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NO and Chronic Venous Insufficiency (CVI): Immunohistochemical Evaluation of iNOS and eNOS Isoforms in the Venous Ulcers. Clinical and Therapeutical Possible Implications

[1]V. Tessitore, [1]M.L. Uzzo, [1]G. Bonaventura, [3]F. Carini, [2]G. Milio, and [1]G.F. Spatola
[1]DI.ME.S. Sezione di Istologia e Embriologia
[2]Dipartimento di Medicina Interna e Malattie Cardiovascolari, Università di Palermo, Italia
[3]DI.ME.S. Sezione di Anatomia umana, Università di Palermo, Italia

Abstract: The pathophysiologic mechanism related to pathogenesis of cutaneous trophic disorders in Chronic Venous Insufficiency (CVI) find different explanations. The increase in the number of capillaries and the subsequently stasis, the fibrin cuffs deposited around the capillaries and leucocytes migration associated at inflammatory phenomena in fact try to explain the etiology of venous ulceration. In this study, we have evaluate the immunohistochemical presence of iNOS and eNOS in cutaneous bord of venous ulcers to evaluate the role of Nitric Oxide (NO) in pathophysiology of these trophic disorders. In addition, using the image analisys method, we have evaluate the increase of capillary numbers respect to normal skin. We have studied immunohistochemical distribution of iNOS and eNOS in human normal skin and in peripheral bord of venous ulcers in 20 subject affected by CVI. The specimens have been taken during a surgical treatment. All of these are fixed in Bouin's mixture and processed with eNOS (Transduction N30020) and iNOS (Transduction N32030) antibodies. After the immunostaining procedures all specimens are studied with Nikon microscope and Lucia system of image analisys. Moderate eNOS immunoreactivity are expressed in the same way both in the endothelium of a dermic capillaries and in the spinous epithelium of the normal skin such as in the pathological specimens. iNOS immunopositivity is more expressed in the spinous epithelium and in the capillaries endothelium of a bord of venous ulcer than in a normal skin. At the image analisys the capillary numbers in the venous ulcers is remarkably higher respect the normal skin. These data provide a morphological basis to explain a possible pathogenesis in the cutaneous trophic disorders in CVI. In fact the strong iNOS immunoreactivity, expressed in this inflammatory phenomenon, may be implicated in microcirculatory stasis.

Key words: Immunohistochemical, CVI, NO, microcirculatory stasis, pathophysiologic mechanism

INTRODUCTION

Chronic Venous Insufficiency (CVI) and venous leg ulcer are a widespread problems with a prevalence of 1 to 2% of the population in Western societies (Korthuis and Schonbein, 2000; Kurz *et al*., 1999). The pathophysiology of this damage is not totally clear at this moment. Surely the elevated pressure values in the peripheral venous system (Bjordal, 1971; Stanley *et al*., 2005) the defective valves into capillary network, the increase of permeability and the capillary neogenesis (discharge of fluids in the extracellular compartment? oedema), the fibrinogen discharge and the subsequently formation of pericapillary "fibrin cuff" are the most important mechanism related to the hypoxia and the cutaneous ischemia represents the first step of the formation of the leg ulcers (Korthuis and Schonbein, 2000; Junger *et al*., 2000; Milio *et al*., 2005; Coleridge, 1995; Dormandy and Thomas, 1989; Browse *et al*., 1993; Burnard, 1999). During ischemia the white blood cells may cause damage to the tissues by release of the free radicals, proteolytic enzymes and cytokines. In addition several authors (Coleridge-Smith, 1995; Dormandy and Thomas, 1989) suppose a "White cell trapping" hypothesis responsible for endothelial injury that results in an additional damage to the microcirculation. The NO perform in the circulatory system its influence in different manner: In the physiological condition NO is produced enzymatically in small amount and for a short time in endothelial cells (eNOS) and in nitroxidergic neurons that innervate the vessels (nNOS) (Moncada *et al*., 1991; Pollock *et al*., 1991; Bredt and Snyder, 1992; Tessitore, 1998); in the pathological conditions the production of NO is inducible in the macrophages (iNOS) by bacterial

Corresponding Author: Giovanni Francesco Spatola, Via M.se di Villabianca, 82-90143, Palermo, Italy

LPS and/or by white cells (Coleridge-Smith, 1995; Dormandy and Thomas, 1989) cytokines and is produced in big quantity and for a long time (Stuehr *et al*., 1990). The results of its action is a vasodilatation, but this, in the inflammatory disease, cause a microcirculatory stasis. We have study the immunohistochemical presence of iNOS and eNOS in cutaneous bord of venous ulcers to evaluate the role of Nitric Oxide (NO) in pathophysiology of these trophic disorders. In addition, using the image analysis method, we have evaluate the differences of capillary numbers, their diameter and colorimetric differences between normal and pathological skin.

MATERIALS AND METHODS

We enrolled 20 patients carriers of venous ulcers of the lower limbs, which were defined as any non-healing wound of the skin of the lower extremities caused by impaired venous return, without complications.The patients, males and females, were between 52 and 68 years old.

The inclusion criteria were the presence of chronic venous insufficiency, evident clinically and with echo-color-doppler evaluation and an Ankle-Brachial Index (ABI) ratio >0.90 for both limbs.

The exclusion criteria included evidence of arterial, diabetic or neurotrophic ulcers and all possible other conditions that cause peripheral ulcers, such us haematologic and neurological diseases and vasculitis; active infection of the ulcers and poor patients compliance.

In these patients we have studied immunohisto-chemical distribution of iNOS and eNOS in peripheral bord of venous ulcers, in comparison with the distribution of the same enzymes in human normal skin obtained during a non traumatic orthopaedic surgical treatment of the lower limbs in 15 patients. All the specimens were fixed in Bouin's mixture. After Bouin fixation the tissue was dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin. Sections of 8 μ were cut on to Leica microtome RM2145, dried overnight at 37°C and then stored at room temperature. On the day of the experiment, slides were dewaxed and rehydrated by sequential immersion in a graded series of alcohols. Slides were then transferred into water for 5 min; for the endogenous peroxidase inhibition slides were treated with 3% hydrogen peroxide in hydrated incubation enclosure at RT. After, the slides were transferred in PBS buffer (PBS: Na2HPO4, KH2PO4, KCl, NaCl pH 7.4 - 7.6) at RT.

The following protocol was used with the kit Ultraystain Polyvalent Strept ABC-HRP YLEM code AFN600: After rinsing with PBS buffered, the tissue sections were blocked with Super Block reagent (normal goat serum in phosphate buffered saline containing carrier protein) for 10 min and then rinsed with PBS for 4 min.

Anti iNOS (Transduction laboratories code N32030), anti eNOS (Transduction laboratories code N30020) were then added at 10 μg mL^{-1} in PBS with BSA and incubated overnight at 6C°.

After the incubations any excess antibodies was removed by washing, the slides were rinsed 2X with PBS, 5 min each. Next we have added the Ultraystain Polyvalent Antiserum (biotinylated goat anti-mouse IgG and goat anti-rabbit IgG in PBS) for 10 min at RT, unbound antibody was removed by washing (2X with PBS, 5 min each) and subsequently we have applied Ultraystain Streptavidin for 20 min at RT. After incubation unbound enzyme was removed by rinse procedure (2X with PBS, 5 min each). The chromogenic development reagent, AEC chromogen in substrate buffer (3-amino-9-etil carbazole in N,N dimetilformamide) was then added for 5-15 min and we have stopped in DI water. We have removed slides from the water and we have applied one drop of aqueous mounting medium containing 15 mM sodium azide (DAKO Faramount) to the tissue and we have applied a coverslip. After the immunostaining procedures all specimens are studied with Nikon optic microscope and Lucia by Nikon system of image analisys.

The protocol was approved by the ethics committee of the University Hospital "P. Giaccone" of Palermo and all patients gave informed consent.

RESULTS

Moderate eNOS immunoreactivity are expressed in the same way both in the endothelium of a dermic capillaries and in the spinous epithelium of the normal skin such as in the pathological specimens (Fig. 1 and 2).

iNOS immunopositivity is more expressed in the spinous epithelium and in the capillaries endothelium of a bord of venous ulcer than in a normal skin. In addition in the venous ulcers, near the capillaries, are present a few number of positive cells (macrophages) (Fig. 3 and 4).

At the image analysis the capillary numbers in the venous ulcers is remarkably higher respect to normal skin and their diameter in the venous ulcers is smaller respect to normal skin (Table 1 and 2).

The study of a colorimetric density shows a statistical significative increase of iNOS in the venous ulcers respect the normal skin. Other parameters are not significative at the statistic T student test (Table 3).

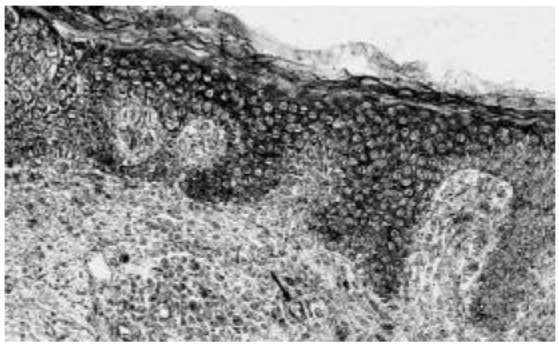

Fig. 1: eNOS immunoreactivity in the epithelium of normal skin. In the dermic capillaries is present a moderate immunopositivity (arrow) (20x)

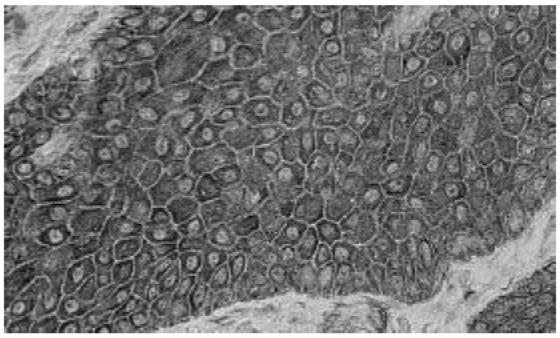

Fig. 2: eNOS immunoreactivity in the epithelium of venous ulcers (40x)

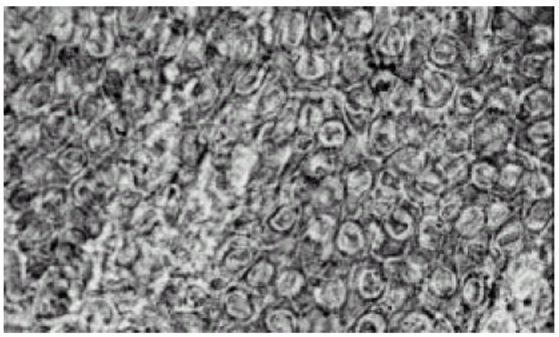

Fig. 3: iNOS immunoreactivity in the epithelium of normal skin (63x)

Table 1: Image analisys: Capillary numbers in the venous ulcers is remarkably higher respect to normal skin

Normal	5±2
Venous ulcer	9±6

(Media calculate on the area 35×35 μ in the image of 40x magnification)

Table 2: Image analisys: capillary diameter in the venous ulcers is smaller respect to normal skin

Normal	2.66μ±0.80
Venous ulcer	1.77μ±0.99

(Media calculate in μ on the area related at the calibration of 40x magnification t student =1.58)

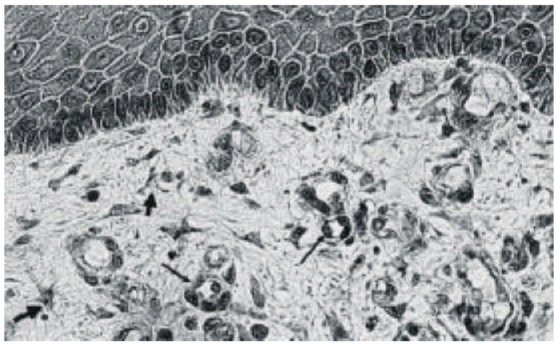

Fig. 4: iNOS immunoreactivity in the epithelium of venous ulcer. In the dermic capillaries is present a strongly immunopositivity (slim arrow) and a few positive macrophagic cells (big arrow)

Table 3: Image analisys: Colorimetric density in the epithelium and in the capillary endothelium

	Colorimetric density in epithelium	
	Normal skin	Ulcer
eNOS	84.36±29.36	-105.0±11.80
iNOS	-129.35±29.97	83.36±16.87
	Colorimetric density in caillary endothelium	
eNOS	-123.43±19.49	-136.92±14.99
iNOS	-152.22±12.13	86.10±1708

T student: eNOS = 4,2 not significative – iNOS = 21.31 significative

DISCUSSION

In this study, we have evaluate the presence of eNOS and iNOS immunoreactivity in the peripheral bord of the venous ulcers of the lower limbs in comparison with the human healthy skin of the lower limbs.

eNOS immunoreactivity is present, as expected, in the vascular endothelial cells but, surprisingly, is present also in cutaneous epithelial cells with the same intensity in normal and in pathological conditions. The presence of an extraendothelial eNOS immunoreactivity has been displayed by us in several previous studies in other organic districts (Tessitore *et al*., 1998, 2003 a, b) but the interpretation of this extraendothelial localization in the skin is difficult to explain and may represent the aim for further researches. At this moment is possible to suppose that epidermal NO is implicated, somehow, in epidermal cytomorphosis process. iNOS immunoreactivity is sparely present, as expected, in the normal skin while in the ulcers appears significantly increased both in the dermic stroma, in perivascular structures and, characteristically, in the germinative layer of epidermis. These data are in agreement with other authors (Shimizu *et al*., 1997; Abd-El-Aleem *et al*., 2000). The image analysis data concerning the increase of vascular number and the reduction of diameter (that indicate the presence of opened real capillary) support the hypothesis of a massive outbreak

in the capillary network (NO induced) and a subsequently microcirculatory stasis because of venous drainage failure.

These data provide a immunohistochemical basis to explain the functional role of NO in the venous ulcers related to Chronic Venous Insufficiency (CVI). The production of NO induced by iNOS may take part in early stages of this pathology causing a massive capillary vasodilatation responsible for microcirculatory stasis which is the cause of subsequent ischemic phenomena.

CONCLUSION

The evaluation of the iNOS presence made by immunohistochemical study therefore, can turn out an useful method to monitor the evolution of the pathology and the effectiveness of the therapy, a decrease of the iNOS presence would evidently be correlated in how much both to a decrease of the inflammatory state and a restoration of a normal cutaneous circulation.

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