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Non-invasive method of measuring airway inflammation: exhaled nitric oxide

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Nitric oxide (NO) was well known to be a component of air pollution, often in the form of nitrogen dioxide (NO₂). However its importance in biological systems altered dramatically with the discovery in 1987 that it was the 'endothelial-derived relaxing factor'. Since then there has been an explosion of research on NO demonstrating that this gaseous molecule was a widespread physiological mediator and was simultaneously recognised as a vital component of immune function contributing to macrophage-mediated cytotoxicity. NO was therefore a key molecule in modulating inflammation, including airway inflammation.

The aim of this thesis was:
1. To adapt a NO chemiluminescence analyser from measuring airway pollution to measuring exhaled air in human subjects.
2. To measure NO levels in exhaled air in adult subjects.
3. To evaluate whether altering measurement parameters altered the levels of NO obtained.
4. To adapt this technique from adults to measure exhaled NO in children.
5. To compare levels of NO from healthy children to groups of asthmatic children on either bronchodilator therapy only, or on regular inhaled corticosteroids.
6. To compare the levels of NO in a pilot group of asthmatic children before and after commencement of inhaled corticosteroids.

Methods

A Dasibi Environmental Corporation Model 2107 chemiluminescence analyser was adapted specifically requiring a reduction in response time, which was achieved by modification of the circuitry and re-routing of the analogue signal directly to a chart recorder, achieving a reduction of the response time by 80%. Addition of a number of analysers allowed the measurement of exhaled NO, carbon dioxide (CO₂), mouth pressure and flow for each exhalation from total lung capacity. Twenty adult subjects (in total) were then studied looking at direct (NO, CO₂, mouth pressure) versus t-piece (with the addition of flow) measurements making five exhalations from total lung capacity, at 3-minute intervals (direct/t-piece/direct or t-piece/direct/t-piece in series). The area of NO under the curve versus the peak of the NO trace was compared and the exhalation pattern of NO versus CO₂ was compared. Measurement conditions were altered to evaluate the effect of individual parameters on the exhaled NO result. This included separately assessing different expiratory flows, different expiratory mouth pressures, the effect of a high versus a low background NO level and the
effect of drinking water (of varying temperatures) prior to exhalation. Healthy control children were then enrolled to the study from a local school (Park Walk Primary School) and compared with asthmatic children enrolled from outpatient clinics at the Royal Brompton Hospital. The asthmatic children were further divided into those on bronchodilator treatment only and those on regular inhaled corticosteroid therapy. NO was also measured before and two weeks after commencing inhaled corticosteroid therapy in previously steroid-naive asthmatics.

Results

It was possible to modify a chemiluminescence analyser to enable measurement of exhaled NO. In 12 healthy subjects (mean age 32 years, 6 males) peak direct NO levels were 84.8 parts per billion (ppb) (standard error of the mean (SEM) 14.0ppb), significantly higher than 41.2ppb (SEM 10.8ppb) measured via the t-piece system. The exhaled NO rose to an early peak and plateau while the CO₂ levels continued to rise to peak late in the exhalation. The mean times to peak NO levels were 32.2 seconds (s) (direct) and 23.1s (t-piece), which was significantly different from the mean times to peak CO₂ levels at 50.5s (direct) and 51.4s (t-piece, p<0.001). At peak NO level, the simultaneous CO₂ level of 4.9% (SEM 0.14%, direct) and 5.2% (SEM 0.18, t-piece) were significantly lower than the peak CO₂ achieved of 5.8% (SEM 0.21%, direct, p< 0.001) and 6.2% (SEM 0.28, t-piece, p<0.001). There was no difference between repeat direct or t-piece measurements.

With regard to varying measurement conditions, the mean peak concentrations of NO decreased by 35ppb (95% confidence intervals 25.7-43.4) from a mean of 79ppb (SEM 15.4ppb) at an expiratory flow rate of 250mls/min to 54.1ppb (SEM 10.7ppb) at 1100mls/min. The mean peak concentration of NO did not change significantly when mouth pressure was increased in eight of ten subjects, although in two it did decrease in the highest pressure. The mean NO concentration with machine and subjects sampling from a low NO reservoir was 123ppb (SEM 19.4), which was an increase from results obtained before at 81.9ppb, SEM 10.2ppb, p=0.001 95%, CI -19.9 to -62.7) and after at 94.2ppb (SEM 18.3ppb, p=0.017, 95% CI 6.0-5.18) sampling with high ambient NO levels. The mean peak NO concentration decreased from 94.4ppb (SEM 20.8) to 70.8ppb (SEM 16.5, p=0.002 95% CI 12.9-33.1) with water consumption.

In 39 healthy pre-pubertal children with a mean age of 9.9 years (range 9-11 years, 23 girls) the mean direct exhaled NO level was 49.6ppb (SD 37.8ppb, range 11.5-197.2ppb) compared with mean exhaled NO via t-piece sampling of 29.2ppb (SD 27.1ppb, range 5.1-141.2ppb).
There was no significant difference between boys and girls for either the direct or the t-piece recordings. In comparison with normal children, 15 asthmatic children on bronchodilator therapy only had much higher levels of exhaled NO at 126.1ppb via the direct system (SD 77.1ppb, p<0.001) and 109.5ppb via the t-piece system (SD 106.8ppb, p<0.001). In 16 asthmatics on regular inhaled corticosteroids the mean peak exhaled levels were significantly lower at 48.7ppb via the direct method (SD 43.3ppb, p<0.001) and via the t-piece system at 45.2ppb (SD 45.9ppb, p<0.01). There was no difference between the normal children and the asthmatic children who were on regular inhaled corticosteroids (p=0.9 direct, p=0.2 t-piece). There was no significant difference in CO₂, mouth pressure, duration of expiration and expiratory flows between the three groups or between the two methods (direct and t-piece). In six asthmatic children the mean peak exhaled NO levels fell from a medium peak level of 124.5ppb to 48.6ppb when measured before and two weeks after commencement of inhaled corticosteroids on treatment.

**Discussion**

This research showed it was possible to modify an NO chemiluminescence analyser to enable measurement of exhaled NO in adult and paediatric subjects. Furthermore, it was possible to measure both healthy and asthmatic children. There were significant differences between the exhalation pattern of NO and CO₂ suggesting that NO was produced in the airways, not at alveolar level, unlike CO₂. The measurement of exhaled NO required a standardised approach as exhaled NO levels decreased with increasing expiratory flow, when measuring at a time of high ambient NO concentration, and with consumption of either hot or cold water immediately preceding exhalation (such as might be given if a subject was coughing). The findings with expiratory mouth pressure were less certain, with a difference seen in only two of ten subjects.

The levels of exhaled NO measured in children aged 9-11 years were lower than that measured in the adult subjects. There was no difference between boys and girls, or with other parameters such as having a personal history of atopy, a family history of atopy, or the presence of a smoker or furry pets within the house-hold. These findings may have altered with increased numbers in this group and could possibly be a type two statistical error. The results of exhaled NO in asthmatic children on bronchodilator therapy only were significantly elevated compared to both normal children and asthmatic children treated with regular inhaled corticosteroids. The exhaled NO level also fell significantly by two weeks following the commencement of inhaled corticosteroid treatment in steroid-naive asthmatic children.
results suggested that the methods of measuring exhaled NO required standardization and that it could potentially be a non-invasive measure of airway inflammation to follow - particularly in children with asthma who were commencing inhaled steroid treatment.
Dedication

This is Dedicated:

To the strong women in my family from my great, great grandmother

to my sister Angela who always believes in me.
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United Kingdom

Professor Andrew Bush invited me to a research position at the Royal Brompton Hospital and has been supportive from day one for both my research career and clinical training. His own approach I seek to emulate with his excellent clinical acumen, approach to children and their families. In addition, he maintains a huge research output and when working with him in this capacity his attention to detail and editing ability is superb. I, like most of the paediatric respiratory fraternity, count Andy, and his wife Sue Bush, as friends.

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Personal

My family has always been completely supportive in everything that I do and my parents, Daphne Mary Pemberton Byrnes and Brian Liston Dominic Byrnes, were thrilled to see me start at medical school all those many years ago, although, sadly, were not alive to see me graduate. And my special Aunt, Veronica Commins, who has supported my sister and myself through our careers and also, sadly, died before seeing this dedication to her years of generosity. My brothers, sisters and their families all offer their special supports – I feel lucky to be part of a large whanau, and I hope they will be pleased to see me emerge from my study.

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And to my own support network: Dr Sue Armstrong-Wahlers, Dr Michael Wahlers and Trudi Fava.
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<td>&lt;</td>
<td>less than</td>
</tr>
<tr>
<td>&gt;</td>
<td>greater than</td>
</tr>
<tr>
<td>ABPA</td>
<td>allergic bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
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<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
</tr>
<tr>
<td>C3, C5</td>
<td>complement factor 3, complement factor 5</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 3', 5' monophosphate</td>
</tr>
<tr>
<td>CAT1, CAT2, CAT2B, CAT 3</td>
<td>cationic amino acid proteins</td>
</tr>
<tr>
<td>CB</td>
<td>chronic bronchitis</td>
</tr>
<tr>
<td>CD3+</td>
<td>cell marker for T lymphocyte</td>
</tr>
<tr>
<td>CD4+</td>
<td>cell marker for T helper lymphocyte</td>
</tr>
<tr>
<td>CD8+</td>
<td>cell marker for T cytotoxic lymphocyte</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cNOS</td>
<td>constituent nitric oxide synthase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Co</td>
<td>cobalt</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
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<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSF</td>
<td>central spinal fluid</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECMO</td>
<td>extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
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<tr>
<td>ECRHS</td>
<td>European Community Respiratory Health Survey</td>
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<td>EDRF</td>
<td>endothelial derived relaxing factor</td>
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ELISA  Enzyme-linked immunosorbent assay
eNOS  endothelial nitric oxide synthase
EPN/EPX  eosinophilic neuraminidase
EPO  eosinophilic peroxidise
ERS  European Respiratory Society
ERSTF  European Respiratory Society Task Force
FAD  flavin adenosine dinucleotide
Fe  iron
FEF_{25-75\%}  forced expiratory flow at 25 to 75 percent of forced vital capacity
FEV₁  forced expiratory volume in 1 second
FEV₀.₅  forced expiratory volume in half a second
FMN  flavin mononucleotide
FVC  forced vital capacity
GINA  Global Initiative for Asthma
GM-CSF  Granulocyte macrophage – colony stimulating factor
GTP  guanosine 5-triphosphate
H₂O  water
H₂S  hydrogen sulphide
HNO₂  nitrous acid
HRCT  high resolution computerised tomography
ICAM1  intracellular adhesion molecule 1
IFNγ  interferon gamma
IgA  immunoglobulin A
IgG  immunoglobulin G
IgE  immunoglobulin E
IHCS  inhaled corticosteroids
IL1, IL2, IL3, IL4, IL5, IL6, IL8, IL9, IL10, IL11, IL12, IL13, IL17  interleukin 1, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, interleukin 17
iNOS  inducible nitric oxide synthase
ISAAC  International Study of Asthma and Allergies in Childhood
Kb  kilobases
L/min  litres per minute
LABA  long acting β₂ agonist
LADA  N"N" dimethyl L-arginine
LFA1 lympocyte function-associated antigen 1
L-NAME N" arginine methyl ester
L-NMMA N" monomethyl L-arginine
L-NOARG N"-nitroarginine
LNIO N- imino-ethyl-ornithine
LPC lysophosphotidylcholine
LPS lipopolysaccharide
LTC4, LTD4, LTE4 leukotriene C4, leukotriene D4, leukotriene E4
MBP major basic protein
mg/ml milligrams per millilitre
mls/min millilitres per minute
Mn manganese
mRNA messenger ribonucleic acid
N2 nitrogen
N2O nitrous oxide ("laughing gas")
N2O3 dihydrogen trioxide
N2O4 dihydrogen tetraoxide
NADPH nicotinamide adenosine di-nucleotide phosphate
NANC non adrenergic, non cholinergic (nerves)
NF-kB nuclear factor-kappa B
NHANES 1 National Health and Nutrition Examination Survey 1
nL/min nanolitres per minute
nNOS neuronal nitric oxide synthase
NO nitric oxide
NO + nitrosoium cation
NO- nitroxyil anion
NO2 nitrogen dioxide
NO2- nitrite
NO3- nitrate
NO2Tyr 3-nitrotyrosine
NOS nitric oxide synthase/s
NOx nitrogen oxides (usually NO, NO2 and NO3)
NZPAG New Zealand Paediatric Asthma Guidelines
O2 oxygen

XX
O$_2^-$: superoxide anion
O$_3$: ozone
OH$: hydroxyl anion
ONOO$: peroxynitrite
ONOOH: peroxynitrous acid
PaO$_2$: pulmonary artery oxygen
PCD: primary ciliary dyskinesia
PC$_{20}$: provocation concentration producing 20% fall in the forced expiratory volume in one second
PD$_{20}$: provocation dose producing 20% fall in the forced expiratory volume in one second
PEF: peak expiratory flow
Pmo: mouth pressure
ppb: parts per billion
ppm: parts per million
PM$_{10}$: particulate matter with a diameter of less than 10$\mu$m
redox: reduction oxidative reactions
ROC: receiver-operator curve
RS: sulphur thios
RSV: Respiratory Syncytial Virus
SD: standard deviation
SEM: standard error of the mean
sGC: soluble guanylate cyclase
SIGN: Scottish Intercollegiate Guidelines Network
SLE: systemic lupus erythematosis
SO$_2$: sulphur dioxide
TB: tuberculosis
TGF$\beta$: transforming growth factor beta
T$_{h1}$: T helper lymphocyte cell type 1
T$_{h2}$: T helper lymphocyte cell type 2
T$_{h3}$: T helper lymphocyte cell type 3
TNFa: tumour necrosis factor alpha
TNF$\beta$: tumour necrosis factor beta
type I (nNOS): Type I neuronal nitric oxide synthase
type II (iNOS): Type II induced nitric oxide synthase
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<td>type III (eNOS)</td>
<td>Type III endothelial nitric oxide synthase</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>µg/ml</td>
<td>micrograms per millilitre</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations International Children’s Fund</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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<tr>
<td>UV light</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>V/Q</td>
<td>ventilation and perfusion ratio</td>
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<td>Zn</td>
<td>zinc</td>
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