Materials and Methods
MATERIALS AND METHODS

Specific pathogen free guinea pigs (n=66), weighing 400-800 gm housed in aluminum pans in the VPCI animal house were used as experimental animals. They were fed on commercially available feed and were given food and water *ad libitum*. The temperature of the animal house was maintained at 25°C. The study was approved by the Institutional Animal Ethics Committee.

5.1. Study Design

To achieve the objectives of the present study, the following experimental protocols were designed:

- To record afferent activity (single nerve fiber activity) from the vagus originating from slowly adapting receptors (SARs) and rapidly adapting receptors (RARs) in the airways of guinea pigs.
- To record the changes in the activity of SARs and RARs and pulmonary mechanics in guinea pigs sensitized with ovalbumin for 4 weeks followed by ovalbumin challenge for determining early asthmatic response.
- To record the changes in the activity of SARs and RARs and pulmonary mechanics 24 hr after ovalbumin challenge in guinea pigs previously sensitized with ovalbumin for 4 weeks, for determining late asthmatic response.
- To record the changes in activity of SARs and RARs and pulmonary mechanics to *in vivo* generated reactive oxygen species (ROS).
- To record the changes in the activity of SARs and RARs and pulmonary mechanics, in guinea pigs supplemented with dietary antioxidants, and sensitized with ovalbumin for 4 weeks followed by an ovalbumin challenge for determining the effects of antioxidants in early asthmatic response.
- To record the changes in the activity of SARs and RARs and pulmonary mechanics 24 hr after ovalbumin challenge in guinea pigs previously...
supplemented with dietary antioxidants and sensitized with ovalbumin for 4 weeks to determine the effects of antioxidants in late asthmatic response.

- To record the changes in activity of SARs and RARs during administration of a bronchoactive agent- histamine, in the above models.
- To estimate the oxidants and antioxidants in all the groups under study.
- Histopathological examinations of the lung tissue of all the groups under study.

5.2. Methods

5.2.1. Recording of Single Nerve Fiber Activity

5.2.1.1. Surgery

Animals of all the groups were anesthetized with urethane (1 g/kg, i.p., Merck, Germany). Anesthesia was maintained by periodic injections of urethane (0.25 g/kg, i.p.), as and when required. The adequacy of anesthesia was examined by pinching the paw and checking the corneal reflex. Polyethylene cannulae (i.d. 0.86 mm) were introduced into the jugular vein and carotid artery. The venous cannula was utilized for giving infusions and drugs. Its tip was advanced as far as the right atrium (position was confirmed at postmortem). The carotid arterial cannula was connected to a pressure transducer (P23, Becton Dickinson, USA) and was used for recording arterial blood pressure. It was utilized for periodic withdrawal of arterial blood samples for the measurement of arterial blood gases also (Nova Biomedical, USA).

The trachea was cannulated and an uncuffed endotracheal tube was introduced into it. The animals were artificially ventilated with a ventilator (Model 683, Harvard apparatus, USA) at the rate of 38 breaths/min and a tidal volume of approximately 0.75 ml/100 mg body wt. The animals were paralyzed by gallamine triethiodide (1 mg/kg i.v., Sigma). The adequacy of anesthesia was checked first before giving gallamine. Gallamine triethiodide injections were repeated every hour after checking the depth of anesthesia and administered after giving the anesthetic first. The inspired air was supplemented with 40% oxygen. The arterial blood gases were monitored and maintained in the normal range- PO₂~100 mm Hg, PCO₂~40 mm Hg and pH~7.4. The
PCO$_2$ and pH were kept at the normal range by adjusting the tidal volume and by infusing sodium bicarbonate (8.5% w/v). A polyethylene cannula (i.d. 0.86 mm) connected to a differential pressure transducer (MPX, Harvard apparatus, USA) was introduced through the tracheal cannula for the measurement of airway pressure. A pneumotachograph (DP 45-14, Harvard apparatus, USA) was introduced in between the ventilator and the tracheal cannula and it was used for measuring airflow. From the pressure and flow measurements, airway compliance and resistance were obtained electronically using a pulmonary mechanics analyzing system (Pulmodyn, Harvard apparatus, USA).

5.2.1.2. Preparation of Vagus Nerve

The right cervical vagus nerve was separated from the carotid sheath and prepared for recording afferent activity originating from SARs and RARs using conventional techniques (Paintal, 1955). Briefly, the nerve was separated from the carotid sheath 4-5 cm in the neck region and the neck skin was retracted such that it was sufficient to make a pool. This pool was filled with paraffin oil to make the environment around the nerve non-conducting and to keep the nerve active by preventing it from drying. The nerve separated from the carotid sheath was gently placed on a platform held by magnetic stand. The vagus nerve was desheathed by fine forceps under a dissecting microscope (Carl Zeiss, Germany). Thin slits of the nerve fiber were cut from cranial end and teased further and placed on the recording electrodes placed in the pool for recording of action potentials.

5.2.1.3. Recording Set-up

Action potentials were recorded using bipolar platinum electrodes. The neural signals were amplified by a pre-amplifier (Tektronix TM 503, USA) and fed into a thermal array recorder (WindoGraf, Gould, USA) and audio amplifier (Ahuja CA15, India) connected to loud speaker. The diagrammatic overview of the recording set-up is shown in Fig. 1.
The SARs were identified by their characteristic respiratory rhythm and their slow adaptation to a maintained inflation (3 times of the tidal volume) of the lungs (Paintal, 1973) as shown in Fig. 2.

The RARs were identified by their irregular resting discharge and rapid adaptation to a maintained hyperinflation (3 times of the tidal volume) of the lungs (Kappagoda et al. 1987) as shown in Fig. 3.
Materials and Methods

5.2.1.4. Localisation of the Receptor

The location of the receptor was ascertained at the end of each experiment by gently probing of the lungs and airways externally using a blunt glass rod of 3 mm diameter.

5.2.1.5. Arterial Blood Pressure

The carotid arterial cannula was connected to a p23dc pressure transducer (P23, Becton Dickinson, USA) for recording of arterial blood pressure, and fed into the thermal array recorder (WindoGraf, Gould, USA). The pulsatile arterial blood pressure was recorded and from this, the mean arterial blood pressure was obtained by electronic damping of the pulsatile signals.

5.2.1.6. Electrocardiogram (ECG)

An electrocardiogram (Lead II) was also recorded. One lead was placed in right arm and the other lead in left leg of the animal. The animal was grounded. ECG was recorded on thermal array recorder (WindoGraf, Gould, USA).

5.2.1.7. Temperature

The temperature of the animal was constantly monitored by a rectal thermometer. It was maintained between 37-38 °C using heating pads.
5.2.2. Airway Sensitization followed by Inhalation Challenge

Guinea-pigs were sensitized as per standard procedures (Santing et al., 1994). For sensitization, 100 mg aluminium hydroxide (Al(OH)₃) (Sigma) mixed with 100 µg ovalbumin (Sigma) per ml of normal saline was used. 0.5 ml of the antigen-adjuvant solution was injected intraperitoneally and 0.5 ml was injected subcutaneously, dividing the amount equally among 7 different sites near the lymph nodes. This protocol results in sensitization by the end of 4 weeks (Santing et al., 1994).

For early asthmatic response, the sensitized animal was challenged with 0.2% ovalbumin for one minute using an ultrasonic nebulizer (Hico-Ultrasonat, 806 EH, Germany), connected to the ventilator. To observe the late asthmatic response, the sensitized guinea pigs were placed in the guinea pig body box and challenged with 0.2% ovalbumin. These animals were kept under observation and utilized for the receptor study 24 hrs later.

5.2.3. In vivo Generation of Reactive Oxygen Species by Inhalation of Xanthine – Xanthine Oxidase

Xanthine (MP Biomedicals, USA) and xanthine oxidase (Sigma) were dissolved in phosphate buffered saline to a volume of 10 ml (Katsumata et al., 1990). Successive inhalations of xanthine (0.1%) for 1 min and xanthine oxidase (1U/ml) for 1 min were given using the ultrasonic nebulizer.

5.2.4. Administration of Bronchoactive Agent (Histamine)

Histamine (Sigma) inhalation was given using the ultrasonic nebulizer connected to the ventilator, starting with the dose of 0.04 mg/ml histamine in phosphate buffered saline and doubling the concentration. The maximum concentration of histamine administered was not more than 5 mg/ml. An interval of 15 min was given between successive injections to avoid tachyphylaxis.

5.2.5. Antioxidant Supplementation

Vitamin E was obtained in the form of capsules (Evion-400 IU, Merck). The oil was removed from the capsule using a syringe, and emulsified by sonification in
presence of gelatin as stabilizing agent. The final concentration was adjusted to 1 ml in water. The animals were given 1 ml of the suspension per day, orally.

Vitamin C was obtained in the form of tablets (Celin-500 mg, Glaxo Smithkline). It was dissolved in water and the final concentration was adjusted to 1 ml. The animals were given 1 ml of the solution per day, orally.

5.2.6. Biochemical Assays

Blood from anesthetized animals was drawn in heparinized syringe by cardiac puncture for in vitro studies. Plasma and red blood corpuscles were separated by centrifugation (6000 rpm) and stored along with whole blood at -80°C for various biochemical assays.

5.2.6.1. Estimation of Oxidative Stress

The following techniques/methods were followed for determining different oxidative stress parameters. All the determinations were done in triplicate spectrophotometrically (Spectroscan, UV-Vis double beam spectrophotometer, Cyprus).

**Lipid Peroxidation**

This assay was carried out by precipitation of lipid peroxides in orthophosphoric acid and was measured as thiobarbituric acid reactive substances (TBARS) formed as described by (Hida et al., 1994). Assay mixture containing 200 µl plasma, 200 µl distilled water, 50 µl (5 mM) butylated hydroxy toluene (BHT), 400 µl (0.2 M) orthophosphoric acid (OPA) and 150 µl thiobarbituric acid (TBA) was incubated at 90 °C for 45 min. The reaction was stopped by immersing the assay tubes in ice cold water. 1000 µl of n-butanol was added, mixed and then centrifuged at 12000 rpm for 5 min. The absorbance of supernatant was read at 535 nm and the amount of TBARS formed was calculated based on the molar extinction coefficient of 156000/M/cm. The results were expressed as nM/ml.

**Superoxide Generation by Peripheral Blood Leucocytes**

Leukocytes were separated according to the method of Baron and Ahmed (Baron and Ahmed, 1969). Viability of isolated cells was tested by Trypan blue dye
exclusion method (Tolani, 1975). Measurement of superoxide ion (O$_2^-$) production by leucocytes was done by discontinuous assay (Lehmeyer et al., 1979). 0.7 ml of cell suspension was taken into 2 test tubes. 10 µl of superoxide dismutase (SOD) was added to one test tube and 10 µl of water to the other. After incubating at 37°C for 2 min, 0.05 ml of cytochrome c followed by 0.75 ml of pre-warmed (370°C) FMLP (N-formyl-methionine-leucine-phenylalanine) was added to each test tube. The mixture was incubated at 37°C for 15 min in a shaking water bath. The reaction was stopped by placing the tubes in ice and cells were removed by centrifuging at 1500 g for 5 min at 4°C. Finally cytochrome c reduction was measured spectrophotometrically at 550 nm.

5.2.6.2. Estimation of Antioxidant Status in Plasma and Red Blood Corpuscles

**Glutathione Peroxidase**

GSH-Px was determined by the method described by Little et al., (1970). This enzyme is coupled to NADPH via glutathione reductase with hydrogen peroxide as substrate. Washed red blood cells were lysed by ice-cold distilled water and stored at -80°C. Before the assay, Drabkin's reagent was added in hemolysate, and further dilutions were made in potassium phosphate buffer (50 mmol/L, pH-7.0). The assay mixture contained 100 µl of diluted hemolysate, 100 µl of EDTA (1 mmol/L), 100 µL NADPH (1.5 mmol/L, 100 µl glutathione reductase (10 Units/mL), 100 µl reduced glutathione (10 mmol/L) and 500 µl potassium phosphate buffer. The reaction was started by the addition of 100 µl cumene hydroperoxide (1.5 mmol/L) and the decrease in absorbance was recorded at 340 nm for 5 min. The change in absorbance/min was used to calculate enzyme activity. The results were expressed as µmoles of NADPH oxidized/min/gm Hb.

**Catalase Activity**

The assay of catalase was done as described by Aebi (Aebi, 1984). Heparinized blood (drawn by cardiac puncture) was centrifuged and the plasma and leukocyte layers were removed. Erythrocyte sediment was washed thrice with isotonic saline. A stock hemolysate was prepared containing 5 g hemoglobin (Hb)/100 ml distilled water. A 1:500 dilution of this hemolysate was prepared with phosphate buffer. The absorbance of the sample containing 2 ml hemolysate and 1 ml H$_2$O$_2$ at 20°C was read at a wavelength of 240 nm. The decrease in absorbance was followed for about 30 sec.
Total Antioxidant Status

The method (Benzie and Strain, 1996) followed is described below. Briefly, 300 mM of acetate buffer, pH 3.6, 10 mM of TPTZ (2, 4, 6 – tripyridyl-s-triazine) in 40 mM HCl (hydrochloric acid) and 20 mM FeCl$_3$.6H$_2$O (ferric chloride) were mixed in the ration of 10:1:1 to give the working FRAP (Ferric Reducing /Antioxidant Power) reagent. 2.9 ml of the FRAP reagent was mixed with 100 µl of test sample (plasma) and after 6 min, the absorbance was read at 593 nm.

5.2.6.3. Measurement of Nitrosative Stress

Plasma Nitrate and Nitrite Levels

Plasma nitrate and nitrite levels were determined using the method described by Grisham et al. (Grisham et al. 1996). Briefly, 100 µl of sample was incubated for 30 min at 37°C in the presence of 0.2 U/ml Aspergillus nitrate reductase, 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, 5 µM FAD (flavin adenine dinucleotide) and 0.1 mM NADPH (nicotinamide adenine dinucleotide phosphate-reduced) in a total volume of 500 µl. Following incubation, 5 µl of lactate dehydrogenase and 50 µl of 100 mM pyruvic acid were added to oxidize any unreacted NADPH. 1 ml of Greiss reagent was then added and after 10 min incubation at room temperature, the absorbance was determined at 543 nm.

Nitrotyrosine Estimation

Nitrotyrosine levels were determined with a commercially available ELISA kit (Hycult Biotechnology, b.v., Uden, Netherland) and following the procedure indicated by manufacturer according to the method described by Ter Steege et al. (1998).

5.2.6.4. Measurement of Blood Levels of α-tocopherol (Vitamin E) and Ascorbic Acid (Vitamin C)

Vitamin E Estimation

Plasma Vitamin E levels were estimated by the method of Baker and Frank (Baker and Frank, 1968). Briefly, 1.5 ml of plasma was taken. To this an equal amount
of absolute ethanol was added and mixed. Then 1.5 ml of xylene was added to the samples. After mixing, the tubes were centrifuged at 3000 rpm. 1 ml of xylene layer was then transferred into another tube. Next 1ml of \( \alpha\alpha \)-bipyridyl reagent (1.20 g/l in n-propanol) was added and mixed. The absorbance of the reaction mixture was taken at 460 nm. Then 0.33 ml of ferric chloride solution (1.20 g/l in absolute ethanol) was added, mixed and absorbance was read at 520 nm after exactly 1.5 min. Simultaneously, blanks and standards were also treated similarly and absorbance was taken at the same wavelengths.

**Vitamin C Estimation**

Plasma Vitamin C levels were estimated as described by Omaye et al (1979). Briefly, 1 ml of plasma was mixed with 1 ml ice cold buffer, mixed thoroughly and centrifuged for 20 min at 3500 g. 0.5 ml of the supernatant was mixed with 0.1 ml of DTC (dinitrophenylhydrazine/thiourea copper) solution and incubated for 3 h at 37\(^{0}\)C. 0.75 ml of ice cold 65% \( \text{H}_2\text{SO}_4 \) was added and mixed well and the solutions were allowed to stand at room temperature for 10 min and absorbance was taken at 540 nm.

**5.2.7. Hemoglobin Estimation**

Hemoglobin concentration was measured in whole blood spectrophotometrically by the kit provided by Sigma (cat. no. 525-A).

**5.2.8. Histopathological Studies**

For making the histological observations, 2 animals from each group were taken into consideration. The lung specimen from upper and lower lobes were quickly excised and washed in ice cold, physiological saline (pH-7.4). The tissues were then fixed in 10% formaldehyde solution for one week with regular change of the fixative. Transverse sections were taken from the lobes of lung and stained with hematoxylin-eosin. The slides were observed under light microscope. The inflammatory changes were graded using a semiquantitative scale of 0-5 (Table 1) for perivascular and peribronchiolar infiltration, epithelial damage and edema (Underwood et al., 1995).
Table 1: Histopathological scoring system used to assess inflammatory change in the lungs of ovalbumin challenged guinea-pigs

<table>
<thead>
<tr>
<th>Grade</th>
<th>Perivascular and peribronchiolar inflammatory cell infiltration</th>
<th>Edema</th>
<th>Epithelial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Low grade cell influx</td>
<td>Low grade diffuse edema</td>
<td>Low grade cell loss</td>
</tr>
<tr>
<td>2</td>
<td>Low to moderate cell influx</td>
<td>Moderate alveolar and bronchiolar edema</td>
<td>Low grade cell loss</td>
</tr>
<tr>
<td>3</td>
<td>Moderate cell influx</td>
<td>Regional and focal edema</td>
<td>Moderate cell loss</td>
</tr>
<tr>
<td>4</td>
<td>Moderate to high cell influx</td>
<td>Pronounced edema</td>
<td>Moderate cell loss</td>
</tr>
<tr>
<td>5</td>
<td>High cell influx</td>
<td>Pneumonic type edema</td>
<td>Epithelial metaplasia</td>
</tr>
</tbody>
</table>

5.3. Statistical Analysis

Group data was expressed as mean ± SEM. p value < 0.05 was accepted as significant. RAR/SAR activity was counted on a breath to breath basis. For obtaining the baseline activity, the RAR/SAR activity was counted for 10 breaths, averaged and expressed as impulses/ breath. An increase in activity by 20% from the base line activity was considered as stimulation. To determine the responses to various inhalations, the RAR/SAR activity was counted for 100 breaths and averaged.

For all the parameters, comparisons among the groups were made using unpaired-t test. The comparisons within the groups were made with the corresponding controls using paired-t test. Unpaired t-test and ANOVA were used to compare the biochemical estimations.