

# Increased metabolic activity of neutrophils in patients with chronic obstructive pulmonary disease

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## ABSTRACT

**Aims:** To compare the metabolic activity of peripheral neutrophils in patients diagnosed with chronic obstructive pulmonary disease (COPD) with that of healthy, nonsmoking volunteers. **Materials and Methods:** Venous blood samples were taken from patients diagnosed with COPD as well as from healthy nonsmokers. Each sample was subjected to the nitro blue tetrazolium (NBT) test in which neutrophils exhibiting elevated metabolic activity were detected by light microscopy. The test was repeated after stimulation with *Escherichia coli* (*E. coli*) endotoxin with fresh samples. Neutrophils showing dye uptake were then counted in each case. **Results:** We found that the mean numbers of activated neutrophils without and with the addition of endotoxin were 19% and 23%, respectively, in the control group and 56% and 62%, respectively, in the test group. Two-sample *t*-test statistic revealed that there was a significant ( $P < 0.01$ ) increase in neutrophilic metabolic activity in patients with COPD as compared to that in healthy volunteers. This significance remained even after stimulation using *E. coli* endotoxin. **Conclusion:** The results hint at a potentially relevant pathogenic mechanism in COPD related to the metabolic activity of neutrophils. By exhibiting enhanced metabolic activity, neutrophils in the COPD patients are more likely to be involved in damaging lung tissues.

**KEY WORDS:** Chronic obstructive pulmonary disease pathology, neutrophil function, nitro blue tetrazolium, respiratory burst

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## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) refers to chronic bronchitis and emphysema, a pair of commonly coexisting diseases of the lungs, in which the airways become narrowed. COPD is characterized by mild chronic inflammation throughout the airways, parenchyma, and pulmonary vasculature.<sup>[1]</sup> The pathogenesis of COPD is attributed to the destruction of alveolar walls due to the increased release of proteases and reactive oxygen species (ROS). Neutrophils that are normally sequestered in peripheral capillaries tend to accumulate in the alveoli and release their granules resulting in tissue damage. During exacerbations of COPD, there is an enormous

increase in the release of chemoattractants,<sup>[2]</sup> thus increasing the accumulation of neutrophils in the alveoli and worsening the damage.<sup>[3]</sup> Compared with healthy nonsmokers, the number of neutrophils in patients with COPD is more, in both sputum and bronchoalveolar lavage fluid.<sup>[4]</sup> Granulocyte-macrophage colony-stimulating factor (GM-CSF) is increased in bronchoalveolar lavage fluid during exacerbations. This cytokine stimulates differentiation of granulocytes and macrophages and can activate them directly.<sup>[5]</sup> The proinflammatory cytokines—tumor necrosis factor (TNF)-alpha and TNF-beta—may play an important role in the pathogenesis of COPD. These cytokines cause release of neutrophils from the bone marrow and activate them.<sup>[6]</sup>

Various aspects of neutrophils have been studied and differences have been found in the COPD patients with respect to the total neutrophil count,<sup>[4]</sup> adhesion properties of neutrophils,<sup>[7]</sup> neutrophil chemotaxis,<sup>[8]</sup> and the role of cytokines in the pathogenesis of COPD.<sup>[5,6]</sup> However, the metabolic and respiratory burst activities of the neutrophils are yet to be clarified. Flow cytometry studies have shown that respiratory burst activity was increased in neutrophils

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from patients with COPD compared to those with normal lung function.<sup>[9]</sup> Hardly any studies have used other techniques such as the nitro blue tetrazolium (NBT) test to assess this property of neutrophils in COPD patients, especially in the Indian population. By evaluating the metabolic activity of neutrophils in the COPD patients, we will get a deeper insight into the changes occurring in these “COPD neutrophils” that will help us uncover the precise role of neutrophils in the pathogenesis of COPD.

The objectives of our study are as follows: (a) to assess or compare the phagocytic and metabolic activity of systemic neutrophils in the COPD patients and that of neutrophils in healthy volunteers and (b) to compare the tendency for activation of neutrophils on exposure to *Escherichia coli* (*E. coli*) endotoxin of COPD patients and healthy individuals.

## MATERIALS AND METHODS

### Study particulars

Approval from the Institutional Ethics Committee was obtained prior to the commencement of this pilot case control study. Patients who were diagnosed with COPD at our tertiary hospital and who were willing to participate in the study were included in the study group after signing an informed consent form. For this pilot study, a total sample size of 30 with a patient-to-control ratio of 1:1 was decided initially with an option to increase the sample size depending on the significance of the results. As a majority of COPD patients in our setup were males, the study was restricted to male patients. An age range of 30–80 years was selected for this pilot study. Patients previously diagnosed with chronic granulomatous disease were excluded from the study.

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria were used to make a diagnosis of COPD—a FEV<sub>1</sub>/FVC ratio of less than 70% in a patient with a post-bronchodilator FEV<sub>1</sub> of less than 80% of the predicted value.<sup>[10]</sup>

For the control group, healthy volunteers coming for “Master Health Checkup” camp held at our urban tertiary hospital were selected and were gender-matched by restricting the gender to male and the age group to 30–80 years. Only nonsmokers were selected for the control group. Volunteers with symptoms/signs of any illness were excluded.

Along with the informed consent document, patients were subjected to a questionnaire and data were collected regarding age, reason for hospital visit, smoking history, current medications, and history of any concurrent illnesses.

### Assessing neutrophil function

Blood samples (2 mL each) from the antecubital vein were taken from the COPD patients as well as the healthy volunteers and were stored in ethylenediaminetetraacetic

acid (EDTA) tubes. To assess neutrophil function, the NBT test was performed on each of the samples and the data were analyzed. The NBT test was repeated after stimulation of neutrophils with fixed quantities of *E. coli* endotoxin. These tests were performed for each blood sample individually within 15 min of collection, to avoid degeneration of neutrophils in the sample.

In the NBT test,<sup>[11]</sup> neutrophils are exposed to the yellow dye NBT. Inactive neutrophils do not ingest this dye, but if stimulated, they take the dye into phagosomes, and intracellular reduction by superoxide free radical converts them to insoluble, blue crystals of formazan. These crystals are visible in the light microscope and can be counted. The test gives information about both metabolic and respiratory burst activities.

## RESULTS

The COPD patients and the healthy volunteers in the study group were all residents of urban Bangalore. The 15 COPD patients were aged 45–80 years and healthy volunteers were aged 42–60 years. The mean age of the COPD patients in our sample population was found to be 64 years.

Healthy volunteers who had a history of smoking were not included in the study. However, as this is based on history, there is a possibility that there were volunteers who actually had a history of smoking but refused to admit it. On the other hand, 87% (13/15) of the COPD patients had a history of smoking. Duration of smoking history ranged from 0 years to 50 years of smoking. The average smoking history was 26 years.

All the patients admitted had a history of sudden increase in breathlessness with 40% (6/15) presenting with fever of less than 1 week duration that led to their admission in the hospital. Clinical examination revealed hyperresonant lungs on percussion and bilateral rhonchi and crepitations on auscultation in all of our COPD patients. Healthy volunteers were found free of any significant clinical findings. All patients had a history of usage of B<sub>2</sub> agonist (salbutamol), with 33% (5/15) concurrently using doxophylline. The blood count report indicated relative neutrophilia in 87% of patients with average neutrophil percentage of 85%. Radiography revealed findings of lung hyperinflation (emphysema) in all the patients. Findings in the 15 healthy volunteers were insignificant.

Using the NBT test, the number of activated neutrophils in COPD patients was found to be significantly higher as compared to healthy volunteers (55.8% vs 18.6%). After activation with *E. coli* endotoxin, the difference in levels of activated neutrophils remained significant with a higher *t*-value (11.84 without endotoxin vs 12.62 with endotoxin) [Table 1, Figure 1a and b].

**DISCUSSION**

The results indicate that there was a significant increase in the percentage of neutrophil activation in patients with COPD as compared to healthy volunteers [Table 1]. This signifies that neutrophils in patients with COPD tend to show an overall increased metabolic activity. Our finding corroborates with studies that have utilized flow cytometry to measure respiratory burst activity of neutrophils in COPD patients.<sup>[9]</sup> By using the NBT dye technique, we have further evidenced that neutrophils in COPD patients indeed behave differently in terms of metabolic activity. Although time-consuming, the NBT test gave us an alternative and inexpensive method that gives us an idea of the activity of the neutrophils. By reducing the dye to insoluble formazan crystals that could be detected by light microscopy, neutrophils in patients with COPD seem to be much more effective in producing ROS. The significance of this phenomenon is unclear. However, despite the fact that our COPD patients are from diverse age groups and are with varying stages of the disease, the neutrophil function in them remained consistently higher as compared to our healthy subjects. However, there is a chance that some of the volunteers in our control group may have withheld their smoking history. Whether this neutrophil “transformation” is the result of smoking or an indicator of pathogenesis of COPD is yet to be ascertained.

Thus, the results hint at a possible pathogenic mechanism in COPD related to the metabolic activity of neutrophils.

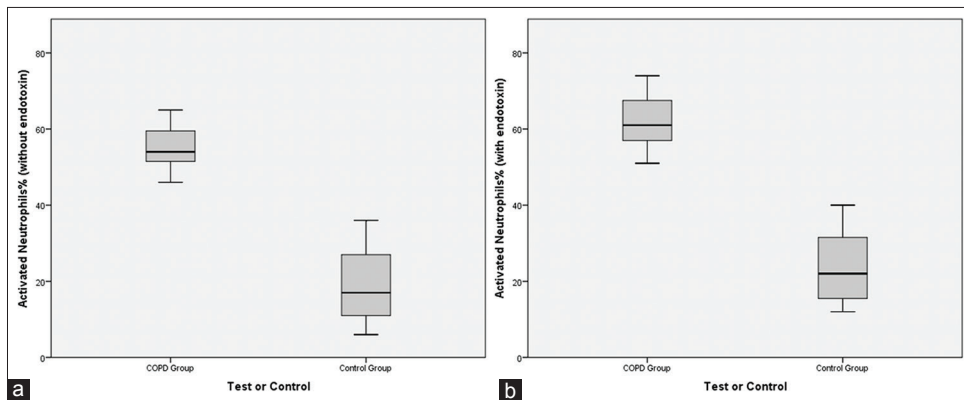
**Table 1: Activated neutrophil percentages for the COPD patients and the control group**

	Mean	SD	IQR	t	P
Unstimulated					
COPD	55.80	6.75	9	11.84	<0.001
Control	18.67	10.10	17		
Stimulated <sup>a</sup>					
COPD	62.00	7.38	12	12.62	<0.001
Control	23.27	9.32	17		

<sup>a</sup>With *E. coli* endotoxin, SD: Standard deviation, t: 2-tailed student's t-test value, IQR: Interquartile range, COPD: Chronic obstructive pulmonary disease

This increase in neutrophil activity may be explained by the release of large amounts of various inflammatory mediators, such as leukotriene B4, Il-8, and TNF, that occur in the circumstance of chronic inflammation throughout the pulmonary airways, parenchyma, and vasculature. These mediators lead to leukocyte recruitment and activation. Macrophages may play an important role in driving the inflammatory process in the COPD patients and may release neutrophil chemotactic factors as well as proteolytic enzymes. Thus, the entire mechanism can be likened to be a sort of snowball effect. Exposure to irritants such as cigarette smoke over a long period of time invokes the inflammatory response leading to the release of various chemical mediators.<sup>[12]</sup> These mediators in turn recruit neutrophils and other leukocytes, and these leukocytes themselves release more mediators, until the neutrophils in the peripheral blood get accustomed to the high level of inflammatory activity and they get perpetually activated. Thus, when triggered by, for example, a bacterial infection, the symptoms of COPD get accentuated and present as acute exacerbations. The outcome of this phenomenon is progressive worsening of COPD and increased suffering for the patients.

Having completed this case control study, we have obtained further evidence of the varied metabolic and respiratory burst activity of COPD neutrophils. A large number of studies on a wider spectrum of COPD patients need to be performed to further elucidate this property. Although the implications of this finding are unclear, the NBT test may possibly be used as a screening test for COPD. As we have obtained statistically significant data with our small sample size, we feel that this technique can be applied in larger studies especially in institutions where expensive equipment for techniques such as flow cytometry may not be available. Further tests need to be done to study this aspect of neutrophils at different stages of the disease and to possibly correlate neutrophil activity with severity of COPD. By targeting the metabolic action of neutrophils, it may be possible to develop alternate treatment modalities for the COPD patients.



**Figure 1:** (a and b) Distribution of activated neutrophil percentages in the COPD patients and the control group

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