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Quantification of NETs formation in neutrophil and its correlation with the severity of sepsis and organ dysfunction

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ABSTRACT

Background: Previous study from this lab has discerned oxidative, nitrosative stress and their relationship with cytokines contributing to the severity of sepsis and organ dysfunction. Cytokines are known to induce neutrophil extracellular traps (NETs) formation via free radicals generation. Hyper-activation of neutrophil leads to the increased NETs formation or ineffective clearance of NETs would likely increase the risk of auto-antibody generation against NETs components and being partly responsible for the sepsis severity and organ dysfunction. The present study was undertaken to further assess the status of NETs formation and their correlation with severity of sepsis, with the cytokines and organ dysfunction.
Methods: The level of NETs formation, DNA release, elastase release, and inflammatory cytokines was determined in 80 sepsis patients and 45 healthy volunteers. Their linearity with organ parameters and associations with sepsis severity were also assessed.
Results: NETs formation experiment was carried out and it was significantly higher in sepsis (70%) compared to control (30%). NETs % were positively correlated with severity of sepsis and organ dysfunction. Pearson's correlation coefficient demonstrated a direct relation between NETs components and organ parameters with Sepsis severity scores.
Conclusion: NETs formation is significantly higher due to which it is contributing to the sepsis severity and organ failure.

1. Introduction

Neutrophils are the crucial part of the innate immune system being one of the primary cells to defend the host. During the episode of infections, it migrates from blood vessels to the site of infections, where it executes destruction of pathogens by employing numerous mechanism such as phagocytosis and degranulation [1]. Neutrophils contribute to pathogen clearance but, simultaneously, are accountable for hyper-inflammatory state causing tissue damage [2]. A novel mechanism of neutrophils was reported by Brinkmann [3], neutrophil extracellular traps (NETs) formation, to eradicate attacking pathogens. NETs give the impression of being precisely intended to arrest and destroy invading micro-organisms extracellularly. NETs comprise of decondensed chromatin fibers form a trap shell adorned with antimicrobial factors. It is circulated by the granular protein which releases proteases and generates free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) that destroy different microbes [4], this entire process is termed as NETosis [5]. All through hyper formation of NETs, histones act as damage-associated molecular pattern proteins, triggering the immune system and initiating further cytotoxicity [6].

Sepsis is often manifest as the systemic inflammatory response syndrome (SIRS) begun by the incapability of the host to confine an infection [7]. Consequently, it became an imperative cause of major health economic problem, through more patient deaths in ICU of hospitals due to sepsis related complications [8]. One of the major cause of mortality in sepsis patients is organ dysfunction [9]. Literature suggest that approximately 33% of sepsis patients have at least one organ (kidney, lung, liver) failure (severe sepsis; [8]) where neutrophil hyperactivation causes tissue damage [10,11]. Even though the role of hyper-activated neutrophils in the sepsis physiology is known for decade now [1], the destructive aspects of neutrophil functions as NETs formation has been underrated due to the poor knowledge of its

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mechanism.

ROS/RNS are the free radicals produced by normal metabolism in the aerobic condition [11]. Activated neutrophils produce large amounts of superoxide via phagocytic NADPH oxidase complex. Superoxide dismutates to hydrogen peroxide leading to the formation of a variety of toxic oxygen derivatives capable of destroying healthy tissues [12]. High concentration of nitric oxide ($NO^{\cdot}$) in sepsis has been reported, where it generates peroxynitrite which leads to cell cytotoxicity [13]. In our previous studies, we reported that oxidative and nitrosative stress due to the ROS and iNOS-derived $NO^{\cdot}$ in neutrophil with the help of inflammatory mediators also contribute to organ dysfunction which is a major cause of sepsis-induced morbidity [14,15]. Moreover, previous study reports that neutrophils level gets elevated but did not establish augmentation of NETs in sepsis and their relationship with the severity. Role of inflammatory cytokines (Tumor necrosis factor [TNF-α], Interleukin-1Beta [IL-1β], and Interleukin-8 [IL-8]) in eliciting neutrophils oxidative burst and NETs formation has been reported in SIRS patients [16]. Cytotoxic effects of inflammatory cytokines, ROS/RNS directly affects the function of several organ systems in later stages for example, lung injury, liver dysfunction, and kidney failure. Consequently, altogether it is called as multiple organ dysfunction syndrome (MODS) [17], which is ultimately leads to death of patients.

2. Materials and methods

2.1. Selection of patients and healthy subjects

This study was performed in the ICU at SMS Hospital, Jaipur. A total of 80 consecutive adult sepsis patients and 45 healthy subjects (controls) were registered in this study. All of them had a positive diagnosis of sepsis according to the sepsis guidelines [18]. The study design was accepted by the institutional ethics committee board and proper written consent was obtained from each patient as well as control. Patients > 75 y and < 16 y as well as patients who received antibiotics within 72 h of the onset of sepsis were excluded. Pre-existing diabetes or any chronic diseases such as arthritis, cancer was an additional exclusion criterion. The Acute Physiology and Chronic Health Evaluation (APACHE) and the Sepsis-related Organ Failure Assessment (SOFA) score at the time of admission in ICU were the important parameter to evaluate the severity of sepsis.

2.2. Collection of samples and neutrophil isolation

All the patients' data were encrypted, and very much confidential. Blood samples were collected in heparinized (sodium heparin) tube within 2 days of sepsis diagnosis. The collected blood was quickly transported to the laboratory for downstream work. Neutrophils were isolated on a percoll density gradient as described by Kumar et al. [14], and plasma samples were stored at −80 °C.

2.3. NETs experiments

Neutrophils from control and sepsis patients (2×10^6 cells/ml) were seeded on poly-L-Lysine coated petri plates. The cells were washed once with Hank's Balanced Salt Solution (HBSS) and incubated with or without Diphenyleneiodonium chloride (DPI) for 5 min followed by induction with peroxynitrite for 3 h. Cells were fixed with paraformaldehyde and stained with elastase antibody (1:250 dilutions) for overnight. Cells were washed 5 times with HBSS and stained with anti-rabbit alexa fluor 488 antibody (1:10,000 dilutions) for 4 h at room temperature in the dark. This was washed 5 times with HBSS. Anti-fading agent containing Hoechst was added and incubated for 15 min. The sample was loaded onto the confocal microscope (Nikon A1Rsi) and the images were captured at 63× objective lens. As a negative control sample was processed without using primary antibody.

NETs sample was centrifuged at 10,000 rpm for 10 min and cell free suspension was collected. Elastase was estimated in the liquid as per manufacturer's instructions mentioned in the elastase assay kit (Sigma Aldrich). Similarly, cell free DNA was assessed using Sytox green dye (Invitrogen) after treated with 500 m unit per ml of micrococcal nuclease. Plasma samples of both healthy volunteers and patients with sepsis were used to estimate the levels of TNF-α, IL-8 and IFN-γ according to the company's information mentioned in the ELISA assay kit (Sigma Aldrich).

2.4. Statistical analysis

The complete patient data were stored by using Excel (Microsoft Corp.). Data are presented as means ± SD and are the result of minimum four independent experiments. Pearson correlation coefficient was used to assess the correlation between the continuous variables. Statistical significance was considered at $P < .05$.

3. Results

3.1. Comparison of demographic, hematological and biochemical between sepsis patients and controls

Table 1 shows the demographics features and the baseline laboratory findings for the sepsis patients and controls Amongst sepsis patients and controls, the changes in age, sex ratio, respiratory rate, heart rate, MAP, WBC counts, neutrophil counts, pH, HCO_3, total bilirubin,

Table 1
Demographic, hematological and biochemical characteristic of sepsis patients and controls (mean ± standard deviation).

	Control (n = 45)	Sepsis (n = 80)	Reference ranges	P-value
Age (y)	40 ± 11	49 ± 17		NS
Temperature (°C)	37.18 ± 0.39	38.35 ± 0.42		–
Male/female ratio	34/11	58/22		NS
Respiratory rate (breaths/min)	19 ± 1	28 ± 3	12–20	< 0.001
Heart rate (beats/min)	82 ± 4	111 ± 10	60–100	< 0.001
MAP (mm Hg)	77 ± 7	92 ± 14	70–100	< 0.001
WBC counts ($10^3/mm^3$)	7.9 ± 0.63	16.9 ± 1.83	4–11	< 0.001
Neutrophil count (%)	67 ± 3	84 ± 7	40–75	< 0.001
Platelets ($10^6/mm^3$)	273 ± 18	151 ± 9	140–450	0.001
pH	7.39 ± 0.05	7.12 ± 0.05	7.35–7.45	< 0.001
HCO_3	30.39 ± 1.5	19.79 ± 1.5	21–28	< 0.001
Total Bilirubin (μmol/l)	18.1 ± 1.2	30.65 ± 2.3	10–70	< 0.001
Creatinine (μmol/l)	140 ± 5	197 ± 5	70–140	< 0.001
Creatinine clearance	–	0.208 ± 0.053		
TNF-α (pg/ml)	5.33 ± 0.44	166 ± 10.32		< 0.001
IFN-γ (pg/ml)	4.15 ± 0.17	142.87 ± 5.29		< 0.001
IL-8 (pg/ml)	3.78 ± 0.187	139.47 ± 6		< 0.001

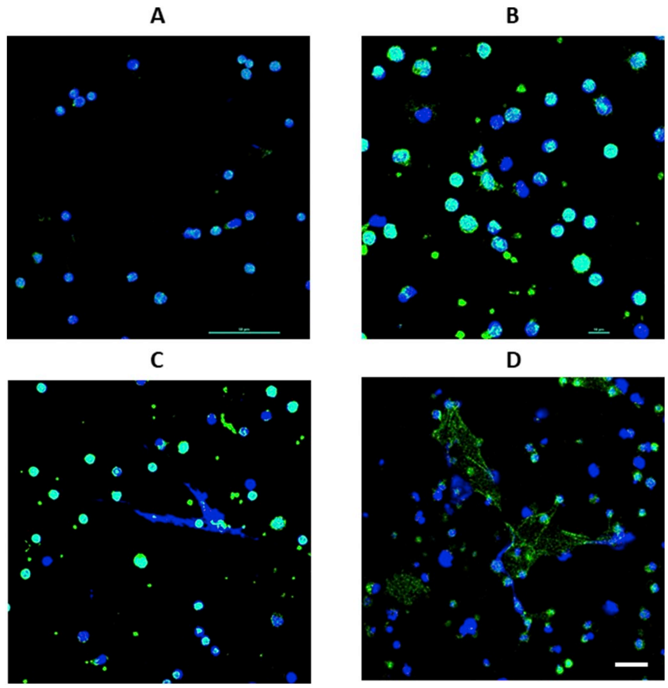

Fig. 1. (A) Control resting neutrophils (B) Sepsis resting neutrophils (C) Control activated neutrophils (D)Sepsis activated neutrophils. Bars represent 10µm.

creatinine, TNF-α, IFN-γ and IL-8 were significantly higher in sepsis patients while platelets was significantly reduced (Table 1).

3.2. Assessment of NETs % formation

Incidence of NETs release was measured in neutrophils of control and sepsis patient using confocal microscopy. NETs release was characterized by elastase in green and DNA in blue color. Under resting conditions, neutrophils isolated from controls were multilobulated (Fig. 1A). However, neutrophils isolated from sepsis patients were larger than neutrophils isolated from controls (Fig. 1B). Upon activation with peroxynitrite for 180 min neutrophils of sepsis patient released more NETs (Fig. 1D) compared with control (Fig. 1C). Percentage of NETs formation was estimated in neutrophils of control and sepsis patient. The NETs formation activity was found to be significantly higher ($P < .001$) in neutrophils of sepsis patient (70%) as compared to the control (30%) (Fig. 2A). The components of NETs including DNA and elastase were estimated in neutrophils of control and sepsis patient. The DNA release was significantly higher in neutrophils of sepsis patient (22 µg/ml) compared to the control (7 µg/ml) ($P < .001$) (Fig. 2B). Similarly, significant ($P < .001$) increase in elastase was reported in neutrophil of sepsis patient (14%) compared to the control (5%) (Fig. 2C).

3.3. Assessment of organ parameters

In sepsis patients, the PaO_2/FiO_2 ratio was used to assess lung function and it was significantly lower 300.38 [21.09]. Creatinine clearance is a potent parameter to assess kidney function showing significant value 0.208 [0.05] ml/min/kg. Total bilirubin is the clinically used marker for liver function and it was also significant higher in sepsis patients 30.65 [2.3]. SOFA 9.4 [1.9] and APACHE II 24.5 [4.2] scores were significantly higher in sepsis. The concentration level of TNF-α, IFN-γ and IL-8 were evaluated through ELISA in plasma of controls and sepsis patients. In sepsis patients and controls, these were TNF-α 166 vs 5.33, IFN-γ 142 vs 4.15 and IL-8139 vs 3.78 pg/ml, respectively, on the day of ICU admission (Table 1). The difference in

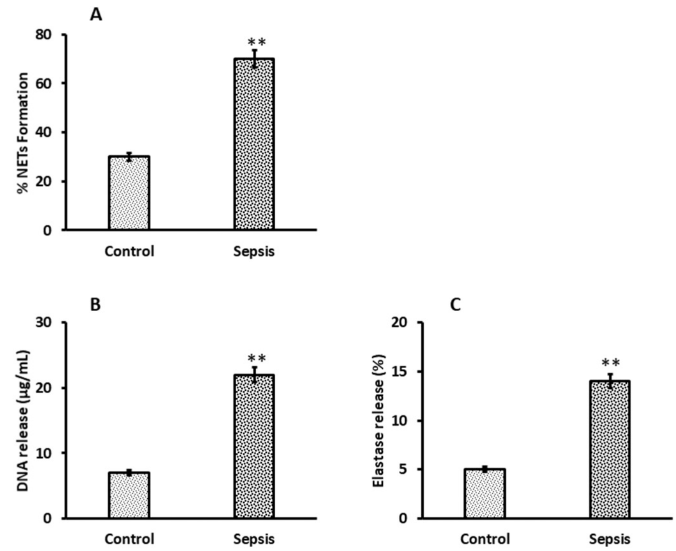

Fig. 2. Bar diagram showing mean NET % level in sepsis and control.(B) Bar diagram showing mean DNA release levels in microgram per litre in sepsis and control. (C) Bar diagram showing mean elastase level in sepsis and control. Differences in mean plasma levels among each group were statistically significant (**$P < 0.01$).

these cytokines values in plasma of control and sepsis patients was statistically significant ($P < .001$).

3.4. Assessment of correlation between NETs components and selected parameters

The SOFA and APACHE II score recorded at the time of admission in ICU correlated positively with NETs formation ($r^2 = 0.908$; $r^2 = 0.281$, $P < .001$) as determined by Pearson's correlation analysis. The NETs formation in the early stage of sepsis were correlated with different organ parameters such as PaO_2/FiO_2 ratio, creatinine clearance and total bilirubin. Pearson's correlation coefficient showed a negative relation between PaO_2/FiO_2 ratio, creatinine clearance and NETs formation ($r^2 = 0.775$; $r^2 = 0.605$, $P < .001$), whereas linear relation with total bilirubin content ($r^2 = 0.666$, $P < .001$). NETs formation in the initial stage of sepsis were positively correlated with TNF- α ($r^2 = 0.691$, $P < .001$), IFN-γ level ($r^2 = 0.559$, $P < .001$) and IL-8 ($r^2 = 0.570$, $P < .001$) (Table 2).

Furthermore, SOFA and APACHE II correlated positively with DNA release ($r^2 = 0.830$; $r^2 = 0.274$, $P < .001$) as determined by Pearson's correlation analysis. The DNA release were correlated with different organ parameters such as PaO_2/FiO_2 ratio, creatinine clearance and total bilirubin. Pearson's correlation coefficient showed a negative relation between PaO_2/FiO_2 ratio, creatinine clearance and DNA release ($r^2 = 0.639$; $r^2 = 0.539$, $P < .001$), whereas linear relation with total bilirubin content ($r^2 = 0.601$, $P < .001$). DNA release in the sepsis

Table 2
NETs component correlation analysis in patient with sepsis.

	NETs Formation	DNA release	Elastase release
SOFA	0.908	0.830	0.766
APACHE II	0.281	0.274	0.264
PaO2/FiO2 ratio	0.775	0.639	0.589
Creatinine clearance	0.605	0.539	0.482
Total bilirubin	0.666	0.601	0.582
TNF-α	0.691	0.672	0.642
IFN-γ	0.559	0.515	0.488
IL-8	0.570	0.494	0.511

Data are correlation coefficients (r^2). All $P < .001$.

were positively correlated with TNF- α ($r^2 = 0.672$, $P < .001$), IFN-γ level ($r^2 = 0.515$, $P < .001$) and IL-8 ($r^2 = 0.494$, $P < .001$) (Table 2).

Similarly, the SOFA and APACHE II correlated positively with elastase release ($r^2 = 0.766$; $r^2 = 0.264$, $P < .001$) as determined by Pearson's correlation analysis. The elastase release was correlated with different organ parameters such as PaO_2/FiO_2 ratio, creatinine clearance and total bilirubin. Pearson's correlation coefficient showed a negative relation between PaO_2/FiO_2 ratio, creatinine clearance and elastase release ($r^2 = 0.589$; $r^2 = 0.482$, $P < .001$), whereas linear relation with total bilirubin content ($r^2 = 0.582$, $P < .001$). elastase release in the sepsis were positively correlated with TNF- α ($r^2 = 0.642$, $P < .001$), IFN-γ level ($r^2 = 0.488$, $P < .001$) and IL-8 ($r^2 = 0.511$, $P < .001$) (Table 2).

4. Discussion

Neutrophils are having the key role to play in innate immunity against any infections. These cells are rapidly fascinated to the site of infection [19]. ROS have been well known inducer of NETs formation, as it is generated through the assembly of NADPH oxidase sub-units at the site of inflammation during sepsis [20]. We aimed to assess the incidence of NETs formation in neutrophils derived from controls and sepsis patients under ex vivo condition. The enhanced NETs contain extracellular DNA and elastase. DNA traps the pathogen and elastase degrades or hydrolyzes the pathogen [21] along with histone [22]. In the present study we found enhanced cell free DNA in NETs from sepsis patient than control, suggesting the generation of free radicals causing NETs progression.

Acute lung injury (ALI) succeeding severe sepsis is a frequent medical repercussion with substantial illness and mortality [23], as lung is the prominent organ to get targeted during the episode of inflammation [24]. In an animal study, the unwarranted proclamation of NETs has been shown the positive significant correlation to the liver, lung and heart injury [25]. Pathogenic effects of aberrant formation of NETs is most vital in lung imjuries because of constructive manner of lungs where neutrophil spends lot of time, in which surely indulgence the dispersion of chromatins, consequently enhancing tissue damage and lung dysfunction [25,26].

Damage-associated molecular pattern molecules (DAMPs) are endogenous peril molecules released at the site of tissue damage under conditions of cell stress. These damages initiate the release of DAMPs where neutrophils endure NETosis. There is an increasing quantity of evidence that DAMPs are principally cytotoxic and not only be contingent on the involvement of inflammasomes during NETosis [27], which intensify tubular epithelial cell injury and interstitial inflammation which is often linked to acute kidney injury [28]. A common path for the development of sepsis begins with pathogens stimulating cytokines, which would, in turn, activate neutrophils to express NETs and their components (NE, PR3 and MPO) onto their cell surface. This phenomenon causes hyper activation of neutrophils and leads to increased expression of adhesion molecules, resulting in vascular endothelial injury in the kidneys [29].

Accretion of neutrophil in irregularly shaped blood vessel in the liver improved clearance of circulating bacteria from liver. Whereas, process of pen down of bacteria is possibly reliant on the proclamation of NETs inside the blood vessels [30]. Besides their crucial part in stopping of flow of blood, platelets contributes to the pathology of ischemia reperfusion (I/R) injury by the means of severe inflammation [31]. Platelet binds to the granulocytes, which results in granulocyte activation. This activation causes formation of NETs at the site of infection and does the tissue damage [32].

Neutrophils generating ROS is depends on the efficient ability of NADPH oxidase [33]. In sepsis patients, improper function of NADPH oxidase might be a factor in the decreased generation of NETs [34]. Conversely, TLR4 activates the platelets which intermingle with neutrophils and gets instigated to generate NETs in abundant form in existence of inflammatory stimuli [35]. This causes liver cirrhosis, and characteristically shows low levels of thrombocytes [36].

In this study we further correlated all these cytokines (TNF-α, IFN-γ and IL-8) levels with the quantity of NETs. As we know neutrophils releases cytokines abundantly after the hyper activation, as it is potent mediator of inflammation helps in envisaging the sepsis severity [37]. Activated neutrophils prompts the NETs formation, where with the help of granular enzymes it contributes to the free radical generation [38]. Schulte et al. [39] also by their study suggested that concentration of cytokines defines the episode of inflammation in sepsis.

5. Conclusion

We support the hypothesis that elevated NETs formation is linked to the episodes of sepsis. This study delivers strong evidence of contribution of NETs to the sepsis severity. Further studies demonstrating the molecular mechanisms of NETs involved in different organ injury in case of sepsis which is mediated by the inflammatory cytokines.

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