Molecular basis of superoxide dismutase alterations in Spirometry proven bronchial asthma

Syed Hafeezul Hassan,1 Rubina Ghani,2 Muhammad Sarwar3

Abstract
Objective: To study alterations in superoxide dismutase at molecular level in spirometry-proven bronchial asthma.
Methods: This pilot study was conducted at Baqai Medical University Hospital, Karachi, from June to December 2013, and comprised spirometry-proven asthmatics. The allele frequencies of missense polymorphisms of the exon-intron of a superoxide dismutase, copper-zinc superoxide dismutase were included in the analysis and compared with their age- and gender-matched healthy controls.
Results: Of the 45 participants, 30(66.7%) were cases and 15(33.3%) were controls. The mean age of cases and controls was 37.77±11.95 and 37.27±11.81 years, respectively. The case population showed significant mean baseline and predicted spirometric values (p<0.05). The mean serum superoxide oxide dismutase in cases and controls was 62.53± 15.23 and 55.65± 15.87, respectively. The superoxide dismutase genetic variants studied for the intronic polymorphism in copper-zinc superoxide dismutase showed increased risk of asthma compared with non-asthmatic controls.
Conclusion: Levels of serum superoxide dismutase were elevated with concomitant amplification of copper-zinc superoxide dismutase gene.
Keywords: SOD, Asthma, Spirometry. (JPMA 67: 1393; 2017)

Introduction
Asthma is characterised by chronic inflammation of the airways, associated with airway obstruction, airway hyper responsiveness (AHR) and remodelling.1 Allergic asthma is characterised by mucus hyper secretion and the accumulation of eosinophils in the airways in response to inhaled allergens.2,3 Recent genetic studies revealed that several cellular events are involved in the progression of asthma with subsequent damage to bronchial epithelium.4 These include complex interaction of cells and mediators, which generate increased amounts of reactive oxygen species (ROS).5 The presence of high levels of ROS, or chemically reactive free radicals and peroxides, adds to the pathogenesis of allergic asthma.6,7

Superoxide dismutase (SOD) are the only enzymes that convert superoxide radicals to hydrogen peroxide.8 Current regimens for asthma therapy usually maintain normal to near-normal pulmonary function and prevent chronic symptoms, but in rare cases asthma is severe or refractory to therapies including corticosteroids.9 The reasons for variable severity of asthma are unclear, but evidence suggests that ROS and reactive nitrogen species (RNS) excess and antioxidant deficiency in the lung are related to severity of airflow limitation and hyper reactivity.10 Although in some experimental systems of lung inflammation, antioxidants increase in response to oxidative stress and minimise oxidant-induced damage.10 Antioxidant enzymes crucial for protection of the airway against oxidative stress include superoxide dismutase whose activity is reduced in the oxidant-rich environment of the asthmatic airway. During asthma exacerbation, further loss of SOD activity occurs with enhanced production of oxygen radicals by inflammatory cells.11

Moreover, SOD activity in the lung is related to airway hyper reactivity and airflow limitation.12 In spite of evidence that localised inactivation of SOD activity occurs within the inflamed asthmatic airways, the relationship of systemic levels of SOD activity to quantitative measures of asthma severity is unknown. It was hypothesised that asthmatic individuals with higher levels of oxidative stress may have greater loss of SOD activity, which would be reflected systemically in loss of circulating SOD activity and clinically by the development of severe asthma and/or worsening airflow limitation. Also the genetic variability of antioxidant enzymes may play a role, not only in the primary oxidant resistance of the airways, but also in the development of asthma and the progression of the disease.
The current study was planned to reveal the alterations in systemic SOD activity to be inherent genetically or acquired as a disease process.

**Patients and Methods**

This pilot study was conducted at Baqai Medical University Hospital, Karachi, from June to December 2013. Approval was obtained from the institutional ethics review committee. Adult patients of both genders between the ages of 16 and 70 years who presented with dyspnoea and history of bronchial asthma or subsequently diagnosed on spirometry were evaluated. Pure asthmatics who were not suffering from any other co-morbid condition were included in this study. All other causes of dyspnoea, including pneumothorax, pneumonia, pleural effusion, pulmonary fibrosis, anaemia, cardiac failure and functional cases of dyspnoea, were excluded. All cases were carefully evaluated on clinical grounds, including detailed history and examination. The relevant investigations, including chest X-ray, arterial blood gases, electrocardiogram, complete blood count, blood urea nitrogen and random blood sugar were done where appropriate. Moreover, healthy adults of both genders without any significant past medical/surgical history were taken as controls and subjected to spirometry and their blood samples were drawn for SOD analysis. Their deoxyribonucleic acid (DNA) were extracted and analysed for SOD gene abnormality.

Portable, handheld electronic spirometer (Micro Medical Plus) was used. The instrument was calibrated using 3.0-litre calibration syringe (Vitalograph Limited) prior to spirometry. Spirometry variables were measured for a series of at least three acceptable forced expiratory readings.\(^\text{13}\) The guidelines by the American Thoracic Society (ATS) were followed for obtaining satisfactory spirometric values.\(^\text{14}\)

The DNA purification from whole blood collected in ethylenediaminetetraacetic acid (EDTA) was carried out by using Epicentre Kit (cat# MCD85201) and the protocol was followed accordingly. In 1.5 ml Eppendorf cup, 150 µl of whole blood was taken and 600 µl red blood cell (RBC) lysis solution was mixed and kept for 10 minutes at room temperature. After centrifugation for 10 minutes, the supernatant was discarded. The pellet was re-suspended by adding 300 µl of tissue and 50 µg/µl cell lysis solution. Proteinase K was also added. All the sample tubes were incubated at 65°C for 45 minutes. At the end of incubation, all the sample tubes were placed on ice for 3-5 minutes and were cooled and 200 µl of protein precipitation reagent was added to the sample, vigorously vortexed and centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes. The supernatant was transferred to isopropanol tube. The tubes were inverted 30-40 times to recover the DNA from supernatant and then centrifuged. The pellet was obtained and washed with 100% ethanol by centrifugation. The tubes were inverted slowly without disturbing the pellet to remove all the ethanol and air dried. The pellet was re-suspended in 35 µl of Tris-EDTA (TE) buffer.

The polymerase chain reaction (PCR) was carried out containing 50 µl of a reaction mixture made up of the following components: 0.6 mM of each forward and reverse primer. The type specific primer sequence was used for identifying the samples with two functional variants of the superoxide dismutase genes in patients having history of asthma.

The following primer pairs were used for amplification:

**Forward:** 5'CTA TCC AGA AAA CAC GGT GGG CC 3'

**Reverse:** 5'TCT ATA TTC CAA ATG CTA CAA AAC C 3'

PCR amplifications were performed by using 200 µM of four deoxynucleotides, 0.3U of Taq polymerase and 10 x PCR buffer containing 2 mM magnesium chloride (MgCl2). Moreover, 10 ng of DNA template was added in the master mix. The thermal cycler was programmed to first incubate the sample for 5 minutes for 94°C, followed by 45 cycles consisting of 94°C for 50 seconds, 61°C for 55 seconds and 72°C for 1 minute with final extension for 10 minutes at 72°C. The 3 µL of gel loading buffer was added to the amplified products, and then loaded on a 2% agarose gel. The gel was set at 100 volts for 1 hour and stained with ethidium bromide. After staining, the bands were visualised under ultraviolet (UV) light and different mutations were analysed with the sizes of PCR-amplified product and estimated according to the migration pattern of a 100-bp DNA ladder mutations of superoxide dismutase gene. The amplification product 278 bp PCR product of third intron of copper-zinc (Cu-Zn) SOD gene were estimated.

**Results**

Of the 370 patients evaluated, 30 (8.1%) were included.

**Table-1:** Mean age with number of males and females participants.

<table>
<thead>
<tr>
<th></th>
<th>CASE n=30</th>
<th>CONTROL n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age (years)</td>
<td>37.77± 11.95</td>
<td>37.27± 11.81</td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

SD: Standard deviation.
Besides, there were 15 controls. So the number of participants was 45. Moreover, 27(60%) participants were males and 18(40%) females. The mean age of cases and controls was 37.77±11.95 and 37.27±11.81 years, respectively (Table-1). The mean baseline and predicted forced expiratory volume in first second (FEV1) among cases was 1.58± 0.74L and 2.96± 0.65L, respectively, showing 53% decrease, forced vital capacity (FVC) was 1.88± 0.83L and 3.49± 0.81L, respectively, showing 52.3% decrease, percentage ratio FEV1/FVC was 84.67± 5.02% and 81.13± 2.37%, respectively, and peak expiratory flow rate (PEFR) was 223.63±123.06 L/minute and 440.97±82.63 L/minute, respectively, showing 50.7% change. The control population showed mean baseline and predicted FEV1 of 2.66±0.83L and 3.16 ± 0.49L, respectively, FVC 2.96±1.03L and 3.77±0.70 L respectively, FEV1/FVC 90.80±8.29% and 81.53±2.87%, respectively, and PEFR of 379.87±141.05 L/minute and 454.40±70.40 L/minute (Table-2).

The mean SOD was 62.53±15.23 among cases and 55.65±15.87 among controls (Table-3).

Table-2: Baseline and predicted spirometry values in case and control groups.

<table>
<thead>
<tr>
<th>SPIROMETRY</th>
<th>CASE n=30</th>
<th>Predicted CASE n=30</th>
<th>CONTROL n=15</th>
<th>Predicted CONTROL n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 (L)</td>
<td>1.58 ± 0.74</td>
<td>2.96 ± 0.65</td>
<td>2.66 ± 0.83</td>
<td>3.16 ± 0.49</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>1.88 ± 0.83</td>
<td>3.49 ± 0.81</td>
<td>2.96 ± 1.03</td>
<td>3.77 ± 0.70</td>
</tr>
<tr>
<td>FEV1/ FVC (%)</td>
<td>84.67 ± 5.02</td>
<td>81.13 ± 2.37</td>
<td>90.80 ± 8.29</td>
<td>81.53 ± 2.87</td>
</tr>
<tr>
<td>PEFR (L/min)</td>
<td>223.63±123.06</td>
<td>440.97±82.63</td>
<td>379.87±141.05</td>
<td>454.40±70.40</td>
</tr>
</tbody>
</table>

*All values in litres
FEV1: Forced expiratory volume in first second
FVC: Forced vital capacity
PEFR: Peak expiratory flow rate.

Table-3: Mean SOD in cases and controls.

<table>
<thead>
<tr>
<th>Mean ± SD</th>
<th>CASE n=30</th>
<th>CONTROL n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/ml)</td>
<td>62.53 ± 15.23</td>
<td>55.65 ± 15.87</td>
</tr>
</tbody>
</table>

SOD: Superoxide dismutase
SD: Standard deviation.

Figure-1a: DNA-extracted sample. Lane 1 is the control sample C1 and lane 2-16 are the blood identified for the positive super oxide dismutase. Lane 17 is the DNA ladder of 50 bp.

Figure-1b: DNA-extracted sample. Lane 1-10 and lane 13 are the blood identified for the positive super oxide dismutase. Lane 11-12 and Lane 14 are the control sample labelled as C2, C3 and C4. Lane 15 is the DNA ladder of 50 bp.

Figure-1c: DNA-extracted sample. Lane 1, 2, 6-12, 15-16 are the control sample C5-C15. Lane 3-5 labelled 27-29 and lane 13-14 labelled 30 and 5 are the blood identified for the positive super oxide dismutase. Lane 17 is the DNA ladder of 50 bp.

Discussion
Airway injury and repair is a major feature of chronic inflammation occurring in asthma and is proposed to be the main target of asthma treatments. Disease control is a priority according to Global Initiative for Asthma (GINA) guidelines.12

Many cells and cellular elements are affected and play a role in the progression of the disease. Host antioxidant systems are generally activated in response to the oxidant attack, but individuals have different capacities.
of antioxidant defence, which are in part genetically determined.\textsuperscript{15} Therefore, asthma may result into oxidant/anti-oxidant imbalance like most other chronic illnesses. The toxicity of oxidants which are directly inhaled such as cigarette smoke and air-pollution or generated through inflammatory process such as in response to allergen and viral infection, is normally balanced by the protective activity of an array of endogenous antioxidant defence system which may be functionally dependent on adequate supply of nutritional antioxidants as well as production of endogenous antioxidants.\textsuperscript{16} Observational epidemiologic studies have provided evidence of a positive association between dietary antioxidant intake and lung function, with stronger effects in cigarette smokers.\textsuperscript{17}

This study was conducted to detect alterations in antioxidant enzymes like SOD at molecular level and in serum in spirometry proven asthmatics.

All the participants in our study were subjected to spirometry which showed a decrease in all parameters in asthmatic as compared to the healthy controls with a ratio of FEV\textsubscript{1}, FVC, per cent ratio and PEFR of 0.59:1, 0.63:1, 0.93:1 and 0.59:1, respectively. This is consistent with our previous study in which all the lung parameters were significantly lowered in asthmatics.\textsuperscript{18}

SOD catalyses the dismutation of superoxide to hydrogen peroxide. In our study, the SOD activity was increased in asthmatics with a value of 62.53± 15.23 as compared to 55.65± 15.87 in age- and sex-matched healthy controls with a ratio of 1.12:1. This is also in agreement to our previous study in which there was 17% increase in patients extracellular Cu Zn-SOD as compared to the controls.\textsuperscript{18} In contrast to these results, a study by Comhair et al. showed significantly decreased SOD activity measured in the bronchoalveolar lavage fluid of asthmatics.\textsuperscript{19} The increase in serum SOD and subsequent decrease in bronchoalveolar lavage may in fact reflect changes in expression, localisation, and/or activity of extracellular SOD.

In our study, the DNA extracted samples of cases showed amplification for superoxide dismutase as compared to the controls. Similar observation was made by Tang et al. who studied genetic variations in antioxidant enzymes and longitudinal change in lung function.\textsuperscript{17}

One limitation of our study was that it was a pilot study, therefore, the sample size was small. Further larger-scale studies may be carried out in the future.

**Conclusion**

There are elevated levels of serum SOD with concomitant amplification of SOD gene suggestive of molecular basis of SOD alterations in spirometry-proven asthmatics. Further work is required to develop therapeutic agents which can alter the expression of SOD genes to bring cure for asthma.

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**Conflict of Interest:** None.

**Source of Funding:** None.

**Reference**

14. [No authors listed]. American Thoracic Society. Standardization of