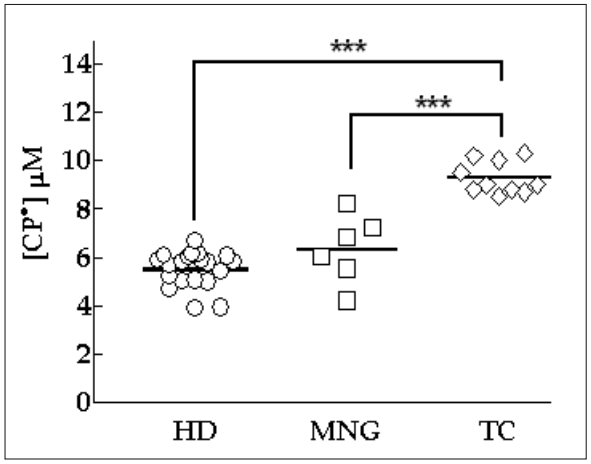

Figure 4. EPR analyses of CP• concentration detected in whole blood of patients enrolled for the study.

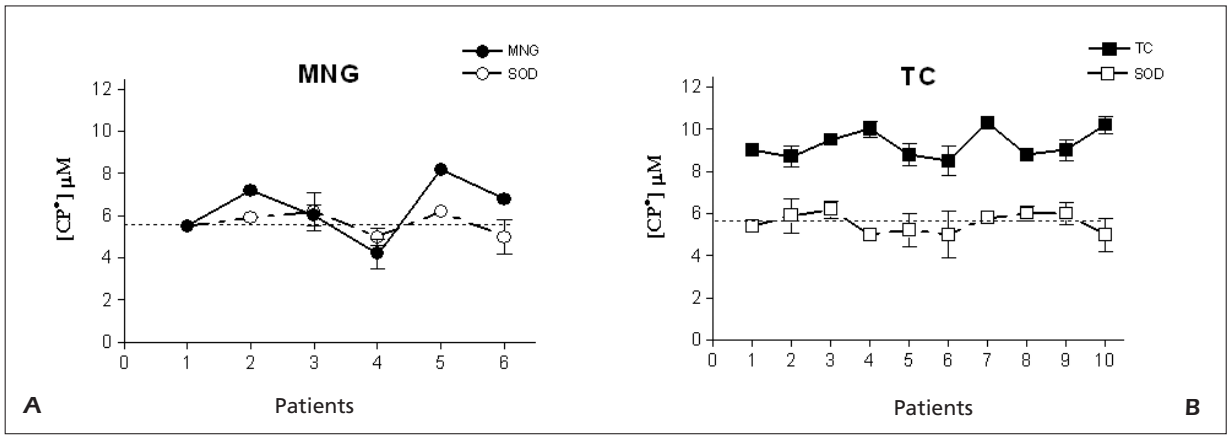

Figure 5. Effect of the treatment with SOD of whole blood of patients with MNG and TC. The exposition to with SOD induced a significant decrease of CP• values in patients with TC. The dashed line show the mean values of CP• of HD.

3.90±0.15 and 6.70±0.13 µM, with an average value of 5.41±0.01 µM, while in MNG (n=6) was within a range of 4.00±0.22 to 8.20±0.18 µM, with a mean value of 6.29±0.9 µM. However, no significative statistical difference was detected between HD and MNG patients. Interestingly, the concentration of CP• in TC patients was significantly increased with respect to HD ($p \leq 0.0001$) and, moreover, the mean value was even significantly higher than the mean value of MNG (9.29±1.74 vs 6.29±0.9 µM, $p \leq 0.0001$).

Identification of ROS and RNS responsible for oxidative stress in patients with MNG and TC.

To understand the mechanism responsible for the oxidation of CPH in the blood of patients with MNG and TC, we verified the possible involvement of $O_2^{\bullet-}$, treating all samples with SOD, a well known scavenger of $O_2^{\bullet-}$ (Figure 5). No change was observed in blood of MNG patients after the exposure to SOD, while the same treatment induced a significant decrease of CP• values of patients with TC ($p \leq 0.0001$). These findings underline the critical role played by $O_2^{\bullet-}$ in the oxidation of CPH, confirming that this oxidant is the major responsible of oxidative stress in patients with thyroid cancer. The results obtained in the same patients 20 days after thyroidectomy (Figure 6), showed that the removal of the tumor results in a normalization of the CP• values.

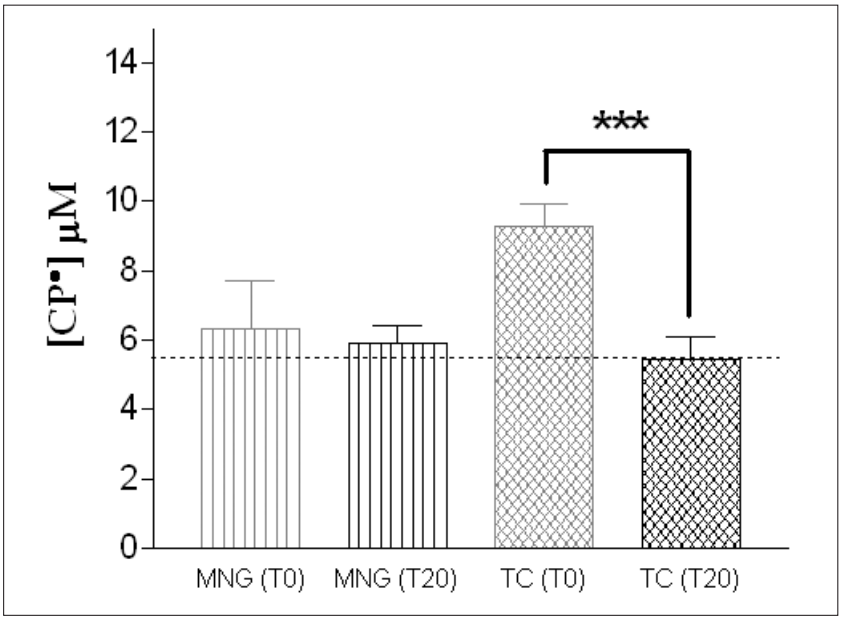

Figure 6. CP• values detected before (T0) and after (T20) thyroidectomy (20 days later).

Discussion

The coupling of the EPR-spin probing technique with the CPH can be used to assess the pro-oxidant state of human blood, both in physiological and pathological conditions. In this preliminary study we have shown that the main mechanism of CPH oxidation in the blood of patients with thyroid cancer is due to the superoxide anion $O_2^{\bullet-}$, a free radical produced during physiological cellular metabolism and inflammation. In particular, it is important to underline that $O_2^{\bullet-}$ is able also to induce irreversible damage to DNA if present in excessive amounts. The pretreatment of the blood of patients with thyroid disease with SOD, which rapidly catalyzes the transformation of $O_2^{\bullet-}$ to H_2O_2, was able to prevent the increase of CPH oxidation, as suggested by the values of CP• comparable to those found in the HD. The pro-oxidant behavior of $O_2^{\bullet-}$ could be due to its overproduction and/or its reaction with other oxidants (such as NO to form peroxynitrite), or to its inadequate removal from the blood. In fact, a quantitative and/or qualitative deficit in the antioxidant system, could be responsible for the establishment of this oxidative stress condition found in our patients. Moreover, a very interesting point of our study is the significant decrease of CP• formation, in all subjects with thyroid cancer, after the removal of neoplastic tissue. These findings show, unequivocally, the involvement of oxidative stress in neoplastic diseases, in particular in thyroid cancer. The EPR technique used for this study has two peculiar aspect: to be able to detect and identify the oxidizing species produced and to be extremely sensitive to the changes in the redox state of the blood. The preliminary results obtained, encourage the use of whole human blood as tissue to study the formation of ROS in some pathological conditions, such as thyroid disease, also due to the lack of invasiveness of the method here described. However, other studies are needed to complete the identification of all the other molecules involved in oxidative stress in thyroid diseases. Considering now the indisputable role of oxidative stress in cancer, it would be desirable for the future, to relate all the changes typically induced by oxidative stress with those found in the blood of the same patients. This would allow to better understand the pathological path that binds ROS to the neoplastic disease and then to take into account, as a possible additional "therapeutic weapon", the antioxidant therapy.

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