

Enzymatic activity analysis of MMP-2 and 9 collected by swab from lower limb venous ulcers

Análise da atividade enzimática de MMP-2 e 9 coletadas por swab em úlcera venosa de membro inferior

Flávio Santos da Silva¹, Diego Neves Araujo¹, João Paulo Matos Santos Lima², Adriana Augusto de Rezende³, Bento João da Graça Azevedo Abreu⁴, Fernando Augusto Lavezzo Dias^{1,5}

Abstract

Metalloproteinases play a role in repair of venous ulcers of the lower limbs. The great majority of studies of metalloproteinase enzyme activity conducted to date have employed material from biopsies of ulcers. We evaluated the viability of using zymography to measure the enzyme activity of metalloproteinases 2 and 9 in samples of venous ulcer exudate collected on swabs. The method chosen for processing the samples proved viable in terms of its ability to provide adequate protein concentrations for analysis. Using zymography, we observed that the parameters that provided the best results for analysis of gelatinolytic activity were 0.125 to 0.5 μg of total protein content in the gels and enzymatic activation time of 19 hours (at 37 °C). Collection of venous ulcer fluid using swabs proved to be a simple, rapid and effective method for obtaining samples for measurement of gelatinolytic activity with a minimum degree of invasivity.

Keywords: venous ulcer; metalloproteinases; gelatinases.

Resumo

As metaloproteínas participam do reparo das úlceras venosas de membros inferiores. Até o momento, estudos de atividade enzimática utilizaram, em sua maior parte, biópsia das úlceras. Objetivamos avaliar a viabilidade da mensuração da atividade enzimática de metaloproteínas 2 e 9, extraídas por swab, em amostras de exsudato de úlcera venosa, através de zimografia. O método de processamento da amostra coletada mostrou-se viável, visto que foi possível obter concentração proteica adequada para análise. Através de zimografia, observamos que as quantidades de proteína total das amostras carregadas nos géis entre 0,125 e 0,5 μg , além do tempo de ativação enzimática de 19 horas (a 37 °C), foram parâmetros adequados e de melhor resultado para a análise da atividade gelatinolítica. A coleta através de swab mostrou-se um método simples, rápido e eficaz para a coleta de fluido de úlcera venosa com o objetivo de mensuração da atividade gelatinolítica com grau mínimo de invasividade.

Palavras-chave: úlcera venosa; metaloproteínas; gelatinases.

¹Universidade Federal do Rio Grande do Norte – UFRN, Graduate Program in Physical Therapy, Natal, RN, Brazil.

²Universidade Federal do Rio Grande do Norte – UFRN, Department of Biochemistry, Natal, RN, Brazil.

³Universidade Federal do Rio Grande do Norte – UFRN, Department of Clinical and Toxicological Analysis, Natal, RN, Brazil.

⁴Universidade Federal do Rio Grande do Norte – UFRN, Department of Morphology, Natal, RN, Brazil.

⁵Universidade Federal do Paraná – UFPR, Department of Physiology, Curitiba, PR, Brazil.

Financial support: This study was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Norte (FAPERN) and CT-INFRA, affiliated with MCT/CNPq (PPP 2009), by the Brazilian Ministry of Science and Technology and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) (UNIVERSAL 2010), and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (MSc scholarships received by FSS and DNA).

Conflicts of interest: No conflicts of interest declared concerning the publication of this article.

Submitted: 03.22.14. Accepted: 05.27.14.

The study was carried out at Hospital Universitário Onofre Lopes (collection stage) and at the Department of Biochemistry of the Center of Biosciences (analysis stage) at Campus Central, UFRN, Natal-RN, Brazil.

■ INTRODUCTION

Cutaneous venous ulcers (VUs) secondary to chronic venous insufficiency are characteristically recurrent and slow to heal and can cause edema, pain, discomfort, restricted mobility and impaired quality of life.^{1,2} Venous ulcers account for 70 to 80% of ulcers of the lower limbs and the cost of treating them places a heavy burden on health service budgets.^{3,4} Both treatment of these ulcers, aiming for complete healing, and determination of their prognosis for healing are difficult.

The metalloproteinases (MMPs) are involved in regulation of the process of tissue repair.⁵ They are a family of zinc-dependent enzymes that break down several different molecules in the extracellular matrix (ECM). The equilibrium between production of ECM proteins and MMP activity is the primary determinant of tissue remodeling both in homeostasis and in the response to injury.⁶ However, when expression and activity kinetics of MMPs are uncontrolled, due to high rates of synthesis or impaired regulatory or inhibitory mechanisms, they can have harmful effects. This dysregulation can contribute to destruction of connective tissue and the loss of its normal mechanical properties, with the result that wounds become chronic.^{7,8}

Studies have detected widespread increase in both activity and expression of MMPs in VUs^{7,9} and MMPs 2 and 9, which are also known as the gelatinases, have received special attention. Specific tissue expression of MMP-9 has been identified as a prognostic marker of healing of chronic ulcers.¹⁰ A previous study has demonstrated high levels of both MMP-2 and MMP-9 in chronic VUs of the lower limbs,⁹ suggesting that these two MMP isoforms play a critical role in healing of venous ulcers. In view of this, these enzymes' expression and activity could serve as markers of the effectiveness and mechanism of action of therapeutic interventions that are intended to accelerate the healing process of chronic ulcers.

The activity of MMPs 2 and 9 can be evaluated using zymography, which is a practical, reproducible and low-cost method. However, the method hitherto employed to collect biological material for MMP assays in studies involves tissue biopsies, which makes serial testing more difficult and involves discomfort and risk to patients, in addition to not being technically practical for all classes of health professionals. Other techniques, such as aspiration of exudate, require use of specific dressings and are time consuming.¹¹

The objective of the present study was to evaluate the viability of assaying MMP 2 and 9 enzyme

activity using a modified zymography protocol to test samples of lower limb VU exudate collected with swabs. We have conceived of a simple method that is rapid, minimally invasive and inexpensive, for serial collection (at different stages of healing) of this type of biological material and analysis of MMP activity.

■ METHOD

Participants

Samples were collected from six patients with chronic VU of lower limbs who were patients of the local University Hospital's outpatients service. Procedures were approved by the Research Ethics Committee (Protocol 376/09). The results are preliminary data from registered clinical trial number RBR-44xmsx. Patients had had VU for at least 8 weeks, with no clinical signs of infection or peripheral arterial occlusive disease, tested using the ankle-brachial index.

Sample collection and processing

Samples of fluid from VUs were taken using sterile cotton buds (swabs), using a rolling motion, from border to border, after light irrigation with sterile saline solution. This procedure was conducted during primary dressing changes. Next, the tips of the swabs bearing the samples were removed and placed into 2 mL microtubes, fast-frozen in liquid nitrogen and stored at -80°C until processing for protein extraction, from 2 to 3 weeks later.

To achieve homogenization, 250 μL of homogenization buffer was added to each tube containing a swab tip. The buffer comprised 50 mM Tris-HCl (pH 7.4); 3.1 mM Sucrose; and 0.1% Triton X-100, with no reduction agent, at a temperature of 4°C . Samples were mixed in a vortex five times for 3 s each time, with a 15 s interval at rest between each mixing. Swabs were then inverted in their microtubes and centrifuged (2 minutes, 10,000 rpm, 4°C). The samples were kept in ice throughout these procedures. Figure 1 summarizes these stages.

Protein concentration was determined using the Bradford method and quantified in an ELISA reader using standard controls made from bovine serum albumin. Readings were taken at 595 nm, 10 minutes after the assay, against a reference white.

Determination of enzyme activity of MMPs by zymography

Samples were separated in polyacrylamide gels with a concentration of 8% Acrylamide/Bisacrylamide 29:1 (Bio-Rad®), 0.1% SDS and a

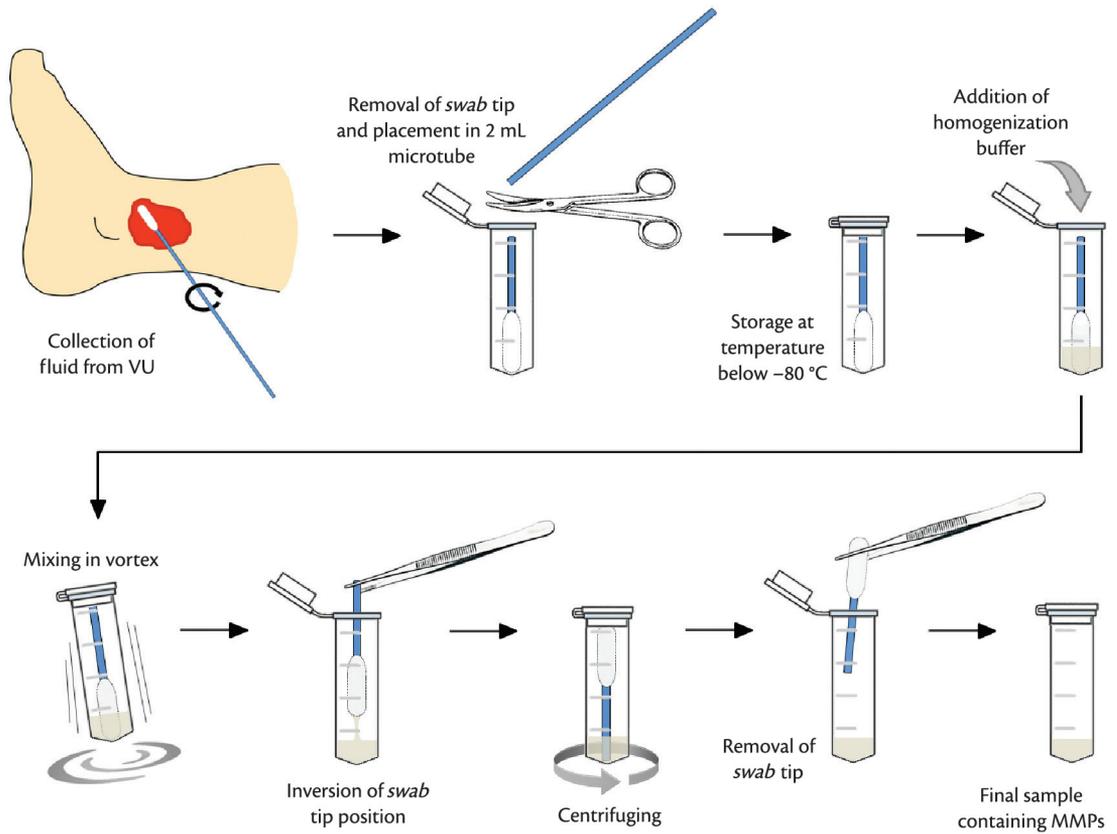


Figure 1. Flow diagram of procedures involved in collecting and processing samples showing steps from collection of venous exudate with a swab to acquisition of the final sample containing MMPs, ready for subsequent analyses.

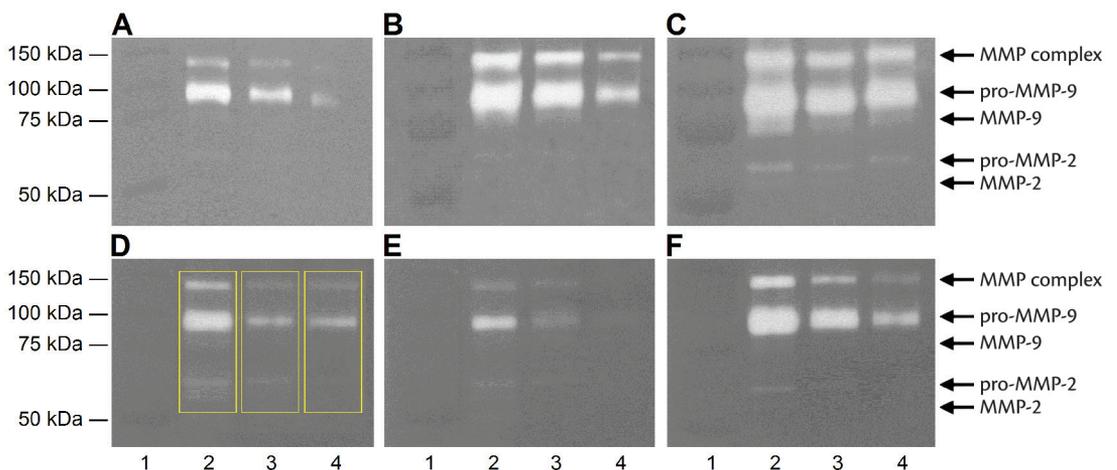


Figure 2. Zymography gels for six samples collected using swabs. On the left are shown the molecular weights of standard markers; on the right are shown the isoforms of metalloproteinases (MMPs) observed in the gels. Pane D shows how the set of bands from each lane was selected for densitometry using the ImageJ software program. MMP complex (130 kDa); pro-MMP-9 (92k Da); MMP-9 (84 kDa); pro-MMP-2 (72 kDa); MMP-2 (62 kDa). Lane 1: Markers, lanes 2, 3 and 4: 0.5 mg, 0.25 mg and 0.125 mg, respectively.

pH of 8.8, containing 1 mg/mL of gelatin (from pig skin, type A, G2500 - Sigma-Aldrich®). The stacking gel had a concentration of 4% and pH of 6.8 and contained 0.1% SDS. Proteins were separated under a constant voltage of 100 V in a buffer with the following concentrations: 192 mM Glycine; 25 mM Tris; 0.1% SDS.

After electrophoresis, the gels were immersed and agitated in Triton solution (2.5% Triton X-100; 50 mM Tris, pH 7.4; 5 mM CaCl₂) to remove the SDS (three changes in 60 minutes).

Gels were then immersed at a temperature of 37 °C for 19 hours in an incubation solution consisting of 50 mM Tris (pH 7.4); 5 mM CaCl₂; and 150 mM NaCl, in order to activate MMPs. After incubation, staining was performed with Coomassie Brilliant Blue G-250 (0.5% Coomassie blue in 30% methanol and 10% acetic acid). Gels were submerged in the dye for 30 minutes, under agitation, before being destained (35% Methanol; 10% acetic acid) for 30 minutes and then placed in deionized water.

Gels were digitized on a bench scanner to enable densitometric analysis. Metalloproteinase enzyme activity was determined by the intensity of the pale bands against the stained background and MMPs were identified by their molecular weights, as described elsewhere,^{9,12} using ImageJ 1.46 software (NIH, <http://imagej.nih.gov/ij/>). The program was first used to transform the image into 8-bits and then a rectangular area was selected containing the bands (areas of enzyme digestion) and part of the stained gel background. The contrast between these two elements could then be demonstrated graphically, indicating the degree to which the gelatin in the gel had been broken down.¹³

RESULTS

Initially, we observed that an adequate quantity of VU fluid protein had been collected on the swabs, using the Bradford method on ELISA plates (Table 1).

We tested the polyacrylamide gel concentration in order to determine the best conditions for viewing and quantification of bands, defining the ideal concentration as 8%, the ideal incubation time for enzymatic activation as 19 hours and the ideal quantity of total protein loaded in each well on the gels as from 0.125 µg to 0.50 µg.

Figure 2 illustrates the zymography results. Bands representing the forms of pro-MMP-9 and MMP-9, and pro-MMP-2 and MMP-2 were identified with the aid of previous descriptions^{9,12} and molecular mass calculations. It was also possible to observe

gelatinolytic activity between 100 kDa and 150 kDa (~130 kDa), which has been described in the literature as an MMP complex that probably contains MMP-9.^{9,12} Bands corresponding to the latent and active forms of each enzyme were selected together (model shown in Figure 2D) and quantified by densitometry (Figure 3) for illustration.

DISCUSSION

This article has described a viable and reproducible method for extraction of MMPs from VU exudate collected on swabs and for analysis of enzyme activity by zymography. As far as we know, this is the first description of this minimally invasive form of extraction of MMPs and the first demonstration of the viability of biochemical analysis of these small sample volumes.

There are descriptions of abnormal activation of MMPs and imbalances between MMP isoforms in chronic venous insufficiency patients, which contribute to formation and maintenance of VU.^{7-9,14} Among the different MMPs, MMP-9 appears to be a prognostic marker of healing of chronic ulcers.¹⁰ Furthermore, the course of the healing process in chronic wounds is associated with reduced expression of certain MMPs.¹⁴ In view of this, development of a simple technique for collection of biological material for analysis of MMPs makes it possible to conduct serial evaluations of the process of ulcer healing and opens a wide range of applications for clinical research.

This study has demonstrated that collecting VU exudate on swabs is a viable and reproducible method for evaluation of the enzyme activity of MMP-2 and MMP-9 using zymography. Zymography is a relatively simple technique that is effective and sensitive for identification of MMPs by degradation of their preferred substrates and by their molecular weights, making quantitative results possible.¹²

The technique described here is based on a minimally invasive procedure that is sterile, rapid and easy to conduct, making it an attractive alternative to

Table 1. Protein concentrations in samples.

Patient	[protein] (mg/µL)
1	0.452
2	0.376
3	0.468
4	0.425
5	0.211
6	0.329

invasive methods used in earlier studies that involve biopsies and histopathological analysis.^{9,15} Although biopsy can provide topographical information, for example, when tissue from the wound border and from the ulcer bed are compared, the technique requires local anesthesia and causes discomfort to patients, which makes serial analysis of wounds difficult. Using exudate obtained by aspiration, when possible, or by collection on swabs, as described in this study, provides a more specific reflection of the activity of MMPs in the ulcer bed. Although the results of these two collection methods have not been compared for venous ulcers, there is evidence that

they correlate in the case of diabetic ulcers.¹⁶ Future studies could be designed to evaluate the agreement between the swab-based method and other methods, such as biopsy, with venous ulcers.

Finally, the technique described in this study also offers the advantage that it allows samples to be collected by many different classes of health professionals, since it does not demand local anesthesia, incisions or punctures, thereby facilitating analysis of the biological effectiveness of therapeutic interventions.

CONCLUSIONS

Collection of fluid from VU using swabs proved to be an effective, minimally invasive, sterile and rapid method offering great ease of use and one that is appropriate when the objective is to extract an adequate quantity of total protein from VU exudate for analysis of the enzyme activity of MMPs 2 and 9 using the zymography protocol proposed in this article.

REFERENCES

- de Aguiar ET, Pinto LJ, Figueiredo MA, Savino Neto S. Úlcera de Insuficiência Venosa Crônica: Diretrizes sobre Diagnóstico, Prevenção e Tratamento da Sociedade Brasileira de Angiologia e Cirurgia Vascular (SBACV). *J Vasc Bras.* 2005;4(Supl.2):S195-200.
- Lopes CR, Figueiredo M, Ávila AM, Soares LMBM, Dionisio VC. Avaliação das limitações de úlcera venosa em membros inferiores. *J Vasc Bras.* 2013;12(1):5-9. <http://dx.doi.org/10.1590/S1677-54492013000100003>.
- de Araujo T, Valencia I, Federman DG, Kirsner RS. Managing the patient with venous ulcers. *Ann Intern Med.* 2003;138(4):326-34. <http://dx.doi.org/10.7326/0003-4819-138-4-200302180-00012>. PMID:12585831
- Wipke-Tevis DD, Rantz MJ, Mehr DR, et al. Prevalence, incidence, management, and predictors of venous ulcers in the long-term-care population using the MDS. *Adv Skin Wound Care.* 2000;13(5):218-24. PMID:11075021.
- Benjamin MM, Khalil RA. Matrix metalloproteinase inhibitors as investigative tools in the pathogenesis and management of vascular disease. *EXS.* 2012;103:209-79. http://dx.doi.org/10.1007/978-3-0348-0364-9_7. PMID:22642194
- Dalton SJ, Mitchell DC, Whiting CV, Tarlton JF. Abnormal extracellular matrix metabolism in chronically ischemic skin: a mechanism for dermal failure in leg ulcers. *J Invest Dermatol.* 2005;125(2):373-9. PMID:16098049.
- Meyer FJ, Burnand KG, Abisi S, Tekoppele JM, van Els B, Smith A. Effect of collagen turnover and matrix metalloproteinase activity on healing of venous leg ulcers. *Br J Surg.* 2008;95(3):319-25. <http://dx.doi.org/10.1002/bjs.5946>. PMID:17854113
- Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med.* 2008;29(5):290-308. <http://dx.doi.org/10.1016/j.mam.2008.05.002>. PMID:18619669

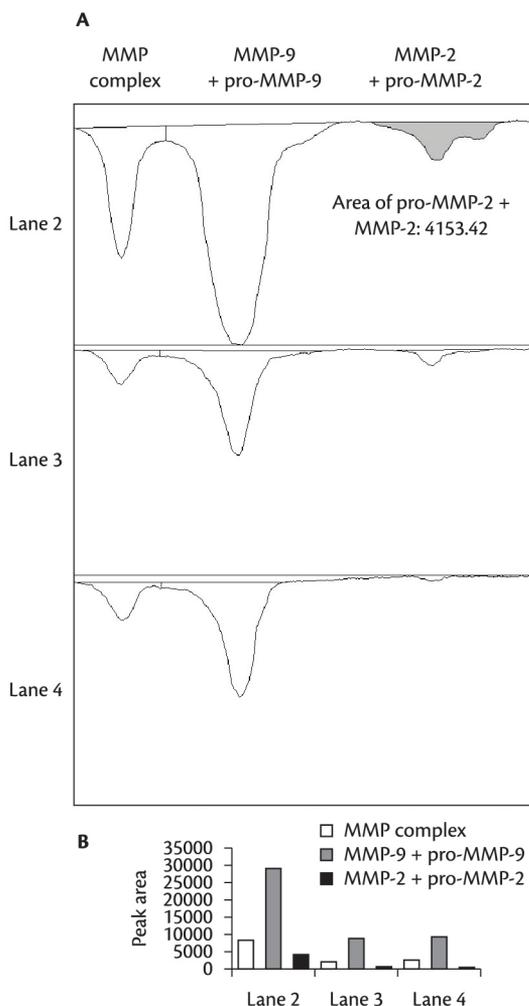


Figure 3. Quantification of metalloproteinases (MMPs) contained in one of the gels analyzed. A: densitometry curves (peaks) for bands in each lane in the gel. The area of peaks indicates the gelatinolytic activity. Note the shaded area in Lane 2, indicating MMP-2 activity. B: measurements for the areas of peaks in arbitrary units, for the purposes of comparative analysis.

9. Wysocki AB, Staiano-Coico L, Grinnell F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol.* 1993;101(1):64-8. <http://dx.doi.org/10.1111/1523-1747.ep12359590>. PMID:8392530
10. Rayment EA, Upton Z, Shooter GK. Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer. *Br J Dermatol.* 2008;158(5):951-61. <http://dx.doi.org/10.1111/j.1365-2133.2008.08462.x>. PMID:18284390
11. Trengove NJ, Stacey MC, MacAuley S, et al. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen.* 1999;7(6):442-52. <http://dx.doi.org/10.1046/j.1524-475X.1999.00442.x>. PMID:10633003
12. Snoek-van Beurden PA, Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques.* 2005;38(1):73-83. <http://dx.doi.org/10.2144/05381RV01>. PMID:15679089
13. Hu X, Beeton C. Detection of functional matrix metalloproteinases by zymography. *J Vis Exp.* 2010;(45):e2445. PMID:21085107.
14. Beidler SK, Douillet CD, Berndt DF, Keagy BA, Rich PB, Marston WA. Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy. *Wound Repair Regen.* 2008;16(5):642-8. <http://dx.doi.org/10.1111/j.1524-475X.2008.00415.x>. PMID:19128259
15. La Rocca G, Pucci-Minafra I, Marrazzo A, Taormina P, Minafra S. Zymographic detection and clinical correlations of MMP-2 and MMP-9 in breast cancer sera. *Br J Cancer.* 2004;90(7):1414-21. <http://dx.doi.org/10.1038/sj.bjc.6601725>. PMID:15054465
16. Schmohl M, Beckert S, Joos TO, Königsrainer A, Schneiderhan-Marra N, Löffler MW. Superficial wound swabbing: a novel method of sampling and processing wound fluid for subsequent immunoassay analysis in diabetic foot ulcerations. *Diabetes Care.* 2012;35(11):2113-20. <http://dx.doi.org/10.2337/dc11-2547>. PMID:22837363

Correspondence

Fernando Augusto Lavezzo Dias
 Universidade Federal do Paraná – UFPR
 Setor de Ciências Biológicas, Departamento de Fisiologia, Centro
 Politécnico
 Avenida Coronel Francisco H. dos Santos, 210 – Jardim das Américas
 CEP 81531-970 – Curitiba (PR), Brazil
 E-mail: fernandoaldias@gmail.com

Author information

FSS and DNA are MSc in Physical Therapy from Universidade Federal do Rio Grande do Norte (UFRN), RN, Brazil.
 JPMSL is a PhD in Biochemistry from Universidade Federal do Ceará (UFC), and an adjunct professor at the Department of Biochemistry at Universidade Federal do Rio Grande do Norte (UFRN), RN, Brazil.
 AAR is a PhD in Biochemistry from Universidade de São Paulo (USP), and an adjunct professor at the Department of Clinical and Toxicological Analysis.
 BJGAA is a PhD in Cellular Biology from Universidade Federal de Minas Gerais (UFMG), and an adjunct professor at the Department of Morphology at Universidade Federal do Rio Grande do Norte (UFRN), RN, Brazil.
 FALD is a PhD in Cellular and Molecular Biology (Physiology) from Universidade Federal do Paraná (UFPR), Adjunct professor at the Department of Physiology at Universidade Federal do Paraná (UFPR), PR, Brazil.

Author contributions

Conception and design: FALD
 Analysis and interpretation: FSS, DNA, JPMSL, AAR, BJGAA, FALD
 Data collection: FSS, DNA
 Writing the article: FSS, DNA, FALD
 Critical revision of the article: JPMSL, AAR, BJGAA, FALD
 Final approval of the article*: FSS, DNA, JPMSL, AAR, BJGAA, FALD
 Statistical analysis: N/A
 Overall responsibility: FALD

* All authors have read and approved of the final version of the article submitted to *J Vasc Bras.*