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NO° and Mitochondrial Complexes in Sepsis

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A B S T R A C T

Septicaemia is consequence of bacterial infection in the body. In this condition endotoxins and cytokines are released in blood inducing platelet nitric oxide synthase to produce nitric oxide. This further forms peroxynitrite and nitrotyrosine which modifies biomolecules. Mitochondrial proteins and complexes may be affected thus affecting their functions. Hence present study was carried out to know the effects of plasma NO°, cellular nitrotyrosine on mitochondrial proteins, total and available thiols and mitochondrial complex activities. The platelets were separated from whole blood. Mitochondria were isolated from 60 experimental and 40 control samples and above parameters were studied on mitochondrial lysate obtained from mitochondria. It was observed that plasma NOx and Nitrotyrosine levels and consequently protein carbonyl levels were increased significantly. As a result of this, significant decrease in mitochondrial thiols and complex activities was observed. These changes were significant in septicaemia without shock and highly significant ($P<0.001$) in septicaemia with shock. Thus it can be concluded from the present study that increased NO° and nitrotyrosine oxidatively modify mitochondrial proteins forming protein carbonyl. This results in decrease in mitochondrial thiols and mitochondrial complex activities. These effects were significant in septicaemia without shock and highly significantly in septicaemia with shock.

Introduction

Septicaemia is fairly common throughout the world. It is more common in third world countries like India owing to poor hygienic conditions septicaemia occurs when a bacterial infection enters the bloodstream. Untreated septicaemia can quickly progress to sepsis. Sepsis causes millions of deaths globally each year (Dellinger *et al.*, 2008). Sepsis is defined as clinical evidence suggestive of infection plus signs of systemic response to infection like tachypnea, tachycardia, hyperthermia or

hypothermia. It is a serious complication of an infection characterized by inflammation throughout the body (Bone *et al.,* 1992; Fry, 2012). This inflammation can cause blood clots and block oxygen from reaching vital organs, resulting in organ failure and death in some severe cases. Overt systemic inflammation in response to infection may inhibits mitochondria. As a consequence, organs become unable to produce enough energy to maintain their normal activities: the organs enter into a hypometabolic state and lose their functions (Singer *et al.,* 2004; Protti and Singer, 2007). Sepsis may also lead to a drop in blood pressure, resulting in shock sepsis is caused by the immune system's response to a serious infection, most commonly bacteria, but also fungi, viruses, and parasites in the blood, urinary tract, lungs, skin, or other tissues. It is now clearly established that bacterial endotoxin, a lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria, is the major mediator of the high morbidity and mortality rates characteristic of Gram-negative septic shock. Endotoxins produced from bacteria and cytokines, particularly TNF, IL-1 and IL-6, can activate the procoagulation factors in the endothelium, leading to endothelial damage. This damaged endothelial surface inhibits anticoagulant properties as well as increases antifibrinolysis, which can lead to intravascular clotting, microvascular thrombosis and multiple organ failure (Marianne and Brilli, 2003).

In the advanced stage it is associated with shock thus increasing mortality. Approximately 20–35% of people with severe sepsis and 30–70% of people with septic shock die. When immune cells like macrophages, T cells, B cells etc. 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°. Circulating platelets are rich in mitochondria and can be obtained easily even from critically ill patients (Alessandro Protti *et al.,* 2015). They are best involved in hemostasis however they also have role in immunity. NO° when synthesised in high concentration acts as bactericidal, fungicidal and viricidal. It is oxidant and reacts with various biomolecules in the vicinity. Mitochondria are the major target of NO° however it cannot act all alone. It reacts with superoxide also generated at the same site to form peroxynitrite. It also has oxidant property and mediates the alterations in the vascular system and the tissue damage observed in septic shock and multiple organ failure.

Platelets are involved in immunity, have large number of mitochondria, can be obtained easily even from critically ill patients and less studied in sepsis. In this view the present study was carried out to see the effect of NO°, nitrotyrosine on mitochondrial proteins and activity of mitochondrial complexes in septicaemia

Materials and Methods

The blood samples under study were collected from 80 patients of sepsis and 40 normal ones after ethical approval and prior consent. The samples from patients were grouped in two. Group II (n = 40) as patients of sepsis without shock and Group III (n= 40) as patients of sepsis with shock. 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 (Wolfe and Shulman, 1969). Mitochondria were lysed and lysate was used for estimation of proteins (Hudson and Hay, 1989), protein carbonyl (Levine *et al.,* 1990) and mitochondrial complex I, II, III and IV

activity (Zeng *et al.,* 1990; Darley Usmar *et al.,* 1984; Benecke *et al.,* 1993), total and available thiol concentrations (Habeeb, 1972) and fornitrothiol (Cook *et al.,* 1996) estimation. The cell lysate was used for estimation of nitrotyrosine (Crow and Ischiropoulos, 1996). NOx levels were estimated in plasma samples (Moshage *et al.,* 1995).

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 $p < 0.001$.

Results and Discussion

Approximately 20–35% of people die with severe sepsis and 30–70% of people with septic shock. Thus it is a serious complication of an infection characterized by inflammation throughout the body. This inflammation in response to infection may inhibits mitochondrial functions in many cells. Platelets are important circulating cells rich in mitochondria and known to be involved in immunity. During immune response, cytokines produced, stimulate iNOS activity in platelets (Lorente *et al.,* 1993; Ochoa *et al.,* 1991). This results in increased production of NO°. In the present study the levels of plasma NOx were found to be increased seven times in septicaemia without shock while it is 1000% in septicaemia with shock. This may be responsible for hypotension observed in sepsis. It is known that mitochondria are the major site for generation of superoxide. NO° reacts with superoxide to form peroxynitrite. It is highly potent and oxidises proteins, react with iron sulphur centers, oxidise thiol groups and nitrosylate tyrosine residues leading to formation of nitrityrosine. Nitrotyrosine which is footprint of increased plasma NOx is also significantly increased. This nitrotyrosine along with NO° modify mitochondrial thiols. Thus there is slight decrease in total thiols in group II as compared to group I however it is significant in group III. The levels of available thiols in the study are decreased significantly in group II and highly significantly 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 are increased 144% while that in group III are increased 240%. When compared with group I. This is in agreement with previous work (Kantrow *et al.,* 1997; Isabella Dalle-Donne *et al.,* 2003). Significant increase in plasma NOx and protein carbonyl supports this fact. However nitrothiols are not detected in control as well as experimental group probably because of its unstable nature.

There is negative correlation between plasma NOx concentration and mitochondrial complex I, II and IV activity in septicaemia without shock and septicaemia with shock. However there is no correlation between plasma NOx concentration and mitochondrial complex III in septicaemia without shock but again negative correlation between two in septicaemia with shock and is highly significant in septicaemia. Mitochondrial complex I activity is decreased about 89% in sepsis without shock and is decreased 58% in sepsis with shock thus decrease in complex I activity is significant in septicaemia without shock and is highly significant in septicaemia without shock. Mitochondrial complex II activity is decreased about 91% in sepsis without shock and is decreased 51% in sepsis with shock thus decrease in complex II activity is significant in septicaemia without shock and is highly significant in septicaemia without shock. Mitochondrial complex III activity is

not affected in sepsis without shock and is decreased 96% in sepsis with shock thus decrease in complex III activity is not significant in septicaemia without shock and is significant in septicaemia with shock. Mitochondrial complex IV activity is decreased about 88% in sepsis without shock and is decreased 60% in sepsis with shock thus decrease in complex IV activity is significant in septicaemia without shock and is highly significant in septicaemia without shock. I, II, III and IV in group II and group III. Inhibitors of iNOS may be used to control production of NO° and nitrotyrosine and avoid its effects

Thus it can be concluded that platelet iNOS is activated during sepsis, 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 and decrease in total and available thiols. As a result of this there is decrease in activity of respiratory chain complexes.

Table.1 Shows no. of groups and number of participants

Groups	Number
Group I (control)	40
Group II (septicaemia without shock)	40
Groups III (septicaemia with shock)	40

Table.2 Shows parameters estimated in the present study and the methods used for the estimation (Hudson and Hay, 1989; Levine *et al.*, 1990)

Parameters	Method
Plasma NOx concentration	Moshage *et al.* (1995) and Granger *et al.* (1996)
Nitrotyrosine	Crow and Ischiropoulos (1996)
Nitrothiols	Cook *et al.* (1996)
Mitochondrial total thiol	Modified Habeeb (1972)
Mitochondrial available thiol	Modified Habeeb (1972)
Mitochondrial complex I activity	Zeng *et al.* (1990)
Mitochondrial complex II activity	Darley Usmar *et al.* (1984)
Mitochondrial complex III activity	Zeng *et al.* (1990)
Mitochondrial complex IV activity	Benecke *et al.* (1993)

Table.3 showing activities of plasma NOx, Nitrotyrosine, in patients with sepsis and control

Groups	Plasma NOx (µmol/L) Mean ± SD	Nitrotyrosine (µmol/L) Mean ± SD
Group I	33.52 ± 5.58	Not detected
Group II	46.07* ± 15.42	15.11** ± 3.65
Groups III	337.31** ± 66.63	48.73** ± 9.07

($P < 0.05$* significant, $P < 0.001$** highly significant)
Nitrothiols were not detected.

Table.4 showing activities of protein carbonyl, total and available thiols in patients with sepsis and control

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	1.37* ± 0.70	1.44 ± 0.31	1.08 ± 0.26
Groups III	2.30 ** ± 1.42	1.17 ** ± 0.44	0.82** ± 0.33

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

Table.5 showing activities of mitochondrial complexes in patients with sepsis and control

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	28.74* ± 3.39	35.18* ±7.01	140.31 ± 05.06	52.03* ±10.84
Groups III	18.67** ± 1.19	19.74** ± 2.12	134.64* ± 11.16	35.30** ± 10.49

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

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