

The tissue oxidant/antioxidant balance – implications in the pathogenesis of venous leg ulcer

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Abstract. Background and aim: In chronic venous insufficiency (CVI), important changes in both the serum and tissue oxidant/antioxidant balance have been evidenced. Iron stored in the skin of patients with CVI has been studied as an alternative source of reactive oxygen species (ROS). The aim of this study is to demonstrate the role of malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG) and the GSH/GSSG ratio as tissue markers of the presence and intensity of tissue oxidative stress in the pathogenesis of venous leg ulcer. Material and methods: Tissue fragments were collected from the ulcer margin (n=40) and the ulcer base (n=40) and the healthy skin of the same lower limb (n=40) in 40 patients with CVI stage 6 of CEAP classification. The oxidant/antioxidant balance markers present in the ulcer base and margin and in healthy skin were determined. Results: An increase of oxidative stress, manifesting by an increase of MDA (a marker of the presence of oxidative stress) was found in the samples taken from the base of the venous ulcer in comparison with ulcer margin (p= 0.0078) and in comparison with healthy skin (p=0.0004). GSH and the GSH/GSSG ratio as markers of antioxidant capacity were not significantly changed in the studied groups. Conclusions: High MDA concentrations found in the ulcer base suggest that oxidative stress plays an important role in the pathogenesis of venous leg ulcer in patients with CVI. The presence of iron in the form of hemosiderin in ulcers can lead to an increase of ROS; this study provides indirect evidence of the implication of iron in the etiopathogenesis of venous ulcer in CVI, supporting the oxidative stress theory.

Key Words: chronic venous insufficiency, venous ulcer, iron, malondialdehyde, reactive oxygen species.

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Introduction

Chronic venous disease includes all clinical manifestations related to a functional or morphological abnormality of the venous system, caused by valvular insufficiency or superficial or deep venous obstruction. Active venous ulcer is the most advanced stage of chronic venous disease (stage C6 of CEAP classification). Varicose leg ulcer, active or closed, is present in approximately 1% of the population of developed countries (Abbade et al 2005).

Several studies showed that reactive oxygen species (ROS) play an important role in the development of chronic venous insufficiency, which required the investigation of the mechanism leading to the increase of free radical production. Starting with 1988, when Ackerman et al (1988) reported an increase of iron concentration in the skin of patients with venous leg ulcer in chronic venous insufficiency (CVI), the role of this element as an alternative source of ROS has been studied.

Iron is an indispensable element, involved in many vital processes, but it can act as a prooxidant, through Fenton's reaction, by generating an extremely reactive hydroxyl radical, under the conditions of increased deposits (Budzyn M et al 2011).

The role of antioxidants in chronic wounds, particularly in venous leg ulcer, has been little studied.

Divalent iron (Fe II) reacts with H₂O₂, forming HO. (the Haber-Weiss reaction). If trivalent iron (Fe III) is bound to specific proteins, transferritin or ferritin, the reaction no longer takes

place. Thus, the key element for the formation of the hydroxyl radical (OH.) is the presence of sufficient amounts of divalent iron (Brisson et al 2012). The local increase of free divalent iron results in cellular degradation and finally, in apoptosis or necrosis (Kury et al 2007).

The aim of this study is to demonstrate the role of malondialdehyde (MDA), a marker of lipid peroxidation, reduced glutathione (GSH) as an antioxidant, oxidized glutathione (GSSG) and the GSH/GSSG ratio as tissue markers of the presence and intensity of tissue oxidative stress in the pathogenesis of venous leg ulcer.

Material and methods

We performed a prospective study on 40 patients, admitted to the Surgical Clinic II, Cluj-Napoca, with the diagnosis of chronic venous insufficiency, stages C6 of the CEAP clinical classification (presence of active ulceration), in the period November 2013 – November 2014. The approval of the Ethics Commission of the “Iuliu Hatieganu” University of Medicine and Pharmacy Cluj-Napoca was obtained and an informed consent was signed by each patient included in the study after a clear explanation of the required procedures.

The selected patients met the following inclusion criteria: presence of a form of trophic disorder in CVI (open ulceration). Diagnosis was made based on clinical data – presence of trophic disorders, and on imaging data (Doppler ultrasound of the deep and superficial venous system). The presence of diabetes

mellitus, peripheral arterial disease, an immobilized patient, heart, liver, kidney or lung failure, and chronic corticoid administration were considered as exclusion criteria.

Tissue fragments were collected from the ulcer margin (n=40) and the ulcer base (n=40) and the healthy skin taken from Scarpa triangle of the same lower limb (n=40), during surgery for the operative management of the primary chronic venous disease. The studied groups were as follows:

- group I – ulcer base (n=40)
- group II – ulcer margin (n=40)
- group III – healthy skin (n=40)

Biochemical determinations for the tissue oxidant/antioxidant balance indicators were carried out in the Laboratory for the Study of Oxidative Stress of the Department of Physiology of the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca. Histopathological examination was performed at the Department of Pathomorphology of the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca.

Reduced glutathione (GSH) was measured by fluorescence with a Perkin Elmer LS 45 fluorescence spectrometer. This determination is based on the fact that GSH forms with o-phthalaldehyde a fluorescent product (Vats *et al* 2008).

Oxidized glutathione (GSSG) was determined using the Vats (2008) method with a Perkin Elmer LS 45 fluorescence spectrometer (purchased with CEEX 15/2005 funds) (1994).

Malondialdehyde (MDA) was measured by fluorescence with a Perkin Elmer fluorescence spectrometer. Malondialdehyde concentration is determined based on a calibration curve with known MDA concentrations processed in the same way (Conti 1991). The samples for histopathological examination were fixed in 10% formol solution and processed for hematoxylin-eosin staining and Perl's staining. This technique allows to evidence hemosiderin deposits in the skin (Tan *et al* 2003).

To test normal distribution, the Shapiro-Wilk test was used. Kruskal-Wallis non-parametric test in the case of non-uniform distribution data or ranks was used. In the case of two unpaired samples, for normal distribution data, the (Student) t test was used, and in the case of non-uniform distribution values or ranks, the Mann-Whitney (U) non-parametric test was conducted. A p value < 0.05 was considered statistically significant.

To determine the correlation between two quantitative continuous variables with normal (uniform) distribution, Pearson's correlation coefficient (r) was used. In the case of non-uniform distribution variables, Spearman's rank correlation coefficient (ρ) was used. The analysis of correlation coefficients was performed using Colton's rule.

Statistical processing was performed with the StatsDirect v.2.7.2. software and the Excel application (Microsoft Office 2007).

Results

The characteristics of the patients are presented in Table 1.

Comparative analysis of the tissue oxidant/antioxidant balance indicators

a) Comparative analysis of MDA values (Table 2)

An increase of oxidative stress, manifesting by an increase of MDA (a marker of the presence of oxidative stress) was found in the samples collected from the venous ulcer base.

Table 1. Demographic characteristics of patients and their classification into CEAP clinical classes

Variables	Frequency (%)
Sex (M/F)	18/22
Age (years) (median ±SD)	58.3±2.23
Clinical class CEAP C6	40

The mean MDA concentration in patients with venous leg ulcer was significantly increased in samples collected from the ulcer base compared to samples taken from the ulcer margin and healthy skin samples. No statistically significant differences were found between the mean MDA values of the ulcer margin samples and the healthy skin samples (Table 2).

b) Comparative analysis of GSH, GSSG, GSH/GSSG values
GSH and the GSH/GSSG ratio as markers of antioxidant capacity were not significantly changed in the studied groups (Table II). The statistical analysis of GSH values, considering all three groups, showed no statistically significant differences between the groups (p = 0.5385). The statistical analysis of GSH values, for unpaired samples, evidenced no statistically significant differences between the groups (p < 0.05).

The statistical analysis of GSSG values, considering all three groups, indicated no statistically significant differences between the groups (p = 0.3541). The statistical analysis of GSH values, for unpaired samples, revealed no statistically significant differences between the groups (p > 0.05).

The statistical analysis of the GSH/GSSG ratio values, considering all three groups, evidenced no statistically significant differences between the groups (p = 0.0663). The statistical analysis of the GSH/GSSG ratio values, for unpaired samples, showed no statistically significant differences between the groups (p > 0.05).

c) Correlation analysis (table 3)

Statistical analysis of the correlation between the oxidant/antioxidant balance indicators evidenced a good negative correlation between MDA – GSH/GSSG, GSH – GSH/GSSG for group II and a very good positive correlation between MDA – GSH.

d) Hemosiderin deposits

Perl's staining (Berlin blue) evidences hemosiderin deposits (a form of deposit of ferric ion Fe⁺⁺⁺) in tissues (3.1.). Tissue hemosiderin was qualitatively assessed.

The samples collected from the ulcer base and margin, n=80, were positive for hemosiderin (Perl's staining). In healthy skin, n=40, no hemosiderin deposits were evidenced.

Discussion

Reactive oxygen species (ROS) play an important role in the pathogenesis of diseases associated with vascular dysfunctions through endothelial injuries, resulting in the loss of endothelial integrity (Hull *et al* 1984). In CVI, venous endothelial changes are probably caused by ROS overproduction (Budyin *et al* 2011). Our study demonstrated an increase of MDA, a marker of lipid peroxidation, in tissues collected from the venous ulcer base of patients with CVI. The obtained data are in accordance with literature data, which detected high MDA values in the plasma and tissue homogenates of patients with CVI. Kozka *et al.* evidenced high plasma MDA concentrations in patients with CVI with varicose veins (CEAP classes C2 and C3) compared to healthy patients (Kozka *et al* 2009). High MDA concentrations

Table 2. Comparative analysis for the values of the oxidant/antioxidant balance indicators (nmol/mg protein) in the studied groups and statistical significance

Indicator	Group	Mean	SD		p	
MDA	I	2.41	1.018	I-II	0.008	I-II-III
	II	1.03	1.269	I-III	0.000	0.001
	III	0.49	0.280	II-III	0.489	
GSH	I	0.99	0.551	I-II	0.297	I-II-III
	II	1.19	0.502	I-III	0.489	0.539
	III	1.38	1.078	II-III	0.640	
GSSG	I	0.86	0.314	I-II	0.963	I-II-III
	II	0.85	0.534	I-III	0.231	0.354
	III	1.4	0.965	II-III	0.231	
GSH/GSSG	I	0.75	0.555	I-II	0.050	I-II-III
	II	3.85	4.242	I-III	0.730	0.066
	III	0.93	1.131	II-III	0.050	

Table 3. Correlation coefficients between the oxidant/antioxidant balance indicators in the studied groups

Indicator	Group I		Group II		Group III	
	r	p	r	p	r	p
MDA-GSH	0.5	0.178	0.683	0.050	0.814	0.008
MDA-GSSG	0.104	0.791	0.667	0.059	0.433	0.250
MDA-GSH/GSSG	0.472	0.200	-0.733	0.026	0.3	0.437
GSH-GSSG	0.367	0.336	0.864	0.003	0.667	0.059
GSH-GSH/GSSG	0.667	0.059	-0.633	0.067	0.317	0.410
GSSG-GSH/GSSG	-0.378	0.316	-0.933	0.000	-0.35	0.336

were also reported in great saphenous vein homogenates from patients with varicose veins or with healed venous ulcer (CEAP clinical classes C3 and C4) (Karatepe et al 2010). Similar results – increased MDA concentrations – were also found in the blood collected from the limb affected by CVI compared to the healthy limb of the same subject (Krzysciak et al 2009).

In CVI, the toxic effect of iron (ferric ion from hemosiderin deposits) has been demonstrated, which is involved in the pathogenic chain that leads to the lysis of the extracellular matrix, the generation of free radicals, the activation of metalloproteinases (Wenk et al 2009) or the down regulation of tissue metalloproteinase inhibitors (Cagiatti et al 2010).

Hemosiderin deposits were evidenced by Perl's staining in all patients included in the study both in LDS plaque biopsies (2 patients) and in active venous ulcer biopsies (38 patients), in accordance with the literature data (Tan et al 2003, Caggiati et al 2010).

The relationship between iron and CVI was clearly demonstrated by Zamboni (2006), who showed that in patients with a mutation in the HFE gene, which encodes an iron regulatory protein frequent in patients with hemochromatosis, the risk of venous ulcer in primary CVI is increased. Another study evidenced a significant difference in iron concentration in CVI ulcer exudate compared to acute wound exudate (Wenk et al 2001). Moreover, a high concentration of iron deposits was found by measuring serum ferritin, by determining iron in ulcer

exudates and ulcer biopsies (Perl's staining) from patients with CVI (Zamboni et al 2005).

In this study, we tested the hypothesis that tissue iron accumulation in patients with venous ulcer in primary CVI contributes to the local formation of ROS: the relationship between iron deposits in the form of hemosiderin and oxidative stress markers (MDA, GSH, GSSG, GSH/GSSG). A significant increase of ROS was found in the ulcer base (through an increase of MDA) compared to ulcer margins or healthy skin. A lack of concordance between iron deposits from the ulcer margins or lipodermatosclerotic skin and ROS production does not exclude the ROS-generating ability of iron. This means that other iron metabolites should be evaluated, particularly free, non-transferrin-bound iron (NTBI), a molecular iron complex able to initiate ROS formation (Le Lan et al 2005). In CVI, the diapedesis of erythrocytes is followed by their lysis, with the subsequent release of hemoglobin, which is a form of prooxidant iron (Cagiatti et al 2008). It seems that non-protein bound iron becomes a catalyzer for the formation of the hydroxyl radical (Alayah et al 2001).

In the patients included in the study, we found normal serum iron levels, which supports the tissue accumulation of iron in the form of hemosiderin and its local prooxidant effect.

Conclusions

High MDA concentrations found in the ulcer base suggest that oxidative stress plays an important role in the pathogenesis of

venous leg ulcer in patients with CVI. The presence of iron in the form of hemosiderin in ulcers can lead to an increase of ROS; this study provides indirect evidence of the implication of iron in the etiopathogenesis of venous ulcer in CVI, supporting the oxidative stress theory.

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Citation Constantinescu D, Constantinescu I, Mironiuc A. The tissue oxidant/antioxidant balance – implications in the pathogenesis of venous leg ulcer. *HVM Bioflux* 2015;7(4):290-293.

Editor Ștefan C. Vesa

Received 4 September 2015

Accepted 23 September 2015

Published Online 24 September 2015

Funding None reported

**Conflicts/
Competing
Interests** None reported