

ROLE OF HYPOXIA ON PLATELET FUNCTION IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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ABSTRACT

Aim: Objective of our study is to establish the presence of platelet hyperactivity in hypoxemic patients with COPD.

Methods: Platelet hyperactivity in patients with COPD as compared to age and sex matched controls was determined by following experiments. Thrombin was used on washed human platelets suspension and ADP on platelet rich plasma to show that there was raised light transmittance in the presence of platelet activation. Free intracellular calcium was measured in a fluorescence spectrophotometer after pre-treatment with calcium chloride. Analysis of leukocyte contamination eliminated the role of leukocyte mediated inflammatory processes in platelet activation. The study was approved by the Institutional Ethics Committee and due informed consent was obtained from participants in the study.

Results: 22 hypoxemic patients were selected for the study based on their diagnosis of COPD (from history and spirometry) along with age and sex matched controls. Presence of comorbidities and other factors that cause platelet activation were excluded. Level of platelet aggregation was determined by several experiments. By an aggregometer using platelet agonists (thrombin and ADP) it was found that platelet aggregation was significantly higher in hypoxemic COPD patients than normal healthy controls. Fluorescence spectrophotometer was used to measure intracellular calcium as a marker of platelet activation and it was found that hypoxemic COPD patients had significantly higher platelet aggregation than normal healthy controls. However no significant difference was found in other markers of platelet activation studied namely P-selectin exposure and PAC-1 binding between the two groups.

Conclusion: There is increased platelet aggregation in hypoxemic COPD patients as determined by platelet agonist studies and increased intracellular calcium. Possible role of intracellular calcium as marker of thrombotic risk. In conclusion hypoxemia due to COPD has a direct atherothrombotic effect independent of other prothrombotic factors.

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INTRODUCTION

GOLD 2017 defines COPD as “Chronic Obstructive Pulmonary Disease (COPD) is a common preventable and treatable disease, is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases.”(1)

COPD is one of the leading causes of morbidity and mortality worldwide. Hypoxemia is caused by ventilation perfusion (V/Q) mismatch which occurs in COPD due to airflow limitation. Since it is an inflammatory disease it is known to

cause many complications in cardiovascular system such as cor pulmonale, pulmonary arterial hypertension and also leads to thromboembolic complications. Very few studies have been carried out exploring the relation between COPD and the increased propensity towards thromboembolic disorders. Such studies have to some extent elucidated on platelet and its activation during chronic normobaric hypoxia. Such a correlation can help in the therapeutic establishment of a role of antiplatelets in prevention of thromboembolic complications of COPD.

COPD is a leading cause of morbidity and mortality worldwide.(1) The role of COPD in causing systemic inflammation by spill-over of the localized pulmonary compartment inflammation into the systemic circulation though debated is well-known. The opponents of the theory

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proposes that the non-pulmonary compartment itself undergoes an enhancement in the production of inflammatory mediators.(2). However no debate exists in the understanding that there is an associated increased propensity for vascular diseases in COPD.(3)Arterial and venous thrombotic events are more frequent in COPD patients with worsening disease. (3). The worsening disease state in COPD can be quantified in terms of decreasing FEV1/FVC ratio. The spectrum of cardiovascular diseases such as right ventricular (RV) dysfunction, pulmonary hypertension (PH), coronary artery disease (CAD), Venous thromboembolism (VTE) and arrhythmias associated with COPD has been recognized for many years.(4) Alterations in gas exchange, vascular biology and remodeling of vasculature all contribute to the pathogenesis of pulmonary vascular diseases and its sequelae in COPD. (4)

Previously it has been shown that platelets have a role in lung inflammation in addition to hemostasis, atherosclerosis and cardiovascular events. It had been demonstrated that increased platelet counts in acute exacerbations of COPD is associated with increased mortality independent of other factors. It was concluded that platelets in COPD patients are hyper responsive and may play a role in lung inflammation and increased cardiovascular risk profile.(5)

MATERIALS AND METHODS

COPD patients between the age group of 40 to 60 years presenting to SS Hospital were screened for the exclusion criteria and selected for the study. Diagnosis of COPD was made based on history, physical examination, chest x-ray and spirometry results. The PaO₂ was less than 60 mmHg. All participants had quit smoking at least more than 1 year prior to the study.

Exclusion criteria included pregnancy, active smokers, alcoholics, drug abusers, patients on anticoagulant and antiplatelet medication, patients on long term steroids and domiciliary oxygen therapy and patients with comorbidities of hypertension, diabetes mellitus, inflammatory diseases, infections, coagulopathies, renal diseases and malignancies.

Patients meeting the inclusion criteria were asked at OPD if they were willing to participate and informed consent was taken before enrolling in the study. Initial ABG was done immediately after admission in Chest Ward. Patients if on inhaled bronchodilators were informed to withhold them for at least 12 hours prior to the performance of spirometry. The best three efforts were summed electronically and those who had FEV₁ less than 70% with no post bronchodilator (salbutamol) reversibility were included in the study. Patients were further categorized with use of modified MRC dyspnea scale. Blood sampling was then carried out under aseptic conditions from a large vein in the antecubital fossa without the use of a tourniquet for routine examinations and platelet function studies. For platelet function studies 5 ml blood was collected in a vial containing 0.7 ml acetate-citrate-dextrose (ACD) and contents mixed by gentle tilting.

After collection the blood sample was immediately sent to biochemistry laboratory for processing.

Stock concentrations and storage conditions for reagents

Reagents stored at -20 °C

1. Acetyl salicylic acid (1M) in ethanol
2. Fura-2 AM
3. ADP
4. Thrombin

Reagents stored at 4 °C

1. Buffer A (10X): 1X contains 20 mM HEPES (pH 7.4), 138 mMNaCl, 2.9 mMKCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄, 1 mM ethylene glycol tetra-acetic acid supplemented with 5mM glucose.
2. Buffer B (10X): 1X contains 20 mM HEPES (pH 7.4), 138 mMNaCl, 2.9 mMKCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄ supplemented with 5mM glucose.
3. Glucose (1 M)
4. Calcium chloride (100 mM)
5. Acetate-citrate-dextrose (ACD)

Reagents stored at room temperature

1. EDTA (0.5 M); pH 7.4 in type I deionized water (18.2 MΩ.cm, Millipore)
2. EDTA (0.1 M), pH 7.4 in type I deionized water (18.2 MΩ.cm, Millipore)
3. Type I deionized water (18.2 MΩ.cm, Millipore).
4. Isotone

METHODS

Platelet preparation

Platelets were isolated by differential centrifugation from fresh human blood, as already described (Sonkaret *et al.*, 2014). Briefly, blood from healthy volunteers was collected in acetate-citrate-dextrose and centrifuged at 180 x g for 10 min. Platelet-rich plasma (PRP) was incubated with 1 mM acetylsalicylic acid for 15 min at 37°C. After addition of ethylene diamine tetra-acetic acid (EDTA) (5 mM), platelets were sedimented by centrifugation at 600 x g for 10 min. Cells were washed in buffer A (20mM HEPES, 138 mMNaCl, 2.9 mMKCl, 1mM MgCl₂, 0.36 mM NaH₂PO₄, 1 mM ethylene glycol tetra-acetic acid (EGTA), supplemented with 5mM glucose. Platelets were finally re-suspended in buffer B (pH 7.4), which was same as buffer A, but without EGTA. Final cell count was adjusted to 2.5-4.0 x 10⁸ cells per ml. All steps were carried out under sterile conditions and precautions were taken to maintain cells in resting state. Ethical clearance was obtained in accordance with the guidelines.

Cell counting and analysis of leukocyte contamination

Platelets were diluted in isotonic solution and counted by using Beckman coulter counter Multisizer 4. Analysis of leukocyte contamination were performed according to size distribution of cells in Beckman coulter counter Multisizer 4.

Platelet aggregation

Platelet aggregation was monitored turbidimetrically using an optical lumi-aggregometer (Chrono-log model 700-2; Wheecon instruments, India). We performed aggregation study both in platelet rich plasma (PRP) as well as in washed human platelets. Both PRP and washed human platelets (300 µl) were incubated at 37°C for 1 min under constant stirring

(at 1200 rpm) before addition of agonists. Aggregation was recorded as percent light transmitted through the sample as a function of time, while blank represented 100% light transmission. (Medispec, India) and quantified using VisionWorks LS software (UVP).

Measurement of intracellular free calcium

Platelet rich plasma (PRP) was incubated with 2 µM Fura-2 AM for 45 minutes at 37°C in the dark. The Fura-2 loaded platelets were washed and finally re-suspended in buffer B. Equal cell counts were maintained both in control as well as in COPD. Fluorescence was recorded in 400 µl aliquots of platelet suspension, both control as well as in COPD, at 37°C under non-stirring condition. Excitation wavelengths were 340 and 380 nm and emission wavelength was set at 510 nm in Hitachi fluorescence spectrophotometer (model F-2500). Changes in intracellular free calcium concentration, Ca²⁺, in control and COPD cases were monitored from the fluorescence ration (340/380) using Intracellular Cation Measurement Program in FL Solutions software. F_{max} was determined by lysing the cells with 40 µM digitonin in the presence of saturating CaCl₂. F_{min} was determined by the addition of 2 mM EGTA. Intracellular free calcium was calibrated according to the deviation of Grynkiewicz *et al.* (6)

Inclusion criteria

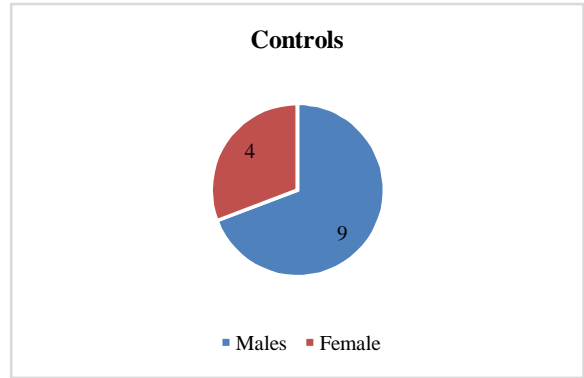
COPD patients with:

- FEV₁/FVC < 70%
- Post bronchodilator reversibility < 200ml and < 12%
- Hypoxemia:
ABG with mild and moderate hypoxemia. (mild hypoxemia → PaO₂ 60-80mmHg; moderate hypoxemia PaO₂ 40-60 mmHg)
- Aged 40-60 years.

Exclusion criteria

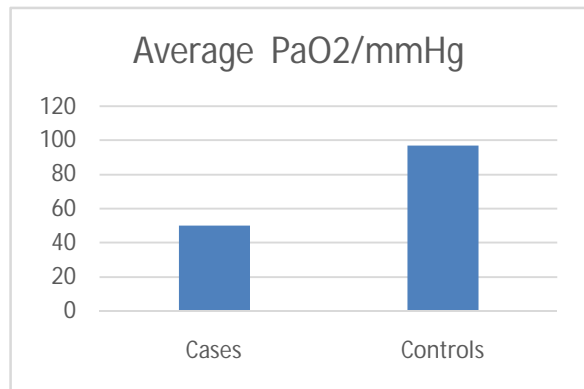
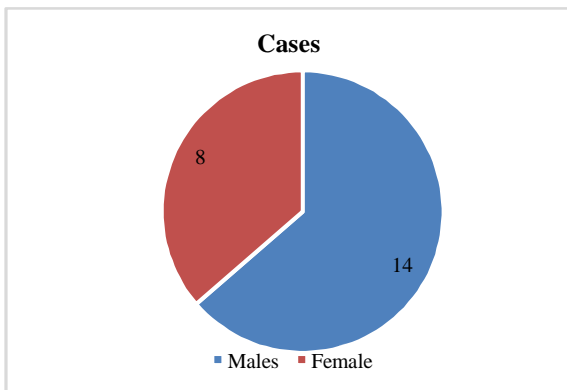
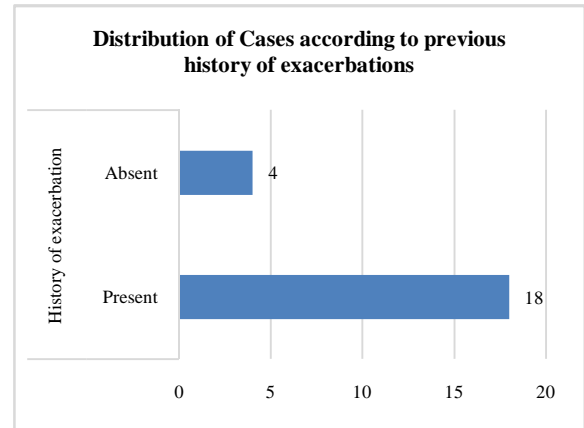
- Domiciliary oxygen therapy
- Hypertension
- Diabetes mellitus
- Inflammatory diseases
- Infections
- Coagulopathies
- Renal diseases
- Liver diseases
- Malignancies
- Long term steroids use
- Anticoagulant and antiplatelet medication use
- Drug abuse
- Alcoholics
- Active smokers
- Pregnancy

OBSERVATIONS AND RESULTS



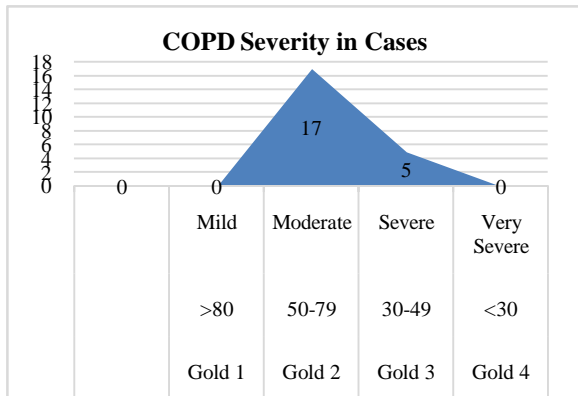
22 patients were selected based on their diagnosis of COPD from history and spirometry according to that which is defined in GOLD.(1) They were further matched to 13 controls with respect to age and sex. Most of the selected candidates were in their fifties which is due to the natural history of COPD which takes decades to develop the typical manifestations of persistent respiratory symptoms and airflow limitations.(1) There was an overall male predominance among the cases due to social circumstances which leads to more smoke exposure in them.

Previous history of exacerbations is important because it is associated with an increase in prevalence of exacerbations, hospitalizations and risk of death. There was no fixed correlation between the GOLD severity classification and the increased risk of exacerbations. The most important predictor of future exacerbations are previous history of treated exacerbations itself.(1)



Almost all the cases selected had one or the other form of smoke exposure. As the main etiology behind the airway abnormalities that occurs in COPD is due to exposure to such noxious particle or gases this is entirely expected.

As severe exacerbations may be associated with acute respiratory failure this provided us with the opportunity to study the effects of hypoxemia induced platelet dysfunction in COPD patients.



RESULTS

Cell counting and analysis of leukocyte contamination

Platelets were diluted in isotonic solution and counted by using Beckman coulter counter Multisizer 4. Equal cell counts were maintained in both control and COPD. Analysis of leukocyte contamination were performed according to size distribution of cells in Beckman coulter counter Multisizer 4 and we found that there was no leukocyte contamination in platelet isolates (Figure 1).

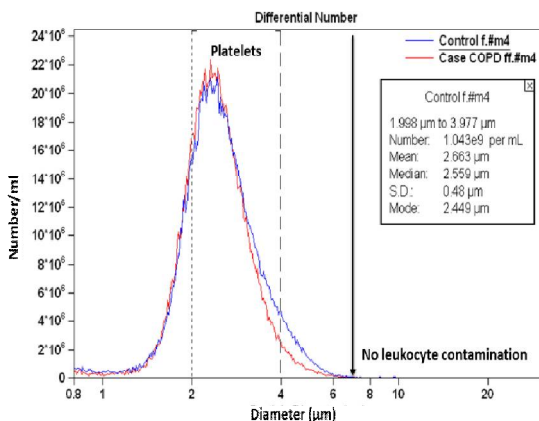


Figure 1 Cell counting and analysis of leukocyte contamination in platelet isolates

Graphical representation demonstrating number per ml of 1.5-4.0 μ m and 7.0-20 μ m-sized particles in cell suspension. Number per ml of 7.0-20 μ m-sized contaminants, reflective of no leukocyte contamination.

Platelet aggregation study in patients with COPD

Platelet aggregation study was performed both in platelet rich plasma (PRP) as well as in washed human platelets under stirring condition (at 1200 rpm) at 37°C in an aggregometer. Addition of thrombin (1 U/ml, a strong platelet agonist) to washed human platelets suspension (300 μ l) induces rise in light transmittance to 100 \pm 10 % in COPD cases as compared to healthy controls (50 \pm 5%) (Figure 2A). Next, we also

determined the platelet hyperactivity in COPD cases by using another platelet agonist ADP (2.5 μ M). Addition of ADP (2.5 μ M) to platelet rich plasma also induces rise in light transmittance to 90 \pm 5% in COPD cases as compared to healthy controls (15 \pm 5 %) (Figure 2B). Thus, above data underscored that platelet are hyperactive in patients with COPD as compared to healthy controls.

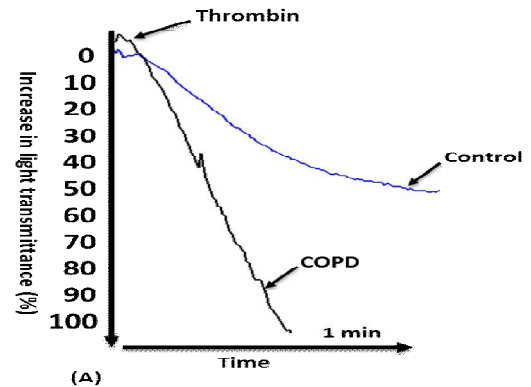


Figure 2 A Platelet aggregation study in healthy controls and COPD cases.

(A) Shows thrombin-mediated (1 U/ml) platelet aggregation (percent light transmittance) in washed human platelets isolated from healthy controls and COPD cases. (B) Shows ADP-mediated (2.5 μ M) platelet aggregation (percent light transmittance) in platelet rich plasma (PRP) isolated from healthy controls and COPD cases.

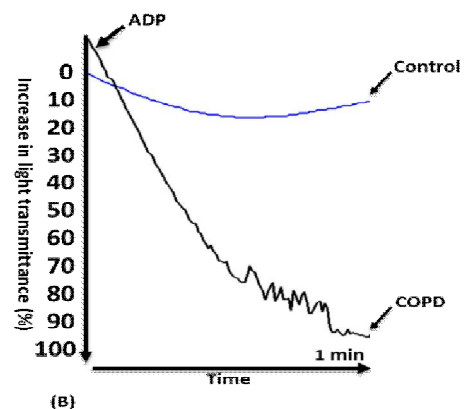


Figure 2 B Platelet aggregation study in healthy controls and COPD cases

(A) Shows thrombin-mediated (1 U/ml) platelet aggregation (percent light transmittance) in washed human platelets isolated from healthy controls and COPD cases. (B) Shows ADP-mediated (2.5 μ M) platelet aggregation (percent light transmittance) in platelet rich plasma (PRP) isolated from healthy controls and COPD cases.

Measurement of free intracellular calcium in platelets from patients with COPD

As platelet activation is linked with increase in concentration of cytosolic calcium. We examined the intracellular calcium level in platelets from patients with COPD as well as in healthy controls. For this we pre-treated platelets with calcium chloride (1 mM) for 5 min at room temperature and free intracellular calcium was measured in a fluorescence spectrophotometer. We found that, platelets from patients

with COPD have increased intracellular calcium level as compared to healthy controls.

Fura-2 stained platelets were pre-treated with calcium (1 mM) for 5 min at room temperature. Intracellular calcium was measured as described above.

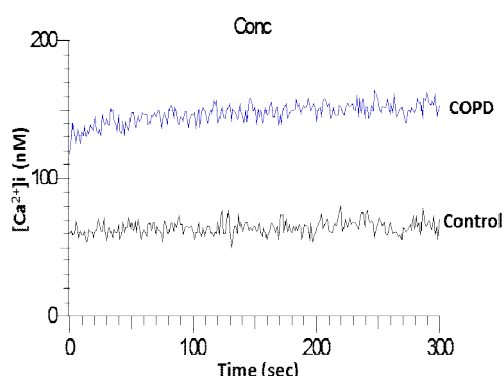


Figure 3 Measurement of free intracellular calcium in platelets from patients with COPD and in healthy controls

DISCUSSION

We demonstrated that platelets are hyperactive in hypoxemic COPD patients by using platelet agonists on unstimulated platelets to show that they have increased aggregability. Thrombin was used on washed human platelets suspension and ADP on platelet rich plasma to show that there was raised light transmittance (platelet aggregation) in hypoxemic COPD patients as compared to matched controls. This established that there is indeed platelet hyperactivity in patients with COPD.

Indirect methods of measuring platelet activation include urinary thromboxane B₂, (7) Monocyte platelet aggregate (8) and P-selectin (9) measurements which have been already studied. These studies suffered from the lack of specificity of the selected parameters for platelet activation alone.

A previous study done in our institute explored the possibility of platelet hyperactivity underlying the increased thrombotic events in COPD. It was demonstrated by flow cytometry that the level of plasma microparticles was significantly elevated in COPD patients as compared to normal healthy individuals. In the same study no significant difference was observed in markers of activated platelets including P-selectin exposure, PAC-1 binding and cytosolic reactive oxygen species between the two groups. (10)

We have measured intracellular calcium levels as a marker of platelet activation in COPD patients. No previous studies has been undertaken to demonstrate this phenomenon so far and also takes advantage of the fact that a high level of specificity exists for increased levels of intracellular calcium in activated platelets.

In our experiments leukocyte contamination was eliminated as stated and hence gives more strength to our results by eliminating effectively the role of leukocyte mediated inflammatory processes that may lead to platelet activation.

SUMMARY AND CONCLUSION

In accordance with the criteria formulated for patient selection 22 cases of COPD satisfying the GOLD definition was included in the study along with 13 age and sex matched

controls. Among the 22 cases 14 (63.63%) were males and 8 (36.36%) were females. All of the COPD cases were above 50 years of age with a mean age of 59 years. As expected majority of the cases were elderly males due to the high prevalence of COPD among this group. It is mainly due to the natural history of the disease which is chronic in nature and develops over decades. Furthermore smoking and exposure to other noxious particles and gases are more common in men.

Among the COPD patients selected 70.59% had moderate COPD and 29.41% severe COPD.

All cases and control with hypertension, diabetes mellitus, inflammatory diseases, infections, coagulopathies, renal diseases, liver diseases, and malignancies were excluded from the study. Long term steroids use, anticoagulant and antiplatelet medication use, history of drug abuse, alcoholism, active smoking, domiciliary oxygen therapy and pregnancy all resulted in exclusion from the study. All the cases had one or the other form of previous smoke exposure. All the cases had PaO₂ less than 60 mmHg.

All samples were checked for leukocyte contamination and equal cell count was maintained in both controls and cases. The initial step of leukocyte contamination is important to ensure that leukocytes and their inflammatory mediators do not act as a confounding factor in our results since inflammation also leads to platelet activation.

Platelet aggregation was determined by addition of agonists like ADP and thrombin. Such samples from COPD cases were compared with age and sex matched controls. There was rise in light transmittance (platelet aggregation) to significant levels in hypoxemic COPD patients in both experiments as compared to controls. This concludes that hypoxemia due to COPD has a direct atherothrombotic effect independent of other prothrombotic factors.

Platelet activation is linked with increase in concentration of cytosolic calcium. (11) It acts as a surrogate for platelet activation in our experiment in which we examined the intracellular calcium level in platelets from patients with COPD as well as in healthy controls. It was found that patients with COPD have increased intracellular calcium level as compared to healthy controls. This also proves that platelet activation occurs independently of other prothrombotic factors as a result of COPD.

Since the most common cause of mortality in COPD is due to cardiovascular complications it is of paramount importance that we elucidate the exact mechanism involved in the platelet activation in COPD. Our study had established that hypoxemia associated with COPD was directly responsible for platelet activation as compared to healthy age and sex matched controls. Thus it further consolidates the importance of maintaining normoxia in COPD patients and as exacerbations are the main reason for acute hypoxic episodes it elucidates the significance of preventing exacerbations in COPD patients. It further adds weight to the already established fact that oxygen supplementation acts a therapeutic intervention that reduces mortality in COPD patients.

Further studies will be needed to find out the exact mechanism in which hypoxia caused by COPD leads to increased level of platelet activation. This will pave the way

for interesting new innovations in therapeutic intervention to prevent atherothrombotic complications in COPD.

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