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Research Article

EFFECTS OF NITRIC OXIDE AND NITROTYROSINE ON MITOCHONDRIA IN TUBERCULOSIS AND MILIARY TUBERCULOSIS

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ABSTRACT

Background: Due to increasing incidence of human immunodeficiency virus infections, the incidence of tuberculosis is increasing. Haematogenous spread of tubercle bacilli results in miliary tuberculosis. Endotoxins and cytokines thus released in blood. These are part of innate immunity and may further stimulate synthesis and secretion of other mediators of innate immunity like nitric oxide (NO^o) indifferent cells including platelets. Hence present study was carried out to know the effects of plasma NO^o and cellular nitrotyrosine on mitochondrial proteins, total and available thiols and mitochondrial complex activities in tuberculosis.

Method: The platelets were separated from whole blood. Mitochondria were isolated from 70 experimental and 40 control samples and mitochondrial lysate was used to study above parameters

Results: In present study plasma NOx and Nitrotyrosine levels and consequently protein carbonyl levels were increased significantly and mitochondrial thiols and complex activities were decreased significantly ($P<0.001$) in miliary tuberculosis. However no significant changes were observed in tuberculosis.

Conclusion: Thus to conclude the present study showed increased nitric oxide synthesis and nitrotyrosine which oxidised mitochondrial proteins forming protein carbonyl. There was also decrease in mitochondrial thiols and mitochondrial complex activities. These effects were highly significant in military tuberculosis and were not significant in tuberculosis without military appearance.

INTRODUCTION

Tuberculosis is known to human since antiquity. Even today, despite many developments in the field of medicine, tuberculosis remains major cause of health problem. It was found that, with one third of the world population being infected with Mycobacterium tuberculosis. Due to increasing incidence of human immunodeficiency virus (HIV) infections, the incidence of tuberculosis is increasing. Classic tuberculosis is the result of infection with Mycobacterium tuberculosis, and atypical tuberculosis is the result of infection with atypical mycobacteria. The pathology and course of the disease depends on the sensitivity of the host. Primary tuberculosis is the first infection in an unsensitised host, and secondary, post primary, or chronic tuberculosis results from reactivation of previously acquired infection or, rarely, reinfection of a sensitized host. Haematogenous spread of tubercle bacilli results in miliary tuberculosis[1]. In children it is often the consequence of primary infection of old disseminated foci. The lesions are usually yellowish granulomas 1-2 mm in diameter that resemble millet seeds. So the term miliary was coined by pathologists in nineteenth century. There is initial activation of alveolar macrophages which contribute to nonspecific or innate immune response. It is followed by specific or cell mediated immune response. Nitric oxide (NO°) is important mediator of innate immunity and is thought to be involved in killing tubercle bacilli inside the macrophages.[2] When immune cells are stimulated by pathogens or antigens, they secrete cytokines. Many of the cytokines stimulate inducible nitric oxide synthase (iNOS) in variety of cells to produce NO°. The platelets are one of them and are rich in mitochondria.. NO° when synthesised in high concentration oxidises various biomolecules in the vicinity. Mitochondria is the major target of NO° and is also a site for superoxide generation. NO° reacts with superoxide to form peroxynitrite which further forms nitrotyrosine.

Platelets are less studied and have role in immunity. So the present study was carried out to see the effect of NO°, nitrotyrosine on mitochondrial proteins and activity of mitochondrial complexes in septicaemia

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MATERIAL AND METHOD

The blood samples under study were collected from 70 patients and 40 normal ones after ethical approval and prior consent. The samples from clinically and radiologically confirmed patients were grouped in two. Group II (n = 32) as patients of miliary tuberculosis and Group III (n= 38) as patients of tuberculosis without military appearance. The samples were collected in tubes with EDTA as an anticoagulant. The platelet coat was isolated after centrifugation and platelets were lysed. Mitochondria were isolated[3]. Mitochondria were lysed and lysate was used for estimation of proteins[4,] protein carbonyl[5] and mitochondrial complex I,II,III and IV activity[6-8], total and available thiol concentrations[9]and fornitrothiol[10] estimation. The cell lysate was used for estimation of nitrotyrosine[11]. NOx levels were estimated in plasma samples[12,13].

The samples were run in duplicate and for each sample; the mean of the two values was taken. The statistical significance was calculated by Mann –Whitney U test by using NCSS-PASS statistical software. Statistical significance was chosen as P< 0.05 and highly significant as P< 0.001.

Table 1 Shows no. of groups and number of participants

Groups	Number
Group I (control)	40
Group II (miliary tuberculosis)	32
Groups III (tuberculosis without millet appearance)	38

Table 2 Shows parameters estimated in the present study and the methods used for the estimation [9-18].

Parameters	Method
Plasma NOx concentration	Moshage et al and Granger et al.
Nitrotyrosine	Crow and Ischiropoulos
Nitrothiols	Cook et al
Mitochondrial total thiol	Modified Habeeb
Mitochondrial available thiol	Modified Habeeb
Mitochondrial complex I activity	Zeng et al
Mitochondrial complex II activity	Darley Usmar et al
Mitochondrial complex III activity	Zeng et al
Mitochondrial complex IV activity	Benecke et al

OBSERVATION AND RESULTS

Table 3 showing activities of plasma NOx, Nitrotyrosine, in control and patients with military tuberculosis and tuberculosis without millet appearance

Groups	Plasma NOx (µmol/L) Mean ± SD	Nitrotyrosine (µmol/L) Mean ± SD
Group I	33.52 ± 5.58	Not detected
Group II	51.39* ± 2.66	13.36** ± 4.61
Groups III	38.04 ± 6.38	Not detected

(P < 0.05* significant, P < 0.001** highly significant)
Nitrothiols were not detected in experimental as well as control samples.

DISCUSSION

Tuberculosis is a major infectious disease responsible for high mortality throughout the world. It is a condition of severe infection and inflammation. This inflammation in response to infection may inhibits mitochondrial functions in many cells. Platelets play important role in immunity. Cytokines are produced as a result of immune responce which stimulate iNOS activity in platelets. There is increased production of NO° as seen in table 3, as it is a important mediator of activated immunity against pathogens and transformed self-cells. However, excess NO° is toxic to normal, healthy cells also. In the present study the levels of plasma NOx were not increased in group III. This could be because it includes patients with highly localised infection and may not lead to activation of wide spread immune response. However the levels were found tobe increased in group II. As miliary tuberculosis is characterised by haematogenous spread of tubercle bacilli from primary focus of infection, the spread may expose the bacilli to circulating immune cells leading to activation of the cells and synthesis of TNF α. The consequent activation iNOS in all cells including platelets leads to increased production of NO°. It is known that mitochondria is the major site for generation of superoxide. NO° reacts with superoxide to form peroxynitrite. It is highly potent anion causes nitrosylation and oxidation of proteins, react with iron sulphur centers, oxidise thiol groups and nitrosylate tyrosine residues leading to formation of nitrotyrosine. Nitrotyrosine which is footprint of increased plasma NOx is not produced and detected in group III while it is increased significantly in group II. This nitrotyrosine along with NO° modify mitochondrial thiols. Thus there was 83% decrease in total thiols and 85% in available thiols in group II as compared to normal.

Table 4 showing activities of Protein carbonyl, Total and available thiols in control and patients with military tuberculosis and tuberculosis without millet appearance

Groups	Protein carbonyl (nmol/mg) Mean ± SD	Total thiol level (nmol/mg) Mean ± SD	Available thiol level (nmol/mg) Mean ± SD
Group I	0.96 ± 0.17	1.58 ± 0.36	1.2 ± 0.30
Group II	1.19* ± 0.16	1.30* ± 0.36	1.02 ± 0.29
Groups III	1.04* ± 0.21	1.49 ± 0.38	1.15 ± 0.29

(P < 0.05* significant, P < 0.001** highly significant)

Table 5 showing activities of mitochondrial complexes in control and patients with military tuberculosis and tuberculosis without millet appearance

Groups	Activity of mitochondrial complexes in nmol/min/mg of mitochondrial proteins			
	Complex I Mean ± SD	Complex II Mean ± SD	Complex III Mean ± SD	Complex IV Mean ± SD
Group I	32.13 ± 3.08	38.34 ± 4.30	140.61 ± 11.18	58.82 ± 04.51
Group II	28.52* ± 2.44	36.54* ±6.23	140.94 ± 05.38	44.05** ±4.30
Groups III	30.75 ± 2.27	37.41 ± 5.91	140.82 ± 5.02	60.37 ± 4.01

(P < 0.05* significant, P < 0.001** highly significant)

This was significant decrease in thiols in group II however this decrease was slight in group III. This may be due to oxidative modification of mitochondrial proteins. Protein oxidation leads to formation of protein carbonyl. In group II the levels of protein carbonyl were increased 125% while that in group III are increased 119% when compared with group I. Thus in both the groups NO° may be responsible for protein carbonyl formation in platelet mitochondria. This was in agreement with previous work [21,22]. Significant increase in plasma NOx and protein carbonyl supports this fact[14,15]. However nitrothiols were not detected in control as well as experimental group probably because of its unstable nature. As per table 5 there was negative correlation between plasma NOx concentration and mitochondrial complex I, II and IV activity in group II and no such correlation in group III. However there was no

correlation between plasma NOx concentration and mitochondrial complex III in both the groups. Mitochondrial complex I activity was not decreased in group III and was decreased about 89% in group II thus decrease in complex I activity is significant in groupII. Mitochondrial complex II activity is not decreased in group III and is decreased significantly in groupII. Mitochondrial complex III activity was not affected in both the groups II and III. Mitochondrial complex IV activity was not affected in group III while it was decreased about 75% in group II and was highly significant. Thus in group III, Plasma NOx was found tobe normal and nitrotyrosine was not detected. Protein carbonyl levels were increased while thiol status of mitochondrial proteins was not affected and nitrothiols were not detected in platelet mitochondria. Platelet mitochondrial complex I, II, III and IV activities were in normal range. This could be because the infection and hence the immune response was restricted to one particular site in group III. While in group II there was increased plasma NOx and nitrotyrosine was detected. Platelet mitochondrial protein carbonyl levels were significantly increased and total thiols were significantly decreased although nitrothiols were not detected, indicating protein damage. Platelet mitochondrial complex I, II and IV activities were decreased significantly[16,17]. This could be because group II includes patients of military tuberculosis. It is characterised by haematogenous spread of mycobacterium tuberculosis where exposure of bacteria to immune cells may activate cells to release cytokines. These cytokines may stimulate iNOS to produce NO° in large amount. Thus inhibitors of iNOS may be used in miliary tuberculosis to control production of NO° and nitrotyrosine and avoid its effects on mitochondrial proteins. The elevated levels of NO° and nitrotyrosinecan assist to differentiate of tuberculosis with and without miliary appearance.

Thus it can be concluded that platelet iNOS is activated during miliary tuberculosis, leading to generation of NO° in large amount and subsequent production of peroxynitrite and nitrityrosine. This may be responsible for oxidation of proteins forming protein carbonyl. As a effect of this there is decrease in activity of respiratory chain complexes.

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