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 P. 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 anti-oxidant (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 anti-oxidant 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 studies^{15–20} 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.

Table 1 General values of d-ROMs (OS status) and PAT (AO activity) in the study group. Considering mean values of d-ROMs (275 (range: 250–300) U.Carr) and PAT (2500 (range: 2200–2800) U.Cor) in healthy populations, both values were significantly different from normal values ($p < 0.001$).

	d-ROMs	PAT
Mean	401	1966
SD	97	491
Median	403	2074
Max	684	2895
Min	209	798
Asymmetry	0.2	−0.7

To evaluate differences between sexes, we first excluded significant differences between males and females with regard to ulcer type, DM, or smoking habits (chi-squared test, $p = 0.104$) (Supplementary Table I). Similarly, males and females were homogenous with regard to age (Supplementary Table II). Comparison of data between sexes (Table 2) revealed no significant differences ($p = 0.273$) regarding d-ROMs (OS status) values, whereas PAT (AO activity) values were significantly lower ($p = 0.030$) in females compared with males (Fig. 2).

No significant differences in oxidant (d-ROMs) and AO (PAT) values were found between patients with arterial ulcers or those with venous ulcers (Supplementary Tables III and IV). With regard to the correlation between ulcer type (arterial or venous) and DM, no significant differences ($p = 0.22782$) were observed for d-ROMs (OS status) values (with or without DM) (Tables 3 and 4). However, a significant difference ($p = 0.00545$) was found in AO (PAT) values of DM patients with chronic venous ulcers compared with non-DM patients with chronic venous ulcers (Fig. 3). That is, DM patients with chronic venous ulcers had significantly lower AO values compared with non-DM patients, thereby suggesting impaired AO defenses. No significant differences were observed when evaluating oxidant/AO values and smoking habits (Table 5).

Figure 1 General oxidant (d-ROMs) and anti-oxidant (PAT) values were significantly different from normal values in healthy populations ($p < 0.001$). n = normal; u = patients with chronic vascular skin ulcers.

Table 2 Differences between sexes. There were no significant differences ($p = 0.273$) for d-ROMs (OS status) values, whereas PAT (AO activity) values were significantly lower ($p = 0.030$) in females compared with males.

	Females		Males	
	d-ROMs	PAT	d-ROMs	PAT
Mean	411	1878	386	2092
SD	83	485	114	479
Median	420	1987	347	2175
Max	589	2669	684	2895
Min	209	892	232	798
Asymmetry	−0.5	−0.6	0.9	−1.2

Discussion

AO capacity in plasma is a measure of the physiologic, environmental, and nutritional factors that determine redox status, and can underline OS conditions in the progression/development of many pathologic mechanisms. AOs are the main barrier of defense against damage due to ROS. If depleted in chronic wounds, endogenous AO defenses are overwhelmed by free-radical production, resulting in OS. Neutrophils, fibroblasts and macrophages, activated by proinflammatory cytokines, use an NADPH cytochrome b-dependent oxidase to produce superoxide anions from molecular oxygen.¹⁰ These anions are dismutated by numerous types of superoxide dismutase (as Mn- and Cu/Zn-dependent variants) to H_2O_2 . The latter is not a radical but, in the presence of transition metals, gives rise to the highly reactive $\bullet OH$, so must be inactivated rapidly. Elevated concentrations of ROS inhibit the migration and proliferation of cells. Mustoe et al⁷ found an increased rate of entry into senescence (with reduction in the phosphorylation of pro-survival kinases and cell viability) in dermal human fibroblasts exposed to H_2O_2 . Expression of ROS-scavenging enzymes (e.g., peroxidases, catalases) might (at least in part) counteract these deleterious effects.¹⁰

Figure 2 Anti-oxidant (PAT) values were significantly lower ($p = 0.030$) in females compared with males.

Table 3 Correlations between arterial and venous ulcers, and diabetes mellitus. No significant differences with regard to d-ROMs (OS status) values between groups were observed. VD = venous + diabetes; VND = venous without diabetes; AD = arterial + diabetes; AND = arterial without diabetes.

	VD	VND	AD	AND
Mean	381	410	395	410
SD	63	91	128	75
Median	359	424	358	420
Max	497	528	684	552
Min	305	232	209	267
Asymmetry	0.60	−0.72	0.69	−0.25
n	12	30	26	17
t test VD-VND	0.312021			
t test AD-AND	0.640905			

Our data showed increased OS and reduced AO capacities in patients with CVSUs compared with the healthy population.^{16,17}

Most chronic ulcers occur in the elderly. Interestingly, our results did not reflect a significant correlation in plasma levels of oxidants and AOs taking into account the age and smoking habits of patients.

Diminished AO activity was observed in female patients, as well as in patients with chronic venous ulcers associated with DM. Precise reasons why the association of DM and venous (but not arterial) ulcers was correlated with defective AO status in plasma are not known. Ischemia–reperfusion injuries have been hypothesized to occur in the development and chronicity of venous ulcers through pathways causing OS, complement/leukocyte activation, and microvascular dysfunction.⁷ Venous hypertension causes sequestration of leukocytes that, with reperfusion (e.g., after leg elevation), are activated and release ROS. Thus, the cellular and functional damage caused by venous ulcers and DM in a synergistic way

Table 4 Correlations between diabetes and ulcer type.

Significant differences between venous ulcers with or without diabetes with regard to PAT (AO activity) values were observed. VD = venous + diabetes; VND = venous without diabetes; AD = arterial + diabetes; AND = arterial without diabetes.

	VD	VND	AD	AND
Mean	2182	1842	2073	1871
SD	243	475	470	609
Median	2174	1986	2094	1987
Max	2584	2550	2895	2618
Min	1742	843	936	798
Asymmetry	−0.25	−0.73	−0.56	−0.60
n	11	29	26	17
t test VD-VND	0.00545			
t test AD-AND	0.22782

Figure 3 A significant difference ($p = 0.00545$) was found in the anti-oxidant (PAT) values of patients with diabetes mellitus (DM) with chronic venous ulcers compared with non-DM patients with chronic venous ulcers.

could increase microvascular dysfunction. Anyhow, it should be stressed that our oxidants and anti-oxidants measurements were from serum and not from the local wound environment.

Cumulative evidence supports the hypothetic role of AO supplements in chronic wounds. Several studies have addressed the role of low-molecular-weight AOs (e.g., ubiquinones, vitamin C/E, lipoic acid, phenolic compounds, glutathione, carotenoids, uric acid) in chronic skin ulcers.¹ AO overdosing does not necessarily enhance antioxidative defense, so increased antioxidative capacity does not always correlate positively with the degree of protection.¹⁵

In several recent studies,^{21–31} we have highlighted some characteristics of adipose-derived mesenchymal stem cells (MSCs). Several MSCs could aid regeneration in chronic wounds by reducing the degree of OS, in addition to their pro-neo-angiogenesis and immunomodulatory capabilities. Future use of these cells is promising, but detailed clinical trials are needed to support this hypothesis.

Table 5 Oxidant levels and smoking habits. No significant differences between smokers and non-smokers were noted.

	d-ROMs (OS status)		PAT (AO activity)	
	Smoker	Non-smoker	Smoker	Non-smoker
Mean	413	402	1808	1982
SD	132	90	650	468
Median	402	403	2104	2068
Max	684	622	2584	2895
Min	232	209	798	892
Asymmetry	0.672	0.030	−0.832	−0.627
n	11	73	10	72
t test d-ROMs	0.7104753			
t test PAT	0.2979195			

Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jccw.2017.02.001>.

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