Profile of oxidative stress in response to treatment for Type 1 leprosy reaction

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Accepted for publication 11 February 2015

Summary

Objective: To measure oxidative stress in Type 1 leprosy reaction, and to document the effect of anti-leprosy multidrug therapy (MDT) and anti-reaction drugs on measures of oxidative stress.

Materials and methods: A prospective study was carried out at a teaching hospital involving consecutive patients with Type 1 reaction. MDA (malondialdehyde), FRAP (ferric reducing ability of plasma) and GSH (reduced glutathione) were measured in venous blood samples as measures of oxidative stress and compared at inclusion, after 4 weeks of initial therapy (following standard guidelines including MDT, NSAIDS, and systemic steroids), and 4 weeks after clinical remission.

Results: The final study cohort included 40 patients with Type 1 reaction (different treatment arms) after excluding for confounding factors such as prior treatment, smoking, NSAID use or concurrent illness requiring therapy. Measures of lipid derived oxidative stress assessed by MDA showed a significant rise with 4 weeks of therapy and a trend towards decline after clinical resolution. In contrast, the other two measures of anti-oxidants namely GSH and FRAP, showed a significant decrease ($P < 0.05$) at 4 weeks of treatment followed by a significant increase after 4 weeks of clinical remission of reaction.

Conclusion: MDT and anti-reactional treatment is associated with significant increases in FRAP and GSH levels, reflecting a reduction in the oxidative stress in patients treated for Type 1 reaction. However, lipid peroxidation as measured by MDA is only partially controlled with treatment.

Keywords: oxidative stress, leprosy reaction, malondialdehyde, FRAP, GSH
Introduction

Leprosy patients often develop acute inflammatory responses known as reactions which reflect abrupt changes in the host immunity to *Mycobacterium leprae* manifesting clinically as acute exacerbations. Reactions based on immunological undercurrents are classified into Type I reactions – typically seen in borderline patients and due to an increase in cell-mediated response to *M. leprae*, and Type II reactions or erythema nodosum leprosum (ENL), encountered almost exclusively at the lepromatous pole of leprosy and an immune-complex syndrome (an antigen-antibody reaction involving complement). The overall prevalence of leprosy reactions has been reported to be around 30% from hospital-based data in India. Reactions may ultimately result in nerve damage, deformity and disability in the leprosy patients. Leprosy was declared eliminated from India in 2005, yet the recognition and management of these reactions continues to be the most significant and challenging task in the post elimination era. Despite effective MDT, morbidity due to reactions remains significant. There remains an unmet need for effective therapy to prevent or mitigate the nerve damage due to reactions.

The activation of monocytes and neutrophils in leprosy leads to the production of Reactive Oxygen Species (ROS). ROS have been implicated as a major mechanism of antimicrobial effector function in leprosy. They induce a state of local oxidative stress in the tissues. The role of oxidative stress in leprosy is well described, but oxidative status in leprosy reactions, with or without the effect of therapeutic drugs used in its treatment, has been evaluated in only a few studies so far. Therapeutic benefit with antioxidant therapy has been demonstrated in some diseases such as chronic idiopathic pancreatitis. In this context the present study was conceived to assess the profile of oxidative stress as measured by malondialdehyde (MDA), reduced glutathione (GSH) and the ferric reducing ability of plasma (FRAP) assays. Since a comparative trial of NSAIDS, clofazimine and steroids is not possible due to ethical reasons, the present study was designed as a prospective observational study to determine the effects of the combination of anti-leprosy multidrug therapy (MDT) and anti-reaction drugs on the parameters of oxidative stress in patients with Type 1 reaction.

Materials and methods

This prospective study was conducted in a tertiary care centre in North India from December 2006 to March 2008. The study was approved by the Institutional Ethics Committee. Written informed consent was given by all patients before their inclusion in the study. Untreated adult multibacillary (MB) leprosy patients, who were diagnosed clinically according to the WHO classification, were enrolled. Since new Type 2 reactions are rarely seen, we included only patients with Type 1 reaction. Patients who had received non-steroidal anti-inflammatory drugs (NSAIDs) in the previous week, or corticosteroids in the previous two weeks, and those with history of smoking, co-infections, diabetes mellitus, any other systemic diseases, were excluded. Pregnant and lactating women were also excluded.

CLINICAL AND HISTOLOGICAL ASSESSMENT

The diagnosis was made on clinical grounds, slit-skin smear test, and histopathology. Clinical details included the number and distribution of lesions, the pattern of nerve involvement, and
complications including reactions, neuritis, and deformities. Two slit skin smears, one from a representative lesion and the other from an earlobe, were obtained and stained by modified Ziehl-Neelsen technique. A minimum of 100 oil immersion fields of the smear were examined for the presence of acid-fast bacilli, and bacterial index (BI) was calculated. A 4-mm punch skin biopsy from the most representative lesion was stained with both haematoxylin and eosin and modified Ziehl-Neelsen stain. The patients were categorized according to the Ridley-Jopling (RJ) scale based on clinical, histological and bacteriological criteria. The study included 40 clinically diagnosed and biopsy confirmed leprosy patients with Type 1 reaction.

**BIOCHEMICAL PARAMETERS**

Blood samples were collected from the patients on three occasions: firstly at inclusion, secondly after four weeks, and thirdly, four weeks after clinical remission. Oxidative Stress parameters (FRAP, GSH, MDA) were measured in these samples, and compared. Clinical remission of reaction was defined as patients being afebrile, regression of erythema and oedema in skin lesions, absence of pain/tenderness in the nerves and regression of other manifestations. The patients in this study served as their own controls for baseline biochemical parameters. Patients in our study group were expected to present at different stages of evolution of the reactional episode, therefore, blood samples were taken on three separate occasions: the first two samples were taken at an interval of 4 weeks to define the initial trend of oxidative stress, and the third sample after clinical remission to interpret the effect of drugs on the oxidative stress.

**ANALYTICAL PROCEDURES**

Blood samples (5 mL) were collected from the cubital vein. The serum was separated and stored at 20°C and assayed within 2 months. For measurement of blood glutathione, venous blood was collected in heparinized tubes.

Total antioxidant capacity of plasma was determined by measuring the ability to reduce Fe3+ to Fe2+ which is known as the FRAP test. At low pH, ferric tripyridyltriazine (Fe+ 3-TPTZ) complex is reduced to the ferrous (Fe+ 2) form, and an intensive blue colour with an absorption maximum at 593 nm develops. The reaction was monitored for 4 minutes. Aqueous solutions of known Fe+ 2 concentrations in the range of 100–1000 mol/L were used for calibration.

The GSH levels were determined by the method described by Teitze. This method is based on the development of a yellow colour with 5, 5′ dithiobis-2-nitrobenzoic acid (DTNB), which is measured at 432 nm, using a spectrophotometer.

MDA levels were measured as an index of lipid peroxidation using the colorimetric method described by Satoh. The thiobarbituric acid (TBA) assay was used as an indicator of lipid peroxidation in serum. The assay is based upon the reaction of TBA with MDA, one of the aldehyde products of lipid peroxidation. The absorbance of the MDA-TBA adduct thus produced, was measured at 532 nm, using a spectrophotometer.

The patients were given treatment according to the severity of the reaction. All the patients received NSAIDS (diclofenac) in addition to MDT. Higher doses of clofazimine (100 mg TDS) were given to those patients who did not improve with NSAIDS. Systemic steroids in the form of prednisolone (1 mg/kg) were given to patients who had neuritis or any recent onset muscle weakness. However, the biochemical parameters were not compared in the subgroups who received different treatment based on severity, due to the small size of the subgroups.
STATISTICAL ANALYSIS

Repeated measures ANOVA with Tukey’s test at the 5% level of significance was used for comparison of oxidative stress parameters. Differences associated with $P < 0.05$ were regarded as statistically significant. Results are reported as means ± SD.

Results

The final study cohort comprised of 40 patients. Based on the RJ classification, 70% ($n = 28$) of the patients were classified as BL leprosy and 30% ($n = 12$) as BT leprosy. Only 10 patients presented with Type 2 reaction during the study period, and all of them were already on MDT, so they could not be included in the study. All 40 patients completed the study.

The mean age of patients in the BT group was 35.46 ± 16.08 years, and the mean age of patients in BL group was 30.83 ± 10.53 years. Both the groups were statistically comparable as regards age ($p < 0.05$). The males outnumbered females with a ratio of 1.86:1 (Table 1).

The slit skin smear was positive in 15 patients in the BT group (53.75%) and 12 patients in the BL group (100%). The average Bacteriological Index (BI) in the BL group was significantly higher ($3.67 ± 1.23$) as compared to BT group, where average BI was $0.93 ± 1.09$ ($P = 0.001$).

The duration of treatment (to reach clinical remission from the leprosy reaction, with complete withdrawal of anti-reaction drugs), in the BT group varied from 1.5 to 12 months with a mean of 4.02 ± 2.67 months, and in the BL group from 2 to 8 months, with a mean of 4.71 ± 1.94 months.

MDA was measured as an index of lipid peroxidation in serum. The mean value of MDA was significantly higher after 4 weeks of therapy as compared to the levels at presentation. After 4 weeks of clinical remission, the mean MDA was lower than that at presentation but this difference was not statistically significant ($P > 0.05$) (Table 2).

Anti-oxidant levels were measured in terms of GSH in whole blood and FRAP in plasma. The mean value of both GSH and FRAP showed a significant decline ($P < 0.05$) after 4 weeks of treatment, as compared to the baseline levels, whereas the mean value of both the parameters increased significantly ($P < 0.05$) 4 weeks after full clinical remission of the reaction (Table 2).

All the patients received NSAIDS ($n = 100$%). Nine patients were controlled with NSAIDS and clofazimine ($n = 22.5$%). Fifteen patients were given steroids ($n = 37.5$%).

Table 1. Important features of patients in the present study

<table>
<thead>
<tr>
<th>Features</th>
<th>BT with type 1 reaction</th>
<th>BL with type 1 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (%)</td>
<td>12 (30%)</td>
<td>28 (70%)</td>
</tr>
<tr>
<td>Mean age of patients</td>
<td>35.46 ± 16.08 years</td>
<td>30.83 ± 10.53 years</td>
</tr>
<tr>
<td>No. of females</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>No. of males</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>SSS* +ve cases</td>
<td>53.75%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean BI**</td>
<td>0.93 ± 1.09</td>
<td>3.67 ± 1.23</td>
</tr>
<tr>
<td>Mean duration of treatment</td>
<td>4.02 ± 2.67 months</td>
<td>4.71 ± .94 months</td>
</tr>
</tbody>
</table>

* SSS – Slit Skin Smear
** BI – Bacteriological index
The trend of oxidative stress parameters in these treatment subgroups are shown in Tables 3 and 4. Out of the three parameters measured in these groups, only GSH showed a significant increase at the time of clinical remission. FRAP and MDA were not significantly affected after treatment of reaction.

### Discussion

This was a prospective observational study conducted to determine the trends in oxidative stress parameters during and after treatment for T1R. However, the effect of individual drugs could not be commented compared, since treatment was provided based on the severity. Therefore, baseline oxidative stress in these patients was varied. The trends in individual patients were compared with their own baseline levels. Antioxidant levels in the form of FRAP and GSH were increased significantly after treatment and clinical subsidence of the reaction. The level of MDA was not significantly decreased after treatment.

The microbicidal ability of phagocytes through reactive oxygen species (ROS) such as hydrogen peroxide (H2O2), superoxide anion and hydroxyl radical is a basic defense mechanism of the human host against microbial infection, including infection with *Mycobacterium leprae*. ROS can also damage lipids, proteins and nucleic acids of the host. Prime targets of peroxidation by ROS are polyunsaturated fatty acids (PUFA) in membrane lipids. PUFA is degraded by free radicals to form malondialdehyde (MDA). The induction of macrophages in response to *M. leprae* infection could contribute to an increase in MDA levels.

#### Table 2. Biochemical profile of patients at presentation, at 4 weeks and at the end of the study, 4 weeks after clinical remission (n = 40)

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/ml)</th>
<th>FRAP (nmol/ml)</th>
<th>GSH (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD*</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.623</td>
<td>0.997</td>
<td>743.30</td>
</tr>
<tr>
<td>After 4 weeks</td>
<td>2.212</td>
<td>1.301</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>At end</td>
<td>1.488</td>
<td>0.985</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

*SD – Standard deviation

The trend of oxidative stress parameters in these treatment subgroups are shown in Tables 3 and 4.

Out of the three parameters measured in these groups, only GSH showed a significant increase at the time of clinical remission. FRAP and MDA were not significantly affected after treatment of reaction.

### Table 3. Biochemical profile of patients who took NSAIDS and high dose clofazimine (n = 9)

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/ml)</th>
<th>FRAP (nmol/ml)</th>
<th>GSH (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD*</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.707</td>
<td>1.015</td>
<td>856</td>
</tr>
<tr>
<td>After 4 weeks</td>
<td>2.113</td>
<td>1.083</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>At end</td>
<td>1.583</td>
<td>0.835</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

*SD – Standard deviation

### Table 4. Biochemical profile of patients who took high dose clofazimine (n = 9)

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/ml)</th>
<th>FRAP (nmol/ml)</th>
<th>GSH (μmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD*</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.707</td>
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<td>After 4 weeks</td>
<td>2.113</td>
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<tr>
<td>At end</td>
<td>1.583</td>
<td>0.835</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>
Oxidative stress has been extensively studied in both PB and MB leprosy and in RJ subtypes. Bhadwat and Borade\textsuperscript{17} reported a progressive increase in mean serum MDA levels along RJ subtypes with minimum increase at the tuberculoid end. Reddy \textit{et al}.\textsuperscript{6} noted a significant increase in serum MDA levels in MB leprosy but the changes were non-significant in the PB group. Lima \textit{et al}. observed increased MDA levels and decreased vitamin A levels with statistical significance in the lepromatous pole of leprosy as compared to controls.\textsuperscript{18} Jyothi \textit{et al}. have detected significantly higher levels of MDA in leprosy patients as compared to controls, decreased SOD levels especially in MB leprosy, and a significantly elevated MDA/SOD ratio in MB patients.\textsuperscript{19} Abdel-Hafez \textit{et al}. detected increased oxidative stress (in the form of decreased SOD activity and increased MDA activity) in both tissues and blood of MB patients, whereas in PB patients the increases were seen in tissues, but not in blood.\textsuperscript{20} A significantly low red blood cell GR (glutathione reductase) activity in leprosy as well as respective increased and decreased blood GSH content in tuberculoid and lepromatous leprosy have been reported by Khaire and Magar.\textsuperscript{21} Prasad \textit{et al}. have observed that GSH, GSH-Px (glutathione peroxidase) and GR were significantly lower in leprosy patients as compared to the control group ($P < 0.001$).\textsuperscript{22} A progressive decrease in GSH level and enzyme activities (GSH-Px, GR) was noted along the leprosy spectrum from TT to LL. A progressively decreasing trend in GSH level and enzyme activities was also noticed along the leprosy groups with advancing levels of BI.

Reports on oxidative stress in leprosy reactions are scarce. Mohanty \textit{et al}.\textsuperscript{7} suggested that the NO/NOM excretion is increased in leprosy patients during ENL episodes. With anti-reactional therapy (steroids) and clinical improvement the levels are reduced. Prednisolone treatment and clinical improvement in leprosy reactional patients (mainly with Type 1 reactions) was found to be associated with a rapid decrease in urinary nitric oxide metabolites.\textsuperscript{23} Similar findings of a higher level of urinary nitric oxide metabolites during Type 1 reaction, which decreased with a high dose of prednisolone therapy and clinical improvement, supports the suggestion that reaction pathology is related to raised NOM levels. One of the reasons for this increased level could be up regulation of pro-inflammatory cytokines, as has been observed in both ENL and RR reactions.\textsuperscript{24} However, there are no published studies evaluating the levels of MDA, red cell reduced glutathione and total plasma antioxidant potential in Type 1 reaction.

NSAIDs inhibit cyclooxygenases (COX), preventing the formation of prostaglandins, prostacyclin and thromboxane. Therapeutic plasma levels of classical NSAIDs are able to suppress the oxygen-dependent anti-microbial or oxidative functions of neutrophils, by inhibiting the MPO-chlorinating activity and also by scavenging HOCl. Diclofenac has also

### Table 4. Biochemical profile of patients who took systemic steroids ($n = 15$)

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/ml)</th>
<th>FRAP (nmol/ml)</th>
<th>GSH ($\mu$mol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD*</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.409</td>
<td>0.929</td>
<td>947.333</td>
</tr>
<tr>
<td>After 4 weeks</td>
<td>1.853</td>
<td>0.999 &lt; 0.05</td>
<td>714.533</td>
</tr>
<tr>
<td>At end</td>
<td>1.362</td>
<td>0.920 &gt; 0.05</td>
<td>1051.333</td>
</tr>
</tbody>
</table>

* SD – Standard deviation

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Trend of oxidative stress in Type 1 leprosy reaction
been shown to suppress the production of superoxide anion, by disrupting activation of NADPH oxidase in phagocytes.\textsuperscript{25,26}

Glucocorticoids have been reported to inhibit the production of cytokines in LPS-stimulated monocytes, endothelial cells and fibroblasts. One of the studies evaluating the role of steroids on oxidative stress found that monocytes from leprosy patients receiving prednisone therapy responded to lower concentrations of IFN-gamma in vitro with enhanced superoxide anion release when challenged with \textit{M. leprae} or BCG, than did monocytes from healthy subjects and other leprosy patients.\textsuperscript{27} It has also been shown that glucocorticoids inhibit iNOS and NO production directly through inhibition of the translocation of nuclear factor kappa beta as well as indirectly by suppressing the production of cytokines such as TNF-\textalpha. The main mechanism by which prednisolone exerts its effects in reactional leprosy patients remains to be established, as the drug affects many inflammatory mechanisms.\textsuperscript{23}

In our study the level of MDA was found to be higher 4 weeks after treatment which may be explained on the basis of ongoing tissue damage and a poor response to 4 weeks of therapy. However after clinical remission and completion of anti-reaction therapy, MDA levels decreased as compared to baseline (although not significantly) which means that cellular damage in the form of lipid peroxidation is not fully reversed by the treatment of leprosy reaction.

Increased lipid peroxidation of the cell membrane can cause leakage of cytosolic antioxidant enzymes.\textsuperscript{28} Lipid peroxidation products can inactivate the enzymes.\textsuperscript{29} Low blood GSH content in leprosy could be related to low glutathione reductase (GR) activity, its increased utilization for direct scavenging of free radicals\textsuperscript{30} and/or its utilisation for replenishment of other crucial antioxidants such as vitamin E and C, which would get oxidized during the course of their antioxidant action.\textsuperscript{31} The low blood glutathione level and the decreased total antioxidant capacity of plasma activities suggest oxidative stress associated with insufficient antioxidant defense potential in leprosy. In the present study, the mean value of FRAP and reduced glutathione decreased after 4 weeks of treatment, but rose significantly after clinical remission. The initial decrease in blood GSH content, followed by an increase in GSH levels in the present study could be related to an increased lipid peroxidation initially, followed by decrease at clinical remission.

In the treatment subgroups of patients who received high dose of clofazimine and who received steroids in addition to NSAIDS, only GSH showed significant increment. However, since the number of patients in these subgroups is small, no conclusions can be drawn. One reason might be that these patients had more severe reactions with greater oxidative stress, which responded poorly to treatment. Also the clinical remission of severe reactions might not correspond to significant decreases in oxidative stress.

In conclusion, at clinical remission, the standard treatment for leprosy reactions decreases the oxidative stress to a large extent. However, there was a residual oxidative stress at the time of clinical remission of the reaction in the form of lipid peroxidation products. This residual oxidative injury can be explained by the ongoing disease process of leprosy \textit{per se}, despite clinical subsidence of the reaction. Also the ongoing production of ROS (in the form of MDA), and depletion of free radicals (in the form of FRAP and reduced glutathione) in patients with Type 1 reaction was not affected after 4 weeks of treatment in our study, which means that the drugs used (including multidrug therapy) are not sufficient to reduce oxidative stress within 4 weeks. This suggests a potential need to evaluate the role of anti-oxidants as a therapeutic strategy in Type 1 reaction. Additionally, the lipid membrane damage is only...
partially controlled, suggesting the need to add lipid soluble antioxidant therapy to the patients with leprosy reactions.

We acknowledge the limitations due to the small sample size. However a careful screening to exclude confounders like smokers, prior treatment and concurrent illness was warranted due to the wide range of factors which influence oxidative stress. Further, the role of the diet of the participant subjects could have been studied, since there are various nutritional antioxidants which prevent lipid peroxidation and DNA damage, including carotenoids (especially beta carotene), lycopene, flavonoids, tea polyphenols, selenium, vitamins C and E. Vitamin E functions as a chain breaking antioxidant in the lipid phase and as a first line of defense against peroxidation of membrane lipids. The effect of individual anti-reactional drugs could be observed if larger subgroups based on the severity and treatment chosen could be included.

References

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