Original Article

# Hydrogen peroxide in exhaled breath condensate: A clinical study

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## ABSTRACT

**Objectives:** To study the ongoing inflammatory process of lung in healthy individuals with risk factors and comparing with that of a known diseased condition. To study the inflammatory response to treatment. **Background:** Morbidity and mortality of respiratory diseases are raising in trend due to increased smokers, urbanization and air pollution, the diagnosis of these conditions during early stage and management can improve patient's lifestyle and morbidity. **Materials and Methods:** One hundred subjects were studied from July 2010 to September 2010; the level of hydrogen peroxide concentration in exhaled breath condensate was measured using Ecocheck. **Results:** Of the 100 subjects studied, 23 were healthy individuals with risk factors (smoking, exposure to air pollution, and urbanization); the values of hydrogen peroxide in smokers were 200-2220 nmol/l and in non-smokers 340-760 nmol/l. In people residing in rural areas values were 20-140 nmol/l in non-smokers and 180 nmol/l in smokers. In chronic obstructive pulmonary disease cases, during acute exacerbations values were 540-3040 nmol/l and 240-480 nmol/l following treatment. In acute exacerbations of bronchial asthma, values were 400-1140 nmol/l and 100-320 nmol/l following treatment. In cases of bronchiectasis, values were 300-340 nmol/l and 200-280 nmol/l following treatment. In diagnosed pneumonia cases values were 1060-11800 nmol/l and 540-700 nmol/l following treatment. In interstitial lung diseases, values ranged from 220-720 nmol/l and 210-510 nmol/l following treatment. **Conclusion:** Exhaled breath condensate provides a non-invasive means of sampling the lower respiratory tract. Collection of exhaled breath condensate might be useful to detect the oxidative destruction of the lung as well as early inflammation of the airways in a healthy individual with risk factors and comparing the inflammatory response to treatment.

**KEY WORDS:** Ecocheck, exhaled breath condensate, hydrogen peroxide

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## INTRODUCTION

Airway inflammation plays an important role in various respiratory lung diseases, including recurrent wheezing, asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Several attempts have been made therefore to detect and monitor inflammatory changes and mediators using non-invasive methods. Analysis of exhaled breath condensate (EBC), a novel and a non-invasive method for studying the composition of airway lining fluid, has the potential for assessing airway inflammation.[1] Analysis of EBC is also useful for assessing the response to treatment.[2] This study helps to validate the analysis of EBC by measuring hydrogen peroxide ($H_2O_2$) concentration in healthy non-smokers, smokers, diseased, and also comparing the response to treatment. Inflammatory cells release $H_2O_2$, which can be detected in EBC. Elevated levels of $H_2O_2$ have been found in a number of respiratory disorders, thus $H_2O_2$ is considered to be a possible biomarker of airway inflammation.

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## MATERIALS AND METHODS

In this hospital-based study, conducted between July 2010 and September 2010, 100 randomly selected subjects were analyzed with EBC $H_2O_2$. Sputum positive tuberculosis patients, pregnant women, children less than 12 yrs and

immunocompromised patients were excluded from the study. EBC was collected and analyzed using Ecocheck-Ecosreen (Jaeger, Hoechberg, Germany) device in all 100 subjects. The subjects were instructed to clean the oral cavity with water and then breathe through a mouth piece and a 2 way non-breathing valve, which also serve as a saliva trap. They were asked to breath at a normal frequency and tidal volume wearing a nose clip for a period of 15 min. About 1-3 ml of EBC was collected at −2 to −4°C [Figure 1]. The collected EBC was diluted with equal quantity of dilution buffer. The diluted sample was analyzed in a measuring chamber containing biosensors.[3] The results were analyzed statistically using *t* test. The amount of condensate generated per exhalation varies among individuals. Minute ventilation remains the major determinant of the amount of condensate over time. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate.[4]

## RESULTS

Of the 100 cases studied, 23 were healthy individuals with risk factors, like smoking, exposure to air pollution and urbanization. The values of $H_2O_2$ in smokers were 200-2220 nmol/l and in non-smokers values were 340-760 nmol/l [Table 1, Figure 2]. In 10 smokers the standard deviation was 643.135 and in 13 non-smokers standard deviation was 217.279I with significant *P* value of 0.045 ($P<0.05$) [Table 2]. In people residing in rural areas values were from 20-140 nmol/l in non-smokers and 180 nmol/l in smokers. $H_2O_2$ concentrations were correlated with pack years. In majority of subjects, as the pack years increased, the $H_2O_2$ levels were also found to be increased. However in some of the subjects varied $H_2O_2$ levels were observed irrespective of pack years. For instance, one subject with 8 pack years had the $H_2O_2$ level as high as 2220 nmol/l, whereas in two subjects with 15 pack years, the values were 180 and 340 nmol/l [Table 3].

In patients who are known COPD presented with acute exacerbations (as per anthonisen criteria and GOLD criteria), the predicted $FEV_1$% varied from 32-65% (48±16%) with $H_2O_2$ levels of 540-3040 nmol/l. These patients were treated with bronchodilators and corticosteroids as per treatment protocol. Following treatment, the predicted $FEV_1$% varied from 35-71%(53±18%) and the concentrations of $H_2O_2$ were reduced to 240-480 nmol/l [Table 4, Figure 3]. Before treatment standard deviation was 770.076 and following treatment standard deviation was 94.571 with *P* value of 0.022 [Table 5].

In cases of acute exacerbations of bronchial asthma, the values of $H_2O_2$ were 400-1140 nmol/l and following

**Table 1: $H_2O_2$ concentration in cases of healthy subjects**

| | Rural (*n*) | Urban (*n*) |
|---|---|---|
| Smokers | 180 nmol/l (1) | 200-2220 nmol/l (10) |
| Non-smokers | 20-140 nmol/l (9) | 340-760 nmol/l (13) |

**Table 2: Statistical analysis of $H_2O_2$ concentration in healthy subjects**

| $H_2O_2$ | *N* | Mean | Standard deviation | Standard error mean | *P* value |
|---|---|---|---|---|---|
| Smokers | 10 | 750.00 | 643.135 | 203.377 | 0.045 |
| Non-smokers | 13 | 344.62 | 217.279 | 60.262 | |

In 10 smokers the standard deviation was 643.135. In 13 non-smokers standard deviation was 217.279. *P* value <0.05

**Table 3: Values of $H_2O_2$ in relation to pack years**

| Number of pack years | Range of $H_2O_2$ |
|---|---|
| 40 | 1180-3040 nmol/l |
| 30 | 780-1060 nmol/l |
| 15 | 680-820 (340,180) nmol/l |
| 12 | 340-620 nmol/l |
| 8 | 240-540 (2220) nmol/l |
| 6 | 260-300 nmol/l |
| 5 | 240 nmol/l |
| 2 | 200 nmol/l |

**Table 4: $H_2O_2$ concentration and predicted $FEV_1$% in COPD**

| | Acute exacerbation (*n*) | After treatment (stable COPD) (*n*) |
|---|---|---|
| $H_2O_2$ conc. | 540-3040 nmol/l (10) | 240-480 nmol/l (14) |
| $FEV_1$ (% predicted) | 32-65% (48±16%) | 35-71% (53±18%) |

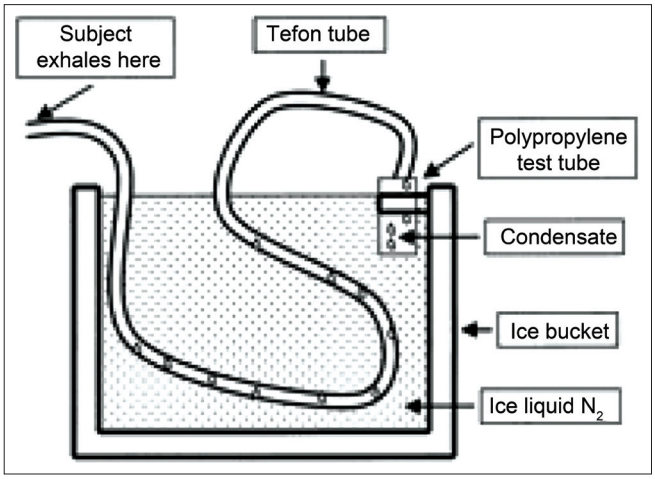


**Figure 1:** Schematic representation of a collection apparatus

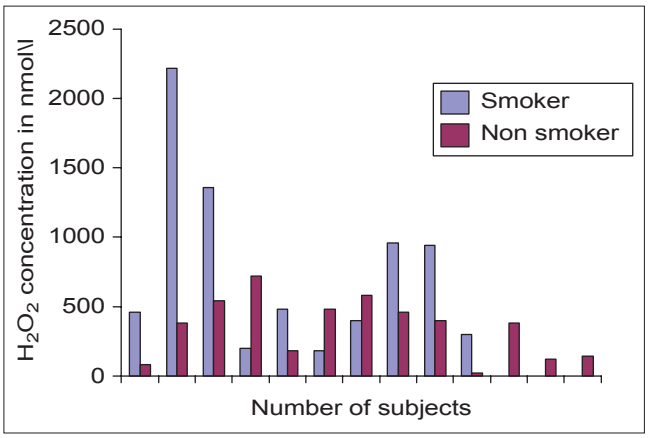


**Figure 2:** Analysis in healthy subjects

treatment the values were reduced to 100-320 nmol/l [Table 6, Figures 4 and 5] with a significant *P* value of 0.002 [Table 7]. In these patients predicted $FEV_1$% varied from 18-62% (40±22%) and following treatment the predicted $FEV_1$% drastically improved to 68-89% (78±10).

In other conditions like bronchiectasis, values of $H_2O_2$ were 300-340 nmol/l and 200-280 nmol/l [Table 8], in pneumonia 1060-11800 nmol/l and 540-700 nmol/l [Table 9], and in patients with interstitial lung diseases 220-720 nmol/l and 210-510 nmol/l [Table 10] before and after treatment, respectively. The *P* values of the above three conditions could not be calculated as the sample size was small. Spirometry was also performed in all these patients but the lung function tests could not be correlated with $H_2O_2$ in all these patients as the sample size was small.

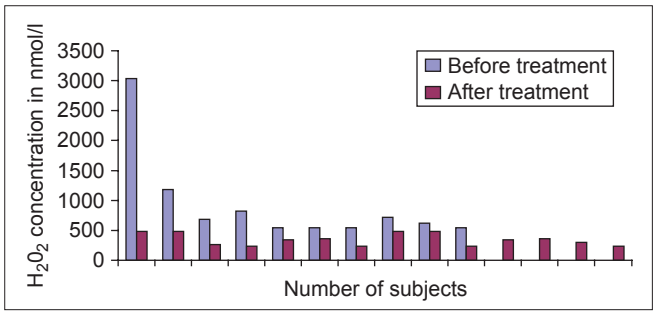


**Figure 3:** Analysis report in COPD

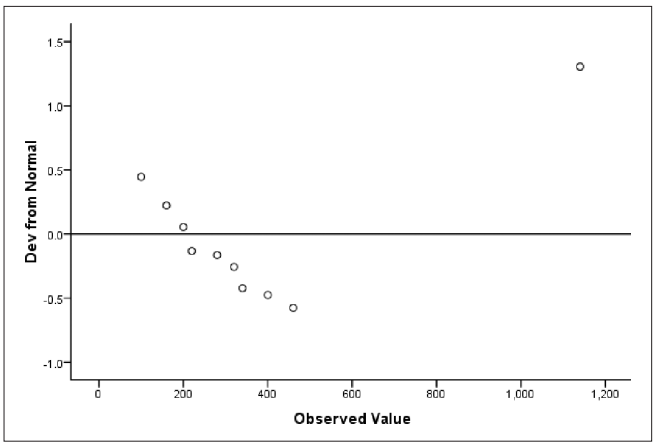


**Figure 4:** Analysis report in bronchial asthma

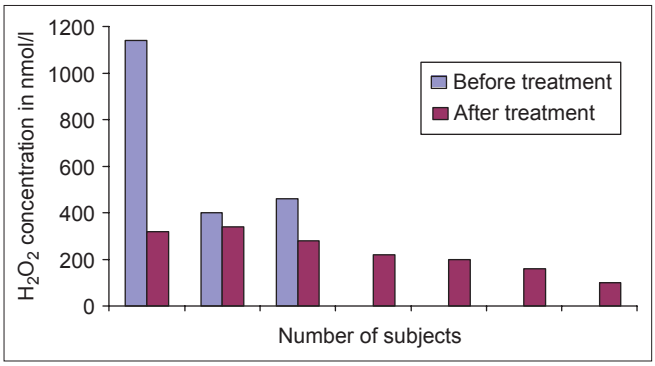


**Figure 5:** Analysis report in bronchial asthma

## DISCUSSION

A variety of inflammatory markers present in EBC have been investigated as possible biomarkers of disease activity.[5] EBC contains aerosolized airway epithelial lining fluid particles and volatile compounds. There is increasing evidence that exhaled markers may reflect biochemical changes in airway lining fluid.[6] Table 11 shows the various markers in exhaled breath. $H_2O_2$ was one of the most commonly studied markers in EBC.[7,8] Lung is constantly exposed to oxygen, so highly susceptible to oxidative stress in the form of reactive oxygen species (super oxide ion, hydroxyl radical, and hydrogen peroxide). These reactive oxygen species produced by active inflammatory cells like neutrophils, macrophages, activated eosinophils, epithelial cells, and endothelial cells.[9] Thus, measurement

**Table 5: Statistical analysis of $H_2O_2$ concentration in COPD**

| $H_2O_2$ | *N* | Mean | Standard deviation | Standard error mean | *P* value |
|---|---|---|---|---|---|
| Stable COPD | 13 | 335.38 | 94.571 | 26.229 | 0.022 |
| COPD exacerbation | 10 | 922.00 | 770.076 | 243.520 | |

After treatment standard deviation: 94.571. Before treatment standard deviation: 770.076. *P* value <0.05(0.022). COPD: chronic obstructive pulmonary disease

**Table 6: $H_2O_2$ concentration and predicted $FEV_1$% in bronchial asthma**

| | Acute exacerbation (*n*) | After treatment (stable) (*n*) |
|---|---|---|
| Smokers ($H_2O_2$ concentration) | 1020 nmol/l | 320 nmol/l (1) |
| Non-smokers ($H_2O_2$ concentration) | 400-1140 nmol/l (3) | 100-280 nmol/l (6) |
| $FEV_1$ (% predicted) | 18-62% (40±22) | 68-89% (78±10) |

**Table 7: Statistical analysis of $H_2O_2$ concentration in asthma**

| Tests of normality | Kolmogorov-Smirnova | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|
| | Statistic | Df | Sig. | Statistic | Df | Sig. |
| $H_2O_2$ | .270 | 10 | .038 | .729 | 10 | .002 |

*P* value <0.05(0.038)

**Table 8: $H_2O_2$ concentration in Bronchiectasis**

| | With secondary infection (*n*) | After treatment (*n*) |
|---|---|---|
| Smoker | 340 nmol/l (1) | 220 nmol/l (1) |
| Non-smoker | 300-340 nmol/l (6) | 200-280 nmol/l (8) |

*P* value cannot be calculated due to small sample size

**Table 9: $H_2O_2$ concentration in Pneumonia**

| Before treatment (*n*) | After treatment (*n*) |
|---|---|
| 1060-11800 nmol/l (4) | 540-700 nmol/l (3) |

*P* value cannot be calculated due to small sample size

**Table 10: $H_2O_2$ concentration In interstitial lung disease**

| Before treatment (*n*) | After treatment (*n*) |
|---|---|
| 220-720 nmol/l (4) | 210-510 nmol/l (6) |

*P* value cannot be calculated due to small sample size

**Table 11: Contents of exhaled breath condensate**

| |
|---|
| Isoprostanes- 8-Isoprostane |
| Leukotrienes- LTB4, LTD4, LTE4, Cys-LTs (LTC4/LTD4/LTE4) |
| Prostanoids- PGE2, PGF2α, PGD2, TxB2, |
| Nitrogen reactive species- Nitrite, Nitrate, S-Nitrosothiols, 3-Nitrotyrosine |
| Adenosine, IGF |
| Hydrogen ions pH |
| Aldehydes |
| Hydrogen Peroxide |
| Glutathione |
| Cytokines- IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, Interferon-γ MIP-1α, |
| PAI-1, RANTES, TGF-β, TNF-α, MIP-β |
| TBARS |
| Electrolytes- sodium, potassium, calcium, magnesium, chloride |

IGF-1: Insulin-like growth factor-1, IL: Interleukin, LT: Ieukotriene, MIP-1: Macrophage inflammatory protein-1, PAI-1: Plasminogen activator inhibitor-1, PG: Prostaglandin, TBARS: Thiobarbituric acid reactive substances, TGF-β: Transforming growth factor-β, TNF-α: Tumor necrosis factor-α, Tx: Thromboxane

of concentration of reactive oxygen species in exhaled breath condensate can reflect the underlying inflammation. In the present study, $H_2O_2$ measurements were evaluated and analyzed.

Exhaled breath condensate was measured in cigarette smokers versus healthy control subjects.[10] Cigarette smokers had a 5-fold higher mean expired breath $H_2O_2$ level than non-smokers.[11,12] In another study that attempted to correlate exhaled breath $H_2O_2$ with $H_2O_2$ generated from the alveolar lining fluid, exhaled $H_2O_2$ was $5 \times 10^4$ times lower than $H_2O_2$ produced in the alveolar lining fluid. This difference was attributed to the presence of antioxidants in the lining fluid of the lower respiratory tract. The above study showed that level of $H_2O_2$ in exhaled breath condensate of smokers is increased half an hour after combustion of one cigarette. In the present study, the levels of $H_2O_2$ were elevated in healthy smokers and also in healthy non-smokers who are residing in urban area compared to those of rural area. These elevated levels can be attributed to constant exposure for vehicle and industrial pollution. The $H_2O_2$ values in healthy individuals with risk factors are more than 180 nmol/l, whereas the healthy individuals residing in rural areas with minimal risk factors had values of $H_2O_2$ varied from 20-140 nmol/l. Hence, the level of $H_2O_2$ below 200 nmol/l can be considered as normal reference value as per our study and needs further studies to support our observation in India.

Dekhuijzen and coworkers demonstrated increased $H_2O_2$ in exhaled breath condensate of patients with stable COPD relative to healthy controls with a further increase noted during an acute exacerbation.[13-16] The effect of corticosteroids on the level of hydrogen peroxide studied by van Beurden *et al*.[17] Levels of $H_2O_2$ also correlated with eosinophils differential counts in induced sputum. In the present study, $H_2O_2$ was increased in all stable COPD patients with further increase during acute exacerbations with reduced predicted $FEV_1$%. Lower the value of predicted $FEV_1$%, higher the elevated $H_2O_2$ concentration. These patients with exacerbations after treatment with bronchodilators, corticosteroids (both inhalational and parenteral) showed reduction in $H_2O_2$ levels with the improvement in predicted $FEV_1$%.

Oxidative stress plays an important pathogenetic role in many inflammatory diseases including asthma. Emelyanov *et al*. studied the correlation between asthma, concentration of $H_2O_2$ and $FEV_1$. They concluded that exhaled $H_2O_2$ may be useful to assess the degree of airway inflammation and oxidative stress in asthmatic patients and significant negative correlation among exhaled $H_2O_2$ and $FEV_1$.[18] In the present study, the $H_2O_2$ levels were elevated during acute exacerbation with decreased predicted $FEV_1$% and reduced $H_2O_2$ levels with significant improvement in predicted $FEV_1$% in all cases following treatment.

Bronchiectasis, a suppurative lung disease, is characterized by significant pulmonary oxidant stress that can be measured using exhaled breath $H_2O_2$. In a study by Loukides and coworkers,[2] patients with bronchiectasis displayed exhaled $H_2O_2$ levels higher than normal controls, and a negative correlation between the $H_2O_2$ levels and $FEV_1$ was documented. In the studied cases of bronchiectasis, $H_2O_2$ was raised significantly with reduction in the levels following treatment.

In the pilot study by Mikuls *et al*., patients with rheumatoid arthritis with interstitial lung diseases had increased levels of exhaled $H_2O_2$ compared with controls, suggesting that EBC $H_2O_2$ is a potentially useful biomarker.[19] In the present study, in patients with interstitial lung disease, pneumonia $H_2O_2$ estimated by EBC were found to be raised and showed decreased values following treatment.

The measurement of the $H_2O_2$ marker in exhaled breath condensate can be used routinely for i) early prediction of the ongoing inflammatory process in healthy individuals who are exposed to risk factors, and for educating them in future, ii) early tool of assessing exacerbation of the lung condition and to reduce the morbidity, iii) as a marker in assessing the inflammatory response to treatment. Hence, the detection of $H_2O_2$ in EBC can be used for routine clinical practice and research activities.

This type of facility is a rare modality available in India, as per our knowledge. The drawbacks of this facility are that the establishment of the unit is quite expensive and sensors need to be changed for every new patient, which are to be imported and of high cost.

## CONCLUSION

Oxidative stress is implicated in various lung diseases. Its assessment with non-invasive technique is of great value. Collection of exhaled breath condensate is a non-invasive method. Measurement of $H_2O_2$ in EBC sample can be used

as a method of measuring oxidative destruction in the lung and inflammation of the airways. Even in healthy individuals with risk factors, elevated $H_2O_2$ levels in EBC is a general marker for airway inflammation and can be used as an early predictor of the ongoing inflammatory process. This measurement can be carried out easily and its application in inflammatory airway diseases has been extensively studied. Most of the clinical studies reported higher levels of $H_2O_2$ in healthy individuals with risk factors and diseased conditions compared to normal subjects without risk factors and also the levels of $H_2O_2$ decreased following treatment.

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