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Aberrant neutrophil function among heavy smokers and chronic obstructive pulmonary disease patients

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Abstract

Background: Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammation disease of the respiratory tract. The aberrant functions of neutrophils has been reported in COPD patients including respiratory burst and phagocytosis. Unfortunately, there is little evidence of neutrophil functions in healthy smokers who are considered a high risk group for COPD.

Objective: To determine the alteration of leukocytic parameters, intracellular reactive oxygen species (ROS) levels and phagocytosis percentages among smokers and COPD.

Method: Sixteen smokers, 17 COPD patients and 10 healthy controls were recruited. The informed consent and recruitment process were ethically approved by the WU-IRB committee. Leukocyte percentages were analyzed by automated cell counter. Polymorphonuclear cells were purified by gradient centrifugation. The cell suspensions were incubated with FITC-conjugated bacteria to allow phagocytosis. Dichlorofluorescin diacetate was loaded to the cells followed by porbol-12-myristyl acetate stimulation to generate ROS. The percentages of phagocytosis and intracellular ROS levels were analyzed using flow cytometry. Mean of leukocyte numbers, mean fluorescence intensities and phagocytosis percentages were compared by t-test using SPSS (version 17).

Results: An increased ROS level of circulating neutrophils was observed among heavy smokers and COPD ($p<0.05$) compared to healthy controls. There was concordance to neutrophil percentages. However, the function on phagocytosis of neutrophils was not abolished in any group of smokers except COPD.

Conclusions: Our findings suggested that the elevation of intracellular ROS generated by circulating neutrophils resulted in pools of inflammatory mediators among heavy smokers, which was suspected as one of the factors causing COPD in high risk people.

Keywords: Chronic Obstructive Pulmonary Disease, Smoking, Neutrophil, Reactive Oxygen Species, Phagocytosis, Flow Cytometry

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a health problem that causes morbidity and mortality globally.[1-3] A large amount of money is needed for healthcare and the treatment of COPD patients. Tobacco smokers appear to be a high risk group. The cardinal features of COPD are chronic inflammation of the respiratory airway, decreased airflow and respiratory obstruction.[4,5] Inflammation is suspected as a major event occurring during disease progression and exacerbation. For example, increasing levels of phagocytosed macrophages within the airway of COPD patients are believed to be associated with the presence of local inflammatory cytokines.[6-8] Neutrophils have been considered to play a major role in COPD pathogenesis.[9-10] The association of aberrant neutrophil

function and COPD pathogenesis has been studied and reported. In the presence of foreign stimuli, circulating neutrophils in the peripheral blood are recruited and attracted to the site of activation through the interaction of chemokines and chemokine receptors. These are confirmed by the presence of chemo-attractants and increased expression of chemokine receptors either on activated circulating neutrophils or neutrophils which reside within the respiratory tract of COPD patients.[11] The activation of neutrophils in COPD results in the production and secretion of enzymes, i.e. neutrophil elastase, cathepsin G and proteinase 3, which are associated with the degree of extracellular matrix destruction in COPD airways.[12,13] Various kinds of aberrant neutrophilic functions have been studied among COPD. Phagocytosis is a crucial step in neutrophils that prime the effector arm of the immune system. Some studies have suggested that phagocytosis diminished in COPD-derived neutrophils and macrophages.[15] However, the results on phagocytosis are under debate due to various methods based on types of stimulants.[8,15] Reactive Oxygen Species (ROS) are oxidative burst molecules augmented after immune recognition and activation. ROS mediates the oxidation of proteins, DNA and lipids of the host cell component. Therefore, the release of ROS is suspected as one of the major factors affecting tissue destruction within the respiratory tract of COPD patients.[16-21] Previous data have reported the enhanced ROS measured by NADPH oxidase observed in circulating neutrophils of COPD patients, by comparing smokers with normal lung function and non-smokers.[16,22] Viatkus and colleagues reported that increased ROS production of peripheral blood and sputum-derived neutrophils among COPD were associated with the exacerbation of COPD.[23,24] Although plenty of data suggest that tobacco use is one of the main factors contributing to COPD and respiratory tract inflammation, approximately 20% of smokers developed disease.[2-4] This work aims to determine the phenotypic change of circulating neutrophils, including intracellular ROS and phagocytosis among different tobacco consumption smokers compared to COPD patients.

Methods

Subject Recruitment

Sixteen healthy smokers, 17 COPD patients and 10 age-matched healthy volunteers residing in Thasala district, Nakhon Si Thammarat were recruited into the study. The diagnosis of COPD-patients was made by physicians at Thasala Hospital, Nakhon Si Thammarat during 2013 - 2014 following Global Initiative for Chronic Obstructive Lung Disease Guideline 2015.[2] COPD patients, smokers and healthy volunteers were tested for lung function by spirometry (**Table 1**). COPD patients who had FEV_1/FVC ratio< 0.7, and possessed underlying diseases associated with chronic inflammation were excluded from the study. The smoking profiles were identified using a questionnaire in healthy smokers and calculated using the Brinkman Index (BI) which is the number of cigarettes smoked per day multiplied by the number of smoking years. The informed consents and questionnaires were approved by the Walailak University Institutional Review Board (WU-IRB) committee. Eight smokers with BI exceeding 400 were categorized as heavy smokers (HS). The remaining 8 smokers were grouped as light smokers (LS). Subjects with other chronic conditions such as diabetes mellitus, recent infections and the previous exacerbation of COPD within 8 weeks were excluded from the study. Heparinized blood and K_3EDTA blood samples were collected from participants to evaluate hematologic parameters and neutrophil functions.

Complete Blood Count

K_3EDTA blood samples were collected to retrieve leukocyte percentages. Neutrophils, lymphocytes, monocytes, eosinophils and basophils were analyzed by automated cell counters (Nihon-Kohden MEK-8222) following the manufacturer's instructions.

Determination of in vitro ROS production.

Endogenous ROS were generated by phorbal-12 13-myristyl acetate (PMA) activation. The quantity of ROS was traced using 2′,7′-dichlorofluorescin diacetate (DCFH-DA).[16,23] Briefly, polymorphonuclear cells were removed from heparinized blood by density gradient centrifugation (Isoprep™). Neutrophils were collected by 3% dextran sedimentation. Red blood cell contamination was lysed by adding 1 mL of hypotonic solution (FACS lysing™) and removed by centrifugation. The pellet was resuspended and washed in 1 mL of 1X HBSS twice and leukocytes were adjusted to 2 x 10^6 cells/μL prior to testing. One microliter of working DCFH-DA (20 μM Sigma, USA) was loaded into a 1 mL cell suspension and incubated at 37°C for 15 minutes. The adjusted cell suspensions were stimulated with 100 μL of PMA at 100 ng/mL (1:9 v/v) to generate ROS production at 37°C for 45 minutes. Mean fluorescence intensities (MFIs) of green fluorescence obtained from flow cytometer (FACSCalibur) acquired by CellQuestPro™ was determined for ROS production on gated neutrophil.

Phagocytosis of Neutrophils

The phagocytic function of neutrophils was determined by flow cytometry. A suspension of *Escherichia coli* at a concentration of 1 x 10^8 CFU/mL was labeled with fluorescence isothiocyanate (FITC) as previously described.[10,27] Five hundred microliters of FITC-conjugated bacteria were opsonized with 500 μL of 20% AB serum and mixed with end-over-end rotation for 30 minutes at 37°C. A hundred microliters of 1 x 10^6 cells/mL cell suspension was incubated with 100 μL of opsonized FITC-conjugated bacteria for 1 hr. The reaction was stopped by adding 200 μL of cold 0.9% NaCl-0.02% EDTA solution. Phagocytic-neutrophils were counted by the expression of green fluorescence emitted at 520 nm. The phagocytosis percentages were calculated by CellQuestPro to determine the phagocytic ability of neutrophils.

Statistical Analysis

The mean leukocyte percentages, phagocytosis percentages and MFIs for ROS generation of smokers (light smokers, heavy smokers) and COPD patients were compared to healthy controls by the Mann-Whitney *U* test. The correlation between

brinkman index and neutrophilic function were compared by Spearman Rank Correlation. The data were computed by SPSS version 17 for statistical significance at 95% confidence interval ($p<0.05$).

Results

Elevation of circulating neutrophil percentages and declining lymphocyte percentages in smokers and COPD patients

Circulating leukocyte numbers retrieved from complete blood count testing represent basic changes in individuals. Mean leukocyte percentages were compared between light smokers (LS), heavy smokers (HS), COPD patients and healthy volunteers (HC). The results showed that neutrophil percentages significantly increased among HS and COPD. In contrast to lymphocytes, lower numbers of lymphocyte percentages were observed among LS, HS and COPD groups (**Figure 1**). However, no alterations of other leukocyte subpopulations including monocytes, eosinophils and basophils were observed.

Table 1. Demographic data of population studied

Subject	Healthy	Light smokers	Heavy smokers	COPD
No. of subject	10	6	9	17
Age (years)	69.8±8.9	58.8±11.1	66.4±7.2	71.7±7.5
Brinkman index	0	248.3±51.7	763.33±211.7	385.0±45.1
FVC, %	90.7±18.7	92.8±26.7	90.1±31.5	85.3±6.1
FEV_1, %	89.2±23.5	89.4±19.2	85.2±23.8	66.5±10.3
FEV_1/FVC, %	0.90±1.8	0.88±1.5	0.87±1.2	0.55±1.5

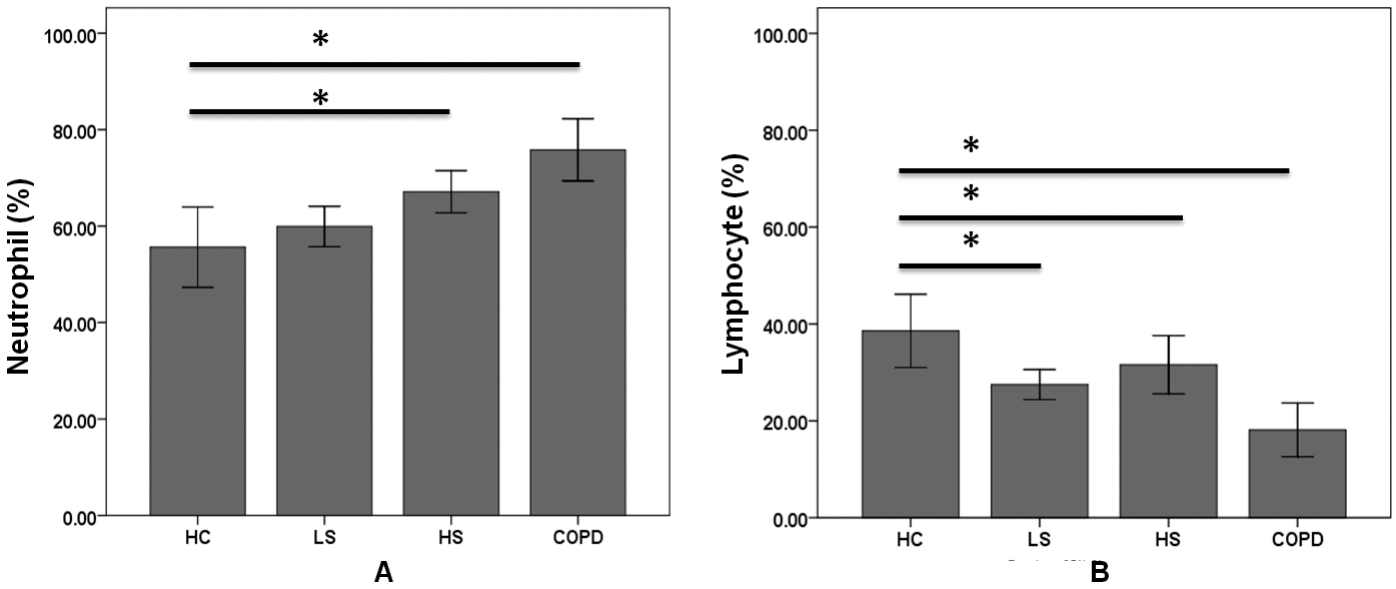

Figure 1. Mean percentage of neutrophil (A) and lymphocyte (B) obtained from peripheral blood of light smokers (LS), heavy smokers (HS) and COPD patients compared to healthy controls (HC). (*p<0.05)

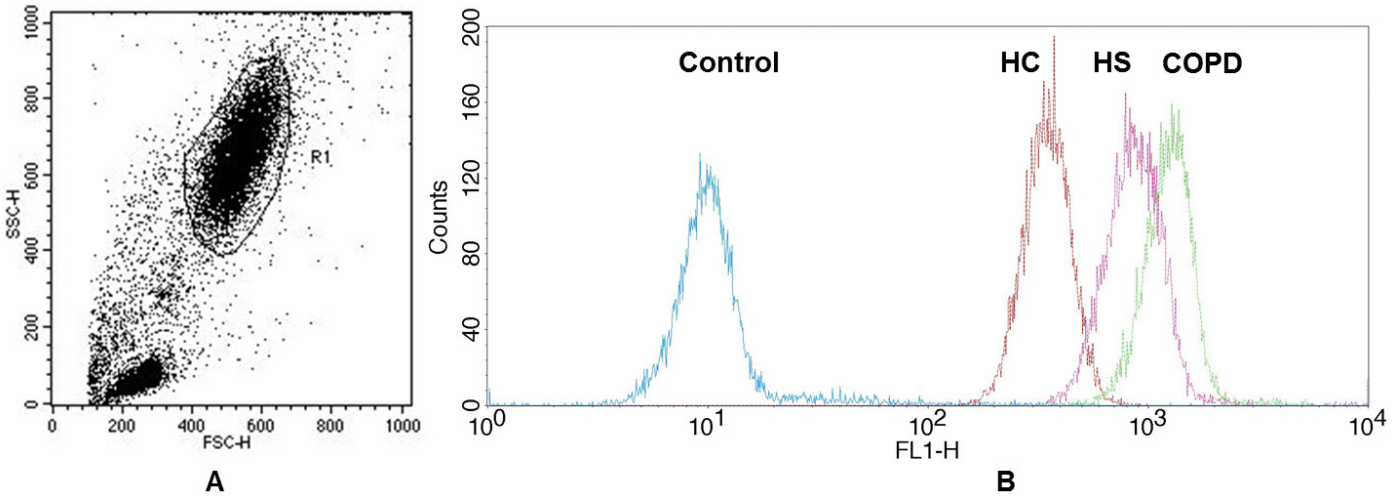

Figure 2. Flow cytometric analysis of *in vitro* ROS levels. Neutrophil (FSC^{high}/SSC^{high}) were gated and showed in R1. The cells in this gate were selected to determine endogenous ROS products represented in mean fluorescence intensities (MFIs). The histogram showed significantly increased MFIs in COPD patients and healthy smokers group (HS) compared to healthy controls (HC).

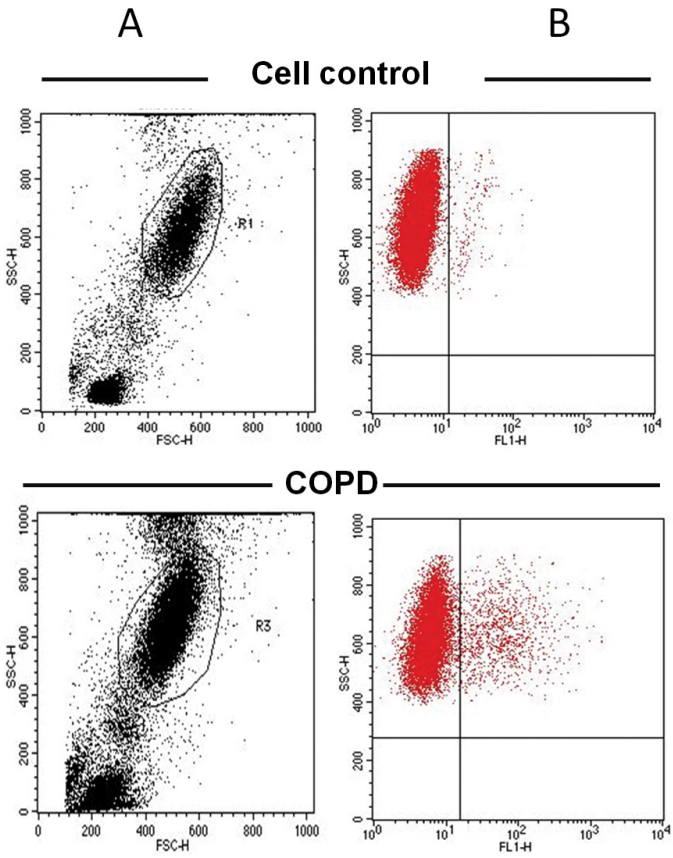

Figure 3. Dot plot representative of flow cytometric approaches to determine phagocytosis percentages. Neutrophils were gated based on FSC and SSC characteristic (FSC^{high}/SSC^{high}) (A). Gated neutrophil were further analyzed for their phagocytic ability by fluorescence intensities of phagocytosed bacteria (B). The cell control was observed for its based line among sample (cell control, upper panel). The increased percentages of fluorescence followed by challenging neutrophils with FITC-conjugated bacteria were obtained and calculated for phagocytosis percentages (COPD, lower panel).

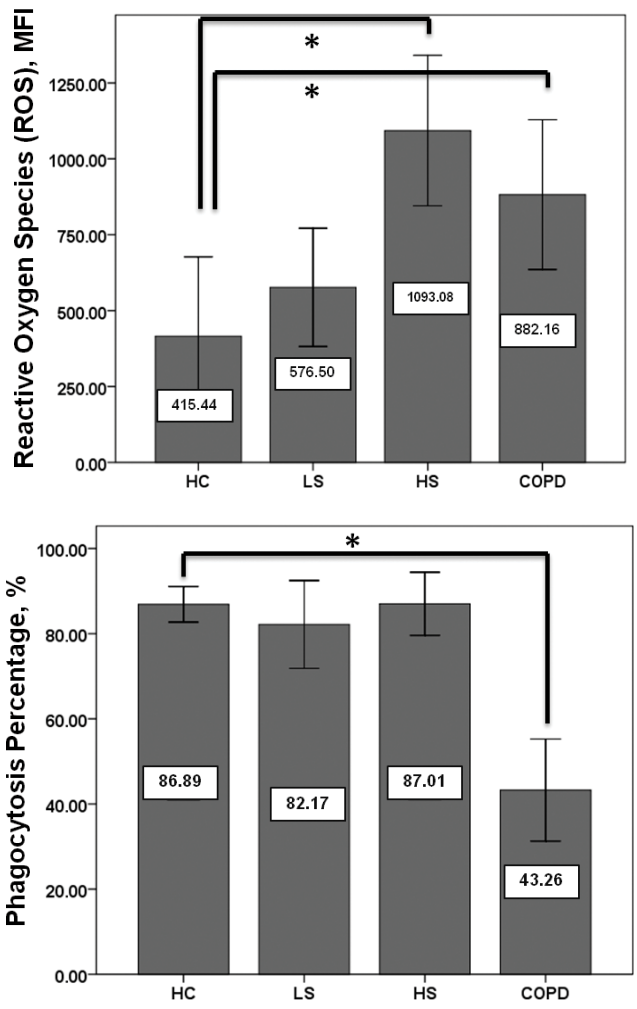

Figure 4. Comparison of neutrophil functions including ROS generation (MFIs, upper panel) and phagocytosis (%, lower panel) among light smokers (LS), heavy smokers (HS) and COPD patients compared to healthy controls (HC). The number in the bar represented means of analyzed. (*p<0.05)

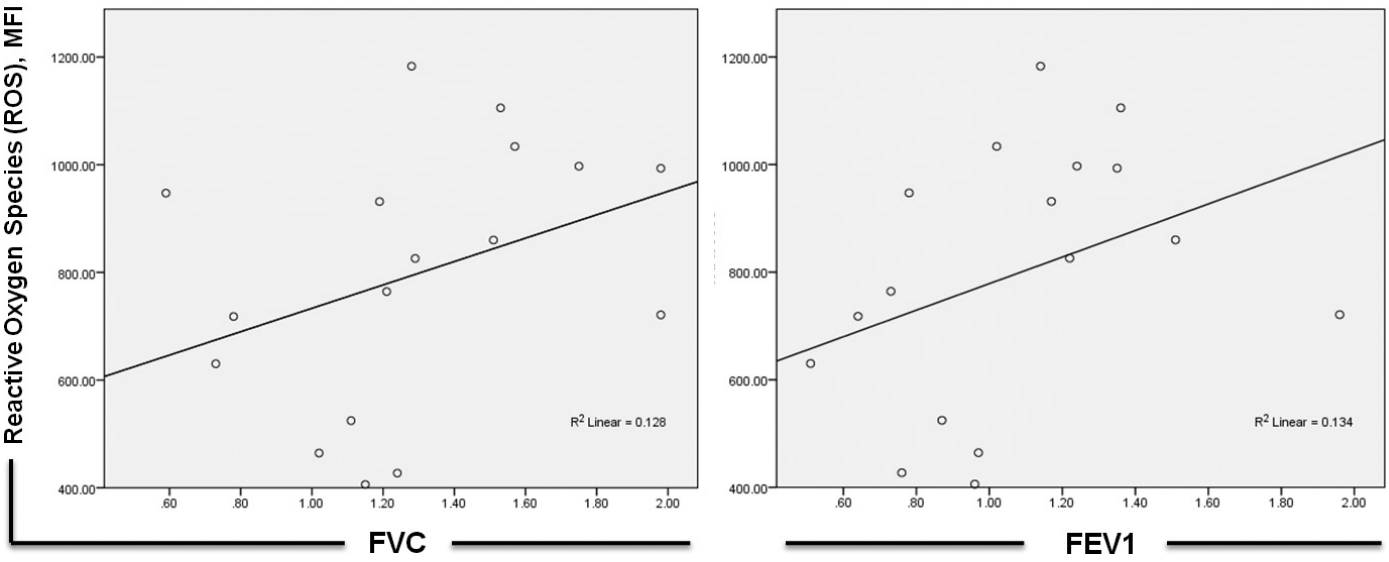

Figure 5. Scatter plot represented the correlation between intracellular ROS generation by blood-derived neutrophil to forced vital capacity (FVC) (left) and Forced expiratory volume in 1 second (FEV_1) (right) in COPD patients (n=17).

Aberrant ROS production in heavy smokers and COPD patients

Intracellular ROS production was observed at 40 minutes of activation. ROS generation represented in MFIs was calculated and the means were compared between groups. The results showed that ROS levels in HS and COPD significantly increased compared to the control group ($p<0.05$) (**Figure 4**). However, no correlation between brinkman index and ROS level was observed (data not shown).

Percentages of phagocytosis in smokers and COPD

Phagocytosis ability of neutrophils was determined by flow cytometry; the results revealed that there was no abnormality in neutrophil phagocytosis percentages among smoker groups either LS or HS. Nevertheless, a decreased phagocytosis percentage was observed among COPD patients (**Figure 4**).

Correlation of lung functions and neutrophil functions

Lung functions including Forced Expiratory Volume at the first second (FEV_1) and Forced Vital Capacity (FVC) were tested among COPD-diagnosed patients. FEV_1/FVC ratio was calculated to define COPD-patients into 4 groups based on disease severity (mild=3, moderate=5, severe=7 and very severe=2). The results showed that there was a statistically significant correlation between lung function tests and ROS generation among COPD patients ($p<0.05$) (**Figure 5**). In contrast to phagocytosis, the correlation between lung functions and the impairment of neutrophilic phagocytosis percentage were not observed in this group (data not shown).

Discussion

Chronic Pulmonary Obstructive Disease (COPD) is a chronic inflammatory condition. The inflammation and defective innate immune responses were suspected as one of immune-mediated mechanisms driving disease progression. There were previous studies which indicated abnormal immune functions in the aspect of inflammation and the changing hematologic parameters. However, the findings of these abnormalities during pre-existing diseases were limited in the view of pulmonary inflammation. In this study, we investigated the inflammatory phenomenon of circulating neutrophils among two groups with different cigarette smoking profiles which were classified as risk groups for COPD. Increased numbers of circulating neutrophil percentages in HS was suspected as a general feature of immune activation associated with inflammation, whereas lower numbers of lymphocyte percentages were supposed to be affected by a relative decrease of percentage-derived calculations.[25] Impaired phagocytosis of neutrophil among the airway of COPD patients has been previously reported. However, these results were controversial and need to be tested using universal methods.[16] The exact mechanisms of decreased phagocytosis are still not clear, and are based on different stimuli. In this study, phagocytosis of neutrophils was induced by *E coli*. There were various kinds of innate immune receptors associated with this stimuli, such as TLR2, TLR4, C-type lectin receptors and complement receptor 1 (CR1).[26] Unfortunately, most studies have reported TLR expression on T-cells or macrophages residing in the airway of COPD patients.[27] Kathleen et al. reported the inhibition of caspase-3-like activity in neutrophils isolated from cigarette smoke extract induction. Their study demonstrated the proposed molecular mechanisms of decayed apoptosis, which is associated with the impairment of circulating-neutrophil phagocytosis ability.[28] However, the expression of innate receptors expressed on neutrophils should be explored to elucidate the exact molecular mechanism-driven pathogenesis of COPD generated by neutrophils. The evidence of aggressive phenotypes in the view of inflammation of neutrophils have been described within the respiratory tract and the possible mechanisms on disease pathogenesis.[29] However, the study by Noguera and colleagues reported that increased intracellular ROS was observed only in COPD patients, whereas expression at the mRNA level was not significantly different.[16] In our study we have shown different phenomena of extra pulmonary inflammation among different smoking consumption profiles. However, the mechanisms of ROS generation in a dose-dependent manner have not yet been described. A previous study reported evidence of dosage effect on neutrophil activation, which indicated that there was extended inflammation in the blood of individuals following airway-induced inflammation.[31] We proposed that the increased consumption of cigarettes is one of the factors affecting intracellular ROS of circulating neutrophils and is associated with a degree of lung function impairment. Interestingly, a few of these smokers progressed to COPD. Thereafter, more evidence is essential to explain variations of immunogenetic molecules in susceptible persons, in particular HLA expression among risk groups and COPD.[31] Moreover, other mechanisms driving ROS secretion such as pathways that excrete these pools of ROS and the effect of neutrophil extracellular traps (NETs) as a result of tissue destruction, translation and post-translation of aberrant cellular signaling on ROS generation should be implemented. The evidence of endogenous ROS production in our study indicated that pools of inflammatory mediators reside within the cell. However, extracellular ROS and associated mechanisms driving the release of ROS should be assessed to elucidate the exact mechanisms driving more advanced stages of inflammation.[32-33] Finally, these findings demonstrated the aberrant phenotype of biomarkers in the pre-COPD stage which may be useful for novel therapies targeted on neutrophils and also for preventive guidance on health promotion strategies.

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