ORIGINAL ARTICLE

Impact of Neutrophil Extracellular Trapping on Hypervirulent *Klebsiella pneumoniae* in Type 2 Diabetes Mellitus

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ABSTRACT

Background: *There is increasing evidence that type 2 diabetes mellitus (T2D) patient has higher risk for hypervirulent Klebsiella pneumoniae (hvKP) infections. Neutrophils achieve different immunological functions such as phagocytosis, and neutrophil extracellular trap (NETs) formation to arrest and eradicate microbes such as hvKp. Physicians have restricted curative approach for treatment of infection due to these superbugs. This necessitates to more understand innate immune arm included in these infections.* ***Objective:*** *This study aimed to investigate in vitro interaction of human NETs (as one of innate immune components) with hvKp in T2D.* ***Methodology:*** *Twenty patients with T2D and twenty age and sex matched healthy persons as healthy controls (HCs) were included in this study. By using clinical-derived hvKp strains, NETs complex testing by immunofluorescence, phagocytosis detection and NETs killing analysis were performed.* ***Results:*** *Direct killing of diabetic patients NETs against hvKp strain significantly decreased compared to NETs of HCs despite NETosis induction was significantly higher in neutrophils of T2D patients, compared to neutrophils of HCs. HvKp showed significantly lower level of phagocytosis compared to the control strain (14.65% vs. 96.43%). However, insignificant difference in the level of phagocytosis was detected between T2D and HCs neutrophils.* ***Conclusion:*** *Our study suggests that impaired NETs bactericidal ability makes diabetic patient more susceptible to develop hvKp invasive infection. This will shed light on a novel NETs dependant therapeutic approach and improved therapeutic plan against hvKP infection in T2D patient.*

INTRODUCTION

Hypervirulent *Klebsiella pneumoniae* is an emerging global health problem, and it has become a dangerous infectious pathogen causing severe infections, like pneumonia, endophthalmitis and pyogenic hepatic abscess[1]. It has been documented that hvKp strains have more virulence factors such as enhanced capsular polysaccharide production with increased anti-phagocytosis and ability to cause distant metastasis. So hvKp is much more invasive than classic *Klebsiella pneumoniae* (cKp)[2]. HvKp is an actual superbug due to higher pathogenicity and multidrug resistance with critical risk to people with underlying diseases[3].

Type 2 diabetes mellitus (T2D) is a metabolic disorder characterized by insulin resistance and improper immune response. T2D prevalence progressively increases in both developed and developing countries over the past decades[4]. Medical observations have suggested a considerable association between diabetes and more vulnerability to infection, especially *K. pneumoniae*. Additionally, there is increasing evidence that T2D patient has higher risk for hvKP infections[5,6]. Really, about 75% of liver abscess patients caused by hvKp had diabetes mellitus or glucose intolerance[7]. Also, there has been significant association between poor glycemic control and further metastatic spread; a typical hvKP infectious syndrome[5].

The immunological response of neutrophil is essential to fight microbial infections firstly. However, *K. pneumoniae* capsular polysaccharide resists neutrophil phagocytosis. Previous studies have found that K1 serotype of hvKp more significantly resists neutrophils- mediated phagocytosis than non-K1 strains[8]. Moreover, Brinkmann et al in 2004 found a novel neutrophil extracellular killing mechanism called NETosis (NET formation)[9]. NETs are extracellular fragile fibers-like structure consists of extracellular DNA fibrils coated by antimicrobial proteins; myeloperoxidase (MPO), histone, neutrophils elastase and α-defensin[10]. Researchers have found that neutrophils die two to four hours following their activations and NETs formation[11]. NETosis is induced by a variety of microbes and proinflammatory agents, such as the strong tumor promoter PMA (phorbol 12-myristate 13-acetate), cigarette smoking, lipopolysaccharides, viruses, bacteria and other environmental mediators. Additionally, chemokines, cytokines, immune complexe and some physiological impulses can induce NETosis[12,13]. Worse than that, hvKp could frequently acquire antibiotic resistance, such as cefoxitin- resistant hypervirulent *K. pneumoniae* and carbapenem-resistant hypervirulent *K. pneumoniae* isolates[14]. Essentially, physicians have restricted curative approach for treatment of infection due to these superbugs. This necessitates to more understand innate immune arm included in these infections. In this study, we investigated in vitro interaction of human NETs (as one of innate immune components) with hvKp in T2D.

METHODOLOGY

Subjects

Twenty T2D patients participated in this research. T2D was diagnosed according to criteria of World Health Organization[15]. Only Patients with glycated hemoglobin/hemoglobin A1c more than 8.5% were included in the study[16]. Twenty age and sex matched healthy persons were included as HCs. HCs had no signs of inflammation or infection or another considerable illness.

Ethical approval

The study was accepted by Institutional Review Board of the Faculty of Medicine, Mansoura University; code number: R.24.12.2973.

Bacterial strain

Clinical hvKp strain was obtained from a previous study done in Mansoura University Children Hospital, Egypt[17]. Hypervirulence trait was confirmed by detection of *peg-344* gene and capsular polysaccharide gene **(**K1) by PCR[18,19].

Neutrophils isolation

Neutrophils were isolated from peripheral blood samples of T2D patients and HCs and used without delay. Neutrophils were isolated using Ficoll Paque (Sigma-Aldrich, USA) with density gradient centrifugation in accordance with the manufacturer instructions. Erythrocytes were sedimented by dextran and lysed by ammonium–chloride–potassium lysing buffer (Thermo Fisher Scientific, USA). The neutrophils purity should be above 95%. Then, neutrophils were wash by PBS and then suspended in RPMI 1640 (free from phenol) as 5×10^6 cells/mL[20].

In vitro NETs induction and visualization

The neutrophils were seeded on poly lysine coated glass cover-slips (Thermo Fisher Scientific, USA) in twenty four wells plate and stayed into cover-slip for half an hour. The NET inducer; 100 nM PMA (Abcam, UK) was added to encourage neutrophils to release NETs. Following 3 hours, cells were fixed with paraformaldehyde at 4°C overnight.

Fixed neutrophils were blocked with the blocking buffer 0.2% gelatin in PBS (Sigma-Aldrich, USA) for 1 hour at 37°C. Subsequently, cells were stained by anti human MPO primary antibody (Bio-Rad Laboratories, USA) for 2 hours at 37°C within humid chamber. Following 3 PBS washes, neutrophils were incubated for additional one hour with secondary donkey anti rabbit IgG antibody conjugated with Alexa Fluor 555 dye (Thermo Fisher Scientific, USA). Hoechst 33342 (Thermo Fisher Scientific, USA) was used to stain the DNA. NETs were visualized on confocal microscopy (Olympus LS, Japan) with low magnification image (40×) was obtained by software on non-overlapping random image (6 distinct fields/cover-slips). NETs were manually quantified on obtained images as Hoechst-positive fibers released from solitary neutrophil with a total length at least double cell diameter and interpreted as the DNA releasing neutrophils percentage[21,22].

Neutrophils phagocytosis testing

The bacteria at exponential phase were heated at 70°C for 60 minutes in water bath, and then washed by PBS. The bacteria were labeled by FITC (Sigma-Aldrich, USA) through incubation with 0.1 mg/mL FITC in 0.1 M sodium bicarbonate (pH 9.0) for 60 min at 25°C. After that, unbound fluorochrome to bacteria were washed by PBS using 3 centrifugation cycles. Both cKp (control strain) and hvKp were evaluated by flow cytometry (BD Biosciences, USA) to be sure that the FITC labeled bacteria percentage was more than 95%. FITC labeled bacteria (4×10^7 CFUs), isolated neutrophils (1×10^6 cells) and normal human serum (10%) were added to PBS to 1 mL final amount. The samples were incubated with continuous agitation at 37°C. Following zero, twenty, and forty minutes incubation, the suspensions were transferred immediately to ice bath. Trypan Blue (100 µl, 0.04%) was added to quench the superficial fluorescent and determine the neutrophils ingesting bacteria percentage via flow cytometer. A totality of 50,000 neutrophils were processed for every sample. Phagocytosis rates

were calculated as the FITC labeled neutrophils percentage[23].

NETs mediated killing assay

Neutrophils (1×10^6 cells) in 500µL RPMI were seeded into 24 wells plate and induced by PMA to release NETs. Following 37°C incubation for 3 hours, 1×10^6 CFUs hvKp were added to wells to be incubated for an hour. Cells were lysed by 0.1% Triton X-100 (Sigma Aldrich, USA) on ice for 15 minutes and hvKp was cultured on MH agar (Thermo Fisher Scientific, USA). Colonies were enumerated after one day and survival percentages were calculated compared with control. The same assay was performed after pre incubation of neutrophils with DNase I (Sigma-Aldrich, USA) for one hour to inhibit NETosis of neutrophils.

Statistical analyses

All tests were done in triplicate. Statistical analysis was done with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was performed by using Student's t-test. *P* value ≤ 0.05 was considered significant.

RESULTS

NETosis induction in diabetic and healthy neutrophils

Neutrophils of T2D patients and HCs were stimulated with PMA at 37°C for 3 hours to induce NETosis. PMA-induced NETosis was significantly higher in neutrophils of T2D patients, compared to neutrophils of HCs (Figure 1).

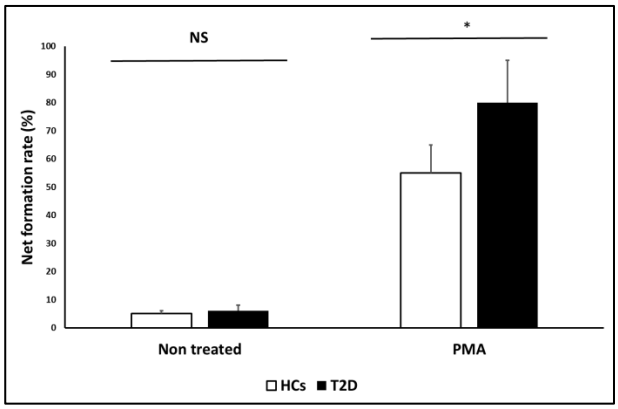

Fig. 1: NETosis induction in diabetic and healthy neutrophils

*$p < 0.05$, ns: no significance

Phagocytosis of hvKp by diabetic and healthy neutrophils

HvKp showed significantly lower level of phagocytosis compared to the control strain cKp (14.65% vs. 96.43%). Insignificant difference in the phagocytic rate level was detected between T2D and HCs neutrophils (Figure 2).

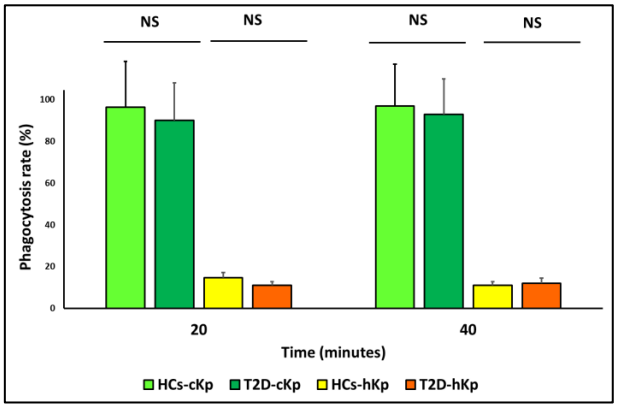

Fig. 2: Phagocytosis of hvKp by diabetic and healthy neutrophils

NETs killing ability by diabetic and healthy neutrophils

HvKp incubated with NETs of T2D patients showed significant higher survival rates compared with hvKp incubated with NETs of HCs. Following one hour incubation with NETs, more than 80% bacteria survived in T2D NETs, but less than 20% bacteria survived with HCs NETs. There wasn't difference in the survival rates of hvKp in T2D and HCs following DNase I treatment of neutrophils (Figure 3).

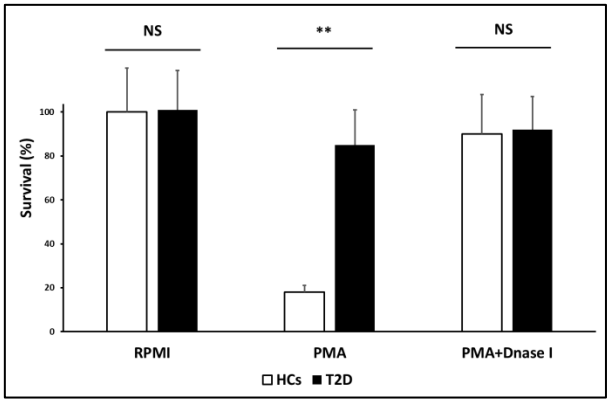

Fig. 3: NETs killing ability by diabetic and healthy neutrophils

**$p < 0.01$, ns: no significance

DISCUSSION

T2D is a major global health alarm. As stated by International Diabetes Federation Diabetes Atlas tenth edition, about 537 million are diabetic worldwide, that will increase to11.3% (643 million) in 2030 and 12.2% (783 million) in 2045 with massive mortality rate and more than 3.96 million people die annually worldwide because of T2D complications including infection. Several researches have been defined the link between diabetes mellitus and the high possibility of infection

with a lot of them pointing to the probability of neutrophil function impairment. Neutrophils achieve different immunological functions such as phagocytosis, degranulation, ROS formation and extracellular trap formation to arrest and eradicate microbes[24]. NETosis has been recognized in the previous decade as novel type of programmed cell death occurring in neutrophil which can be induced by infectious microorganisms and inflammatory stimulants. NETosis is a double edged sword as it provides an innate defense through entrapping bacteria[9]. Nevertheless, deregulated or excessive NETosis also causes some diabetic complications such as delayed wound healing, inflammation, thrombosis and endothelial dysfunctions[25].

Studies have reported that T2D patients significantly exhibit an increased risk of hvKP infection[26]. In this study, we investigated in vitro interaction of neutrophil extracellular trapping with hvKp in T2D. A previous study found that T2D is associated with impaired NETosis, due to delayed formation of unstable or short lived NETs[27]; explaining why T2D patients are more susceptible to hvKP infection. Importantly, it was reported that NETosis is enhanced in T2D, as revealed by increased circulating level of NETs related proteins; DNA-MPO complex and citrullinated histone H3 with subsequent complications in diabetic patients[28,29]. To make clear the diversity of the previous findings regarding NETosis in T2D, neutrophils of HCs and T2D patients were induced by PMA to induce NETs formation, in our research. We found that PMA-induced NETosis was significantly higher in neutrophils of T2D patients, compared to neutrophils of HCs. Confirming our result, Jin et al[30] reported that NETosis increased in T2D and proposed that deficient NETosis is not a cause of increased susceptibility to infection by hvKP in T2D patient.

Neutrophils have an important role in hvKP infection control, because they are attracted rapidly toward the infection site to eradicate attacking microbe by both phagocytosis (intracellular killing) and NETosis (extracellular killing). Phagocytosis is an essential bactericidal mechanism done by neutrophil[31]. HvKp has been found to be very resistant to phagocytosis in several earlier researches[26,32]. In this research, to find out whether increased susceptibility of infection by hvKp in T2D patients was caused by imperfect phagocytosis by neutrophils, phagocytosis testing was done on neutrophils of HCs and T2D patients with FITC-labeled hvKp strain. We found that hvKp showed significantly lower level of phagocytosis compared to the control strain cKp (14.65% vs. 96.43%). Furthermore, insignificant difference in the phagocytic rate level was detected between HCs and T2D neutrophils. Our results are in consistent with another study in which the researchers reported that difference in susceptibility to infection by hvKP between HCs and T2D patients are not attributed to impaired phagocytosis in diabetic patient neutrophils[30].

NETosis is considered a strong antimicrobial mechanism to kill extracellular pathogen[24]. Disagreement of increased NETosis with higher susceptibility to infection by hvKp in T2D pushed us to study whether NETs are unable to kill hvKp in T2D. In this study, hvKp incubated with NETs of T2D patients showed significant higher survival rates compared with hvKp incubated with NETs of HCs. Following one hour incubation with NETs, more than 80% bacteria survived in T2D NETs, but less than 20% bacteria survived with HCs NETs. There wasn't difference in the survival rates of hvKp in T2D and HCs following DNase I treatment of neutrophils. Other studies reported the same result and suggested that NETs have a critical responsibility regarding hvKP killing and this killing ability against hvKp significantly decreased in T2D[27,33]. Of interest, Lee et al[16] found that bactericidal ability of NETosis against cKp was insignificantly impaired in T2D patients in comparison with HCs.

Some researchers found that defective killing by NETs is not because of lack of hvKp within NET cages, but the most important cause is the antimicrobial components of NETs in diabetic patients with restrained bactericidal function compared to healthy controls[27]. In recent times, researchers have been confirmed that the effect of NETosis in different infections is mediated principally by infection associated proteins load, that could be due to the alteration in expression level of neutrophils associated proteins in different infection specific microenvironment[34,35]. Current proteomic research showed that there are differences in 35 expressed proteins between diabetic patients with poor glycemic control and patients with good glycemic control. Interestingly, a number of key bactericidal constituents of NETs, such as MPO, SA100A9, and azurocidin, are down regulated in poor glycemic controlled diabetic patient. These results suggest relationship between defective NETs killing and protein components dysregulation of NETs amongst poorly controlled diabetic patients[36].

CONCLUSION

Our study concluded that direct killing of diabetic patients NETs against hvKp strain significantly decreased compared to NETs of HCs despite NETosis induction was significantly higher in neutrophils of T2D patients, compared to neutrophils of HCs. Our results suggested that impaired NETs bactericidal ability makes diabetic patient more susceptible to develop hvKp invasive infection. This provides an insight to an impaired innate immune reaction against hvKp in diabetic patient. This will shed light on a novel NETs dependant therapeutic approach and improved therapeutic plan against hvKP infection in T2D patient.

Conflicts of interest

- The authors declare that they have no financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript has seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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