

**Title:** Effect of choline chloride in allergen induced mice model of airway inflammation

**Short title:** Anti-inflammatory action of choline in mouse model

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## **ABSTRACT**

Incidence of asthma has increased world over and current therapies for the disease suffer from potential side effects. This has created an opportunity to develop novel therapeutic approaches. Here anti-inflammatory activity of choline was investigated in mice model of allergic airway inflammation.

Choline (1 mg/kg) was administered via oral gavage or intranasal (i.n.) route before and after ovalbumin (OVA) challenge in sensitized mice. Airway hyperresponsiveness (AHR) to methacholine was measured in mice by whole body plethysmography. Th2 cytokines and leukotrienes were estimated in bronchoalveolar lavage fluid (BALF) and spleen culture supernatant by ELISA. Eosinophil peroxidase (EPO) activity in BALF supernatant was also determined.

Choline treatment in sensitized mice before OVA challenge via oral/i.n. routes significantly inhibited eosinophilic airway inflammation and EPO activity. It also reduced IgE and IgG1 production and inhibited the release of Th2 cytokines and leukotrienes. However, the development of AHR was prevented effectively by i.n choline treatment. Most importantly, choline treatment after OVA challenge by both routes could reverse already established asthmatic conditions in mice by inhibiting AHR, eosinophilic airway inflammation and other inflammatory parameters.

This study provides a new therapeutic approach for controlling as well as preventing asthma exacerbations.

**Keywords:** Airway inflammation, animal model, asthma, choline, eosinophils.

**Abbreviations:**

AB/PAS: alcian blue/periodic acid-schiff

ACh: acetylcholine

AHR: airway hyperresponsiveness

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

Cys-LT: cysteinyl leukotrienes

PBS: phosphate buffered saline

ELISA: enzyme linked immunosorbent assay

EPO: eosinophil peroxidase

H & E: hematoxylin and eosin

I.n: intranasal

Mch: methacholine

nAChR: nicotinic acetylcholine receptors

OVA: ovalbumin

RT: room temperature

## INTRODUCTION

Incidence of asthma and allergic disorders has increased 2-fold in the past two decades [1]. Asthma is a chronic inflammatory disease characterized by eosinophilic infiltration in the bronchial mucosa, airway hyperresponsiveness (AHR) and intermittent pulmonary obstruction. It is partly mediated by CD4<sup>+</sup> T-cells that activates numerous airway inflammatory cells such as T-cells and eosinophils leading to morphological changes in airway tissue and increased AHR [2]. Further, cysteinyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>) induce AHR in asthmatics followed by bronchoconstriction, mucus secretion and promoting eosinophil chemotaxis [3, 4].

Several therapeutic interventions have focused on attenuating airway inflammation, such as glucocorticoids. Such drugs relieve symptoms but do not reverse the progression or cure the disease but have potential side effects that limit their usefulness [5]. Leukotriene modifiers, a new class of anti-asthma drugs have been associated with liver toxicity [6]. Therefore, new drugs are required to control immune inflammation with minimal or no side effects.

Choline, a lipotropic factor, plays a role in mobilization of fats in liver, essential for acetylcholine (ACh) formation [7] and used for phosphatidyl choline (PC) synthesis by *de novo* pathway [8]. It has shown anti-inflammatory activity in arthritis animal model [9]. Choline magnesium trisalicylate improved symptoms in aspirin induced asthma patients [10]. Further, tricholine citrate reduced symptoms and drug requirement in asthma [11, 12]. These reports suggest the benefit of choline in asthma but evidence is lacking about its action on the airways. The present study investigates the potential of choline in inhibiting pulmonary infiltration of inflammatory cells and AHR in ovalbumin induced mice model of allergic airway inflammation.

## METHODS

*Sensitization and Treatment:* Female Balb/c mice (n = 100) of 6-8 weeks were obtained from National Institute of Virology, Pune (India). The institutional animal ethics committee approved the study protocol. Choline chloride (Sigma, St. Louis, MO, USA) was administered via oral gavage or intranasal (i.n.) route before and after OVA challenge in sensitized mice in two different protocols. In protocol A, mice were randomly divided in 5 groups (10 mice each). Control group was sensitized, challenged and treated with 100  $\mu$ l of 0.9% NaCl (saline). Experimental groups were sensitized intraperitoneally (i.p.) with 100  $\mu$ g OVA adsorbed on 2 mg of Al (OH)<sub>3</sub> in 100  $\mu$ l of saline on days 0, 7 and 14. They were challenged with 2.5% aerosolized OVA in saline in a plexiglass chamber using nebulizer (Omron, Tokyo, Japan) for 30 minutes on days 25-27. OVA sensitized and challenged mice were treated with (1) 100  $\mu$ l saline, (2) 1 mg/kg of choline in 100  $\mu$ l saline orally 1 hour before each OVA challenge on days 25-27, (3) 1 mg/kg of choline (i.n.) in 50  $\mu$ l saline 30 minutes before each OVA challenge and (4) 1 mg/kg of dexamethasone phosphate (i.p.) in 100  $\mu$ l saline 30 minutes before each OVA challenge. After the last treatment/challenge, AHR was measured on day 28 and mice sacrificed on day 29.

In protocol B, mice were randomly divided in 5 groups (10 mice each) and sensitized as mentioned in protocol A. Further, they were challenged with 2.5% aerosolized OVA in saline for 30 minutes on days 25-30. Twenty-four hours after the last challenge, mice were treated with 100  $\mu$ l of (1) saline, (2) choline (1 mg/kg) orally for 10 days from day 31-40 in saline. Similar doses of (3) choline (i.n.; in 50  $\mu$ l saline) and (4) dexamethasone (i.p.; in 100  $\mu$ l saline) were given on alternate day from day 31-40. Finally, a booster OVA challenge was done on day 38. AHR was measured on day 41 after the challenge/treatment and mice sacrificed on day 42. Control group was sensitized, challenged and treated with 100  $\mu$ l of saline. Mice receiving i.n.

dose were lightly anesthetized (3% isoflurane) before each dose. Dose response analysis was made with choline chloride (Fig. 1) and the dose of 1 mg/kg was selected for all experiments.

*Determination of AHR:* Airway reactivity in response to methacholine (Sigma, St. Louis, MO, USA) was measured in conscious, unrestrained mice in a preconditioned whole body plethysmograph (Buxco Electronics Inc., Troy, NY). Five mice from each group were nebulized with phosphate buffered saline (PBS), followed by increasing concentration of methacholine (Mch; 4-50 mg/ml) to induce bronchoconstriction. Lung functions were recorded and calculated as enhanced pause (Penh) that correlates with pulmonary resistance [13].

*Collection of bronchoalveolar lavage fluid (BALF) and blood:* After last airway challenge, mice were sacrificed using sodium pentobarbital (100 mg/kg i.p.). Blood was collected after cardiac puncture for total and differential cell counts. Sera were separated and used for analysis of serum immunoglobulins. The trachea was cannulated after the collection of blood. The lungs were lavaged three times with 0.5 ml of chilled saline and volume recovered was approximately 1.5 ml from each mouse. Total cell counts in BALF were determined by hemocytometer using light microscope. Each BALF sample was centrifuged at  $400 \times g$  for 10 minutes at 4°C. The cell pellets were resuspended into 1 ml of PBS and BAL cell smears were stained with Leishman. BALF cell eosinophils were identified (%) by counting minimum of 200 cells in high magnification ( $\times 400$ ). Absolute eosinophils were calculated by multiplying total cell counts by percentage of eosinophil in BALF cell pellets.

*Determination of eosinophil peroxidase (EPO) activity:* EPO levels in BALF supernatant were determined as described previously [14]. Briefly, 100  $\mu$ l of substrate solution containing 0.1 mM *o*-phenylene-diamine-dihydrochloride (OPD); (Sigma, St. Louis, MO, USA), 0.1% Triton X-100, 1 mM hydrogen peroxide in 0.05 M Tris-HCl was added to 100  $\mu$ l of BALF supernatant in

microtiter plates and incubated for 30 minutes at 37°C. Reaction was stopped by adding 50 µl of 4 M sulphuric acid and absorbance read at 492 nm.

*Histopathology:* Lungs were inflated and fixed with 10% neutral-buffered formalin. After fixation, lung sections of 4 µm were cut and stained with hematoxylin and eosin (H & E) or alcian blue/periodic acid-schiff (AB/PAS) for histological evaluation, monitoring of inflammatory cells and mucus production. Sections were scanned using light microscope for antigen-induced peribronchial and perivascular inflammation. The eosinophils or lymphocytes infiltration around the airways and the proliferation of goblet cells in the bronchial epithelium were quantified and graded as, 0: not present; 1: very slight, 2: slight, 3: moderate, 4: moderate to marked and 5: marked. An inflammatory reaction affecting <20% of the airways was defined as '1' (<20% goblet cells stained with AB/PAS), '2' was defined as 20-40% of the airways affected, '3' 40-60%, '4' 60-80% and '5': >80% of the airways affected [15].

*Splenocyte culture:* For *in-vitro* experiments, splenocytes ( $5 \times 10^6$  cells/ml) were cultured in RPMI-1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin; Sigma) and stimulated with 100 µg/ml OVA. Cells were cultured for 72 hours at 37°C in a CO<sub>2</sub> incubator. Culture supernatant was collected to measure cytokines and eicosanoids.

*Determination of specific antibody levels and cytokine by ELISA:* OVA-specific serum IgE, IgG1 and IgG2a titres were measured in duplicates by ELISA as described with slight modification [16]. Briefly, microtiter plates (Nunc-Immuno<sup>Tm</sup> modules, Roskilde, Denmark) were coated with 5 µg/ml of OVA in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS and blocked with 3% defatted milk for 3 hours at 37°C. The plates were incubated overnight at 4°C with mice sera for IgE (1:10), IgG1 (1:50) and IgG2a (1:50)

estimation, individually. After washing with PBST (0.05% Tween-20) and PBS, the plates were incubated for 3 hours at 37°C with IgG1-peroxidase and IgG2a-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA).

For IgE, biotinylated anti-mouse IgE (2 µg/ml, BD Pharmingen) was incubated at RT for 90 minutes. Following washing, it was incubated with streptavidin-peroxidase (1:1000; BD Pharmingen) for 30 minutes. The plates were washed and developed using OPD and absorbance read at 492 nm.

IL-4, IL-5 and IFN- $\gamma$  levels were determined in undiluted BALF and culture supernatants in duplicates by ELISA using paired antibodies according to manufacturer's instruction (BD Pharmingen). The detection limit for IL-4, IL-5 and IFN- $\gamma$  was 7.8, 15.6 and 31.3 pg/ml, respectively.

*Eicosanoid Assays:* Undiluted BALF and culture supernatants were assayed in duplicates for LTB<sub>4</sub> and Cys-LT using enzyme immunoassay kits (Cayman Chemical Co. MI, USA) according to manufacturer's instructions. The detection limit for LTB<sub>4</sub> and Cys-LT was 13 pg/ml.

*Statistical analysis:* Groups were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison tests to examine differences between OVA challenged saline treated and choline treated groups. Differences were considered significant at  $p < 0.05$ . Data have been presented as mean  $\pm$  SD for each group. The software package GraphPad InStat was used for data analysis.

## **RESULTS**

*Choline suppresses allergen induced eosinophilic airway inflammation:* Two different schedules of choline administration before (preventive) and after (therapeutic) OVA challenge were used to demonstrate the changes occurred after treatment in allergen induced inflammatory model of

airway disease. A significant reduction in BALF cell count and eosinophils were observed on choline treatment orally ( $p < 0.05$ ) or i.n ( $p < 0.05$ ) as compared to OVA challenged saline treated mice in both the study protocol (Fig. 2A and 3A). Similar results were obtained in peripheral blood count and eosinophils (data not shown). Treatment with dexamethasone also led to significant reduction in eosinophils numbers ( $p < 0.05$ ).

The recruitment of inflammatory cells in the airways was monitored in choline treated groups as compared to OVA challenged saline treated group stained with H and E (Fig. 4 and 5). In OVA challenged saline treated group, numerous eosinophils were observed into the lung interstitium around airways and blood vessels alongwith narrowing of airway lumen (Fig. 4B and 5B). The choline treatment given orally or i.n. significantly reduced the inflammatory infiltrates in both the protocols (Fig. 4C, 4D, 5C and 5D). Mice treated with dexamethasone showed reduced inflammation and eosinophil infiltration in lungs (Fig. 4E and 5E). These results were confirmed by a reduction in total inflammation score in choline treated groups compared to saline treated groups ( $p < 0.05$ ) (Fig. 4F and 5F).

In control groups, either no or very few AB/PAS positive epithelial cells were observed (Fig. 6A and 7A). Contrary to this, many of the epithelial cells were enlarged in OVA challenged mice, alongwith airway narrowing and mucus plugging with increased goblet cell hyperplasia visualized with AB/PAS staining (Fig. 6B and 7B). Choline treatment before challenge by either route (oral/i.n.) inhibited the mucus production and goblet cell hyperplasia (Fig. 6C and 6D). Choline treatment downregulated the accumulation of mucus in the airways and prevented airway obstruction after antigen challenge (Fig. 7A and 7B). There is a substantial reduction in total inflammation score in choline treated mice compared to saline treated mice ( $p < 0.05$ ) (Fig. 6F and 7F).

*Choline reduces methacholine induced AHR:* Methacholine induced airway resistance was measured on day 28 and on day 41 for both the protocols. OVA challenged saline treated mice developed AHR as demonstrated by a dose dependent elevation in Penh in response to Mch (Fig. 2B and 3B). Treatment with oral choline before challenge exhibited a modest decrease in AHR, though it was not significant. However, i.n. treatment demonstrated significant reduction ( $p < 0.05$ ) in Penh at Mch concentrations of 12-50 mg/ml (Fig. 2B).

In protocol B, the Penh values significantly decreased in oral choline treatment (20-50 mg/ml). However, i.n. treatment was more effective and showed statistically significant reduction ( $p < 0.05$ ) in Penh at Mch concentrations of 4-50 mg/ml (Fig. 3B). Dexamethasone treatment inhibited the development of airway hyperresponsiveness in both the study protocols ( $p < 0.05$ ).

*Loss of EPO activity in choline treated mice:* Significantly, high levels of EPO with increased infiltration of eosinophils in BALF were observed in saline treated mice. However, the choline treatment (Fig. 2C and 3C) by both the routes reduced the activity of EPO ( $p < 0.05$ ) and thus reduced inflammation in the airways. Treatment with dexamethasone significantly reduced the EPO activity in both the protocols ( $p < 0.05$ ).

*Effect of choline on serum OVA-specific IgG1, IgE and IgG2a:* Oral and i.n. choline treatment resulted significant decrease in IgG1 and IgE ( $p < 0.05$ ) levels in both the study protocols (Fig. 2D and 3D). Dexamethasone treatment also reduced IgG1 and IgE in mice. However, no significant difference was observed in IgG2a antibody levels by choline treatment.

*Choline treatment reduces Th2 cytokine levels in BALF and culture supernatant:* IL-4 and IL-5 levels were remarkably reduced with choline treatment by oral and or i.n. route ( $p < 0.05$ ) in BALF and in culture supernatants (Fig. 2E and 3E). In protocol A, i.n. choline treatment showed increased IFN- $\gamma$  level in BALF ( $p = 0.035$ ) but not in oral treatment group ( $p = 0.149$ ). Oral

choline treatment induced IFN- $\gamma$  levels significantly ( $p < 0.05$ ) compared to i.n. treatment ( $p = 0.76$ ) in spleen culture supernatant. In protocol B, both the routes showed increase in IFN- $\gamma$  level in BALF as compared to normal mice but this was not observed in culture supernatant.

*Choline inhibits the release of eicosanoids in BALF and culture supernatant:* LTB<sub>4</sub> level was reduced in culture supernatant but not in BALF in oral choline treatment in protocol A (Fig. 2F). However, LTB<sub>4</sub> showed a significant decrease in BALF as well as in culture supernatant ( $p < 0.05$ ) with choline treatment after challenge by either routes (Fig. 3F).

Apart from LTB<sub>4</sub>, choline treatment strongly inhibited the release of Cys-LT not only in BALF but also in culture supernatant (Fig. 2F and 3F). This inhibition of Cys-LT was observed in both the protocols ( $p < 0.05$ ).

## **DISCUSSION**

Identification of effective drugs for asthmatics has been relegated in recent years especially for those who respond poorly to conventional therapy. Glucocorticosteroids, used for asthma treatment, are associated with systemic side effects [17]. Consequently, there is need for the development of new agents to treat airway diseases.

The nicotinic acetylcholine receptors (nAChR) agonist is known for protective effect on the development of airway inflammation through cholinergic anti-inflammatory pathway [18, 19]. Nicotinic acetylcholine receptors (nAChR) are expressed on various inflammatory cells such as eosinophils, lymphocytes, alveolar macrophages and airway smooth cells. Nicotinic acetylcholine receptors agonist like 1, 1-dimethyl-4-phenylpiperazinium (DMPP) has been shown to have a potential down-regulating effect on eosinophils functions by inhibiting LTC<sub>4</sub> and eosinophils migration [20]. Choline, a selective agonist of alpha-7-nicotinic receptors suppressed passive joint anaphylaxis and showed anti-inflammatory in guinea pigs [9, 21, 22],

however, its role in inhibiting eosinophilic inflammation is not known. In the present study, choline was demonstrated as a potent inhibitor of airway inflammation suppressing the accumulation of eosinophils and release of EPO in BALF may be mediated through the activation of alpha-7-nicotinic receptor via cholinergic anti-inflammatory pathway.

Eosinophils on activation release eosinophil cationic protein, major basic protein and EPO that correlates with the disease severity or AHR [23, 24]. In our study, choline administration was effective in inhibiting AHR both before and after OVA challenge. The data from choline treatment is encouraging as the resolution of AHR may be associated with loss of eosinophil activation. Choline administered through i.n. route gives a localized effect whereas oral route gives systemic effect in reducing AHR. Aerosol therapy via i.n. route is the preferred asthma therapy for adults and children. Since it offers a rapid onset of drug action directly into the target organs, requires smaller doses and reduces systemic effects.

Clinical and experimental evidence implicates Th2 cytokines in orchestrating the inflammatory response and AHR in asthma [2]. In our study, the anti-inflammatory effects of choline were at least partly mediated by suppression of Th2 cytokines in BALF and culture supernatant with significant decrease in IgE and IgG1. IL-4/IFN- $\gamma$  ratio in BALF also decreased in choline treated mice (data not shown). The reduction of IL-5, a potential activation and survival factor for eosinophils, was concomitant with the loss of EPO activity in BALF supernatant.

Leukotrienes are the key mediators of allergen-induced airway eosinophil infiltration and mucus release [3]. High levels of LTB<sub>4</sub> and Cys-LT were demonstrated in BALF of asthmatics [25, 26]. Cys-LT<sub>1</sub> receptors are expressed on eosinophils and their activation by Cys-LT induces eosinophil infiltration in airways and thus considered potent eosinophil chemo-attractant [27]. Similarly, choline (nAChR agonist) treatment also inhibited the release of Cys-LT and

eosinophilic inflammation in the allergen-induced airway eosinophil infiltration may be mediated through the activation of alpha-7-nicotinic receptor as evident by nAChR agonist DMPP [20] and interestingly suppressed mucus hyperproduction.

Choline, a dietary component is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neuro-transmission, transmembrane signaling, lipid and cholesterol transport and metabolism. Its deficiency results in loss of membrane PC and induction of apoptosis in PC12 cells *in vitro* [28]. It is involved in eliciting a variety of pharmacological effects in many diseases, including stroke, dementia, Alzheimer's, Parkinson's etc [29-32]. Previously, asthma patients have shown improvement in symptom-drug score on oral choline administration [11, 12] but this lacks experimental verification. Recently, choline has been shown to produce antinociceptive effects against inflammatory pain that is blocked by alpha-7 nAChRs antagonist methyllycaconitine citrate, which strongly supports the involvement of alpha-7 nAChRs in the antinociception of choline [22]. Adverse effects with high intake of choline (more than 10 grams per day) are fishy body odor, hypotension, nausea, sweating and diarrhea reported earlier [31, 33].

Phosphatidylcholine is synthesized by *de novo* and transmethylation pathway, the two being reciprocally compensatory [8]. The activity of transmethylation pathway is coupled to phospholipase A2 activation, calcium influx and arachidonic acid formation with release of leukotrienes, prostaglandins and of lysophosphatidyl choline [34]. Choline supplementation increases membrane PC via *de novo* pathway and inhibits transmethylation pathway thereby decreases mediator release from human basophils [35].

Several of the functions that are governed by ACh are disturbed in allergic airway disease and there is evidence that dysfunction of the cholinergic system is involved in the pathogenesis of

asthma [36]. Acetylcholine synthesis require uptake of choline that is mediated by high affinity choline transporter-1 (CHT1) present in airway epithelial cells. Choline supplementation might restore this damage, as choline uptake via the CHT1 is the rate-limiting step in the ACh synthesis by choline acetyltransferase [37]. However, it is difficult to explain this discrepancy based on the existing data.

The mechanism underlying the modulating effect of choline is still not clear. Various inflammatory cells like lymphocytes express most components of the cholinergic system including ACh, muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) etc which helps in eliciting intracellular Ca<sup>2+</sup> response. Since the smooth muscle contraction induced by methacholine is reduced by choline treatment. One of the major steps of smooth muscle contraction is intracellular calcium mobilization [38]. In this study Ca<sup>2+</sup> level was not checked but it was demonstrated that nicotinic agonist could deplete Ca<sup>2+</sup> level in lymphocytes [39]. Similarly, in rat model of endotoxin shock, choline restrained the increase in calcium influx and release of TNF- $\alpha$  by activated macrophages possibly by increasing membrane PC, thereby preventing lung injury and improving survival [40]. This down regulating effect of nicotinic agonist on Ca<sup>2+</sup> mobilization could be responsible for both anti-inflammatory and bronchoprotective effect of choline.

In conclusion, choline was effective in inhibiting airway inflammation in mice model of airway hyperresponsiveness. However, further studies are required to elucidate its mechanism within preclinical model and possibly subsequent investigations in patients of asthma.

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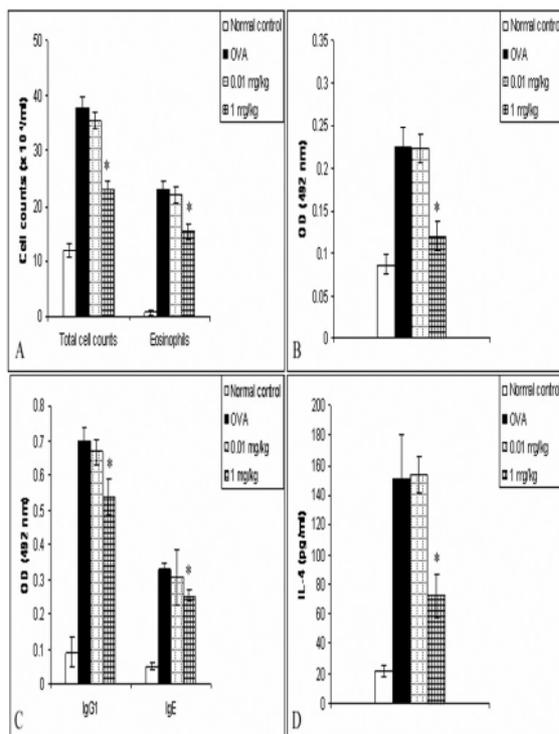
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## FIGURE LEGENDS

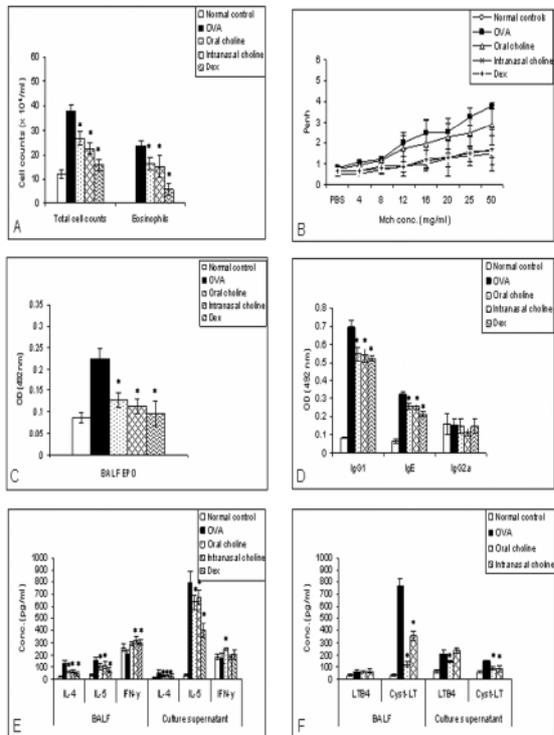
**Fig. 1:** Dose response analysis with lower (0.01 mg/kg) and higher dose (1 mg/kg) of choline chloride. (A) Total cell count and eosinophil number in bronchoalveolar lavage (BAL), (B) EPO activity in BALF supernatant, (C) Serum OVA-specific IgG1 and IgE levels and (D) IL-4 cytokine level in BALF supernatant. Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.



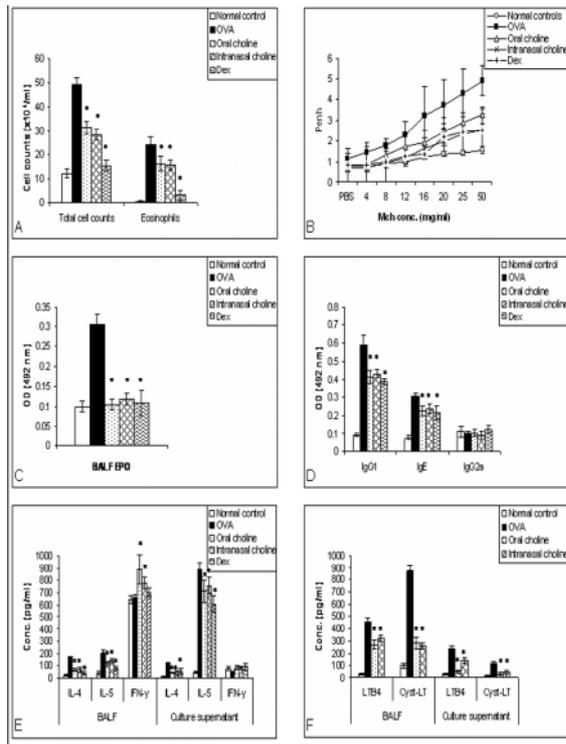
**Fig. 2:** Choline given before challenge prevented AHR and eosinophilic airway inflammation in OVA-sensitized Balb/c mice. (A) Total cell count and eosinophil number in bronchoalveolar lavage (BAL), (B) AHR induced by increasing doses of methacholine in choline and saline treated OVA-challenged mice, (C) EPO activity in BALF supernatant, (D) Serum OVA-specific IgG1, IgE and IgG2a levels, (E) Cytokine levels in BALF and spleen culture supernatant. IFN- $\gamma$  release (p < 0.05) in choline treated in BALF and culture supernatant as compared to normal

control mice. (F) LTB<sub>4</sub> and Cys-LT level in BALF and spleen culture supernatant by EIA.

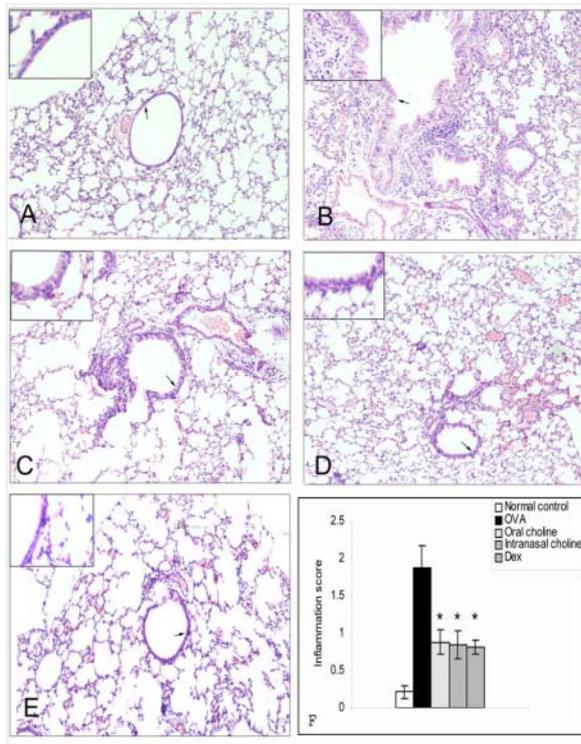
Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.



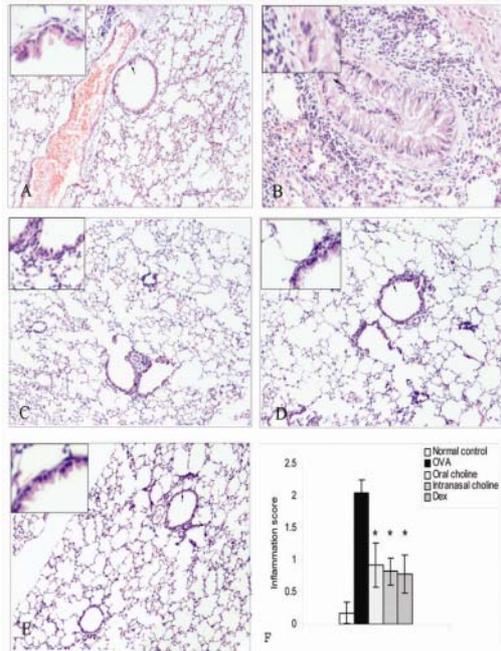
**Fig. 3:** Choline given after challenge on already established mice model of allergic inflammation. (A) Total cell count and eosinophil number in bronchoalveolar lavage (B) AHR of normal controls and OVA-challenged mice treated with saline, choline or dexamethasone (C) EPO activity in BALF supernatant, (D) OVA-specific serum IgG1, IgE and IgG2a levels (E) Cytokine levels in BALF and in spleen culture supernatant. Choline (oral and i.n.) induces significant IFN- $\gamma$  release ( $p < 0.05$ ) in BALF as compared to normal control mice (F) LTB<sub>4</sub> and Cys-LT levels in BALF and spleen culture supernatant. Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.



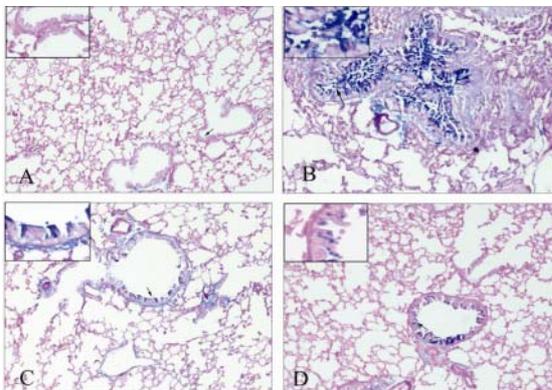
**Fig. 4:** Preventive effect of choline in airway inflammation before challenge. H & E stained sections are representative of lungs from (A) Normal mice, saline sensitized and challenged. (B) OVA-challenged saline treated mice (C) OVA-challenged mice treated with oral choline (D) OVA-challenged mice treated with i.n. choline and (E) OVA-challenged mice treated with dexamethasone (Dex) at 20× magnification. (F) Total Inflammation score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.

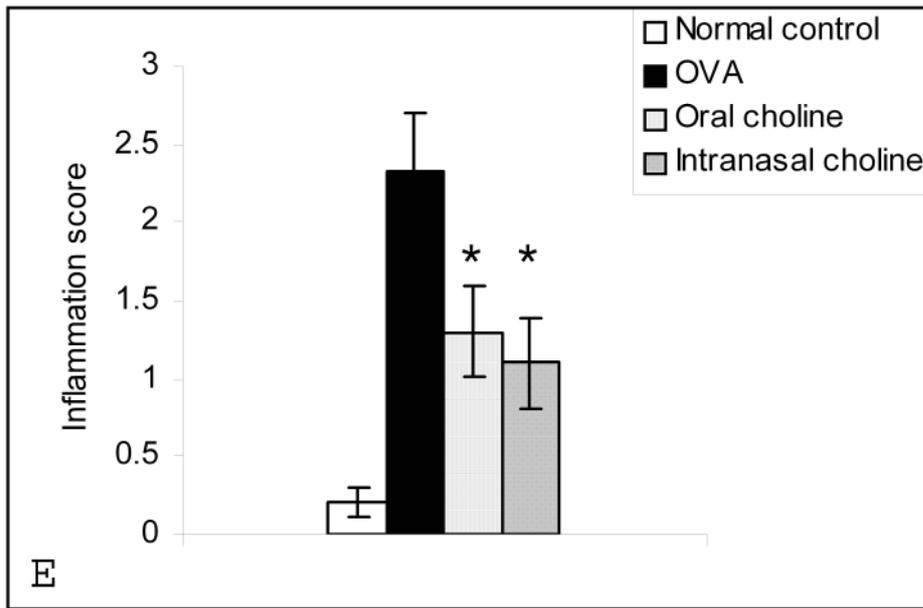


**Fig. 5:** Effect of choline given after challenge. H & E stained sections are representative of lungs from (A) Normal mice, saline sensitized and challenged. (B) OVA-challenged saline treated mice (C) OVA-challenged mice treated with oral choline (D) OVA-challenged mice treated with i.n. choline and (E) OVA-challenged mice treated with dexamethasone (Dex) at 20 $\times$  magnification. (F) Total Inflammation score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.



**Fig. 6:** Alcian blue/Periodic acid-Schiff staining of lung sections from mice in first protocol. Lung histology of (A) Normal mice, saline sensitized and challenged. (B) OVA-challenged saline treated mice (C) OVA-challenged mice treated with oral choline and (D) OVA-challenged mice treated with i.n. choline at 20× magnification. (E) Degree of infiltration of goblet cells was estimated and score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Means  $\pm$  SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.





**Fig. 7:** Alcian blue/Periodic acid-Schiff staining of lung sections from mice in second protocol. Lung histology of (A) Normal mice, saline sensitized and challenged (B) OVA-challenged saline treated mice (C) OVA-challenged mice treated with oral choline and (D) OVA-challenged mice treated with i.n. choline at 20× magnification. (E) Degree of infiltration of goblet cells was estimated and score graded on a scale from 0-4. Score was significantly reduced in choline treated group. Means ± SD of 5 mice per group; \* p value less than 0.05 compared with OVA mice.

