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RESEARCH PAPER

Plasma Oxidative Stress in Patients With Chronic Vascular Cutaneous Ulcers

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KEYWORDS:

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Abstract Oxidative stress is hypothesized to be one of the main causes of the pathophysiologic alterations observed during impaired healing of wounds. In the present study, we aimed to measure systemic levels of free radicals in blood and anti-oxidant (AO) activity in the plasma of patients with chronic ulcers (venous stasis ulcers and arterial insufficiency ulcers) of lower extremities (CULEs). Oxidants and AO activity were measured in eighty-five consecutive patients with CVSUs of the lower extremities as they arrived randomly for routine visits to our ambulatory clinic. Values of oxidant and AO status in patients with CULEs were significantly different from normal. No significant differences in oxidant and AO values were found between patients with arterial ulcers or those with venous ulcers. A significant difference was found in AO values of diabetic patients with chronic venous ulcers compared with non-diabetic patients with chronic venous ulcers. No significant differences were observed when evaluating oxidant/AO values and smoking habits. Precise reasons why the association of diabetes and venous (but not arterial) ulcers was correlated with defective AO status in plasma are not known. Other data were also intriguing: diminished AO activity was observed in female patients, no significant differences in oxidant and values were found between patients with arterial ulcers or those with venous ulcers, no significant correlation was found between age and oxidant, as well as no significant differences were observed when evaluating oxidant/AO values and smoking habits.

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Author's role/participation in the authorship of the manuscript:

Edoardo Raposio: study concept and design, supervised the study in all parts, manuscript preparation. *Francesco Simonacci*: data collection. *Davide Gorni*: data analysis. *Michele Pio Grieco*: contributed to interpretation of data, supervised clinical activities, provided critical revision of data.

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Introduction

Exact mechanisms underlying impaired responses to healing in chronic skin ulcers are poorly understood. Recent studies have provided evidence for the role of oxidative stress (OS) in the pathogenesis of non-healing wounds.¹⁻¹⁴ Reactive oxygen species (ROS) are chemically active molecules containing oxygen; examples include peroxides, superoxide, the hydroxyl radical (\bullet OH), and singlet oxygen.

In a biologic context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during environmental or local stress, ROS levels can increase dramatically, which can result in significant damage to cell structures. In a physiologic context, ROS are generated by monocytes, neutrophils and macrophages during oxidative metabolism in mitochondria as well as in cellular responses to xenobiotics, cytokines, and bacterial invasion. The superoxide anion is the major species generated during the respiratory burst in these cells. ROS are produced within cells through multiple mechanisms and, depending on cell and tissue types, the major sources are complexes of NADPH oxidase in cell membranes, mitochondria, peroxisomes, and endoplasmic reticuli.

OS refers to an imbalance due to excess ROS or oxidants over the capability of cells to mount an effective antioxidant (AO) response. OS results in macromolecular damage and is implicated in atherosclerosis, diabetes mellitus (DM), cancer, neurodegeneration, and aging.²

Levels of mediators of ROS and reactive nitrogen species in affected tissues increase. In particular, toxic byproducts such as hydrogen peroxide (H_2O_2) and $\bullet OH$ appear to damage dermal structures directly.¹³ Indeed, OS is hypothesized to be one of the main causes of the pathophysiological alterations observed during impaired healing of wounds.⁴⁻⁶

Here, we aimed to measure systemic levels of free radicals in blood and AO activity in the plasma of patients with chronic ulcers (venous stasis ulcers and arterial insufficiency ulcers) of lower extremities (CULEs).

Materials and Methods

The present study was conducted in compliance with the Declaration of Helsinki and Guidelines for Good Clinical Practice. Enrolled patients provided written informed consent to participate in the study.

Eighty-five consecutive patients with CULEs were enrolled as they arrived randomly for routine visits to our ambulatory clinic. Exclusion criteria were: patients taking chemotherapeutic agents; wounds with an active, clinically diagnosed infection; ulcers present for <3 months.

Demographic data of patients are summarized in [Supplementary Tables I and II](#). Blood (1 cm^3) was collected immediately upon patient arrival at the ambulatory clinic. AO capacity in plasma was measured by the plasma antioxidant test (PAT). Amounts of oxidants were measured by the plasma reactive oxygen metabolites (d-ROMs) test. Both tests were carried out using a specific colorimetric diagnostic kit (FRAS 4 Evolve system; H&D, Parma, Italy). This apparatus has been validated in numerous studies¹⁵⁻²⁰ as a correct clinical measurement of OS and AO status.

The d-ROMs (OS status) test is a simple assay designed to analyze the total amount of hydroperoxides (as an index of oxidants) in serum *via* the Fenton reaction. After blood was drawn, 20 μL of whole blood was mixed in a pipette with an acetic acid buffer solution (pH 4.8) to stabilize the concentration of hydrogen ions. In an acidified medium, bivalent and trivalent iron from the protein component of the blood are ionized and work as catalysts to break down hydroperoxide groups in the blood into alkoxyl groups and transform peroxy radicals into free radicals. These were transferred into a cuvette containing a colorless chromogen (N,N, diethyl *para*-phenylenediamine), which is oxidized by free radicals into a magenta-colored radical cation. The density of the magenta color reflects the concentration of hydroperoxides in blood, which is proportional to the quantity of ROMs. Using this method, the magenta color is measured using a photometer (505 nm) after centrifugation for 1 min to measure the quantity of hydroperoxides. Various concentrations of hydroperoxides in blood were measured, so values were reported, as usual,¹⁶ in the arbitrary unit U.Carr, whereby 1 U.Carr = 0.08 mg H_2O_2/dL .

The PAT is designed to measure (by means of addition of zirconium salts to the reaction mixture) AO capacity in plasma. In this case, 40 μL of one (R_2) reagent (iron solution) were added to a cuvette containing the other (R_1) reagent (thiocyanate-derivate pre-dosed solution), followed by 10 μL of patient blood. A reading was taken at 505 nm after 1-min incubation at 37 °C using the same FRAS apparatus as described above. PAT values are reported in the arbitrary unit U.Cor, whereby 1 U.Cor = 1.4 $\mu\text{Mol/L}$ of ascorbic acid. Values of the PAT (AO capacity) and d-ROMs (OS status) in healthy subjects have been determined in previous studies^{16,17}: d-ROMs values are considered to be normal at 250–300 U.Carr, and those of PAT at 2200–2800 U.Cor.

Statistical analyses were carried out using Prism software (GraphPad, La Jolla, CA, USA). Data are the mean \pm standard deviation. $p < 0.05$ was considered significant.

Results

General values of d-ROMS (OS status) and PAT (AO capacity) were evaluated ([Table 1](#)). Considering the mean values in healthy populations, values of d-ROMs and PAT in patients with CULEs were significantly different from normal^{16,17} ($p < 0.001$) ([Fig. 1](#)): d-ROMs (OS status) values were augmented significantly, whereas PAT (AO capacity) values were reduced significantly. These were clear signs of unbalanced oxidative status.

In the entire study population, no significant correlation was found between age and oxidant ($r = -0.05252$) or AO ($r = 0.011577$) activity.

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