Serum nitric oxide metabolites in leprosy patients as a parameter of prognostic value
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Background
Leprosy is a chronic infectious disease caused by the obligate intracellular microorganism \textit{Mycobacterium leprae} that tends to infect the skin and peripheral nerves. Nitric oxide (NO) plays a role not only in limiting bacterial growth, but also in limiting the damaging of immunopathological consequences of chronic mycobacterial infection.

Objective
To assess NO metabolites level in sera of leprosy patients across the spectrum of the disease as a possible parameter of prognostic value and as an indicator of disease state.

Patients and methods
This case–control study was conducted on 80 leprosy patients who were selected from Benha Dermatology and Leprosy Clinic, Benha University Hospital, and Abu Zaabal Leprosarium. Twenty age-matched and sex-matched healthy volunteers were included as the control group. Patients were subjected to history taking, clinical examination, and dermatological examination and were divided into groups according to the WHO classification. Blood samples were collected from both patients and controls for assessment of NO metabolites [nitrite (NO\textsubscript{2}) and nitrate (NO\textsubscript{3})] serum levels by ELISA.

Results
Our results showed that comparison of the mean values of serum NO metabolites levels for each group of untreated patients versus the control group revealed increased serum NO metabolites levels in untreated patients, whereas comparison for each group of treated patients versus the control group revealed decreased serum NO metabolites levels in treated patients. The present study also showed that, in paucibacillary leprosy patients, in multibacillary leprosy patients, and in type 1 reaction patients, receiver operating characteristic curve at cutoff value of 155.97, 195.98, and 157.17 \textmu mol of serum NO\textsubscript{2}, respectively, has sensitivity of 80, 67, and 73\% and specificity of 90, 93, and 93\%, respectively.

Conclusion
Serum NO metabolites levels of leprosy patients increase during the disease and decrease after treatment. Serum NO metabolites in leprosy patients can be used as a parameter of prognostic value.

Keywords: leprosy, \textit{Mycobacterium leprae}, nitric oxide, sensitivity, specificity

Introduction
Hansen’s disease is still considered a major health problem in some countries of Asia, Latin America, and Africa including Egypt. According to the WHO, the number of new cases of leprosy during 2010 as reported by 130 countries globally was 228,474. The worldwide registered prevalence at the beginning of 2010 was 192,246 cases [1]. Nerve damage seen across the spectrum of leprosy is the main cause of deformities and morbidity in this disease [2].

Nitric oxide (NO) is a multifunctional reactive gaseous molecule, which may act as a vasodilator, neurotransmitter, and the effectors’ molecule of immune cells [3].

A major component of the antimicrobial activity of effectors’ mechanisms of activated macrophages is the production of reactive nitrogen intermediates (RNI), chiefly NO. Upon activation by cytokines following interaction with pathogens, phagocyte cells such as macrophages, neutrophils, etc. produce NO and other RNI by the induction of nitric oxide synthase (iNOS). These RNI modulate the immune response and control inflammation [4]. Generation of RNI, especially NO, by macrophages is an effective antimicrobial mechanism against intracellular pathogens, including \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium leprae} [5].

High levels of NO are produced by activated macrophages expressing the inducible iNOS in leprosy as a host
Nitric oxide metabolites in leprosy Elesawy et al. 45

response to M. leprae [6,7]. During inflammation, NO may react with the superoxide radical (O$_2^-$) yielding peroxynitrite, an unstable metabolite that rapidly nitrosylates tyrosine residues on proteins to form the stable end product nitrotyrosine. Moreover, there is increased expression of nitrotyrosine in neurofilament aggregations and granuloma-associated macrophages infiltrating dermal nerves, suggesting a possible role for peroxynitrite and NO in the nerve damage following borderline leprosy [8].

The primary aim of this study was to evaluate NO stable end products [nitrite (NO$_2$) and nitrate (NO$_3$)] in sera of leprosy patients across the variety of the disease compared with controls. The secondary aim was to detect whether NO could be considered as a possible parameter of prognostic value and as a marker of disease status.

**Patients and methods**

This case–control study was conducted on 80 leprosy patients who were selected from Benha Dermatology and Leprosy Clinic, Benha University Hospital, and Abu Zaabal Leprosarium from October 2012 to September 2013. Twenty age-matched and sex-matched healthy volunteers were included as the control group.

**Inclusion criteria**

Inclusion criteria were: (i) newly diagnosed untreated paucibacillary (PB), multibacillary (MB), and type 1 reaction (T1R) leprosy patients; (ii) PB and MB leprosy patients who were treated with standard WHO-recommended multidrug therapy (MDT) for not less than 6 months (treated); and (iii) T1R leprosy patients who were treated with standard WHO-recommended MDT for not less than 6 months and prednisolone.

A written informed consent form, which was approved by the Ethics Committee for Human Research in Benha University, was taken from each patient.

**Methods**

All patients were subjected to the following: (i) detailed history taking (personal history, duration of the disease, present history of leprosy, nerve affection, disability, course of reaction if present, and family history of leprosy) and (ii) clinical examination (erythematous or hypopigmented patches, infiltration or nodules, number of the lesions, hair growth over the lesions, presence or absence of sweating, presence of neurotrophic ulcers or visible deformities, and sign of nerve affection). Patients were diagnosed according to the WHO classification of leprosy. Clinical assessment for leprosy patients included the number of skin lesions and nerves involved as the basis for grouping leprosy patients into MB (≥ 5 skin lesions) and PB (≤ 5 skin lesions) leprosy [1]. The patients were divided into four main groups according to the WHO classification: group I that included 20 patients with PB leprosy who were subdivided into two subgroups, IA (10 untreated PB patients) and IB (10 treated PB patients); group II that included 30 patients with MB leprosy who were subdivided into two subgroups, IIA (15 untreated MB patients) and IIB (15 treated MB patients); group (III) that included 30 patients with T1R leprosy who were subdivided into two subgroups, IIIA (15 untreated T1R patients) and IIIB (15 treated T1R patients); and the control group that included 20 normal healthy individuals.

**Laboratory investigation**

Blood samples (5 ml each) were collected by venipuncture under aseptic conditions, centrifuged at 2000 rpm for 15 min, and supernatants were stored at –20°C until biochemical analysis. Total NO (NO$_3$/NO$_2$) level assay was performed using Total Nitric Oxide Kit (catalog number PKGE001) according to the manufacturer (R&D Systems, Minneapolis, MN, USA).

**Principle of the assay**

This assay determines NO concentrations based on the enzymatic conversion of NO$_3$ to NO$_2$ by NO$_3$ reductase followed by colorimetric detection of NO$_2$ as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO$_2^-$ produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is coupled to N-(1-naphthyl)ethylenediamine to form the chromophoric azo-derivative, which absorbs light at 540–570 nm.

**Statistical analysis**

The clinical and laboratory data were tabulated, coded, and then analyzed using Microsoft Office Excel 2010 (Redmond, Washington, USA) software program for figures and IBM statistical package for the social sciences (SPSS, version 19; SPSS Inc., Chicago, Illinois, USA).

In this study, mean ± SD was used for quantitative data and numbers and % were used for qualitative data. Different types of ‘significant tests’ were used to make a comparison between two or more groups of values; in each case we used one of the following tests: the $\chi^2$-test for nonparametric data and the paired $t$-test and the Student $t$-test for parametric data. $P$-value less than 0.05 was considered significant. Receiver operating characteristic (ROC) curve was used to determine a cutoff value for a clinical test.

**Results**

**Clinical results**

The current study included 80 leprosy patients, 45 (56.25%) male and 35 (43.75%) female. Their ages ranged from 15 to 65 years with a mean age of 39.54 ± 12.6 years. Twenty clinically free individuals served as the control group, 11 (55%) male and nine (45%) female; their ages ranged from 16 to 70 years with a mean age of 40.5 ± 16.78 years. Forty (50%) patients had nerve destruction. Slit-skin smear was positive in 28 (35%) patients, whereas it was negative in 52 (65%). Thirty-four (42.5%) patients had positive family history of leprosy.
Laboratory results
Comparison of the mean values of serum NO₂ levels for each group of untreated patients versus the control group (control level is 165.87 ± 13.05 μmol) revealed increased serum NO₂ levels in untreated patients. This difference was statistically significant in the MB group (366.49 ± 263.97 μmol, P = 0.001) and in the T1R group (302.5 ± 198.25 μmol, P = 0.02) (Table 1 and Fig. 1). Comparison of the mean values of serum NO₂ levels for each group of treated patients versus the control group (165.87 ± 13.05 μmol) revealed decreased serum NO₂ levels in treated patients. This difference was statistically significant in the MB group (161.7 ± 20.87, P = 0.001) and in the T1R group (145.9 ± 7.89, P = 0.028), but it was statistically insignificant in the PB group (137.5 ± 14.49, P = 0.86) (Table 2 and Fig. 1).

Among the studied patients’ NO metabolites levels before and after treatment, comparison of serum NO₂ and NO₃ in five patients before and after treatment showed a decrease in the amount of NO metabolites in the serum of these patients after treatment (Table 3).

ROC curve in PB patients was at cutoff value of 195.98 μmol of serum NO₂, with sensitivity of 67%, specificity of 93%, AUC of 0.74, and P-value of 0.021, whereas at cutoff value of 204.21 μmol of serum NO₃, sensitivity was 93%, specificity was 40%, AUC was 0.89, and P-value was 0.003 (Fig. 3).

ROC curve in T1R patients was at cutoff value of 157.17 μmol of serum NO₂, with sensitivity of 73%, specificity of 93%, AUC of 0.73, and P-value of 0.029, whereas at cutoff value of 285.29 μmol of serum NO₃, sensitivity was 73%, specificity was 80%, AUC was 0.74, and P-value was 0.021 (Fig. 4).

Discussion
Activation of macrophages by proinflammatory cytokines such as IFN-γ is known to induce the production of NO through iNOS [9]. The labile nature of NO makes it impossible to analyze its serum level; hence, NO metabolites (NO₂ and NO₃) levels are helpful to measure the level of NO. NO₃ is first reduced to NO₂ and the NO₂ level is considered the marker of NO in body fluids including serum [10,11].

Our results showed that comparison of the mean values of serum NO metabolites levels for each group of untreated patients versus the control group revealed increased serum NO metabolites levels in untreated patients, whereas it revealed decreased serum NO metabolites levels in treated patients. Serum NO metabolites level was significantly higher in untreated MB and T1R patients than in controls. However, it was significantly lower in treated MB and T1R patients than in controls. It can be suggested that serum NO metabolites levels in leprosy patients gradually decrease across the spectrum with clinical improvement in response to MDT. These findings are in agreement with the findings of Boga et al. [12] who reported the highest levels of serum NO metabolites in untreated MB patients and in patient undergoing T1R. This is in contrast to the maximum localization of the iNOS enzyme in skin lesions of tuberculoid leprosy patients [13]. As iNOS is induced during inflammatory responses, its increased expression in localized tuberculoid leprosy skin lesion is expected; however, the serum values reflect the metabolic status of the entire body, and hence NO metabolite levels are

Table 1. Comparison of serum nitrite levels of the untreated leprosy patients groups versus the control group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment status</th>
<th>N</th>
<th>NO₂ (μmol) (mean ± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>20</td>
<td>165.87 ± 13.05</td>
<td>–</td>
</tr>
<tr>
<td>PB leprosy patients</td>
<td>Untreated</td>
<td>10</td>
<td>172.85 ± 30.39</td>
<td>0.425</td>
</tr>
<tr>
<td>MB leprosy patients</td>
<td>Untreated</td>
<td>15</td>
<td>366.49 ± 263.97</td>
<td>0.001*</td>
</tr>
<tr>
<td>T1R leprosy patients</td>
<td>Untreated</td>
<td>15</td>
<td>302.5 ± 198.25</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

MB, multibacillary; NO₂, nitrite; PB, paucibacillary; T1R, type 1 reaction. *P < 0.05, statistically significant.

Table 2. Comparison of serum nitrite levels of the treated leprosy patients groups versus the control group

<table>
<thead>
<tr>
<th>Types of leprosy</th>
<th>Treatment status</th>
<th>N</th>
<th>NO₂ (μmol) (mean ± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>20</td>
<td>165.87 ± 13.05</td>
<td>–</td>
</tr>
<tr>
<td>PB leprosy patients</td>
<td>Treated</td>
<td>10</td>
<td>137.5 ± 14.49</td>
<td>0.86</td>
</tr>
<tr>
<td>MB leprosy patients</td>
<td>Treated</td>
<td>15</td>
<td>161.7 ± 20.87</td>
<td>0.001*</td>
</tr>
<tr>
<td>T1R leprosy patients</td>
<td>Treated</td>
<td>15</td>
<td>145.9 ± 7.89</td>
<td>0.028*</td>
</tr>
</tbody>
</table>

MB, multibacillary; NO₂, nitrite; PB, paucibacillary; T1R, type 1 reaction. *P < 0.05, statistically significant.

Figure 1.
Scattergram comparing serum nitrite levels for each group of leprosy patients across the spectrum versus the control group. MB, multibacillary; PB, paucibacillary; T1R, type 1 reaction.
presumably higher in MB patients with chronic multiple lesions [12]. This is in contrast with the study by Cooper et al. [9] who supported the role of NO as a limiting agent in both lymphocyte response and inflammation during mycobacterial disease.

This study showed that, in the PB, MP, and TIR groups, comparison of serum NO₂ and NO₃ levels between untreated and treated patients showed statistically significant difference. These findings were in accordance with the findings of Boga et al. [12]. This may be due to the effect of the treatment that can return NO metabolites to normal levels, apparently by inhibiting iNOS production and not through suppression of proinflammatory cytokines [14]. The present study also showed that, in leprosy patients, treatment reduced the levels of NO metabolites not only for TIR patients as has been reported earlier by Schön et al. [15] and Little et al. [7], but also for patients without reaction, and this was in agreement with the study by Boga et al. [12]. In this study, follow-up of five leprosy patients from different clinical types across the spectrum confirmed reduction of serum NO metabolites levels after treatment with MDT for a minimum 6 months with or without prednisolone therapy according to patient’s case. However, some other studies documented the association between prednisolone therapy and rapid decrease in urinary NO metabolites level and clinical improvement in leprosy patients with TIR [14]. Little et al. [7] and Atkinson et al. [16] observed that, following prednisolone treatment, there was a decrease in expression of cytokines and iNOS in leprosy skin lesions. Further study with a larger sample size is necessary to confirm our

<table>
<thead>
<tr>
<th>Patient numbers</th>
<th>Clinical status</th>
<th>Nitrite levels (NO₂) Before treatment</th>
<th>Nitrite levels (NO₂) After treatment</th>
<th>Nitrate levels (NO₃) Before treatment</th>
<th>Nitrate levels (NO₃) After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Paucibacillary</td>
<td>154.25</td>
<td>146.91</td>
<td>219.99</td>
<td>131.27</td>
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<tr>
<td>2</td>
<td>Multibacillary</td>
<td>173.5</td>
<td>152.73</td>
<td>628.85</td>
<td>433.97</td>
</tr>
<tr>
<td>3</td>
<td>Multibacillary</td>
<td>545.5</td>
<td>197.64</td>
<td>677.36</td>
<td>241.78</td>
</tr>
<tr>
<td>4</td>
<td>Type 1 reaction</td>
<td>280.1</td>
<td>136.01</td>
<td>218.26</td>
<td>128.26</td>
</tr>
<tr>
<td>5</td>
<td>Type 1 reaction</td>
<td>212.09</td>
<td>141.62</td>
<td>208.16</td>
<td>119.52</td>
</tr>
</tbody>
</table>

Receiver operating characteristic (ROC) curve showing the performance of serum nitric oxide metabolites levels as a parameter among paucibacillary patients. Cutoff value 155.97 μmol of serum nitrite with sensitivity of 80%, specificity of 90%, area under the curve (AUC) of 0.94, and P-value of 0.001. Cutoff value 253.46 μmol of serum nitrate with sensitivity of 80%, specificity of 90%, AUC of 0.89, and P-value of 0.003.
results with respect to ROC curve sensitivity and specificity for NO in leprosy, to be used as a large-scale screening procedure for follow-up of leprosy patients and for monitoring treatment success.

**Conclusion**

It is worthy to mention that serum NO metabolites level could be a valuable parameter for prognosis and follow-up of leprosy patients. In addition, it could be used to monitor treatment success especially in MB and T1R patients.

**Acknowledgements**

Conflicts of interest

There are no conflicts of interest.

**References**