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Research article

Antitubercular therapy decreases nitric oxide production in HIV/TB coinfected patients

A Wanchu*[1], A Bhatnagar[2], M Khullar[2], A Sud[1], P Bambery[1] and S Singh[1]

Address: [1]Department of Internal Medicine, Post-Graduate Institute of Medical Education and Research, Chandigarh, INDIA and [2]Department of Experimental Medicine and Biotechnology, Post-Graduate Institute of Medical Education and Research, Chandigarh, INDIA

E-mail: A Wanchu* - awanchu@glide.net.in; A Bhatnagar - abhat19@yahoo.co.in; M Khullar - madhukhu@yahoo.com; A Sud - asud@icpmr.wsahs.nsw.gov.au; P Bambery - pradeepbambery@hotmail.com; S Singh - surjit51@hotmail.com

*Corresponding author

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Abstract

Background: Nitric oxide (NO) production is increased among patients with human immunodeficiency virus (HIV) infection and also among those with tuberculosis (TB). In this study we sought to determine if there was increased NO production among patients with HIV/TB coinfection and the effect of four weeks chemotherapy on this level.

Methods: 19 patients with HIV/TB coinfection were studied. They were treated with standard four drug antitubercular therapy and sampled at baseline and four weeks. 20 patients with HIV infection, but no opportunistic infections, were disease controls and 20 individuals were healthy controls. Nitrite and citrulline, surrogate markers for NO, were measured spectrophotometrically.

Results: The mean age of HIV/TB patients was 28.4 ± 6.8 years and CD4 count was 116 ± 36.6/mm. Mean nitrite level among HIV/TB coinfected was 207.6 ± 48.8 nmol/ml. This was significantly higher than 99.7 ± 26.5 nmol/ml, the value for HIV infected without opportunistic infections and 46.4 ± 16.2 nmol/ml, the value for healthy controls (p value < 0.01). The level of HIV/TB coinfected NO in patients declined to 144.5 ± 34.4 nmol/ml at four weeks of therapy (p value < 0.05). Mean citrulline among HIV/TB coinfected was 1446.8 ± 468.8 nmol/ml. This was significantly higher than 880.8 ± 434.8 nmol/ml, the value for HIV infected without opportunistic infections and 486.6 ± 212.5 nmol/ml, the value for healthy controls (p value < 0.01). Levels of citrolline in HIV/TB infected declined to 1116.2 ± 388.6 nmol/ml at four weeks of therapy (p value < 0.05).

Conclusions: NO production is elevated among patients with HIV infection, especially so among HIV/TB coinfected patients, but declines significantly following 4 weeks of antitubercular therapy.

Background

Nitric oxide (NO) is an free-radical gas and an important biologically active molecule that participates in host defense against microbes, tumor cells and alloantigens.[1] It is synthesized from L- arginine by a family of three enzyme NO synthase (NOS) proteins, two of which are constitutive (type I and III) and one inducible (type II or iNOS).[2] Citrulline is released by the above reaction.[3] NO is readily transformed into nitrite and nitrate which are excreted into the urine. Type II NOS plays a significant role in various inflammatory processes and also in the functioning of the immune system. [4–8]

Production of NO is elevated among patients with HIV infection. [9–11] These levels may, however, be decreased among patients with advanced disease.[12] Elevated levels of NO among patients with HIV/TB coinfection have also been reported. [13–15] The relevance of NO production in HIV infection lies in the ability of the former to modulate the replication of the latter [1,16,17]. Further, Mycobacterium tuberculosis (Myco. tub.) enhances the replication of HIV.[18,19] In the presence of elevated NO production and Myco. tub. infection among HIV infected the disease tends to progress more rapidly. It is imperative to treat tuberculosis energetically and remove one of the variables that affect HIV replication.

Use of antitubercular medications has been shown to reduce the level of immune activation in the HIV/TB coinfected. [20] There has been one previous study looking at the level of NO metabolites after chemotherapy in patients with HIV/TB coinfection.[21] In that study untreated HIV positive patients with pulmonary TB did not have increased urinary nitrite/nitrate levels as compared with controls. As opposed to this HIV negative patients with pulmonary TB had higher urinary metabolite levels and when some of them were followed after chemotherapy there was a significant reduction in these levels.[21] In view of the conflicting data regarding NO production following chemotherapy among the HIV/TB coinfected, we carried out this study to determine if NO production, as measured by its surrogate markers nitrite and citrulline, is elevated among patients with HIV/TB coinfection and if it changes following four weeks of anti-tubercular therapy.

Methods

Patients

Nineteen patients with HIV infection who were diagnosed on the basis of positivity on a panel of three ELISA's and documented to have active TB as shown by AFB positivity were included in the study. They were administered antitubercular therapy (Rifampicin, INH, Ethambutol and Pyrazinamide) in doses appropriate for weight. Blood samples in these cases were taken at the start and at the end of 4 weeks of therapy, which was continued as per standard guidelines. Twenty patients with HIV infection but with no opportunistic infection were studied as disease controls. The latter was ruled out by absence of clinical features of any opportunistic infection and normal hemogram, routine biochemical parameters and chest x-ray. In addition, 20 healthy individuals served as controls.

Nitrite and citrulline measurement

Measurement of NO production in vivo is difficult because of its short half-life and the need for specialised equipment that uses chemiluminescence detection. Consequently, nitrite and citrulline levels were used as surrogate markers for estimating NO production. Nitrite and citrulline were measured in the serum by methods described previously.[22,23] Samples were stored at -20° C until analysis. For nitrite estimation, 100 ul of serum was reacted with 100 ul of Griess reagent (1% sulfanilide (w/v) in 5% o-phosphoric acid and 0.1% N-1 (napthyl ethylene diamine dihydrochloride and 2% H3PO4). Absorbance was read at 546 nm. Sodium nitrate (10–100 nmol) was used as standard. Citrulline was measured in serum by taking 100 ul of serum, diluting it 20 fold, and deproteinizing it using 0.5 ml of 25% tricarboxylic acid solution. To 0.5 ml of clear supernatant so obtained by centrifugation, 1.5 ml of chromogenic solution was added, mixed vigorously and boiled at 1000 C for 5 min. The tubes were cooled to room temperature and absorbance was read at 530 nm. DL-citrulline (25–300 nmol) was used as standard.

Table 1: Clinical profile of patients and disease and healthy controls

Feature	HIV+/TB+	HIV+/TB-	HIV-/TB-
Number	19	20	20
Males	12	14	15
Age, years	28.4 ± 6.8	26.5±4.6	25.7 ± 3.6
Range	19–48	22–37	21–44
Clinical signs suggesting TB (fever, weight loss, etc)	18	0	0
Radiological abnormality (Chest x-ray/ CT defect)	15	0	0
Disseminated TB	6	0	0
Mean CD4 count, /mm3*	116 ± 36.6	162.4 ± 24.2	
Range	14–456	48–440	

* Mean CD4 counts of HIV/TB coinfected were significantly lower than those of patients with HIV infection alone ($p < 0.05$)

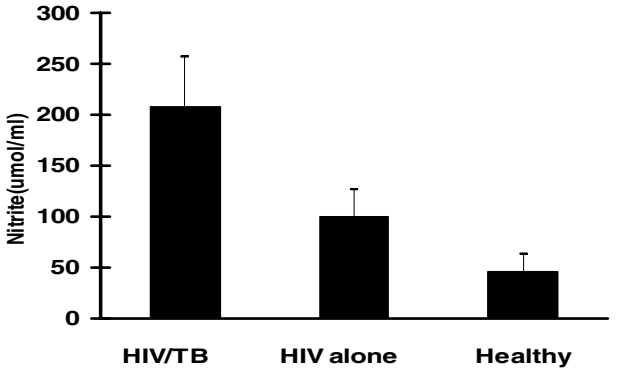

Figure 1
Mean nitrite levels among the HIV/TB coinfected, those with HIV infection alone and healthy controls were 207.6 ± 48.8 nmol/ml, 99.7 ± 26.5 nmol/ml and 46.4 ± 16.2 nmol/ml, respectively. Levels among the HIV/TB coinfected were significantly higher than the other two groups ($p < 0.01$) and those among patients with HIV infection alone were significantly higher than those of healthy controls ($p < 0.05$).

CD4 counts

To determine CD4+ T cell population, 10 ul of anti-human CD4-FITC (Sigma Immunochemicals) was added to 100 ul of whole blood and incubated at room temperature for 15 min. At the end of the incubation, lysing solution was added and incubated for 10 min at room temperature. The washed and fixed cells were then analyzed on the flowcytometer (FACScan, Becton Dickinson, Mountain View, CA). Ten thousand cells were computed and analyzed using Cell Quest program. Dead cells were excluded by forward and side scatter gating. Statistical markers were set using irrelevant isotype-matched controls as reference.

Statistical analysis

The results in the groups were normally distributed as shown by Shapiro-Wilk W test and were analyzed by two-way analysis of variance (ANOVA). Pearson's correlation coefficient was calculated to determine correlation between serum level of nitrite and citrulline and CD4 counts. A p value less than 0.05 was considered significant.

Results

Clinical profile of the two groups of patients and healthy individuals is shown in Table 1. The patients were both out patients and hospitalized ones. They were well matched for age and sex and none of them had evidence of renal insufficiency as shown by normal urine examination and normal blood urea and creatinine values. The mean CD4 counts of HIV/TB coinfected were significantly lower than those of patients with HIV infection alone ($p < 0.05$).

Serum nitrite levels are shown in figures 1 and 2. Mean nitrite levels among the HIV/TB coinfected were 207.6 ± 48.8 nmol/ml and in those with HIV infection alone these were 99.7 ± 26.5 nmol/ml. Healthy controls had mean nitrite levels of 46.4 ± 16.2 nmol/ml. The levels among the HIV/TB coinfected were significantly higher than the other two groups ($p < 0.01$). Levels among patients with HIV infection alone were significantly higher than those of healthy controls ($p < 0.05$). Following 4 weeks of chemotherapy nitrite levels declined to 144.5 ± 34.4 nmol/ml among the HIV/TB coinfected. These were significantly lower than at start of therapy ($p < 0.05$). There was no correlation between CD4 counts and nitrite levels.

Serum citrulline levels are shown in figures 3 and 4. Mean serum citrulline levels in the HIV/TB coinfected were 1446.8 ± 468.8 nmol/ml and those with HIV infection alone were 880.8 ± 434.8 nmol/ml. Healthy controls had levels of 486.6 ± 212.5 nmol/ml. Levels among HIV/TB coinfected were significantly higher than the other two groups ($p < 0.01$) and those with HIV infection alone had significantly higher levels than controls ($p < 0.05$). Therapy for TB among the HIV/TB coinfected for 4 weeks resulted in a significant decline to 1116.2 ± 388.6 nmol/ml ($p < 0.05$). There was no correlation between CD4 counts and citrulline levels.

Discussion

Our results showing that NO production is elevated among patients with HIV infection and still higher in those with coinfection with TB suggest a role for NO

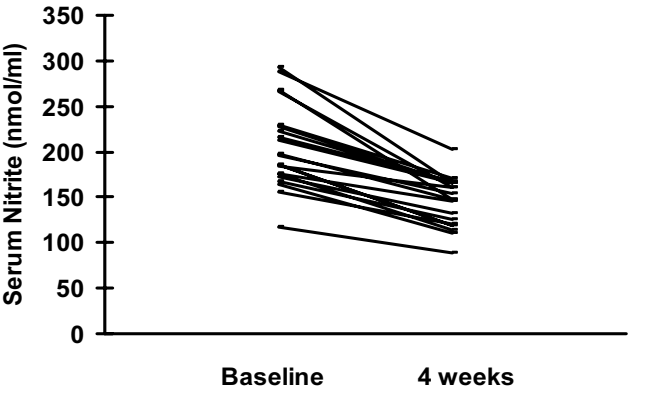

Figure 2
Four weeks of chemotherapy led to decline of nitrite levels from 207.6 ± 48.8 nmol/ml to 144.5 ± 34.4 nmol/ml among the HIV/TB coinfected. These were significantly lower than at start of therapy ($p < 0.05$).

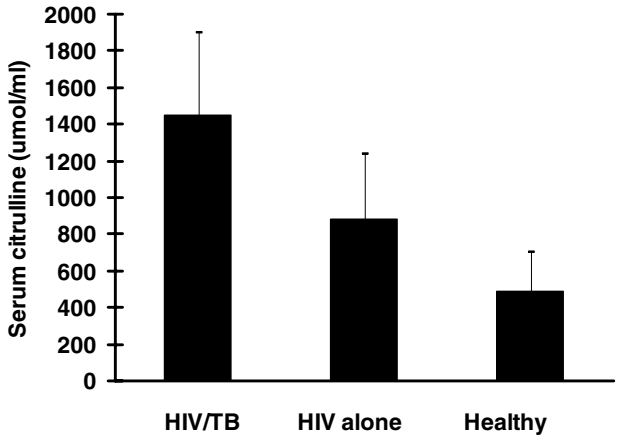

Figure 3
Mean serum citrulline levels in the HIV/TB coinfected, those with HIV infection alone and healthy controls were 1446.8 ± 468.8 nmol/ml, 880.8 ± 434.8 nmol/ml and 486.6 ± 212.5 nmol/ml, respectively. Levels among HIV/TB coinfected were significantly higher than the other two groups ($p < 0.01$) and those with HIV infection alone had significantly higher levels than controls ($p < 0.05$).

among these individuals. Further, we have demonstrated a decline in NO production among the HIV/TB coinfected following four weeks of antitubercular therapy.

NO has been shown to modulate several immune functions.[24,25] There have, however, been some contrasting effects attributed to NO. It upregulates proliferation and increases glucose uptake by T lymphocytes, while other reports have suggested that it inhibits T cell activation.[26,27] HIV infected patients are known to have elevated levels of proinflammatory cytokines, especially tumor necrosis factor (TNF)-α.[11] These are powerful activators of HIV replication.[28] Cytokines produced in response to cell activation can to stimulate iNOS in a autocrine or paracrine manner.

The significance of NO production in HIV infection has been well established. There is an accumulation of NO metabolites, nitrite and nitrate, in individuals who have neurological complications.[29] Nitrite and citrulline levels are elevated among those HIV infected individuals who have no opportunistic infection.[11] There is also evidence to show an association between high levels of virus load and increase NO production in the serum of HIV infected patients.[30] Consequently, production of large amounts of NO by macrophages could be a leading cause of lymphocyte inactivation and induction of persistent immunosuppression.[31]

Evidence for the role of iNOS in TB comes from several studies. NO has been demonstrated to kill 99% Myco. tub. in culture in two days at a concentration of 90 ppm.[32] iNOS inhibitors exacerbate infection in macrophages and mice treated during acute or chronic phase of disease.[33] Myco tub grows rapidly and kills mice that have been rendered selectively deficient in iNOS.[34]

Elevated levels of NO in HIV infection may be due to cytokines, mainly TNF-α and interleukin-6.[35] Both these cytokines play an important role in TB too as NO levels may be stimulated directly by Myco. tub.[36] It is likely that there is some cumulative effect of the increase and, hence, it is easy to see why NO levels are still higher in individuals with the coinfection as demonstrated in this study. It is pertinent to note that NO is involved in HIV-1 replication, especially that induced by TNF-α.[1] There might, therefore, be a self perpetuating mechanism wherein HIV replication leads to increased NO production and the latter increases the rate of HIV multiplication.

Effect of four weeks of therapy in reducing NO production is significant in view of the fact that this coincides with the onset of clinical response. There is reduced immune activation at that time.[20] There are significant clinical implications of this for therapy. Thus, while chemotherapy cures Myco. tub. infection in immunocompetent mice it fails to do so in iNOS-deficient mice.[38] Further, some commonly used drugs that are tuberculocidal in vitro are comparably effective in vivo only with the help of iNOS.[38] In effect chemotherapy might be more beneficial to immunocompromised hosts if accompanied by delivery of a source of NO. Sensitization of tubercle bacilli to NO might reduce the time for chemotherapeutic bene-

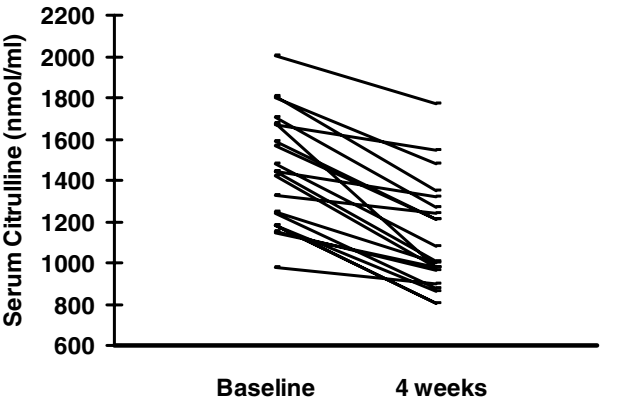

Figure 4
Therapy for TB among the HIV/TB coinfected for 4 weeks resulted in a significant decline from 1446.8 ± 468.8 nmol/ml to 1116.2 ± 388.6 nmol/ml ($p < 0.05$).

fit in immunocompetent hosts and might bring down the incidence of treatment interruption and consequent drug resistance. Since all the patients in the study received rifampicin, the role of the latter as an anti-inflammatory agent needs some consideration. Rifampicin activates the human glucocorticoid receptor.[39] Transient expression of wild-type, deleted or mutated glucocorticoid receptors and sucrose density gradient sedimentation studies have suggested that the drug binds to the receptor with the physiological consequence that this antibiotic acts as an immunosuppressive agent.[39] Four weeks can produce an anti-inflammatory effect to be produced enough to reduce the level of immune activation reflecting in a decrease in NO production. This is likely to have been abetted by a decrease in the level of immune activation due to a decrease in bacillary load and antigenic stimulation.

In conclusion, we have demonstrated that NO production is elevated among patients with HIV infection and is still higher in those with coinfection with TB and that these levels decline with four weeks of anti-tubercular therapy. Taken along with other studies mentioned previously there may be a case to suggest that NO plays a deleterious role in HIV infection. It would be interesting to see what effect NOS inhibitors might produce if combined with standard antitubercular therapy in HIV/TB coinfected.

Authors Contributions

Author 1 AW conceived the study, compiled the clinical data, analyzed part of the data and drafted the manuscript

Author 2 AB carried out the flowcytometry work for the study

Author 3 MK carried out the spectrophotometric analysis for the study

Author 4 AS participated in design of the study and analyzed part of the data

Author 5 PB participated in the design and coordination of the study

Author 6 SS participated in the design and coordination of the study

Competing interests

None for all the authors

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