# Polymorphonuclear leucocyte respiratory burst activity correlates with serum zinc level in type 2 diabetic patients with foot ulcers

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**ABSTRACT**

Patients with diabetes mellitus (DM) are prone to infection, in part due to phagocyte dysfunction and impaired polymorphonuclear (PMN) leucocyte superoxide generation. Another frequently mentioned factor in the pathogenesis of infection in DM patients is altered zinc status. This study aims to evaluate the association between serum zinc level and PMN respiratory burst activity in patients with type 2 DM. Thirty-nine type 2 DM patients (19 with foot ulcers) and 20 healthy controls are studied. Respiratory burst activity is evaluated at baseline and in stimulated states by a nitro blue tetrazolium (NBT) reduction test. Serum zinc level is evaluated by atomic absorption spectrophotometry. Although not statistically significant, PMNs from diabetics with foot ulcers appeared to be slightly hyperactivated at the baseline state. The NBT index was significantly lower in DM patients with foot ulcers after stimulation. Mean serum zinc level was significantly lower in diabetics with foot ulcers compared to those without foot ulcers. A significant negative correlation between serum zinc level and NBT index at baseline was seen in patients with foot ulcers, but this changed to a significant positive correlation after stimulation. These findings may be explained by PMN hyperactivity at baseline and by respiratory burst dysfunction following stimulation in diabetic patients.

KEY WORDS: Diabetes mellitus.
Diabetic foot.
Neutrophil activation.
Respiratory burst.
Zinc.

## Introduction

The incidence of infection is increased in patients with diabetes mellitus (DM) as a result of impaired or altered cellular innate immunity. In addition, some infections are more likely to have a complicated course in diabetic patients.[1–3]

An important mechanism in the elimination of pathogens by the innate immune system is the oxidative burst, which is a coordinated series of metabolic events that takes place when phagocytes are exposed to appropriate stimulants. On stimulation, both macrophages and polymorphonuclear leucocytes (PMNs), which represent the first line of defence against invading pathogens, markedly increase their oxygen uptake.[2] In the course of this activity they produce various reactive oxidising agents that also inflict harm on nearby tissues.[4]

Diabetics are prone to infections, in part due to phagocyte dysfunction and impaired superoxide generation.[5] In general, the killing capacity of diabetic PMNs is lower than that of controls.[1] In DM patients, the formation of oxidative components, including $H_2O_2$, from stimulated PMNs is suppressed.[1,3,5–8] However, at baseline the PMNs are hyperactivated, which leads to overproduction of such components.[6,8–11] This overproduction paves the way for oxidative stress, a condition in which cells are subjected to elevated levels of molecular oxygen or reactive oxygen species (ROS). Research suggests that oxidative stress plays a key role in the pathogenesis of both microvascular and macrovascular complications in DM patients.[2,12–17]

Another frequently mentioned factor in the pathogenesis of infection in DM patients is altered zinc status.[18] Zinc is necessary for normal immune function and it enhances the *in vitro* effectiveness of insulin. Several studies have clarified the potential antioxidant effect of zinc supplementation in type 2 DM.[19,20] Low plasma zinc levels and hyperzincuria have been reported in type 1 and type 2 DM patients.[1,17,18,21,22] In addition, it is reported that zinc can suppress superoxide anion generation ($O^{2-}$) by PMNs from healthy subjects and from experimental animals *in vitro* and *in vivo*.[23,24]

However, a link between serum zinc level, PMN respiratory burst state (as an ROS generator) and the incidence of vascular complications and infection in DM has yet to be clarified. Due to the critical rule of ROS in the pathogenesis of vascular complications, this link seems to be more significant in those DM patients who suffer from foot ulcers.[14,16]

The present study attempts to clarify the situation by

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investigating the zinc state and PMN respiratory burst activity simultaneously in DM patients with and without foot ulcers.

## Materials and methods

*Subjects*

Thirty-nine type 2 DM patients (19 with foot ulcers) without any sign of infections were selected randomly from patients referred to the endocrine ward of Shariati Hospital, regardless of stage or therapy. Twenty healthy volunteers (matched for gender and age) were selected as controls. Heparinised blood (20 mL) was drawn from each patient and control after overnight fast. The study was approved by the local ethics committee. Informed consent was obtained from all subjects.

*Evaluation of glycaemic state*

Glycaemic state was evaluated by fasting serum glucose and HbA1c determinations.[25,26] Average HbA1c ≤7.5 was considered to indicate good control; HbA1c 7.6 to ≤9 was considered to indicate fair control; and HbA1c ≥9.1 poor control.

*Isolation of polymorphonuclear leucocytes*

Polymorphonuclear leucocytes were isolated from blood using Polymorphoprep gradient (Nycomed, Oslo, Norway), as described previously.[27] The cells were resuspended in RPMI 1640 culture medium at a final concentration of $7 \times 10^6$ cells/mL. Cell viability was >95% by trypan blue exclusion staining.

*Nitro blue tetrazolium reduction test*

Evaluation of superoxide production was assessed by the nitro blue tetrazolium (NBT) reduction test, as described previously.[28] Briefly, following stimulation of PMNs and activation of the respiratory burst pathway, superoxide radicals reduce the soluble NBT dye to insoluble formazan granules. Those PMNs containing blue formazan granules were regarded as showing a positive result. N-formyl-methionine-leucine-phenylalanine (FMLP, 100 nmol/L) was used to induce receptor-mediated $O_2$ formation. Phorbol myristate acetate (PMA, 10 µg/mL) was used for direct activation of protein kinase C (PKC)-mediated $O_2$ generation. All chemicals were obtained from Sigma, St. Louis, MO, USA. The percentage of NBT-positive cells was determined for each sample by evaluating 1000 cells both at baseline and in the stimulated state by three skilled observers in a blinded manner.

*Serum zinc levels*

Blood samples were taken from a vein in the antecubital fossa. Sera were transferred immediately to acid-washed Eppendorf tubes and stored at –20°C prior to analysis. Plastic polypropylene tubes, pipettes and Eppendorf tubes were soaked in 1% nitric acid solution for 24 h and rinsed (x6) in deionised water. Samples were diluted (1 in 4) with deionised water. Serum zinc level was determined by flame atomic absorption spectrophotometry (Unicam 929) at 213.8 nm. Measurements were repeated (x2) and a commercial serum control was included.

*Statistical analysis*

Adjustment to normal distribution was tested by the Kolmogorov-Smirnov test. For comparison of means between two independent groups of subjects, the Mann–Whitney U-test was used for non-parametric data. Spearman correlation analysis was also performed. Data are presented as mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.

## Results

Clinical and biochemical characteristics of the study subjects are outlined in Table 1. As the difference between NBT reduction at baseline and on stimulation was significant in all three groups (data not shown), the percentage augmentation in NBT reduction on stimulation was used for comparison and statistical analysis. However, at baseline the percentage NBT reduction was used for analysis.

**Table 1.** Clinical and biochemical characteristics of the study subjects.

| | Healthy controls ($n$=20) | Diabetics without foot ulcers ($n$=20) | Diabetics with foot ulcers ($n$=19) |
|---|---|---|---|
| Gender (male/female) | 11/9 | 11/9 | 10/9 |
| Age (years) | 50.2±6.4 | 52.8±9.5 | 48.9±9.0 |
| Duration of diabetes (years) | – | 5.4±1.5 | 6.6±1.8 |
| Fasting blood glucose (mg/dL) | 72±12 | 158±65* | 189±108* |
| HbA1c (%) | 6.4±1.2 | 9.9±2.7* | 10.6±3.1* |
| Serum zinc level (mg/L) | 0.85±0.24 | 1.02±0.31‡ | 0.79±0.26 |
| NBT index[a] (%) | 81.8±61.3 | 81.0±80.5 | 46.7±59.3† |
| NBT index[b] (%) | 87.5±89.3 | 56.0±66.6 | 42.2±50.8† |
| NBT index[c] (%) | 46.9±16.5 | 49.9±21.7 | 51.0±19.0 |

*$P<0.0005$, †$P<0.05$ (compared to controls); ‡$P<0.05$ (compared to diabetics with foot ulcers).

[a]Respiratory burst activity index on stimulation with PMA.

[b]Respiratory burst activity index on stimulation with FMLP.

[c]Respiratory burst activity in baseline state.

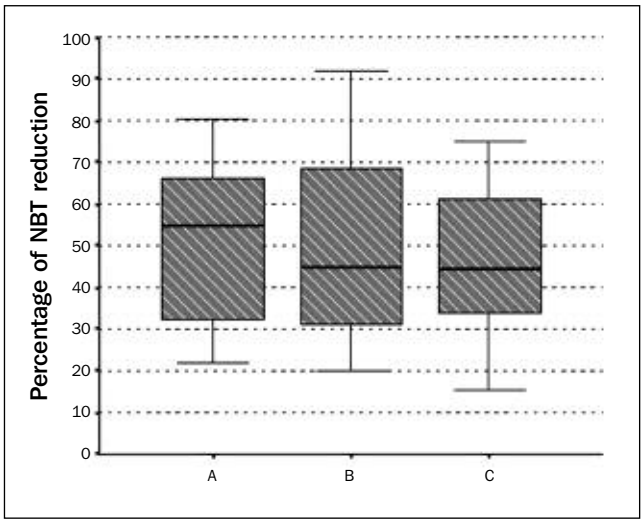


**Fig 1.** Respiratory burst activity in the baseline state: A) healthy controls; B) diabatic patients without foot ulcers; C) diabetic patients with foot ulcers.

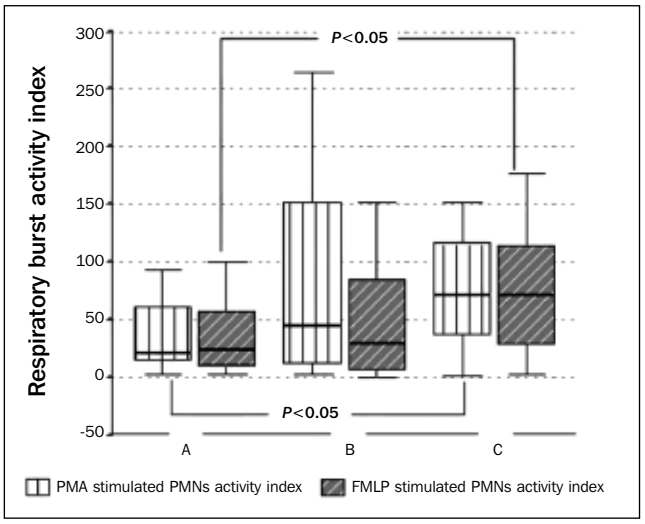


**Fig 2.** Respiratory burst activity index on stimulation: A) healthy controls; B) diabetic patients without foot ulcers; C) diabetic patients with foot ulcers.

Although the PMNs from diabetics with foot ulcers appeared slightly overactive at baseline, there was no significant difference in PMN respiratory burst activity between the two diabetic groups and the control group at baseline (Fig. 1).

On stimulation with FMLP, the NBT index was significantly lower in diabetics with foot ulcers ($P<0.05$) compared to that in the control group. Stimulation with PMA resulted in a significantly lower NBT index in the same group ($P<0.05$). Patients in the diabetic group without foot ulcers showed no significant difference in NBT index compared with results in the control group (Fig. 2).

There was no difference in serum zinc level between the diabetic and control groups; however, it was significantly lower ($P<0.05$) in diabetics with foot ulcers compared to those without foot ulcers. Serum zinc level showed a significant negative correlation ($r=-0.604$, $P<0.05$) with NBT index at baseline, but changed to a significant positive correlation following stimulation with PMA ($r=0.698$, $P<0.01$) and FMLP ($r=0.759$, $P<0.005$). This was not observed in the diabetics without foot ulcers or in the control group.

## Discussion

Diabetes mellitus is associated with several vascular complications and infections. Chronic bacterial ulcer is a frequent cause of the non-healing diabetic foot.[29] Foot infections and the subsequent amputation of a lower extremity are the most common cause of hospitalistion among patients with DM.[30] Recent studies indicate an altered non-specific immune response in patients with diabetic foot syndrome and chronic bacterial infection.[29,31]

It is widely stated that oxidative stress is the major factor in pathogenesis of diabetic vascular complications.[2,12–17] Polymorphonuclear leucocytes play a key role in generating ROS through a respiratory burst pathway, which, in turn, may lead to the onset of oxidative stress.[16] Previous studies report an increase in PMN respiratory burst activity at baseline in DM patients,[6,8–11] which can be an indication of free radical overproduction.

The present study found that baseline reduction of NBT following respiratory burst activity in PMNs from DM patients is slightly increased but not statistically different from controls, which may have been due to the small sample size. Furthermore, it was noticed that baseline NBT reduction is greater in DM patients with foot ulcers (Fig. 1).

Collier *et al*.[32] observed an increase of free radical activity in diabetic patients with microalbuminuria, and suggested that disturbance in the oxygen/redox state might be responsible for the development of diabetic microvascular complications, supporting previous studies.[9,10,13] It is tempting to speculate that this enhanced production of free radicals contributes to progression of vascular damage.

Although unstimulated PMNs from DM patients show increased NBT reduction, many reports indicate that formation of oxidative components by stimulated PMNs via the respiratory burst pathway is suppressed in DM.[1,3,5–8] This may increase the susceptibility of DM patients to infection.

The present study demonstrated a reduced response to FMLP in PMNs from DM patients with foot ulcers; however, the response to PMA and FMLP was unaffected in those without vascular complications. This confirms previous data.[33,34] Moreover, it was shown before that when diabetic cells were suspended in normal glucose conditions, FMLP-stimulated $O_2$ production increased to a point where there was no significant difference from control PMNs.[35]

In contrast, physiologically relevant glucose concentrations present in poorly controlled diabetics cause suppression of receptor-mediated $O_2$ generation in both diabetic and normal PMNs. This may explain the non-suppression of NBT reduction on stimulation by PMA and FMLP in the DM patients without foot ulcers (who showed better glycaemic control) in the present study.

Impaired metabolism of trace elements may be involved in some metabolic dysfunctions and may contribute to the development of vascular complications in DM patients. Zinc deficiency may stimulate PMN leucocyte activation and contribute to the development of vascular complications in type 2 DM patients.[36]

Zinc can suppress superoxide anion ($O^{2-}$) generation by PMNs in healthy subjects and experimental animals *in vitro* and *in vivo*.[23,24] Some studies indicate that physiological concentrations of zinc facilitate not only the ROS generating capacity of human PMNs but also serum opsonic activity. It is suggested that zinc is essential for optimal functioning of the non-specific immune system, and that either lack of zinc or excessive amounts of zinc can impair immune function.[37,38]

The observed paradoxical correlation in the present study may suggest an association between serum zinc level and PMN respiratory burst activity, depending to the state of PMN activity. At baseline, where PMNs tend to be hyperactivated in DM patients, optimal zinc levels may help to reduce respiratory burst activity, whereas, on stimulation, it may increase weak respiratory burst activity.

This concept is supported by Peretz *et al*., who reported that zinc supplementation ameliorated mean PMN phagocytic activity in patients with inflammatory rheumatic disease.[39] In another study, at concentrations not exceeding the physiological level in human plasma, zinc suppressed PMN superoxide anion generation *in vitro*.[38]

Hasegawa *et al*. found that physiological concentrations of zinc facilitated the ROS-generating capacity of human neutrophils *in vitro*, suggesting that this trace element is essential for optimal functioning of the non-specific immune system, and that lack of zinc or excessive amounts of zinc can impair immune function.[37] It has been suggested recently that zinc deficiency may provoke PMN activation, contributing to the development of vascular complications in type 2 diabetic patients.[36]

The present study demonstrates that a negative correlation exists between serum zinc level and PMN respiratory burst activity at baseline in type 2 diabetes complicated by foot ulceration, and that a positive correlation exists in the stimulated state. Although the mechanism behind this is unclear, this finding may be explained by PMN hyperactivity at baseline and by a respiratory burst dysfunction in the stimulated state in diabetic patients.

Clearly, this study should be repeated using leucocyte cytoplasmic zinc content as a better indicator of zinc state in the body. The direct effect of zinc supplementation on PMN respiratory burst activity in DM patients with foot ulcers also requires further investigation and may help to clarify the matter. □

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## References


1 Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM). *FEMS Immunol Med Microbiol* 1999; **26**: 259–65.

2 Llorente L, De La FH, Richaud-Patin Y *et al*. Innate immune response mechanisms in non-insulin-dependent diabetes mellitus patients assessed by flow cytoenzymology. *Immunol Lett* 2000; **74**: 239–44.

3 Inoue S, Lan Y, Muran J, Tsuji M. Reduced hydrogen peroxide production in neutrophils from patients with diabetes. *Diabetes Res Clin Pract* 1996; **33**: 119–27.

4 Babior BM. Phagocytes and oxidative stress. *Am J Med* 2000; **109**: 33–44.

5 Ortmeyer J, Mohsenin V. Inhibition of phospholipase D and superoxide generation by glucose in diabetic neutrophils. *Life Sci* 1996; **59**: 255–62.

6 Delamaire M, Maugendre D, Moreno M, Le Goff MC, Allannic H, Genetet B. Impaired leucocyte functions in diabetic patients. *Diabet Med* 1997; **14**: 29–34.

7 Marhoffer W, Stein M, Maeser E, Federlin K. Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. *Diabetes Care* 1992; **15**: 256–60.

8 Shah SV, Wallin JD, Eilen SD. Chemiluminescence and superoxide anion production by leukocytes from diabetic patients. *J Clin Endocrinol Metab* 1983; **57**: 402–9.

9 Wierusz-Wysocka B, Wysocki H, Siekierka H, Wykretowicz A, Szczepanik A, Klimas R. Evidence of polymorphonuclear neutrophil (PMN) activation in patients with insulin-dependent diabetes mellitus. *J Leukoc Biol* 1987; **42**: 519–23.

10 Kantar A, Wilkins G, Swoboda B *et al*. Alterations of the respiratory burst of polymorphonuclear leukocytes from diabetic children. A chemiluminescence study. *Acta Paediatr Scand* 1990; **79**: 535–41.

11 Nath N, Chari SN, Rathi AB. Superoxide dismutase in diabetic polymorphonuclear leukocytes. *Diabetes* 1984; **33**: 586–9.

12 Ohmori M, Harada K, Kitoh Y, Nagasaka S, Saito T, Fujimura A. The functions of circulatory polymorphonuclear leukocytes in diabetic patients with and without diabetic triopathy. *Life Sci* 2000; **66**: 1861–70.

13 Zozulinska DA, Wierusz-Wysocka B, Wysocki H, Majchrzak AE, Wykretowicz A. The influence of insulin-dependent diabetes mellitus (IDDM) duration on superoxide anion and hydrogen peroxide production by polymorphonuclear neutrophils. *Diabetes Res Clin Pract* 1996; **33**: 139–44.

14 Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. *Diabetes Care* 1996; **19**: 257–67.

15 Ceriello A. New insights on oxidative stress and diabetic complications may lead to a 'causal' antioxidant therapy. *Diabetes Care* 2003; **26**: 1589–96.

16 Bayraktutan U. Free radicals, diabetes and endothelial dysfunction. *Diabetes Obes Metab* 2002; **4**: 224–38.

17 DiSilvestro RA. Zinc in relation to diabetes and oxidative disease. *J Nutr* 2000; **130**: 1509S–1511S.

18 Chausmer AB. Zinc, insulin and diabetes. *J Am Coll Nutr* 1998; **17**: 109–15.

19 Faure P, Benhamou PY, Perard A, Halimi S, Roussel AM. Lipid peroxidation in insulin-dependent diabetic patients with early retinal degenerative lesions: effects of an oral zinc supplementation. *Eur J Clin Nutr* 1995; **49**: 282–8.

20 Anderson RA, Roussel AM, Zouari N, Mahjoub S, Matheau JM, Kerkeni A. Potential antioxidant effects of zinc and chromium supplementation in people with type 2 diabetes mellitus. *J Am Coll Nutr* 2001; **20**: 212–8.

21 Niewoehner CB, Allen JI, Boosalis M, Levine AS, Morley JE. Role of zinc supplementation in type II diabetes mellitus. *Am J Med* 1986; **81**: 63–8.

22 Salgueiro MJ, Zubillaga M, Lysionek A *et al*. Zinc as an essential micronutrient: a review. *Nutr Res* 2000; **20**: 737–55.

23 Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. *Free Radic Biol Med* 1990; **8**: 281–91.

24 Benoni G, Cuzzolin L, Marrella M *et al*. Neutrophil behavior following exposure to *in vivo* or *in vitro* zinc in normal and acutely-inflamed rats: studies on lysozyme secretion, superoxide anion release and platelet adhesion. *Inflammation* 1998; **22**: 175–89.

25 Huggett AS, Nixon DA. Use of glucose oxidase, peroxidase, and O-dianisidine in determination of blood and urinary glucose. *Lancet* 1957; **273** (6991): 368-70.
26 Schifreen RS, Hickingbotham JM, Bowers GN Jr. Accuracy, precision, and stability in measurement of hemoglobin A1C by 'high-performance' cation-exchange chromatography. *Clin Chem* 1980; **26**: 466–72.
27 Venuprasad K, Parab P, Prasad DV *et al*. Immunobiology of CD28 expression on human neutrophils. I. CD28 regulates neutrophil migration by modulating CXCR-1 expression. *Eur J Immunol* 2001; **31**: 1536–43.
28 Segal AW. Nitroblue-tetrazolium tests. *Lancet* 1974; **2**: 1248–52.
29 Jirkovska A, Fejfarova V, Hosova J, Kalanin J, Striz I, Skibova J. Non-specific immune responses in patients with chronic diabetic foot syndrome and chronic bacterial infection. *Vnitr Lek* 2002; **48**: 142–6.
30 Yonem A, Cakir B, Guler S, Azal OO, Corakci A. Effects of granulocyte-colony stimulating factor in the treatment of diabetic foot infection. *Diabetes Obes Metab* 2001; **3**: 332–7.
31 Uchimura K, Nagasaka A, Hayashi R *et al*. Changes in superoxide dismutase activities and concentrations and myeloperoxidase activities in leukocytes from patients with diabetes mellitus. *J Diabetes Complications* 1999; **13**: 264–70.
32 Collier A, Rumley A, Rumley AG *et al*. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. *Diabetes* 1992; **41**: 909–13.
33 Balasoiu D, van Kessel KC, Kats-Renaud HJ, Collet TJ, Hoepelman AI. Granulocyte function in women with diabetes and asymptomatic bacteriuria. *Diabetes Care* 1997; **20**: 392–5.
34 Fuller CJ, Agil A, Lender D, Jialal I. Superoxide production and LDL oxidation by diabetic neutrophils. *J Diabetes Complications* 1996; **10**: 206–10.
35 Tauber AI. Protein kinase C and the activation of the human neutrophil NADPH-oxidase. *Blood* 1987; **69**: 711–20.
36 Karahan SC, Deger O, Orem A *et al*. The effects of impaired trace element status on polymorphonuclear leukocyte activation in the development of vascular complications in type 2 diabetes mellitus. *Clin Chem Lab Med* 2001; **39**: 109–15.
37 Hasegawa H, Suzuki K, Nakaji S, Sugawara K. Effects of zinc on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity *in vitro*. *Luminescence* 2000; **15**: 321–7.
38 Gavella M, Lipovac V, Car A. *In vitro* effect of zinc on superoxide anion production by polymorphonuclear leukocytes of diabetic patients. *Acta Diabetol* 2000; **37**: 135–7.
39 Peretz A, Cantinieaux B, Neve J, Siderova V, Fondu P. Effects of zinc supplementation on the phagocytic functions of polymorphonuclears in patients with inflammatory rheumatic diseases. *J Trace Elem Electrolytes Health Dis* 1994; **8**: 189–94.