Chapter 5

To investigate anti-inflammatory activity of choline chloride in bronchial asthma patients
INTRODUCTION

Asthma is an inflammatory disorder of the lower airways characterized by chronic eosinophilic infiltration, increased bronchial hyperresponsiveness (BHR) and variable airway obstruction (Tattersfield et al., 2002). In asthmatic individuals, inflammation causes frequent episodes of wheezing, breathlessness, chest tightness, and coughing. Many cells and cellular elements play a role in asthma pathogenesis, in particular, mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils, and epithelial cells (Foley et al., 2007). Eosinophils play an important role in bronchial inflammation in asthma. These cells are in part recruited to the site of the inflammation by chemoattractants liberated by the structural cells of the mucosa, especially epithelial cells (Van Wetering, 2007). Cysteinyl leukotrienes (Cys-LTs) released form eosinophils and mast cells are important pro-inflammatory asthma mediators, which give rise to broncho-constriction, mucus secretion, increased vascular permeability, smooth muscle hypertrophy and recruitment of inflammatory cells (Leff, 2001).

Asthma is one of the most common and increasingly prevalent chronic diseases in the world, in both children and adults. Approximately 300 million people suffer from asthma worldwide, and this is expected to increase to 400 million people by 2025 (GINA, 2006). The steering committee of the International Study on Asthma and Allergies in Childhood (ISAAC) recently reported that the prevalence of atopic diseases in childhood (including asthma) has increased considerably in Western industrialized countries. Surveillance of asthma in North America, Western Europe, and Australia has demonstrated approximately 5-18% increase in asthma prevalence. Approximately 20% of these patients have severe asthma, of which 20% is inadequately controlled. Allergic rhinitis affects 10% to 30% of adults and the majority of patients with asthma (60-80%) have rhinitis and 20-40% of patients with rhinitis can have asthma (Peters et al., 2006). Rhinitis symptoms in patients with asthma are associated with worse asthma outcomes and have a significant impact on the quality of life (Lehman and Lieberman, 2007).

The incidence of asthma in India is reported currently in the range of 2.3-16.6% (Chhabra et al., 1998; Gaur et al., 2006; Aggarwal et al., 2006; Jindal, 2007), which is higher than reported (< 1%) earlier (Viswanathan et al., 1966). Studies show prevalence of asthma to be 3.5% with diagnosis, whereas it was 9-12% of symptomatic subjects without diagnosis (Chowgule et al., 1998). The reported prevalence of asthma was 2.3-3.3% in the children from Lucknow (Awasthi et al.,
2004), 2.6% in rural children from Ludhiana (Singh et al., 2002) whereas it was 29.5% in children from Bangalore (Paramesh, 2002).

Anti-inflammatory therapy is central to long-term asthma management. Treatment strategies are aimed at not only neutralizing the effect of inflammatory parameters (sputum eosinophilia, BHR etc) but also on improving symptoms and lung function (Hanania, 2008). Inhaled corticosteroids (ICS) affect a variety of inflammatory pathways in asthma and are recommended as first-line treatment for asthma/allergic rhinitis. The anti-inflammatory effects of ICS are expressed clinically by improved lung function, symptoms, and rescue medication requirements (Hanania, 2008). However, ICS have the potential for systemic side effects that are dependent on the dose, potency, its bioavailability, absorption in the gut, first-pass metabolism in the liver, and the half-life of its systemically absorbed fraction (Rhen and Cidlowski, 2005). It should be emphasized that the abrupt discontinuation of ICS is an important cause of worsening asthma symptoms and asthma exacerbations (Guilbert et al., 2006). A combination therapy of LABA and ICS leads to better symptomatic control in moderate-to-severe asthma and lower exacerbation frequencies than increasing the dose of inhaled steroids which again may be associated with local and systemic side effects (Holgate and Polosa, 2008; Shrewsbury et al., 2000). Approximately 25% of patients using add-on therapy, however may remain inadequately controlled and at high risk of exacerbation (Hanania, 2008).

Leukotriene modifiers act primarily as anti-inflammatory agent with slight bronchodilator effect by inhibiting lipid mediators i.e. leukotrienes that promote vascular leakage, airway smooth muscle contraction and mucus production (Horwitz et al., 1998). However, anti-leukotrienes are not recommended as first-line monotherapy in patients with asthma, except those who have aspirin induced asthma. Patients with concomitant allergic rhinitis may be a good target population for therapy with anti-leukotrienes. Adding a leukotriene modifier to therapy is an alternative to adding LABA (National Asthma Education and Prevention Program, 2003). However, addition of leukotriene modifiers to inhaled corticosteroids produces only a modest improvement in the clinical response, and is not greater to that of add-on long acting beta-agonists (Polosa, 2007).

Researches into the pathogenesis of asthma have led to the development of specific anti-inflammatory treatments, which aimed to target the recruitment or activation of inflammatory cells. Despite the progress made in pharmacotherapy, there
remains a clear need for identification and validation of new anti-inflammatory drugs with minimal or no side effects. Therapy should be aimed at controlling (ideally abolish) symptoms, restore normal or best possible lung function and reduce the risk of severe attacks. These aims should be achieved by use of minimum treatment with the lowest incidence of side-effects.

Choline is a lipotropic agent, involved in maintaining cell structure and movement of fats in and out of the cells (Blusztajn, 1998). Choline is a precursor to acetylcholine and used for phosphatidyl choline (PC) synthesis by de novo pathway (Pelech and Vance, 1984; Blusztajn, 1998). Choline has shown anti-inflammatory activity in arthritis animal model (Ganley et al., 1958). Choline magnesium trisalicylate had been used for treatment of asthma with aspirin hypersensitivity (Szczeklik et al., 1990). Studies with tricholine citrate in asthmatics had shown improvement in symptoms and airway hyperresponsiveness (Gupta and Gaur, 1997; Gaur et al., 1997). Choline was effective in inhibiting antigen induced airway inflammation in mouse model of airway hyperresponsiveness in our previous observation (chapter 2). However, it is still unknown whether it specifically improves airway inflammation in asthmatics. So, the present study was aimed to investigate the effect of choline treatment on immune inflammation and BHR in asthma patients for six months. The change in oxidative stress was also monitored after choline therapy and standard pharmacotherapy.

MATERIALS AND METHODS

Subjects and study design of the study: The patients of bronchial asthma with or without rhinitis (atopic and non-atopic) were recruited for the present study. A total of 90 subjects of either sex, between 15 to 45 years of age were screened by clinical history, pulmonary function test (PFT), skin tests and other relevant investigations at outpatient department, V. P. Chest Institute, Delhi. The diagnosis of asthma and / or rhinitis was confirmed following American Thoracic Society guidelines and ARIA (ATS, 1991; Bousquet et al., 2001), respectively. Bronchial hyperreactivity was assessed by challenge with histamine di phosphate (Sigma). The study protocol was approved by human ethics committee of the institute and written consent from subjects was obtained (in Hindi and English) for inclusion in the study.

Inclusion criteria: The presence of airway obstruction and > 12% reversibility with 200 ml increase in FEV1 (Forced expiratory volume in one second) after 200 µg of
Inhaled salbutamol was considered to assign asthma as per American Thoracic Society guidelines (ATS, 1991).

For the diagnosis of allergic rhinitis, ARIA guidelines were followed (Bousquet et al., 2001). The diagnosis of rhinitis was assigned to patients having two or more of the symptoms- (1) post nasal drip, (2) watery nasal discharge, (3) sneezing, (4) itching of nose, and (5) nasal congestion, most of the times for the last two years’ period.

**Exclusion criteria:** Patients with associated cardiopulmonary disorders, infectious diseases, other respiratory or systemic diseases, steroid dependent, non-cooperative, pregnant or lactating females and smokers were not included in the present study.

**Treatment Protocol:** This is a randomized study which included a 4 weeks run-in period (baseline) followed by 6 months treatment period. At the first visit of patient, peripheral blood counts, chest radiogram and pulmonary function test (PFT) with reversibility were carried out. PFT included forced vital capacity (FVC), FEV1, FEV1 / FVC and reversibility after 200 µg of inhaled salbutamol. Patients were advised to withhold inhaled steroid and inhaled beta-agonist (12 hours) before PFT. Eligible subjects were randomly assigned to receive oral choline chloride (1500 mg twice daily) + pharmacotherapy or standard pharmacotherapy alone for 6 months. The patients in Group A were given oral choline chloride (1500 mg twice per day) and long acting β-agonist (LABA; formoterol fumarate; 6 µg twice daily) and/or SABA (levosalbutamol sulphate; 50 µg)/ other drugs SOS as and when required. Group B patients were treated with standard pharmacotherapy including inhaled steroids (Budesonide; 400 µg twice daily) and LABA and/or SABA/other drugs as and when required. In both the groups mometasone furoate monohydrate nasal spray 50 µg twice a day was given in patients having asthma associated allergic rhinitis.

**Skin reactivity:** Skin prick tests with common allergens were performed at 0 and 6 month of choline therapy by the method followed at V. P. Chest Institute, Delhi. Skin tests were graded on the basis of wheal size of positive control i.e. histamine diphosphate (5 mg/ml) and PBS served as negative control (Dreborg, 1989; Kumari et al., 2006). Atopy was defined by a positive skin prick test to one or more of 60 common aeroallergens including pollens, insects, dust mite, fungi, cat or dog dander tested.

**Symptom/Drug score:** Day to day symptoms were recorded on a diary card (charts) provided to the patient for the entire study. It was collected and assessed every month.
Symptoms were recorded on a scale from 0 to 4 (0 = no problem; 1 = morning/night; 2 = evening; 3 = daytime; 4 = most of the time) for nasal (sneeze, blockage, itching and running nose) and bronchial symptoms (breathlessness, wheeze, chest tightness, coughing). The daily symptom score was calculated as the sum of all individual symptom scores. Patients with worsening episodes of asthma that required additional therapy were treated as and when required basis and score was added to the symptom/drug score accordingly.

The patients were instructed to record the use of drugs for symptoms’ treatment, and a specific score was given in relation to the class of drug. Drugs were scored as follows: 1 = Inhaled short acting β agonist (SABA) / inhaled corticosteroid / inhaled steroid nasal spray / oral antihistamines, 2 = low dose oral SABA / oral theophylline / oral steroid, 3 = LABA + inhaled steroid / high dose oral SABA, 4 = injectible steroid / injectible theophylline / nebulization with LABA / use of antibiotics, 5 = hospitalization (Srivastava et al., 2007). Patients with missing data were considered non-compliant and were dropped from the study.

**Bronchial hyperreactivity:** BHR was measured before starting (0 month) and after the treatment (6 month) using a bronchoconstrictor according to ATS guidelines (Crapo et al., 2000; Srivastava et al., 2007) under the supervision of a clinician at V. P. Chest Institute, Delhi. BHR was defined as a provocative concentration of histamine required to provoke a 20% fall in FEV1 (Forced expiratory volume in one second) from baseline (PC_{20}FEV1). Patients were advised to withhold inhaled steroid and inhaled beta-agonist (12 hours) before BHR test at the second visit. Spirometry was done to obtain baseline FEV1 value of patients. BHR was assessed following 2 min tidal breathing protocol where aerosolized histamine in PBS was administered in increasing 2 fold concentrations from 0.004 mg/ml to 8 mg/ml. FEV1 was done after 30 and 90 seconds and the highest reading was taken. The challenge was stopped when 20% fall in FEV1 from the baseline was reached. Finally, patients were allowed to recover up to baseline and if required, patients were nebulized after the test.

**Serological analysis:** To check the response of the patients, 10 ml of venous blood was drawn from the patients at 0 and 6 month for analysis of various immunological and biochemical parameters. Serum was separated and stored at -20 °C for different assays.
**Total IgE estimation:** Total IgE levels in blood (serum) were measured by enzyme immunoassay (Omega diagnostics Ltd., Scotland, UK). Briefly, 20 µl of samples or standard (six standard set) were dispensed in a microtiter plate along with 100 µl of zero buffer and incubated for 30 minutes at 25°C. After incubation, plate was washed with distilled water and enzyme conjugate was added and incubated for 20 minutes at 25°C. The plate was washed with distilled water and developed using TMB substrate solution and incubated in the dark for 20 minutes at 25°C. Reaction was stopped by adding 2 N H2SO4 and absorbance read at 450 nm. The minimum detectable concentration of total IgE is 5.0 IU/ml. Eosinophil counts in blood were expressed as percentage of cells.

**PBMCs isolation and cytokine analysis on mitogen challenge:** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll Hypaque density gradient centrifugation [Appendix 7]. Heparinized blood (5 ml) diluted 1:1 with PBS was layered onto an equal volume of histopaque (Sigma) and centrifuged at 1000 × g for 30 minutes at 25°C. During centrifugation, the erythrocytes and granulocytes were aggregated by ficoll and sedimented to the bottom of the tube with the PBMCs as a separate band at the plasma / histopaque interface. PBMCs were recovered and washed twice with PBS and resuspended in complete RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma). Cell viability was determined using the trypan blue exclusion test [Appendix 2]. 5 × 10^5 cells/ml were cultured in RPMI-1640 medium in a 96 well plate and stimulated in triplicate with 5 µg/ml of phytohemagglutinin (PHA) and no stimulant was added to negative control. The culture plates were incubated in a 5% CO2 incubator and the supernatant was collected for various immuno-biochemical assays after 72 hours.

**Determination of cytokine levels in PBMC’s cell culture supernatant by ELISA:** Cytokines IL-4, IL-5, IL-10, IFN-γ (BD Pharmingen) and TNF-α (R & D Systems Inc. Minneapolis, MN) were determined in PBMCs culture supernatants by ELISA following manufacturer’s instructions. Briefly, capture antibody (100 µl; 1:250 v/v) for each cytokine was coated separately in the microtiter plate in coating buffer and incubated overnight at 4°C. After washing, the wells were blocked with PBS containing 10% FBS at 25°C for 1 h. After washing, 100 µl of standards (7 serial dilutions) and culture supernatants were added to the wells in duplicates and
incubated at 25°C for 3 h. The plates were washed again and incubated with biotinylated detector antibody labeled with avidin-HRP at 25°C for 1 h. After washing with PBST and PBS, TMB substrate solution was added and plate was incubated in dark at 37°C for 30 min. The reaction was stopped with 2 N H₂SO₄ and absorbance was read at 450 nm. The cytokine concentrations were calibrated from the standards. The detection limit for IL-4, IL-5, IL-10, IFN-γ and TNF-α was 7.8, 15.6, 15.6, 31.3 and 7.8 pg/ml, respectively.

**Determination of leukotriene levels in PBMC’s culture supernatant by EIA:** Leukotriene B₄ (LTB₄) and Cys-LT were determined in PBMC’s culture supernatant using enzyme immunoassay kit (Cayman Chemical) according to manufacturer’s instructions. Briefly, 50 µl of standards (8 serial dilutions) and undiluted culture supernatants were added to the wells in duplicates, followed by adding 50 µl of tracer and antiserum of respective leukotrienes into each well and incubated at 25°C for 18 h. After washing with wash buffer (PBST), substrate was added and plate was incubated in dark at 25°C for 90-120 mins. The absorbance was read periodically at 420 nm until the maximum binding wells have reached a minimum of 0.3 A.U. The detection limit for LTB₄ and Cys-LT was 13 pg/ml.

**Measurement of 8-isoprostanes (8-iso PGF₂α) in PBMC’s culture supernatant by EIA:** PBMC’s cell culture supernatants were assayed in duplicates for 8-isoprostanes using enzyme immunoassay kits (Cayman Chemical) following manufacturer’s instructions. Briefly, 50 µl of standards (8 serial dilutions) and undiluted BAL fluid were added to the wells in duplicates, followed by adding 50 µl of 8-isoprostanes tracer and 8-isoprostanes antiserum into each well and incubated at 4°C for 18 h. After washing with wash buffer, substrate was added and plate was incubated in dark at 25°C for 90-120 min with gentle shaking. The absorbance was read periodically at 420 nm until the maximum binding wells reached a minimum of 0.3 A.U. The detection limit for 8-isoprostanes was 2.7 pg/ml.

**Analysis of lipid peroxidation:** Malanodialdehyde (MDA) concentration was determined in the serum by spectrophotometric assay (Cayman Chemical) at 532 nm with a detection limit of 0.625 µM. Briefly, 100 µl of standards (8 serial dilutions) and undiluted sera samples were added to the glass vials in duplicates, followed by adding 100 µl of SDS and 4 ml of colour reagent containing thiobarbituric acid (TBA), TBA-acetic acid and TBA-sodium hydroxide and boiled at 100°C for 1 h.
After one hour, vials were immediately placed in ice bath to stop reaction and incubated on ice. After 10 minutes, vials were centrifuged for 10 minutes at 1600 × g at 4 °C. Clear supernatant (150 µl) were loaded to plate and absorbance was measured at 532 nm.

**Adverse events:** Adverse experiences by patients were monitored throughout the study. The adverse events such as fever, headache, nausea, vomiting, diarrhea, and skin rashes etc, were recorded during the treatment period.

**Assessment criteria:** The treatment response was assessed based on the clinical parameters such as 1) symptom scores, 2) drug scores, 3) airway reactivity, 4) skin tests, and 5) immunological and biochemical parameters and 6) no. of acute attacks/hospitalization.

**Statistical analysis:** Parametric analysis of data was performed using student’s two-tailed ‘t’ test for symptom/drug score and immunological parameters. Wilcoxon test taking log transformed values was carried out for bronchial hyperreactivity. All other results were analyzed using non-parametric Mann-Whitney rank-sum test and Wilcoxon signed rank test. Paired ‘t’-test was used for intra-group and unpaired ‘t’-test was used for intergroup comparisons. Correlation after treatment was examined by means of Spearman rank correlation coefficients. Values are presented as the mean ± SEM. A p value < 0.05 was considered significant.

**RESULTS**

**Patient’s characteristics:** A total of 90 patients were screened by history and clinical examination for the study at Department of Pulmonary Medicine, V. P. chest Institute, Delhi. Out of 90 patients, 76 patients fulfilled the criteria of inclusion and were recruited for the study including 44 male (57.9%) and 32 female (42.1%) and allocated to respective group (38 each in choline + pharmacotherapy and standard pharmacotherapy group; Table 5.1). Of 38 patients in each group, 30 completed the study in choline + pharmacotherapy [17 male (56.7%) and 13 female (43.3%)] and 26 in standard pharmacotherapy [17 male (65.4%) and 9 female (34.6%)]. In choline + pharmacotherapy, 73.3% patients suffered with asthma + rhinitis, whereas 26.7% suffered with asthma alone. In standard pharmacotherapy group, 76.9% patients suffered with asthma + rhinitis, whereas 23.1% suffered with asthma alone. Eight patients dropped out of the choline treatment group (2 because of personal reasons, 2
did not turned up at the end of six month and 4 were non-compliant). Twelve patients dropped out of the pharmacotherapy group (5 because of personal reasons and 7 were non-compliant or irregular).

Table 5.1: Patients’ characteristics and lung function at baseline (0 month)

<table>
<thead>
<tr>
<th>Demographic variables</th>
<th>Choline + Pharmacotherapy</th>
<th>Standard Pharmacotherapy</th>
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<tbody>
<tr>
<td>n = 76</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Patient completed 6 month study (n = 56)</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Male (%)</td>
<td>17 (56.7%)</td>
<td>17 (65.4%)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>13 (43.3%)</td>
<td>9 (34.6%)</td>
</tr>
<tr>
<td>Age group (years)</td>
<td>15-45 years</td>
<td>15-44 years</td>
</tr>
<tr>
<td>Asthma with allergic rhinitis (%)</td>
<td>22 (73.3%)</td>
<td>20 (76.9%)</td>
</tr>
<tr>
<td>Asthma alone (%)</td>
<td>08 (26.7%)</td>
<td>06 (23.1%)</td>
</tr>
<tr>
<td>FEV1 % predicted, mean</td>
<td>79.6 (± 2.22)</td>
<td>80.8 (± 3.37)</td>
</tr>
<tr>
<td>FEV1/FVC predicted, mean</td>
<td>88.9 (± 3.25)</td>
<td>83.9 (± 2.49)</td>
</tr>
<tr>
<td>Peripheral blood eosinophil counts %</td>
<td>5.45 (± 0.60)</td>
<td>5.09 (± 0.64)</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM.

Clinical parameters:

Symptom/Drug score: The median value of symptom scores in the choline (choline + pharmacotherapy) and standard pharmacotherapy group at baseline was 94 and 83, respectively. After six month of choline and pharmacotherapy, the median values for choline and pharmacotherapy group were 53 and 41, respectively (Figure 5.1). There was a significant decrease in symptom scores of patients receiving choline as well as standard pharmacotherapy from baseline (p < 0.01). However, no significant difference was observed in total symptom score between choline and pharmacotherapy group patients post-treatment (p = 0.3416).

The median value for drug scores of the choline and standard pharmacotherapy group at baseline was 112 and 120, respectively (Figure 5.2). After six months of treatment, the median values for choline and standard pharmacotherapy group were 84 and 138, respectively. There was a significant decrease in drug scores of patients receiving choline from baseline (p < 0.01). Also there was reduction in total drug score from baseline in standard pharmacotherapy group patients, though not significant (p = 0.1485). The change in total drug score from baseline in choline group was
significantly different from the pharmacotherapy group post-treatment (p < 0.01). Further, the requirement of additional drugs was less during choline treatment.

**Bronchial hyperreactivity:** Analysis of spirometric data for both choline and standard pharmacotherapy group patients’ revealed that the effect on FEV1 was invariable over the 6 months’ treatment period. However, BHR decreased significantly in choline treatment group compared to baseline (Figure. 5.3; p < 0.01). A number of the patients (73.3 %) showed significant increase in PC20 FEV1 values compared to baseline values after 6 months of choline treatment. Patients on choline therapy after 6 months required higher concentration of histamine to achieve 20% fall in FEV1 as compared to their baseline indicating improvement in PC20 FEV1. In the pharmacotherapy group, the decrease in BHR was not significant from its baseline (p = 0.6397). Also, the change in BHR in choline therapy group was significantly different from the change in standard pharmacotherapy group post-treatment (p < 0.01).

**Immuno-biochemical parameters**

*Blood Eosinophil counts:* Peripheral blood eosinophil counts have been expressed as percentage against total cell counts (Table 5.2). There was a significant decrease in peripheral blood eosinophil count of patients receiving choline for 6 months from baseline (p < 0.01). Peripheral blood eosinophil counts also reduced in standard pharmacotherapy group significantly from baseline (p = 0.002). The change in eosinophil counts in choline therapy group was not significantly different from the pharmacotherapy group after 6 month of treatment (p = 0.0511).

*Total IgE estimation:* There was a reduction in total IgE levels after 6 month of choline treatment (Table 5.2; p < 0.01). The total IgE levels in pharmacotherapy group also showed reduction after 6 months compared to its baseline (p < 0.01). However, no change was observed in skin test reactivity after 6 months in choline therapy and pharmacotherapy group patients as compared to their baseline.

*Cytokine levels:* The data for Th2 cytokine profile in the peripheral blood of choline and standard pharmacotherapy group are presented (Figure. 5.4 & 5.5). Compared to baseline (mean 108 ± 7.73 pg/ml), the patients in choline group had significantly lower IL-4 levels post treatment (mean 67.1 ± 6.95 pg/ml; p < 0.01). The pharmacotherapy group patients also had lower IL-4 levels (mean 102.4 ± 8.84 pg/ml) than baseline (mean 110.1 ± 6.57 pg/ml), but difference was not significant (Figure 5.4; p = 0.099).
Figure 5.1: Total symptom score at baseline (0 month) and after 6 months’ post-treatment with choline (p < 0.01) and standard pharmacotherapy (p < 0.01). Bars represent median values at baseline and treatment groups. NS: non-significant.

Figure 5.2: Total drug score at baseline (0 month) and after 6 months’ treatment with choline (p < 0.01) and standard pharmacotherapy (NS). Bars represent median values at baseline and treatment groups. NS: non-significant.
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Figure 5.3: Bronchial hyperreactivity (PC$_{20}$ FEV1) of choline and standard pharmacotherapy group at baseline (0 month) and treatment (after six months of therapy). PC$_{20}$ FEV1 before and after treatment with choline (p < 0.01) and standard pharmacotherapy (NS). * p < 0.01; NS: non-significant.

Table 5.2: Inflammatory parameters before and after 6 months of treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Choline + Pharmacotherapy</th>
<th>Pharmacotherapy</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Treatment</td>
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<tr>
<td>Eosinophil count %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>5.45 (± 0.60)</td>
<td>3.90 (± 0.41)</td>
</tr>
<tr>
<td>Total IgE (IU/ml)</td>
<td>642.1 (± 83.18)</td>
<td>529.73 (± 74.98)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>112.20 (± 5.87)</td>
<td>123.13 (± 4.09)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>44.60 (± 2.31)</td>
<td>38.85 (± 3.95)</td>
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</table>

Data are the mean ± SEM; NS, non-significant.
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There was a significant difference in IL-4 levels between choline and pharmacotherapy group patients (p < 0.01) after 6 months of treatment. The patients in choline group had significantly lower IL-5 levels (mean 95.77 ± 13.65 pg/ml; p < 0.01) compared to baseline (mean 208.2 ± 27.07 pg/ml; Figure. 5.5). Pharmacotherapy group patients also showed significantly reduced IL-5 levels (mean 185.6 ± 22.44 pg/ml; p < 0.01) than baseline (mean 229.4 ± 27.38 pg/ml). There was a significant difference in IL-5 levels between choline and pharmacotherapy group (p < 0.01) after six months of treatment (Figure. 5.5). Further, a significant correlation was observed between peripheral blood eosinophil counts and IL-5 levels post treatment in choline group (r = 0.7519; n = 30, p < 0.01).

Compared to baseline (mean 307 ± 21.82 pg/ml), the patients in choline group had significantly lower TNF-α levels after treatment (Figure. 5.6; mean 233.7 ± 18.33 pg/ml; p < 0.01). The pharmacotherapy group patients also showed lower TNF-α levels (mean 301.17 ± 19.565 pg/ml) than baseline (mean 314.75 ± 19.94 pg/ml), but difference was not significant (p > 0.05). There was a significant difference in TNF-α level between choline and pharmacotherapy group patients (p < 0.05) after 6 months of treatment. However, IL-10 and IFN-γ levels did not show significant change between pre and post-treatment in choline or pharmacotherapy group (Table 5.2).

Leukotrienes: The levels of Cys-LT and LTB4 in peripheral blood were increased in asthma patients at baseline in both the treatment groups. The Cys-LT levels reduced significantly in choline group patients compared to baseline (Figure. 5.7; p = 0.03). After 6 months of choline treatment, LTB4 levels also reduced significantly compared to baseline (Figure. 5.8; p < 0.01). There was a significant difference in Cys-LT and LTB4 levels between choline and standard pharmacotherapy group patients after 6 months of treatment (p < 0.01).

Oxidative stress marker: After 6 months of treatment, 8-isoprostanes levels in the peripheral blood reduced significantly in choline group compared to baseline and pharmacotherapy group (Figure. 5.9; p < 0.01). No significant difference in 8-isoprostanes level was observed in pharmacotherapy group patients as compared to baseline (p = 0.4227). MDA concentrations however did not change after choline treatment and standard pharmacotherapy group (data not shown).
Figure 5.4: Levels of IL-4 at baseline and after 6 months’ treatment with choline (p < 0.01) and standard pharmacotherapy (NS). Bars represent median values at baseline and treatment groups. NS: non-significant.

Figure 5.5: Levels of IL-5 at baseline and after 6 months’ treatment with choline (p < 0.01) and standard pharmacotherapy (p < 0.01). Bars represent median values at baseline and treatment groups. NS: non-significant.
Figure 5.6: TNF-α level in peripheral blood mononuclear cell culture supernatant at baseline and after 6 months’ treatment with choline (p < 0.01) and standard pharmacotherapy (p = 0.0525). Bars represent median values at baseline and treatment groups. NS: non-significant.

Figure 5.7: Level of cysteinyl leukotriene (Cys-LT) at baseline and after 6 months’ treatment with choline (p < 0.05) and standard pharmacotherapy (NS). Bars represent median values at baseline and treatment groups. •, filled circle represent the highest and lowest values. NS: non-significant.
Figure 5.8: Level of leukotriene B4 (LTB4) at baseline and after 6 months’ treatment with choline (p < 0.05) and standard pharmacotherapy (NS). Bars represent median values at baseline and treatment groups. •, filled circle represent the highest and lowest values. NS: non-significant.

Figure 5.9: Level of 8-isoprostanes at baseline and after 6 months’ treatment with choline (p < 0.05) and standard pharmacotherapy (NS). Bars represent median values at baseline and treatment groups. •, filled circle represent the highest and lowest values. NS: non-significant.
Adverse effects: Few adverse effects were observed within the patients’ of choline therapy group. Two patients reported symptoms of mild diarrhea/vomiting and 3 with mild abdomen discomforts, but it was self-limiting without any additional therapy. No adverse effects were observed in patients of standard pharmacotherapy group.

DISCUSSION

Airway inflammation, a prominent feature in asthma, needs to be targeted with effective medication to achieve asthma control. Inhaled corticosteroids (ICS) are recommended as first-line treatment for asthma/allergic rhinitis, but they are associated with systemic adverse effects (Holgate and Polosa, 2008). Therefore, new drugs are required which can control immune inflammation with no or minimum side effects. The present study was undertaken to evaluate the effect of choline in reducing airway inflammation and improving BHR in asthma patients. The change in Th2 immunologic parameters and oxidative stress was also assessed after 6 months of choline treatment.

Bronchial hyperreactivity is an important parameter to evaluate the clinical efficacy of asthma treatment (Garcia-Robaina et al., 2006). In the present study, significant improvement in PC_{20} FEV1 was observed in patients treated with choline compared to baseline as well as pharmacotherapy group (p < 0.01). More than 75% of patients in choline treatment had significant improvement in their PC_{20} FEV1. Out of 30 subjects, 10 patients of choline treatment group required more than 10 fold concentration of histamine to provoke a 20% fall in FEV1 compared to baseline. In contrast, pharmacotherapy group patients did not show significant improvement in PC_{20} FEV1 (p = 0.6757). Further, the patients showed significant decrease in symptom score (p < 0.01) post treatment in choline as well as in pharmacotherapy group. Also there was significant decrease in drug requirement in choline group compared to baseline. In previous studies, choline magnesium trisalicylate was well tolerated when administered orally for a maximum of 1 week in asthmatics with aspirin hypersensitivity (Szczeklik et al., 1990). Further, studies with choline have shown reduction in symptoms and drug requirement in asthma patients (Gupta and Gaur, 1997; Gaur et al., 1997). However, the focus of previous studies was primarily towards the clinical outcome with small number of patients.

The eosinophils are one of the most important effector cells of airway inflammation in asthma (Sampson, 2000). Cytokines in particular IL-4 and IL-5
Chapter 5

Anti-inflammatory action of choline in asthmatics

associated with a Th2 like response appear to play a role in disease progression in asthma (Romagnani, 1991; Hasday et al., 1994). IL-4 enhances secretion of IgE by B-cell, contributes towards mast cell growth, and endothelial cell up-regulation of adhesion molecules, which is involved in selective recruitment of eosinophils (Sampson, 2000). IL-4 and IL-5 proteins and the frequencies of cells expressing mRNAs are markedly increased in BAL fluid and lung tissue samples of asthmatic patients compared to healthy subjects (Robinson et al., 1993). Further, mast cells obtained from patients with allergic asthma can synthesize and store cytokines such as IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α that are considered important for chronic inflammatory response in asthma (Okayama et al., 1995). In the present study, IL-5 concentration as well as peripheral blood eosinophil counts were significantly reduced after 6 months of treatment in both the groups. Total IgE and IL-4 levels were significantly reduced compared to baseline in choline group patients. Standard pharmacotherapy also reduced total IgE, but no change was observed in IL-4 level post-treatment. TNF-α is a pleiotropic inflammatory cytokine present in increased concentrations in BAL fluid from the airways of patients with asthma and refractory asthma (Broide et al., 1992; Berry et al., 2006). Furthermore, choline treatment reduced the level of TNF-α, consistent with the previous reports (Deto poloulou et al., 2008). To the best of our knowledge, this is the first report showing the effect of choline in reducing IL-5 as well as peripheral blood eosinophil counts in asthma patients.

Eosinophils may contribute to AHR in asthma through the actions of granule derived basic proteins and the release of membrane derived lipid mediators such as Cys-LT (Robinson et al., 1993). Leukotrienes are produced by mast cells, eosinophils and neutrophils and are implicated in the airway inflammation and airway smooth muscle remodeling (Henderson, 1994). Smith demonstrated inhibition of the release of histamine and slow reacting substance of anaphylaxis (SRS-A) in sensitized guinea pigs (Smith, 1961). In the present study, choline treatment for 6 months significantly reduced Cys-LT and LTB4 compared to baseline (p < 0.01). However, no significant change was observed in pharmacotherapy group, similar to previous findings in asthma patients on corticosteroids treatment (O'Shaughnessy et al., 1993).

Oxidative stress plays a crucial role in the pathogenesis of cystic fibrosis, COPD and asthma. Elevated levels of 8-isoprostanes are considered to be the most reliable biomarker to measure oxidative stress (Montuschi et al., 1999; Bowler and
Crapo, 2002). There was an increased level of 8-isoprostanes, malondialdehyde and H$_2$O$_2$ concentrations in severe asthmatics with airway obstruction, as corticosteroid treatment alone is not sufficient to neutralize oxidative stress (Fitzpatrick et al., 2009). In the present study, choline treatment significantly reduced isoprostanes levels compared to baseline (p < 0.01). In contrast, no change in the level of 8-isoprostanes was observed in standard pharmacotherapy group. Choline deficiency activates production of ROS as one of the pathogenic mechanism of mitochondrial and oxidative damage (Ossani et al., 2007). Further, mice fed with choline deficient diet showed increase in oxidative stress and altered antioxidants defenses (Grattagliano et al., 2000; Yoshida et al., 2006). The results suggest that probably choline mediates its anti-inflammatory effect partially through modulation of the redox status of the cell.

Choline functions as a precursor for acetylcholine, phospholipids and the methyl donor betaine (Zeisel, 2006). Adverse effects with high intake of choline (more than 10 grams per day) are trimethylaminuria, hypotension, nausea, sweating and diarrhea in patients with Alzheimer senile dementia, tardive dyskinesia and cerebellar ataxia (Boyd et al., 1977; Eberhardt et al., 1990). In the present study, no serious side-effects were observed in patients, except two patients reported symptoms of mild diarrhea/vomiting and three with mild abdomen discomforts in choline group. The dosage selected here were 1500 mg/bd for 6 months based on a previous study (Gupta and Gaur, 1997). However, the dose of choline used in present study was below the level of UL (3.5 g/day) set by IOM (Institute of Medicine, DRI, 1998).

The relation between low-grade inflammation and diet is still evolving. Several cross-sectional studies have reported beneficial associations between dietary fish intake, asthma and atopic disease (Hodge et al., 1996; Dunder et al., 2001). Investigations in asthma and dietary lipids suggest that asthma and atopy are a consequence of increasing n-6 polyunsaturated fatty acids and decreasing n-3 polyunsaturated fatty acids consumption that effect on inflammatory mediators and Th cell differentiation (Devereux and Seaton, 2005). There has been considerable interest in the possible modulation of asthma by supplementation of anti-oxidants e.g. hydrophilic and lipophilic antioxidants like vitamin C and E, respectively (Bowler and Crapo, 2002). However, supplementation with vitamin B to healthy subjects which are involved in homocysteine metabolism could lower levels of inflammatory molecules (Ulleaddi et al., 2004) with some exceptions (Solini et al., 2006; Peeters et al., 2007). Choline has been found to decrease homocysteine concentrations (Olthof et
al., 2005) and restrain cytokine levels in animal model (Rivera et al., 1998) and is involved in methyl transport through its oxidation to betaine. High intake of dietary choline was independently associated with a reduction in inflammatory indexes that are believed to have an important role in cardiovascular disease (Detopoulou et al., 2008).

The mechanism underlying the modulating effect of choline in asthmatic subjects remains to be established. However, based on previous reports, two general mechanisms are most likely. First, asthma has been hypothesized as being a metabolic disorder since the patients have altered lipid metabolism (Agarwal, 1987; Johnson et al., 2007; Shore, 2008). Phosphatidylcholine is synthesized by de novo and transmethylation pathway, the two being reciprocally compensatory (Pelech and Vance, 1984). The activity of transmethylation pathway is coupled to phospholipase A2 activation, calcium influx and arachidonic acid formation with release of leukotrienes, prostaglandins and of LPC (Hirata and Axelrod, 1980). Indeed, high serum LPC levels have been associated with increased asthma severity and increased Na+ in PBMCs and leukocytes (Agarwal, 1987; Skoner et al., 1990; Gentile and Skoner, 1997). Choline supplementation increases membrane PC via de novo pathway and inhibits transmethylation pathway thereby decreases LPC and mediator release from human basophils (Morita et al., 1981; Agarwal, 1987). Second, nicotinic acetylcholine receptors (nAChR) agonist is known for protective effect on the development of airway inflammation through cholinergic anti-inflammatory pathway (Wang et al., 2003; Gallowitsch-Puerta and Tracey, 2005). Choline, a selective agonist of alpha-7-nicotinic receptors has been shown to produce antinociceptive effects against inflammatory pain (Papke et al., 1996; Wang et al., 2005), may be mediated through the activation of alpha-7-nicotinic receptor via cholinergic anti-inflammatory pathway.

In conclusion, choline administration for 6 months suppresses immune inflammation and oxidative stress in asthma patients. The results suggest that choline exerts anti-inflammatory effect on the airways and reduces bronchial hyperreactivity in asthmatics. Further, it can be used as an adjunct therapy in the management of asthma.