

Serum nitrite level and adenosine deaminase activity is altered in visceral leishmaniasis

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ABSTRACT

In this study we sought to determine if there is alteration in nitric oxide (NO) production and adenosine deaminase (ADA) activity among patients with visceral leishmaniasis (VL) and the effect of four weeks of chemotherapy on these levels. Fifty-three VL patients diagnosed clinically and by direct demonstration of the LD bodies in the bone marrow smear were studied. They were treated with Sodium Stibogluconate and sampled at the baseline and four weeks. Forty-three healthy individuals coming from the same endemic area were taken as control. Total nitrite (NO₂⁻ and NO₃⁻) as an index of NO production and ADA activity was measured spectrophotometrically. Serum nitrite level decreased significantly in patients as compared to the healthy individuals but significantly increased following 4 weeks of chemotherapy. Conversely, Increased ADA activity was observed in the beginning of treatment and decreased significantly with successive 4 weeks of chemotherapy. It seems a negative correlation between NO level and ADA activity. This result indicates parasite induced evasion of NO and activation of T lymphocytes during immunopathogenesis of VL. Therefore, assessment of NO metabolites may be useful marker in the evaluation of the effector mechanism of macrophages and clinical manifestation of patients.

Keywords: NO, ADA, Visceral leishmaniasis

INTRODUCTION

Intramacrophage infection by *Leishmania donovani* (LD), an obligate intracellular protozoan, results the potentially fatal visceral leishmaniasis (VL) or kalaazar. The elimination of leishmania parasite by macrophage depends upon the mounting of effective cell-mediated immune response. During leishmaniasis infection, the microbicidal interaction between the parasite and host cells involves the presentation of leishmania antigen by macrophage to T-helper (Th) cell through MHC II molecule along with the co-stimulatory molecules (B7-1/B7-2 and CD40).^{1,2} Activation of Th cell causes proliferation of IFN- γ producing CD4⁺ Th subset.³ IFN- γ and TNF- α , the defining cytokines of the Th1 subset in turn activates the macrophage to generate NO which is the most relevant anti-leishmanial oxidant.

Contrary results have been found in the NO level during *L. donovani* infection both *in vitro* and *in vivo* experiments.^{4,5} Also studies reported hitherto have generally originated from *in vitro* culture and animal experiments. However no study has so far been conducted to investigate NO production in patients with visceral leishmaniasis. Therefore we aimed to determine serum nitrite level in VL patients as surrogate marker of NO production and compare it with the healthy subjects. Assessments of NO metabolites may be useful tool in the evaluation of the effector mechanism of macrophage and clinical manifestation of patients.

Adenosine deaminase (ADA) is an essential for the proliferation, maturation and function of T lymphocytes. Its activity increases during antigenic and mitogenic responses of lymphocytes and is considered as an important immunoenzyme marker for assessing cell-mediated immunity in diseases characterized by T lymphocytes proliferation and maturation.⁶

Considering the knowledge of molecular mechanism of the macrophage and T cell interaction in the cell mediated immunity against LD, we attempt to find the correlation between NO synthesized by the macrophage and the ADA activity during the immunopathogenesis of VL. This study is also an attempt to increase the knowledge concerning the immune response against intracellular residing LD parasite.

MATERIALS AND METHODS

Patients: Fifty-three patients with visceral leishmaniasis were taken in this study. As a control group forty-three healthy individuals from the same endemic area i.e. the terai belt of southern Nepal were taken. Routine hematological and biochemical tests were performed from the blood samples obtained from both cases and subjects. They were tested for concurrent infections. Cases of malaria, tuberculosis, and other diseases mimicking

VL were excluded from the study. In practice, VL was diagnosed on the basis of clinical judgment and direct demonstration of LD bodies in the bone marrow smear. The patients were treated with Sodium Stibogluconate (SAG, 20mg/kg body weight/day) for four weeks. All participants gave informed consent for the study. Further blood samples were obtained from the cases after the four weeks of chemotherapy. Serum was aliquoted and stored at -80°C until analysis.

Nitrite and ADA activity measurement: Serum nitrite concentration was determined by employing Griess reagent using the diazotization reaction as a colorimetric method.^{7,8} The ADA activities were measured according to the method described by Giusti and Galanti,^{9,10} based on the Bertholet reaction.

Statistical analysis: Statistical analyses were performed by using Epi Info 2000. The results were analyzed by ANOVA. Pearson's correlation coefficient was calculated to determine correlation between serum nitrite and ADA activity.

RESULTS

There was a matched demographic characteristic of patients and healthy individuals in Table. Serum nitrite level significantly decreased in patients as compared to those in healthy individuals ($21 \pm 6.2 \mu\text{mol/dl}$ vs. $33.53 \pm 12.7 \mu\text{mol/dl}$; $p < 0.001$) (Fig. 1). Out of 53 patients we could follow up only 35 patients. Paired t-test analysis of pre and post treatment samples showed significant increase in serum nitrite level in post treatment samples ($30 \pm 14.3 \mu\text{mol/dl}$; $p < 0.005$) (Fig. 2). Significantly higher serum ADA activity was measured in patients with visceral leishmaniasis than healthy individuals ($52.82 \pm 18.05 \text{ IU/L}$ vs. $17.03 \pm 8.83 \text{ IU/L}$; $p < 0.001$) (Fig. 3) and decreased level estimated after four weeks of chemotherapy ($21.52 \pm 10.25 \text{ IU/L}$) (Fig. 4).

Correlation analysis between serum nitrite and ADA activity among patients before treatment and healthy individuals showed a significant negative correlation.

DISCUSSION

NO is a reactive molecule in aqueous solution with half life estimated to be of few seconds, so it wasn't possible to measure NO directly in human, however measurement of serum nitrite provides an indirect estimate of systemic NO synthesis. Decrease in NO level in visceral leishmaniasis patients is contrary to the findings by Kumar *et al*⁵ which showed nitrite levels in the supernatant of monocytes cultures of VL patients similar to those of healthy individual. The result of this present study showing the decrease NO production in patients and subsequent increase in the NO level after 4 weeks of chemotherapy, suggest role of NO among the patients. This result is in contrary to other in vitro findings by Murraray and Nathan.⁴ The result of the present study signifies the parasite-induced evasion of this potential effector mechanism (NO). Importantly, there may have been down regulation of nitric oxide production by LD enabling considerable parasite replication inside the macrophages.

Effect of four weeks of chemotherapy in increasing NO production is significant in view of the fact that this coincides with the recovery of the clinical symptoms. This observation is nevertheless in agreement with the results of Kumar *et al*⁵ who demonstrated increased nitrite levels in the supernatant of the monocytes cultures of the VL patients. In addition, they found that following four months of treatment, the production of nitrite decreased significantly. Increase in systemic nitrite/NO levels demonstrate increased expression of nitric oxide synthase (NOS) protein. Elevation in NOS protein expression in circulating mononuclear cells have been correlated with increased systemic NO production in Tanzanian children's with anemia.¹¹ The increased in serum nitrite level after 4 weeks of chemotherapy also reflects the increased antileishmanicidal activity of macrophages.

It is well established that ADA levels reflects the activity of stimulated T lymphocytes and its levels are raised wherever cell mediated immunity is stimulated. Its activity has been shown to be elevated in disease characterized by T lymphocytes proliferation and activation. In visceral leishmaniasis, cell mediated immunity is the major immunological response. Hence, ADA level have been estimated in these patients. Elevated level of ADA in VL has been noted only in our previous study¹² and by Gaski *et al*.¹³ These observations are reproduced and further confirmed in this study. Furthermore, decrease in ADA activity after 4 weeks of chemotherapy is likely to have been abetted by a decrease in the level of immune activation due to decrease in parasite load and antigenic stimulation.

NO has been shown to modulate several immune response. There have however been some contrasting effects attributed to NO. It up regulates proliferation of T lymphocytes^{14,15} while others reports have suggested that it inhibits T cell activation.^{16,17} Increased production of NO is one of the mechanisms by which activated macrophage can restrict T cell expansion. The view of NO reproduction by macrophages is a mechanism designed for suppression of harmful immune response i.e. defending the host from it. Large amount of NO may inhibit production of cytokines; smaller amount may induce it.¹⁸ As the ADA is particularly sensitive to stimulation by the growth factors and cytokines during rapid tissue proliferation,¹⁹ this may be the reason for the

decrease in ADA activity with increase in NO level in visceral leishmaniasis after 4 weeks of chemotherapy. Ultimately there is resolution of visceral leishmania infection.

This study should therefore, encourage further investigations on the function of NO and ADA during the immunopathogenesis of visceral leishmaniasis.

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Table-1: A demographic characteristics of patients with visceral leishmaniasis and healthy individuals

Variables	Healthy Individuals	Patients	P value
Age (Mean±SD)			
Years	24±9.70	27.83±13.51	0.1
Sex			
Male	24 (55.8%)	29 (54.7%)	0.5
Female	19 (44.2%)	24 (45.3%)	

FIGURES

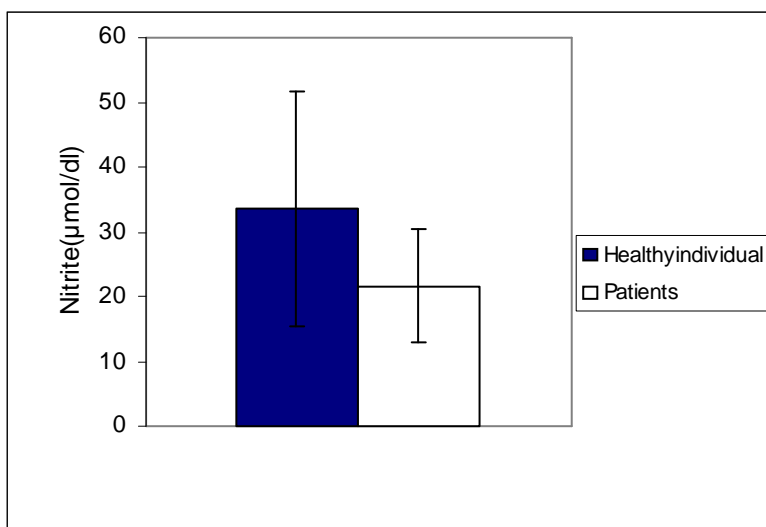


Fig. 1. Serum Nitrite Level in patients is significantly decreased in patients as compared to Healthy individuals (p<0.0001)

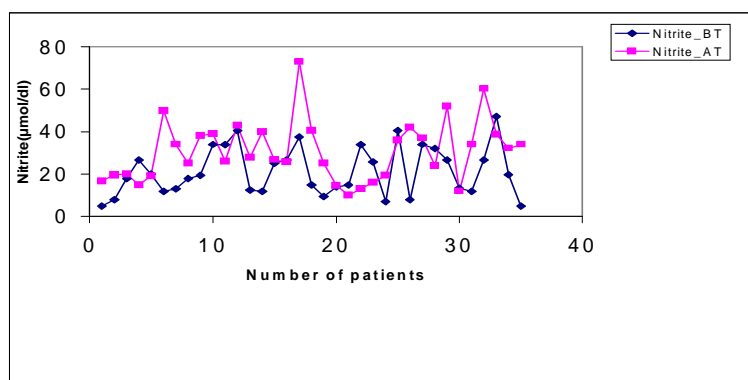


Fig. 2. Serum Nitrite level after treatment(AT) increased significantly as compared to before treatment(BT) sample(p<0.0001)

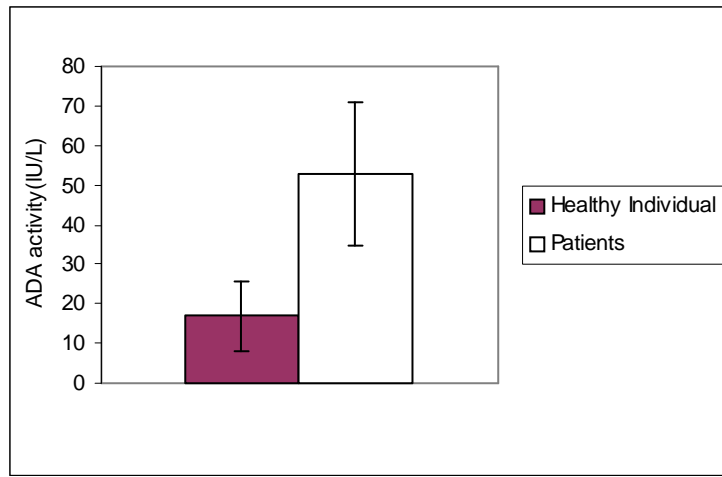


Fig. 3. ADA activity increased significantly as compared to healthy individuals ($p < 0.0001$)

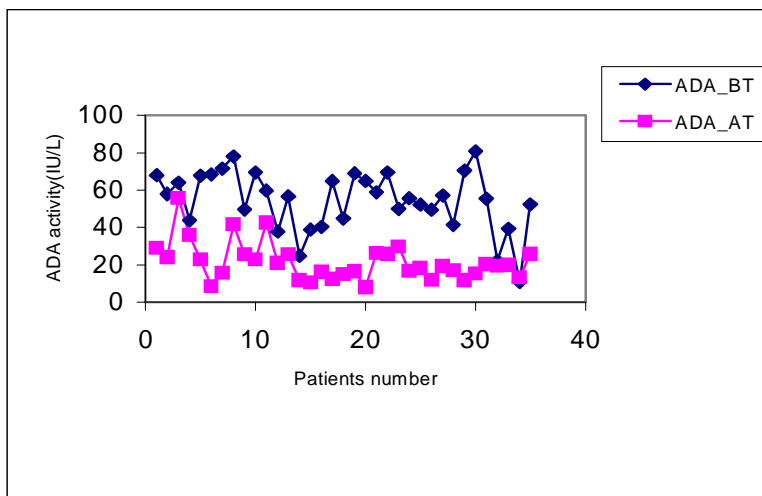


Fig. 4. Serum ADA activity in patients after treatment (AT) decreased significantly as compared to before sample (BT) ($p < 0.0001$)